Université de Montréal

Implications of neuronal cell loss in chronic liver disease

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Thèse présentée en vue de l'obtention du grade de Doctorat
en Sciences Biomédicales

August 2023

Université de Montréal

Programme des Sciences Biomédicales, Faculté de Médecine

Cette thèse intitulée

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Résumé

Contexte: L'encéphalopathie hépatique (EH) est une complication majeure de la maladie hépatique chronique (MHC) caractérisée par des symptômes débilitants, notamment des troubles cognitifs, psychiatriques et moteurs. Il est cru que l'EH, définie comme étant un syndrome métabolique, disparaît après une transplantation hépatique (TH). Cependant, des complications neurologiques persistantes ont été signalées chez jusqu'à 47 % des receveurs de TH. Plusieurs études rétrospectives ont démontré une association entre des antécédents d'épisodes d'EH pré-TH et une mauvaise condition neurologique après la TH. D'autre part, l'alcool est un facteur étiologique fréquent à l'origine de la MHC. Cependant, une consommation excessive d'alcool a également un impact sur le cerveau. À ce jour, l'impact des épisodes d'EH ainsi que de l'alcool sur le développement de l'EH et l'intégrité cérébrale reste indéfini. Par conséquent, nos objectifs étaient 1) d'évaluer l'impact de plusieurs épisodes d'EH (induits par l'ammoniac) et 2) d'évaluer l'effet d'une consommation constante d'alcool sur le déclin neurologique, l'intégrité et les lésions cérébrales chez les rats atteints de MHC induite par la ligature des voies biliaires (LVB).

Méthodes: Pour le premier objectif, des rats LVB ont reçu une injection (s.c.) d'acétate d'ammonium (LVB-Ammoniac), précipitant un épisode sévère d'EH réversible (perte du réflexe de redressement) tous les 4 jours à partir de la semaine 3 post-chirurgie LVB (total ; 4 épisodes). Des rats SHAM ont également reçu une injection d'ammoniaque et des rats témoins BDL/SHAM ont reçu une injection de solution saline. La coordination motrice (rotarod) et la mémoire à court et à long-terme (test de reconnaissance d'objets nouveaux) ont été évaluées une semaine après la dernière injection. Pour évaluer l'intégrité neuronale, une analyse d'immunobuvardage de type Western et d'immunofluorescence a été réalisée dans le cortex frontal, le cervelet et l'hippocampe. Pour le deuxième objectif, des rats LVB ont reçu de l'alcool (LVB-Alcool) deux fois par jour (51 % v/v, dose de 3 g/kg, par gavage) pendant 4 semaines. Les rats SHAM ont également reçu de l'alcool et les rats LVB/SHAM servant de contrôles ont reçu une solution saline. La coordination motrice (rotarod) et le comportement anxieux (champ ouvert et labyrinthe surélevé) ont été évalués une semaine après la dernière administration d'alcool. Des analyses d'immunobuvardage de type Western et d'immunofluorescence ont été effectuées pour étudier l'intégrité neuronale du cortex frontal et du cervelet.

Résultats: Chez les LVB-Ammoniac, des niveaux protéiques plus élevés d'un marqueur astrocytaire (GFAP) et apoptotique (caspase-3 et Bax/Bcl2) ont été trouvés dans l'hippocampe, alors que les niveaux de marqueurs neuronaux (NeuN et SMI311) ont été réduits par rapport à

tous les autres groupes expérimentaux. Les rats LVB-Ammoniac ont présenté des niveaux accrus de stress oxydatif plasmatique par rapport aux rats SHAM/BDL respectifs. Une diminution des niveaux de capacité antioxydante totale (CAT) et une augmentation des protéines modifiées par le 4-HNE ont été observées dans l'hippocampe (et non dans le cortex frontal ou le cervelet) du groupe LVB-Ammoniac par rapport aux rats SHAM/BDL respectifs. Les résultats d'immunofluorescence ont révélé la colocalisation du marqueur apoptotique (caspase-3 clivée) avec un marqueur neuronal (NeuN) dans la région CA1 de l'hippocampe des rats LVB-Ammoniac.

Chez les rats LVB-alcool, il a été démontré une altération de la coordination motrice aux semaines 2, 3, 4 et 5 ainsi qu'une augmentation du comportement anxieux par rapport aux rats SHAM respectifs. Chez les rats BDL-alcool, il a été démontré une diminution des marqueurs neuronaux (NeuN et SMI311), une augmentation de l'activité enzymatique apoptotique (caspase-3 clivée), une augmentation des marqueurs de nécroptose (pRIP3 et pMLKL), une diminution de la CAT et une augmentation des protéines modifiées par 4-HNE dans le cervelet par rapport à tous les groupes. Les résultats d'immunofluorescence ont révélé la colocalisation du marqueur apoptotique (caspase-3 clivée) et du marqueur de nécroptose (pMLKL) dans les neurones de la couche granulaire du cervelet de rats LVB-alcool.

Conclusion: De multiples épisodes d'EH sévère ont entraîné une perte de cellules neuronales dans l'hippocampe des rats LVB et qui est associée à une augmentation du stress oxydatif, à l'apoptose et à une diminution des cellules neuronales. Des niveaux élevés du marqueur astrocytaire (GFAP) dans l'hippocampe insinuent une gliose, pouvant être le résultat d'une perte neuronale. De plus, l'administration d'alcool aggrave les troubles de la coordination chez les rats LVB et qui ont été associés à une augmentation du stress oxydatif, à une diminution des marqueurs neuronaux (NeuN et SMI311) avec l'apoptose et la nécroptose dans le cervelet des rats LVB-alcool. Globalement, de multiples épisodes d'EH sévère ainsi qu'une consommation constante d'alcool, via le stress oxydatif, déclenchent une perte/lésion neuronale qui entraînera par conséquent une mauvaise condition neurologique après la TH.

Mots clés: Encéphalopathie hépatique, Toxicité de l'ammoniac, Alcool, Perte de cellules neuronales, Complications neurologiques, Cirrhose, Ligation de la voie biliaire

Abstract

Background: Hepatic encephalopathy (HE) is a major complication of chronic liver disease (CLD) characterized by debilitating symptoms, including cognitive, psychiatric, and motor disturbances. HE, defined as a metabolic syndrome, is believed to resolve following liver transplantation (LT). However, persisting neurological complications have been reported in up to 47% of LT recipients. Several retrospective studies have demonstrated an association between a history of HE episodes pre-LT and poor neurological outcome following LT. Furthermore, alcohol is a common etiological factor which causes CLD. However, excessive alcohol consumption also impacts the brain. To date, the impact of HE episodes as well as of alcohol on the development of HE and brain integrity remains undefined. Therefore, our aims were to 1) evaluate the impact of multiple episodes (induced by ammonia) and 2) evaluate the effect of constant alcohol consumption on neurological decline, brain integrity and injury in rats with CLD induced via bile-duct ligation (BDL).

Methods: In the first aim, BDL rats were injected (s.c.) with ammonium acetate (BDL-Ammonia), precipitating a reversible overt episode of HE (loss of righting reflex) every 4 days from week 3 post-BDL surgery (total; 4 episodes). SHAM rats were also injected with ammonia and BDL/SHAM rats were injected with saline as controls. To assess the neuronal integrity, western blot and immunofluorescence analysis were performed for frontal cortex, cerebellum, and hippocampus. Motor coordination (rotarod) and short- and long-memory (novel object recognition test) were assessed one week following last injection. In the second aim, BDL rats were administered alcohol (BDL-Alcohol) twice a day (51% v/v, dose of 3g/kg, via gavage) for 4 weeks. SHAM rats also received alcohol and BDL/SHAM rats received saline as controls. Motor coordination (rotarod) and anxiety-like behavior (open field and elevated plus maze) were assessed one week following last alcohol administration. Western blot and immunofluorescence analyses were performed to investigate neuronal integrity in frontal cortex and cerebellum.

Results: In BDL-Ammonia, higher protein levels of astrocytic marker (GFAP) and apoptotic markers (caspase-3 and Bax/Bcl2) were found in the hippocampus, whereas neuronal markers (NeuN and SMI311) levels were reduced compared to all other experimental groups. BDL-Ammonia rats showed increased levels of plasma oxidative stress compared to respective SHAM/BDL rats. Decreased levels of total antioxidant capacity (TAC) and increased 4-HNE modified proteins were found in the hippocampus (not in frontal cortex or cerebellum) of the BDL-Ammonia group compared to respective SHAM/BDL rats. Immunofluorescence results

revealed the colocalization of apoptotic marker (cleaved caspase-3) with neuronal marker (NeuN) in the CA1 region of the hippocampus of BDL-Ammonia rats. BDL-Alcohol rats demonstrated impaired motor coordination at weeks 2, 3, 4, and 5 as well as an increase in anxiety-like behavior compared to respective SHAM rats. BDL-Alcohol rats showed a decrease in neuronal markers (NeuN and SMI311), an increase in apoptotic enzyme activity (cleaved caspase-3), an increase in necroptosis markers (pRIP3 and pMLKL), a decrease in TAC and an increase in 4-HNE modified proteins in the cerebellum compared to all groups. Immunofluorescence results revealed the colocalization of apoptotic marker (cleaved caspase-3) and necroptosis marker (pMLKL) in granular layer neurons of the cerebellum of BDL-Alcohol rats.

Conclusion: Multiple episodes of overt HE led to neuronal cell loss in the hippocampus of BDL rats which was associated with increased oxidative stress, apoptosis and decreased neuronal count. Elevated levels of astrocytic marker (GFAP) in the hippocampus insinuate gliosis, possibly a result of neuronal loss. Moreover, alcohol administration worsens coordination impairments in BDL rats which were associated with increased oxidative stress, decreased neuronal markers (NeuN and SMI311) with apoptosis and necroptosis in the cerebellum of BDL-Alcohol rats. Overall, both multiple episodes of overt HE as well as continuous alcohol consumption, via oxidative stress, triggers neuronal loss/injury which consequently will lead to poor neurological outcome post-LT.

Keywords: Hepatic encephalopathy, Ammonia toxicity, Alcohol, Neuronal cell loss, Neurological complications, Cirrhosis, Bile-duct ligation

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Discussion

Abbreviations

HE: hepatic encephalopathy

CHE: covert hepatic encephalopathy

OHE: overt hepatic encephalopathy

ALF: acute liver failure

CLD: chronic liver disease

LT: liver transplantation

CNS: central nervous system

BBB: blood-brain barrier

GS: glutamine synthetase

GLS: glutaminase

ADH: alcohol dehydrogenase

NMDA: N-methyl-D-aspartate

GABA: γ-aminobutyric acid

ROS: reactive oxygen species

4-HNE: 4-Hydroxynonenal

TAC: total antioxidant capacity

BDL: bile-duct ligation

PCA: portacaval anastomosis

MRI: magnetic resonance imaging

GDH: glutamate dehydrogenase

LPS: lipopolysaccharides

CSF: cerebro-spinal fluid

CNS: central nervous system

UCD: urea cycle disorders

LOLA: L-ornithine L-aspartate

OP: ornithine phenylacetate

GFAP: glial fibrillary acidic protein

MLKL: mixed lineage kinase domain-like protein

RIP: receptor-interacting serine/threonine-protein kinase

TNF: tumor necrosis factor

Acknowledgements

I would like to express my deepest gratitude and appreciation to all those who have supported me throughout this incredible journey of pursuing my PhD. This thesis would not have been possible without the unwavering assistance, encouragement, and contributions of numerous individuals and institutions.

First and foremost, I extend my heartfelt thanks to my supervisor, Dr. Christopher F. Rose, for their invaluable guidance, patience, and mentorship. Their insightful feedback and constant encouragement played a pivotal role in shaping the direction and quality of this research.

I am immensely grateful to the members of my thesis committee, for their insightful suggestions, critical reviews, and expert advice that enriched the content of this thesis.

My gratitude extends to the participants of my study, whose willingness to share their time, experiences, and insights contributed immensely to the depth and validity of my research findings.

I am indebted to the staff and resources of Université de Montréal, for providing a conducive environment for learning and research.

I would also like to acknowledge my friends and family for their unending support and encouragement.

Last but not least, I wish to express my sincere appreciation to all the mentors, colleagues, and peers who engaged in stimulating discussions, provided valuable suggestions, and shared their expertise.

In conclusion, this thesis stands as a testament to the collaborative effort of many individuals and institutions. My gratitude knows no bounds, and I am humbled by the support I have received.

Introduction

Liver

Anatomy

In adults, the liver represents approximately 2% of the total body weight. A healthy liver is smooth on the exterior surface, with its color being reddish-brown. The liver receives blood from two sources: the hepatic artery which delivers oxygenated blood, constituting 20% of the total liver perfusion, while the portal vein delivers 80% of the total blood to the liver, bringing blood from the spleen and mesenteric. Liver is divided into four lobes; right, left, caudate, and quadrate. Externally, a falciform ligament divides the liver into a larger right lobe and a smaller left lobe (Fig. 1A). This ligament attaches the liver to the anterior abdominal wall. The liver lobules consist of hepatocyte plates, which are arranged hexagonally and separated by intervening sinusoids which are small blood vessels similar to capillaries, but with fenestrated endothelium. Sinusoids radiate outward from a central vein. The portal triads are located at the vertices of each hexagon. Within each lobule, the different functions of the terminal branches of the biliary tree, hepatic artery, and portal vein occur. Each lobule drains via a central vein, which joins the hepatic vein away from the liver (Kline et al., 2011). Couinaud classification is a system used to divide the liver into functional and anatomical segments based on its vascular and biliary anatomy, which divides the liver into eight different segments. Each segment has its own portal pedicle, which consists of the hepatic arterial branch, the portal branch, and the bile duct with a separate hepatic venous branch that provides blood outflow (Sibulesky, 2013).

Cellular composition

The liver is composed of different cell types, with hepatocytes representing over 60% of the total cell count and contributing to 80% of the hepatic volume. Hepatocytes are responsible for a variety of cellular functions, such as protein, lipid, and carbohydrate metabolism, immune cell activation, and detoxification. Additionally, they play a crucial role in maintaining liver homeostasis (Gong et al., 2023). There are different types of non-parenchymal cells in the liver. Endothelial cells form a coating along the sinusoids, while cholangiocytes line the bile ducts. Kupffer cells, or the tissue-specific resident macrophages mainly located in the sinusoidal lumina, are in close association with endothelial cells. Interestingly, they constitute 80% of the body's macrophage population and exhibit phagocytic capabilities, engulfing apoptotic and necrotic liver cells, as well as foreign substances transported through the portal vein (Horst et

al., 2016). Additionally, Kupffer cells can serve as antigen-presenting cells, releasing various cytokines and inflammatory agents. Ito cells, or Stellate cells, are the fat-storing cells, and in addition to storing and maintaining fat as well as fat-soluble molecules such as vitamin A, they have a role in the regulation of blood flow in the sinusoidal space and hepatic tissue repair in case of liver injury. In response to liver injury, these cells can become activated, adopting a myofibroblast phenotype and producing collagen. Pit cells, categorized as natural killer/granular lymphocytes specific to liver tissue, contribute to the immune response within the liver. Furthermore, oval cells, possess the remarkable capability of being multipotent stem cells and play a vital role in the proliferation and repopulation of liver cells, particularly hepatocytes, during instances of liver injury.

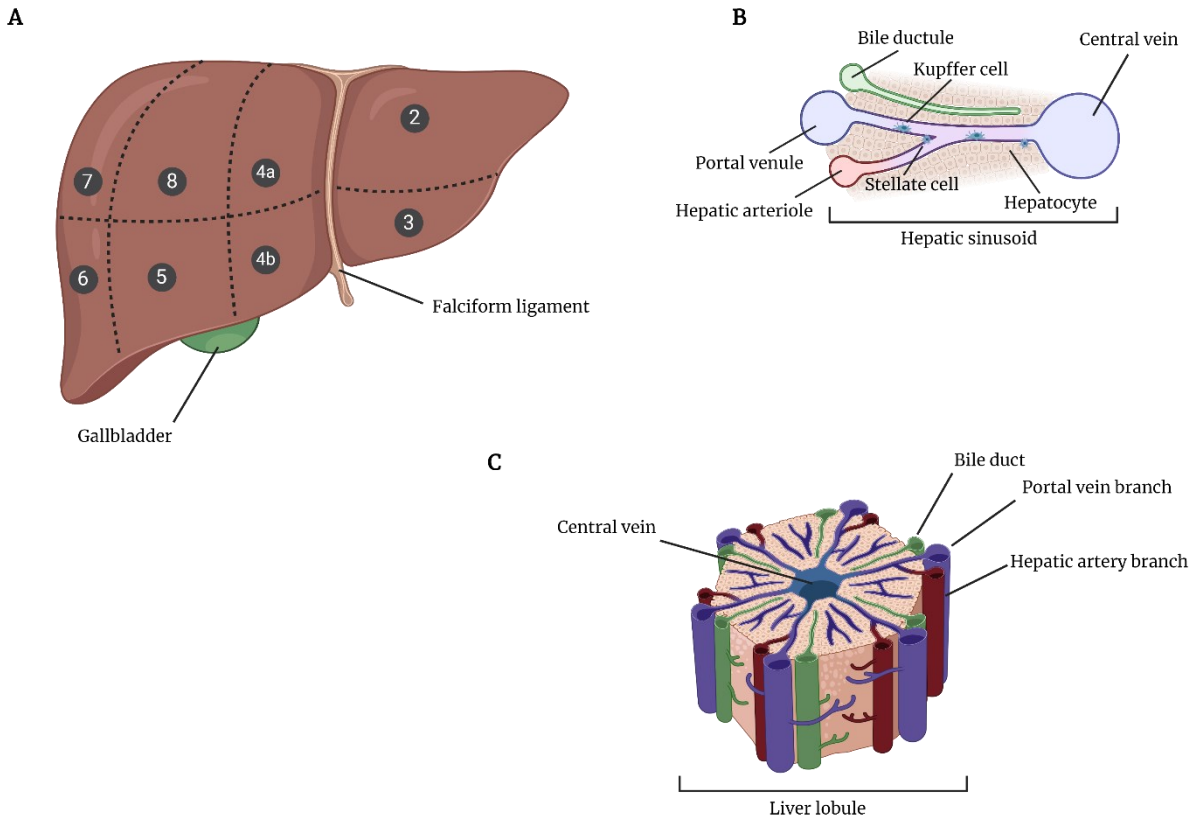

Figure 1: (A) Anterior view of the liver with segmental anatomy (Segment I is situated in the posterosuperior part of the liver); (B) Hepatic sinusoid; (C) Anatomy of a liver lobule.

Functions

Energy metabolism

Liver is one of the major metabolic organs, and its metabolic activity is tightly regulated by insulin and other metabolic hormones. Upon digestion of food in the gastrointestinal (GI) tract, glucose, fatty acids, and amino acids are absorbed into the bloodstream and transported to the liver through the portal vein. Glucose is condensed into glycogen and/or converted into fatty acids in the postprandial state, also known as the fed state, in the liver. In hepatocytes, free fatty acids are esterified with glycerol-3-phosphate to generate triacylglycerol, which is stored in lipid droplets in hepatocytes or secreted into the circulation as very low-density lipoproteins. The breakdown of amino acids provides energy or is utilized for synthesizing proteins, glucose, and/or other bioactive molecules. Although eating occurs at irregular intervals, the energy supplied to the cells remains consistent; for example, in the fasted state or during exercise, fuel substrates (such as glucose and triacylglycerol) are released from the liver into the circulation and metabolized by muscle, adipose tissue, and other extrahepatic tissues (Rui, 2014).

Synthetic properties

As the largest internal organ in the body, liver has a wide variety of functions, including the synthesis and regulation of numerous plasma proteins, vitamins, and hormones, as well as production and secretion of bile. Most plasmatic proteins are produced by liver, such as albumin, fibrinogen, and globulin (Miller and Bale, 1954). In normal physiological conditions, liver hepatocytes produce around 10-15 grams of albumin daily. Albumin is the most abundant protein in the blood stream, as it represents almost 50% of the total protein content of the plasma. Also, albumin provides approximately 70–80% of the total plasma oncotic pressure, being the main modulator of fluid distribution among the compartments of the body. It plays an important role in binding and carrying hydrophobic molecules, transition metal ions, and gas, which has subsequent implications for solubilization, transport, metabolism, and detoxification of these substances (Caraceni et al., 2013). Additionally, liver is responsible for synthesizing coagulation factors, anticoagulants, proteins involved in fibrinolysis, and the regulator of platelet production (Tripodi, 2015). Furthermore, liver has many endocrine functions and mediates metabolic pathways directly by producing hormones such as insulin-like growth factor-1 (IGF-1), angiotensinogen (Rhyu and Yu, 2021), thrombopoietin (Wolber and Jelkmann, 2002) and hepcidin (Nemeth and Ganz, 2023). Other than circulatory factors, liver is responsible for bile production. Bile is an aqueous secretion of the liver that is formed

by hepatocytes and modified by bile duct epithelium cells. Bile consists of 95% water, which contains bile salts, cholesterol, vitamins, amino acids, bilirubin, phospholipids, steroids, enzymes, and heavy metals, as well as environmental toxins, exogenous drugs, and xenobiotics. Bile has several important functions and is a major excretory pathway for potentially exogenous and endogenous lipophilic substances, such as bilirubin and bile salts. Bile salts function as an emulsifying factor for dietary fat and facilitate its intestinal absorption. Additionally, bile is a route to eliminating cholesterol (Boyer, 2013).

Immunological properties

Liver has unique immunological properties by conducting numerous crucial immune-related functions. For instance, the liver has a significant number of resident immune cells, and hepatocytes produce 80–90% of the circulating innate immunity proteins in the body including complement proteins, C-reactive protein, serum amyloid a proteins, and serum amyloid p component. Moreover, in response to receiving signals from pathogens and inflammation, hepatocytes secrete innate immunity proteins into the bloodstream. Additionally, in liver lymphocytes found throughout portal tracts and parenchyma. It is known that human liver contains around 10^{10} lymphocytes, which includes lymphocyte subpopulations of the innate (natural killer T and natural killer cells) and adaptive immune systems (T and B cells) (Zhou et al., 2016)

Central nervous system

Anatomy

The central nervous system (CNS) consists of the brain and the spinal cord, and its role is to receive, process, and respond to sensory information. As a part of the CNS, brain is responsible for movement, emotions, communication, responses, sensation, thought processing, and memory. In general, the brain can be divided into the cerebrum, brainstem, and cerebellum. The cerebrum is the anterior part of the brain, and it consists of mainly grey matter in the cerebral cortex and white matter at its center. The cerebrum, the largest part of the brain, initiates and coordinates the movements, and enables thinking and reasoning, speech, judgment, emotions, learning, and problem-solving. The cerebral cortex is divided into right and left hemispheres, which connect and communicate through a C-shaped structure of white matter called the corpus callosum. Each hemisphere has four lobes: frontal, parietal, temporal, and occipital (Fig. 2A). Each lobe controls specific functions. The frontal lobe is the largest

one and located in the anterior part of the brain and involved in personality characteristics, decision-making, muscle control and movement, and memory storage. The parietal lobe located in the central part of the brain and involved in pain and touch, understand spoken language and spatial relationships. Occipital lobe located in the back and is involved with vision. The temporal lobes, located on the lateral sides of the brain, are involved in speech, musical rhythm, short-term memory, and some degree of smell recognition. The cerebellum is located at the posterior of the brain below the temporal and occipital lobes and above the brainstem, and similar to the cerebral cortex, it consists of two hemispheres. Cerebellum cortex is divided in three layers, the outer layer called molecular layer which consists of granule cells axons, Purkinje cells dendrites, and GABAergic interneurons such as stellate and basket cells. The middle layer or Purkinje layer is consisting of Purkinje neuron cell bodies and Bergman glia (BG) cells. BG cells are unipolar astrocytes which locate their cell bodies around Purkinje neurons. The inner layer or granular layer is consisting granular neuron cell bodies, Golgi cells which are GABAergic interneurons and Purkinje cell axons. Cerebellum is involved in the coordination of voluntary muscle movements and in maintaining posture, balance, and equilibrium. Brain stem connects the brain to the spinal cord, and it consists of the midbrain, pons, and medulla.

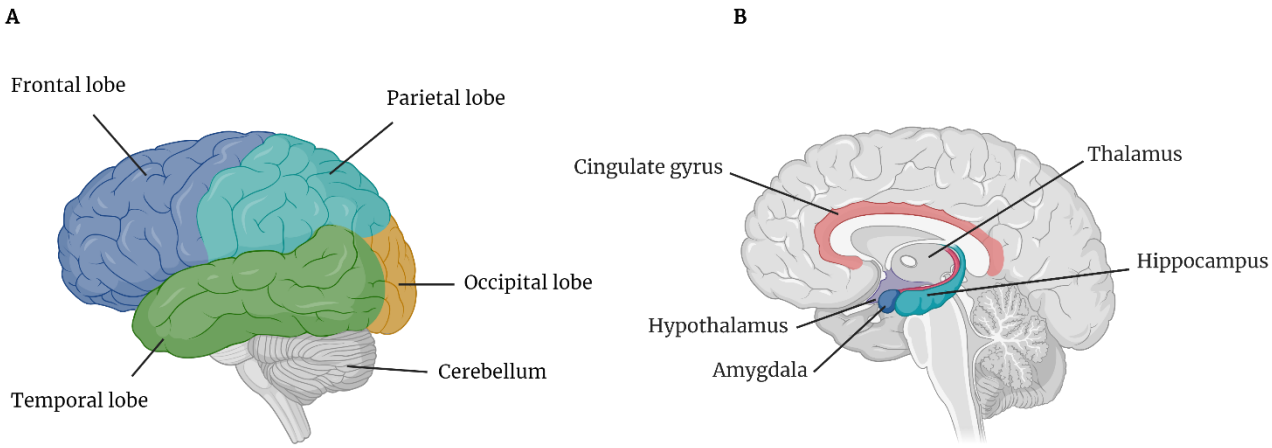

Figure 2: (A) Lateral view of the brain. (B) Limbic system.

Beyond surface-level parts, the brain contains deeper structures, these interconnected brain regions form the limbic system, which includes the hypothalamus, amygdala, hippocampus, thalamus, and cingulate gyrus (Fig. 2B). The hypothalamus regulates and synchronizes sleep patterns and plays a role in some aspects of memory and emotion. In addition, it has a role in

controlling body temperature, hunger, and thirst. The amygdala regulates emotion and memory, and it is associated with the brain's reward system and stress. The hippocampus on the other hand, supports memory, learning, navigation, and perception of space. Thalamus is the relay station, with information including motor and sensory, from body (except smell), first being processed by the thalamus and then directed to the cerebral cortex for interpretation. And finally, the cingulate gyrus is involved in emotional processing, social behavior and decision-making (Lisman et al., 2017).

Cellular composition

Brain is a complex organ made up of different cell types, including neurons and non-neuronal glial cells. Neurons are the building blocks of the brain, sending, and receiving electrical and chemical signals. They transmit information to other neurons, throughout the whole body. Neurons enable cognitive processes, emotional experiences, voluntary movements, and the comprehension of the surrounding world. Neurons and glial cells communicate through various signals, including ion fluxes, neurotransmitters, cell adhesion molecules, and specialized signaling molecules released from synaptic and non-synaptic areas of the neuron. Glial cells are diverse, and their presence is critical for the healthy function of the nervous system. There are many types of glial cells in the brain, including oligodendrocytes, microglia, and astrocytes. Oligodendrocytes wrap around the neuronal axon and have a role in the production of the myelin sheath (Michalski and Kothary, 2015). Microglia are the immune cells and unique residential macrophages of the CNS. Microglia cells are involved in supporting CNS development, contributing to an immune response against infectious agents, sustaining homeostasis of brain micro-environment, neuroinflammation, degenerative diseases, stroke, trauma, and regeneration (Lannes et al., 2017). Astrocytes are one of the most important components of the CNS. They have various functions such as being involved in metabolic, structural, homeostatic, and neuroprotective tasks, including clearing excess neurotransmitters, promoting synapse formation, and stabilizing and regulating the barrier between blood and the brain (Vasile et al., 2017). Moreover, astrocytes have an important role in brain energy metabolism. The main energy source of the brain is glucose, which is metabolized to lactate in astrocytes and supplied to neuronal cells (Weber and Barros, 2015).

Blood brain barrier (BBB)

The extremely selective semi-permeable membrane between the blood and the brain's extracellular fluid is called blood brain barrier (BBB). The BBB is composed of several cell

types, including endothelial cells, capillary basement membrane, pericytes and astrocyte end-feet. The BBB plays an essential role in protecting the brain from foreign substances in the blood and regulating the transport of necessary elements for optimal brain function. Endothelial cells in the BBB are closely sealed with tight junctions, which results in the restriction of passive transmission of molecules. The tight junctions are specific endothelial proteins belonging to the claudin and occludin families. These proteins are connected to cytoskeletal actin through the ZO (zonula occludens) family of proteins. Also, junctional adhesion molecules are found in the cerebral endothelial cells, and they contribute to tight junction development and preservation (Liebner et al., 2018). Astrocytes cover the entire surface of the cerebral capillary with their specialized end feet. It has been shown that appropriate regulation of astrocyte function is considered crucial in enhancing BBB function and reducing BBB disruption following brain damage (Michinaga and Koyama, 2019). Pericytes are mural cells that cover the endothelial walls of the microvasculature. Long cellular processes of the pericytes extend along the abluminal surface of the endothelium and can often span several endothelial cell bodies. They also contain contractile proteins with the ability to control the diameter of the capillary. It's known that pericytes are involved in angiogenesis, deposition of extracellular matrix, regulating immune cell infiltration, wound healing, and regulation of blood flow in response to neural activity (Daneman and Prat, 2015).

Ammonia metabolism

Urea cycle

The urea cycle is a family of enzymes solely found together in the liver and is responsible for ammonia homeostasis in the body. Ammonia is converted to urea through an energy-dependent process (Fig. 3). In the mitochondria of the hepatocytes, ammonia and bicarbonate form carbamylphosphate, catalyzed by carbamylphosphate synthase 1. Within the process, ornithine transcarbamylase generates citrulline from carbamylphosphate and ornithine. Later, through the reactions catalyzed by argininosuccinate synthase 1 and argininosuccinate lyase, citrulline is converted to arginine in the cytosol of the hepatocytes. Finally, arginase breaks down arginine into urea and restores ornithine, completing and allowing the cycle to begin anew. Urea subsequently leaves the hepatocyte cytoplasm and is ultimately excreted in the urine (Ribas et al., 2022).

Glutamine synthetase and glutaminase

Glutamine synthetase (GS) is an ATP dependent enzyme which aminates glutamate forming glutamine. Basically, GS captures the ammonia released during cellular processes, combines it with glutamate, and converts it into glutamine. Therefore, GS is one of the key regulators of circulating ammonia and it's expressed in different organs including kidney, liver, muscle, and brain. In the liver, GS is highly expressed in the perivenous hepatocytes and within the brain it is mainly expressed in astrocytes (Castegna and Menga, 2018). Muscle cells (skeletal and cardiac) express GS. Since the synthesis of glutamine predominantly occurs in skeletal muscle, the activity of GS plays a role in the regulation of glutamine synthesis in the muscle (Biolo et al., 2005). Therefore, in the state of hyperammonemia, muscle play a crucial role in ammonia detoxification and removal (Olde Damink et al., 2009). Glutaminase (GLS) is an enzyme that catalyzes the hydrolysis of glutamine. The hydrolysis of the amide bond results in the separation of the glutamine molecule into two components: glutamate and ammonia. There are two types of glutaminase: the kidney-type GLS and the liver-type GLS2 or LGA. Glutaminase expressed in several tissues including liver, kidney, brain, lungs, and intestine (Katt et al., 2017).

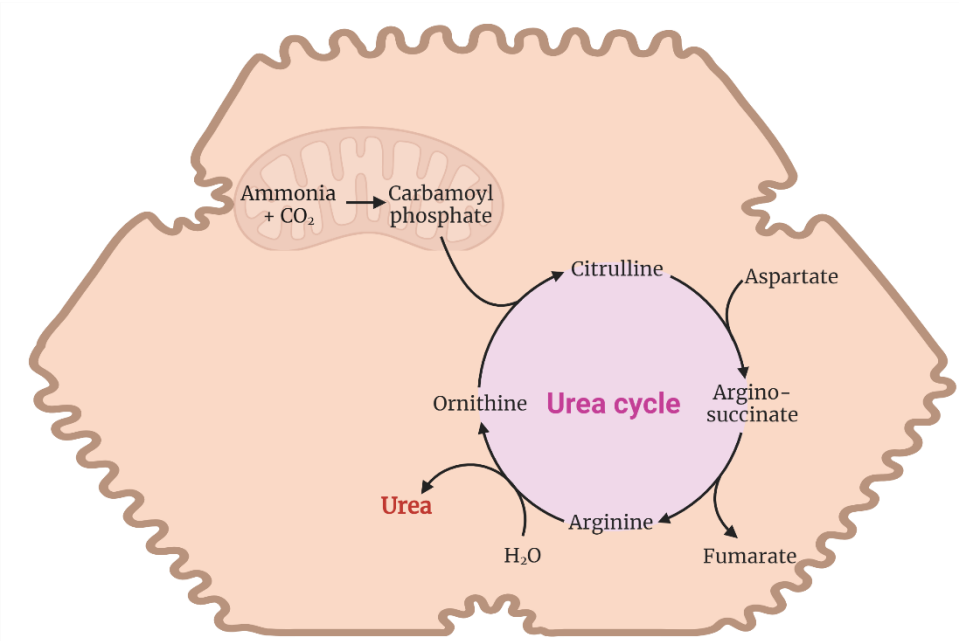

Figure 3: Urea cycle.

Glutamine-Glutamate cycle in CNS

This cyclic metabolic pathway which maintains the adequate neurotransmitter glutamate supply within the CNS. In the physiological state and during the process of glutamatergic

neurotransmission, neurons release glutamate molecules into the extracellular space. Subsequently, astrocyte glutamate transporters efficiently eliminate the released glutamate from the extracellular environment (synaptic cleft). In astrocytes, glutamate is aminated to glutamine via GS, solely expressed in astrocytes. In the process, one molecule of ammonia is removed. The synthetized glutamine in astrocytes is shuttled to neurons where it is metabolized to glutamate via the enzyme GLS, solely expressed in neurons (Bak et al., 2006). Here, ammonia is generated and therefore "one turn" of the glutamate-glutamine cycle no net production or removal of ammonia.

Other ammonia metabolizing enzymes

Glutamate dehydrogenase (GDH) and alanine transaminase (ALT) enzymes are also playing a role in ammonia metabolism. GDH is an enzyme that catalyzes the reversible conversion of glutamate to α-ketoglutarate and ammonia (Plaitakis et al., 2017). Additionally, ALT is an enzyme that participates in the conversion of alanine and α-ketoglutarate into pyruvate and glutamate. This process can lead to the production of glutamate and ammonia (Yang et al., 2009).

Healthy liver = Healthy brain

The brain is a well-isolated organ; however, it is connected to other organs including liver through metabolic, immunological, inflammatory, and hormonal pathways. It has been highly documented that acute liver injury/failure as well as chronic liver disease/failure leads to neurological impairment coined as hepatic encephalopathy (HE) which will be discussed in detail below. The diseased liver can cause an increase in multiple pathogenic factors including hyperammonemia, systemic inflammation and oxidative stress which are believed to negatively impact brain function (Rose et al., 2020). Interestingly, it has been also documented that brain injury can lead to liver impairment (Bordet and Deplanque, 2020). Insults to the brain such as brain stroke can lead to disturbance of hepatic metabolism and homeostasis such as changes in hepatic enzymes, inflammation and cell death in liver (Wang et al., 2014; Balch et al., 2020). Therefore, a healthy brain requires a healthy liver and vice versa.

Liver disease

Liver disease is one of the most common causes of death in the world, accounting for 2 million deaths per year worldwide (Asrani et al., 2019). Cirrhosis is the 10th most common cause of death in Canada, with an estimated 5000 deaths per year (Sherman et al. 2017). Liver disease

can be defined as chronic liver disease (CLD), or acute liver failure (ALF) based on the progression of the disease. ALF is associated with rapid progression of the disease by acute and severe hepatocyte injury without evidence of pre-existing liver dysfunction; the major causes of ALF are drug overdoses such as acetaminophen and viral hepatitis (Bernal and Wendon, 2013).

Chronic liver disease (CLD)

Chronic liver disease is a progressive deterioration of liver functions. Different etiological factors can cause CLD which include toxins, prolonged alcohol misuse, autoimmune disease, infection, genetic and metabolic disorders. In the early stages, hepatic injury leads to steatosis, which progresses to fibrosis and finally cirrhosis (when the liver parenchyma is replaced by scarring tissue) (Friedman, 2008). A healthy liver is able to regenerate and heal itself; however, in CLD which is a continuous process of inflammation, injury, and destruction of liver tissue; the liver's wound-healing response to the repeated injury diminishes and leads to fibrosis (Fausto, 2004). Chronic damage to the liver leads to excess accumulation of extracellular matrix proteins such as collagen, forming fibrous scars that alter the hepatic architecture. Studies have shown liver fibrosis may be reversible to some degree; however, it can progress to advanced bridging fibrosis by forming nodules and eventually cirrhosis (Bataller and Brenner, 2005). In cirrhosis, the scarring tissue replaces the liver parenchyma with fibrotic bands, resulting in vascular distortion and parenchymal nodules. Cirrhosis represents the end-stage of CLD, with a high risk of irreversibility (Dezső et al., 2022).

Etiologies of chronic liver disease

Alcoholic liver disease (ALD)

Globally, alcohol stands as the major cause of cirrhosis, responsible for nearly 60% of cases in Europe, North America, and South America. Also, it is one of the main drivers of hepatic steatosis when consumed excessively (daily consumption higher than 50 g in females and 60 g in males). Furthermore, approximately 35% of individuals with Alcohol Use Disorder (AUD) will develop diverse forms of alcohol-associated liver disease (ALD) (Devarbhavi et al., 2023). Alcohol increases the risk of mortality related to liver disease by 260 times (Arab et al., 2023). ALD often coexists with other types of liver diseases, such as viral hepatitis and steatotic liver disease (SLD) (Rinella et al., 2023).

Viral hepatitis

Viral hepatitis caused by viral infection leading to liver inflammation results in 1.4 million deaths per year. Hepatitis B virus (HBV) and hepatitis C virus (HCV) together are responsible for more than 90% of these fatalities, 48% and 47%, respectively (Jefferies et al., 2018; Bhadoria et al., 2022). Hepatitis B is one of the most common infectious diseases globally, and 15-40% of the chronically infected population is estimated to develop cirrhosis; however, only around 5% of the infected population are chronic carriers (Sharma et al., 2005; Bhadoria et al., 2022). On the other hand, around 71 million people are estimated to have HCV globally, and are at higher risk of developing end-stage liver disease and hepatic cancer (Jefferies et al., 2018). However, according to World Health Organization's report from 2016, 95% of cases of HCV can be cured by antiviral medication, which reduces the risk of cirrhosis and hepatic cancer consequently (Berenguer et al., 2017).

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver disease is one of the most common liver diseases in developed countries and it is often associated with obesity, insulin resistance, diabetes, inactive lifestyle, and western diet (Kořínková et al., 2020). Early in the disease course, hepatocytes start accumulating fat, which is usually described as macro-vesicular steatosis characterized by a single large fat droplet pushing the nucleus to the side of the cell. Fat accumulation greater than 5% of the liver weight is historically defined as non-alcoholic fatty liver disease (NAFLD). In this stage, steatosis is not associated with inflammation. With the progression of CLD, non-alcoholic steatohepatitis (NASH) develops, which is identified as steatosis along with lobular inflammation and hepatocellular ballooning (Fernando et al., 2019). Early stages of CLD, such as NAFLD and NASH, are reversible with proper lifestyle change, exercise, and improving liver fat content (Brunner et al., 2019). NAFLD was introduced by Ludwig in 1980 (Ludwig et al., 1980); however, the word "non-alcoholic" cannot capture the accurate etiology of the disease, and the word "fatty" can be stigmatizing. Over time, the desire to change the terminology considering the role of stigma and to define a more precise description of the underlying cause of the disease increased (Rinella et al., 2023). It is known that metabolic syndrome (a complex disorder defined by a collection of health problems that increase the risk of stroke, diabetes, cardiovascular and heart disease) and NAFLD have a mutual and bi-directional association (Lonardo et al., 2020), therefore the new terminology is recommending the term metabolic dysfunction associated steatotic liver disease (MASLD) rather than NAFLD

and furthermore, the term metabolic dysfunction associated steatohepatitis (MASH) to replace NASH (Rinella et al., 2023).

Steatotic liver disease (SLD)

Steatotic liver disease (SLD) is an umbrella term that includes different etiologies coexisting with steatosis (Fig. 4). SLD can be used when none of the cardiometabolic criteria can be detected or diagnosed; such as ALD, where steatosis is one of the main features, and cryptogenic SLD, which defines those with no metabolic parameters and no known cause (Rinella et al., 2023).

Metabolic dysfunction associated steatotic liver disease (MASLD)

Hepatic steatosis is defined as accumulation of fat intra-hepatically for at least 5% of the liver weight. Long-term hepatic fat storage may cause inflammation and metabolic dysfunction (Nassir et al., 2015). MASLD defines patients with steatosis who have at least one of the cardiometabolic criteria (Fig. 5) with no other discernible cause and ALD. The coexistence of multiple etiologies of steatosis is possible, which can be reported as MASLD+autoimmune hepatitis or MASLD+viral hepatitis (Rinella et al., 2023).

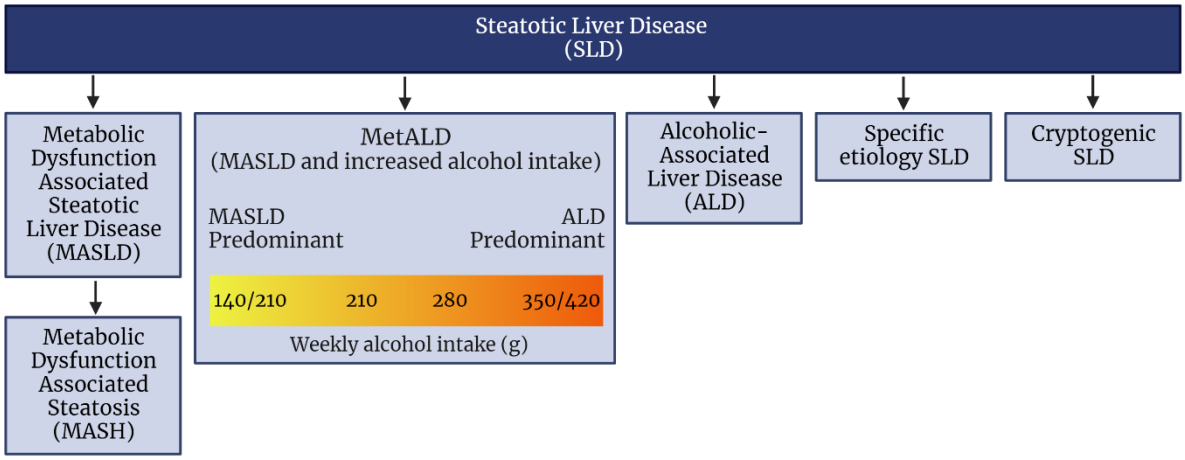

Figure 4: Steatotic Liver Disease sub-classification. Figure is adopted from (Rinella et al., 2023).

Metabolic dysfunction associated steatohepatitis (MASH)

MASH is defined as a sub-classification of MASLD, when steatohepatitis and inflammation coexist with MASLD (Rinella et al., 2023).

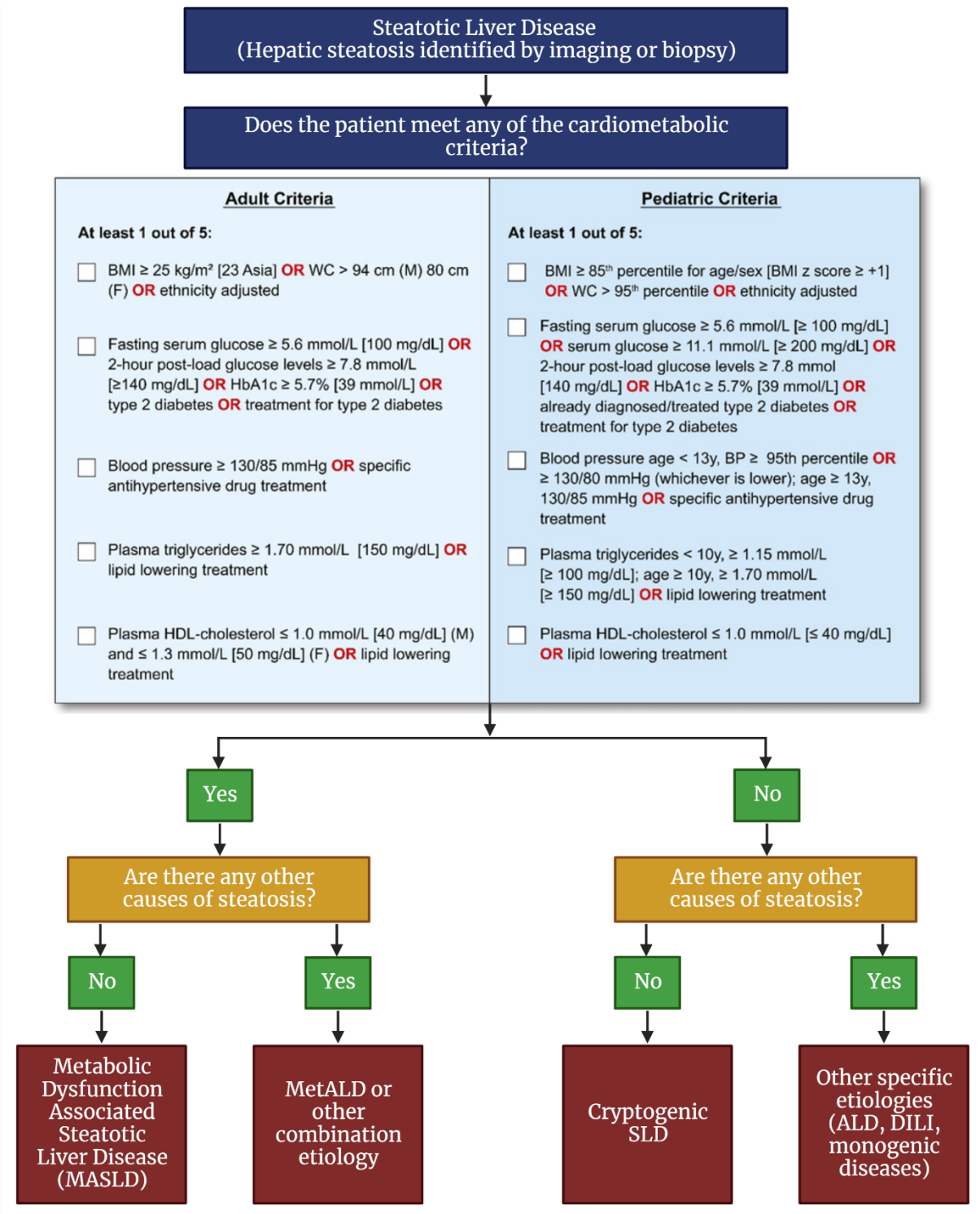

Figure 5: MASLD diagnostic criteria. Figure is adopted from (Rinella et al., 2023).

MetALD

In the case of coexisting larger amounts of alcohol consumption with MASLD, to accurately address the underlying cause of the disease, MetALD classification was introduced, a continuum across which the contribution of MASLD and ALD varies. This term defines patients with MASLD who consume more alcohol weekly (140 to 350 g/week for females and 210 to 420 g/week for males) (Rinella et al., 2023).

Other etiologies

Among other CLD etiologies, genetically inherited and autoimmune liver diseases are observed. Genetically inherited liver diseases are usually metabolic or genetic defects that contribute to liver dysfunction. Most of these deficits are due to the lack or defect of enzymes, proteins, or transporters in the metabolic pathways generating pathogenic effects in the liver, such as Wilson disease, tyrosinemia, urea cycle disorders, and congenital hepatic fibrosis (Scorza et al., 2014). Moreover, autoimmune liver diseases such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and autoimmune hepatitis (AIH) cause persistent hepatic injury by targeting biliary epithelial cells and hepatocytes, respectively. Autoimmune liver diseases are associated with inflammation, liver fibrosis, and ultimately cirrhosis (Liberal and Grant, 2016).

Complications of liver disease

Irrespective of the underlying causes of CLD, the resulting cirrhosis can lead to the development of severe complications. In the compensated state, patients with cirrhosis do not experience or develop any complications. Whereas in the decompensated state, patients develop complications, including portal hypertension, gastrointestinal bleeding, ascites, jaundice, and HE.

Portal hypertension

Clinically significant portal hypertension is defined as a hepatic venous pressure gradient of at least 10 mmHg, results in potential formation of portal-systemic collaterals and esophageal or gastric varices. Portal hypertension is a consequence of increased blood flow to and vascular resistance within the liver. Under physiological conditions, portal blood passes through the liver with minimal resistance. In cirrhosis, the increased resistance is caused by structural alterations including collagen deposition, fibrosis, formation of nodules, and liver stiffness, as

well as dynamic alteration and increased hepatic vascular tone caused by endothelial dysfunction, which initiates cirrhosis-related portal hypertension (Engelmann et al., 2021).

Gastrointestinal bleeding

Gastrointestinal (GI) bleeding is a common complication of cirrhosis and occurs in 60-65% of patients with cirrhosis. Stomach and esophageal varices are responsible for the majority of GI bleeding cases. Portal hypertension is the main factor in the development of these varices, which are expanded sections of blood vessels caused by high blood pressure (Garcia-Tsao and Bosch, 2010). Although upper GI bleeding is more common in cirrhotic cases, lower GI bleeding has also been reported in these patients, and is associated with a higher mortality rate (Khalifa and Rockey, 2020).

Ascites

The accumulation of free fluids in the peritoneal cavity defines ascites, which is the most common complication of cirrhosis (Tsochatzis and Gerbes, 2017). The main factors involved in the formation of ascites are portal hypertension and renal sodium retention. Around 20% of patients with cirrhosis develop ascites at their first presentation, and between 1-4% of patients with decompensated status develop ascites each year (D'Amico et al., 2014). Ascites can be graded based on volume and severity into mild or grade 1 (detectable only by ultrasound), moderate or grade 2 (detectable by physical examination and the volume is usually >500 ml), and large or grade 3 (causes abdominal distension and usually associated with dyspnea and the volume is up to ~5 to 15 L). Ascites can also become refractory to medical treatment, which consequently leads to frequent paracentesis, affecting quality of life. According to EASL (European Association for the Study of the Liver) and AASLD (American Association for the Study of Liver Diseases) guidelines, transjugular intrahepatic portosystemic shunt (TIPS), is the main surgical treatment for patients with refractory ascites (Benmassaoud et al., 2020).

Jaundice

Hyperbilirubinemia, or jaundice, is defines as yellowish discoloration of the skin, eyes, and mucous membranes caused by the accumulation of excess bilirubin. Hyperbilirubinemia occurs when there is an increase in bilirubin production or a decrease in bilirubin excretion. Bilirubin is an end product of heme, consisting of conjugated and unconjugated bilirubin, and an elevation of either form results in jaundice. Bilirubin metabolism highly relies on the liver, and it can be categorized into three main phases, the pre-hepatic, hepatic, and post-hepatic phases

(Wolkoff and Berk, 2011). In patients with cirrhosis, jaundice is one of the most well-known indicators of transitioning from compensated to decompensated stage; therefore, it can be associated with worsening the patient's prognosis (D'Amico et al., 2022).

Spontaneous bacterial peritonitis

Spontaneous bacterial peritonitis (SBP) is one of the most severe complications in cirrhosis patients with ascites, and it is defined as infection of the ascites fluid. The incidence of SBP varies and has been reported in between 7-30% of hospitalized cirrhotic patients with ascites (Marciano et al., 2019), which is associated with a higher mortality rate and a lower long-term prognosis (Lata et al., 2009). Several factors are involved in the pathophysiology of SBP, including intestinal bacterial overgrowth, bacterial translocation, increased gut permeability, as well as immune dysfunction (Marciano et al., 2019).

Malnutrition

Malnutrition and sarcopenia are also associated with cirrhosis, which occurs due to multiple factors including reduced appetite, intestinal malabsorption, medication side effects, and an imbalance between catabolism and synthesis resulting from increased breakdown of protein and elevated energy expenditure (Dhaliwal and Armstrong, 2020). Moreover, in CLD, muscle plays a significant compensatory role in ammonia detoxification due to the presence of GS which forms glutamate from glutamine and ammonia. It is known that the capacity of ammonia removal through muscle is reduced in CLD patients with sarcopenia and poor muscle mass (Jindal and Jagdish, 2019).

Hepatic encephalopathy (HE)

Hepatic encephalopathy is a common and serious complication of both ALF and CLD. It can occur in up to 50% of patients with cirrhosis. HE leads to a broad spectrum of manifestations, from subclinical changes such as cognitive impairments to more severe symptoms such as disorientation, confusion, and coma. HE can be categorized into different types based on the underlying degree and acuity of liver failure (Louissaint et al., 2022). Studies have shown that HE is the most frequently occurring complication of liver disease leading to a high rate of hospitalizations and re-admissions (Hirode et al., 2019). In addition, HE has a significant impact on clinical outcomes and economic costs (Tapper et al., 2016). It has been demonstrated that HE is associated with poor survival and high rates of mortality (approximately 50% at one year) (Bustamante et al., 1999; Cordoba et al., 2014). Also, HE impacts the quality of life in

patients with cirrhosis affecting their ability to perform daily tasks, such as driving and working on a daily basis (Wein et al., 2004; Shaw and Bajaj, 2017; Tapper et al., 2019).

Type A HE

Hepatic encephalopathy associated with ALF is defined as type A HE. This is a rapidly progressing neurological impairment that manifests as severe lethargy, which can evolve to coma, and death which is believed to be strongly associated with cerebral edema and consequently intercranial hypertension and brain stem herniation (Bernal and Wendon, 2013).

Type B HE

Type B HE, or bypass HE is defined as an alteration in behavior and cognitive function in the absence of liver insufficiency which is related to the presence of portosystemic shunts (Vilstrup et al., 2014). The shunts completely or partially prevent the portal blood from undergoing sufficient detoxification by bypassing the liver (Franchi-Abella et al., 2018). Shunts can occur due to several factors, such as congenital (at birth) shunting, obstruction of the portal or hepatic vein, hepatic trauma, hepatic neoplasms, or inflammatory diseases (Wu et al., 2018).

Type C HE

Type C HE is associated with CLD; caused by liver insufficiency, which leads to the accumulation and circulation of toxins in the blood systemically. Type C HE manifests with a wide spectrum of neurological impairments, from mild alteration of cognitive function such as altered psychometric function, psychomotor speed, and electrophysiological brain measures to obvious alterations including personality change, sleep-awake cycle, disorientation, confusion, and in the most severe cases, coma (Vilstrup et al., 2014). The semi-quantitative scoring scale of West Haven criteria has been used for grading HE severity. However, HE is presently classified into covert HE (CHE) and overt HE (OHE) categories based on detection of clinical symptoms and severity (Rose et al., 2020).

Covert HE (CHE)

Covert HE is an umbrella term used for patients who score poorly on neuropsychological tests, including minimal HE (mHE) and Grade I HE. Since CHE is defined as non-clinically detected symptoms, it requires psychometric testing for diagnosis. Some well-known neuropsychological tests used for the diagnosis of CHE include the psychometric hepatic

encephalopathy score (PHES), animal naming test, EncephalApp Stroop, and critical flicker frequency (Rose et al., 2020).

Overt HE (OHE)

Defined by West Haven criteria, OHE covers grade II (lethargy, moderate confusion, asterixis (the intermittent loss of muscle tone when attempting to maintain a set position), motor incoordination, and obvious personality changes), grade III (gross incoordination, somnolence to semi-stupor and confusion), grade IV (coma, unresponsive to pain) (Fig. 6).

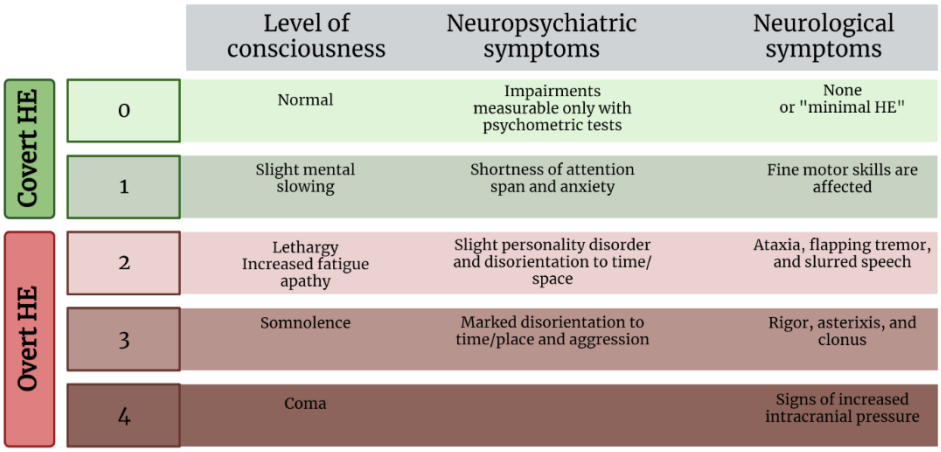

Figure 6: Subjective tool for grading hepatic encephalopathy based on West Haven criteria. Adopted from (Vilstrup et al., 2014).

Pathogenesis of HE

Systemic factors caused by liver dysfunction are the main pathogenic components of the neurological and cognitive impairments in HE. These blood-derived factors impact the permeability and integrity of the BBB. Pathogenic factors, such as ammonia, oxidative stress, and systemic inflammation, are generated as consequences of CLD. Additionally, factors such as blood manganese and bile acids can contribute to HE pathogenesis (Rose et al., 2020).

Ammonia

Although the pathogenesis of HE is multifactorial; the role of ammonia is undeniable. Ammonia toxicity involves different endogenous and exogenous routes. It is naturally produced in the gut as a result of bacterial urease activity, protein digestion, and amino acid deamination. Ammonia molecules exist in both gaseous NH_3 (weak base) and ionic NH_4^+ (weak acid) forms (Bromberg et al., 1960). Ammonia as a gas freely crosses the phospholipid

membrane by simple diffusion; however, because of its similarity with K^+ ions, NH_4^+ can also be transported through other pathways, including different K^+ ion channels and transporters. For instance, increased $NH4^+$ concentrations may interfere with K^+ homeostasis and cause K^+ to compete with it on Na^+/K^+, H^+/K^+, and K^+/Cl co-transporters as well as Na^+/K^+/Cl exchangers which in turn may have an impact on the Nernst potential for potassium and resting membrane potential (Bosoi and Rose, 2009). It's also been reported that specific ammonia transporters play a role in ammonia transportation (Grishin et al., 2020). In physiological conditions and with healthy liver function, excess ammonia is metabolized through the urea cycle in the liver. Moreover, ammonia participates in several metabolic reactions, and a normal range concentration of this metabolite is essential for the proper function of these pathways. However, ammonia at higher concentrations is a well-known neurotoxin, as excess levels of ammonia can directly alter intracellular pH, membrane potential, and cellular metabolism (Bosoi and Rose, 2009). It is known that ammonia metabolism is altered in liver disease conditions, which could lead to hyperammonemia; accumulation of ammonia in the systemic circulation (Aldridge et al., 2015). Also, it is known that hyperammonemia results in inhibition of the neuron-astrocyte glutamate trafficking and affects postsynaptic glutamate receptors (Butterworth, 1993). Moreover, in pathological states, during hyperammonemia glutaminase is inhibited by ammonia (feedback inhibition) and glutamine production is upregulated. Hence, osmotic load of accumulated intracellular glutamine is believed to be implicated in astrocyte swelling since hyperammonemia-induced astrocyte swelling was prevented by inhibition of glutamine biosynthesis (Willard-Mack et al., 1996). It is reported that changes in the morphology of astrocytes could also alter their function, such as uptake of neurotransmitters and regulation of energy metabolism, consequently affecting neuronal function and further manifestations such as HE (Suárez et al., 2002). Although hyperammonemia is associated with liver dysfunction, the direct correlation between hyperammonemia levels, and severity of HE has not been confirmed (Rose et al., 2020).

Although there are multiple pathways for ammonia utilization, the liver represents the main organ responsible for ammonia detoxification through hepatic ureagenesis. Thus, any decrease in liver function due to liver failure/disease causing an imbalance between production and elimination of ammonia will lead to an increase in circulating ammonia. Considering this and the fact ammonia is neurotoxic, it is well-known that increased ammonia levels have harmful effects on the brain (Butterworth, 2002). Effects including disruption of glutamine-glutamate cycle, neurotransmitter alteration, change in astrocyte morphology and induction of brain

edema have been studied extensively over the past years (Bosoi and Rose, 2013b; Cudalbu and Taylor-Robinson, 2019; Zielińska et al., 2022).

Although the severity of HE is poorly correlated with ammonia levels, numerous studies support the critical role of hyperammonemia in neurological impairment following HE since symptoms were improved by ammonia lowering treatments in patients (Sanjo et al., 1985; Quero et al., 1996; Gentile et al., 2005; Jiang et al., 2013; Naderian et al., 2017; Ikeda et al., 2018). In animal models, the severity of HE has been shown to be correlated with blood and brain ammonia levels (Kanamori et al., 1996; Zwirner et al., 2010). Regardless, inconsistent results with the stability of ammonia have been discussed; the lag time until the measurement of ammonia (including on and off ice), the sensitivity of the ammonia measurement assay as well as differences between accurately trained and untrained practitioners in grading patients with HE (Reuter et al., 2018). To help improve ammonia measurements and avoid false positives anticoagulant coated tubes for blood collection are recommended (Goldstein et al., 2017), separating plasma from blood cells as soon as possible considering that blood cells can metabolize ammonia (Hashim and Cuthbert, 2014). Also, for the long-term plasma storage, storing samples in cold condition preferably -70ºC and avoiding freeze-thawing the samples is recommended (Favresse et al., 2018).

Molecular mechanisms of ammonia toxicity

Ammonia exists in two molecular forms NH_3 and NH_4^+ and the ratio in equilibrium ($NH_3 + H^+ \rightleftharpoons NH_4^+$) is pH dependent based on Henderson-Haselbach equation. At physiological pH, 98% of ammonia is present as NH_4^+ which is a positively charged small molecule able to cross cell membranes since NH_4^+ has similar characteristics as potassium ion (K^+). Therefore, NH_4^+ can cross cell membranes via K^+ channels or transporters. Ammonia as a gas (NH_3), can easily diffuse across all lipid membranes. It has been reported that two membrane protein families including rhesus glycoproteins and aquaporins are capable of transporting ammonia and ammonium (NH_3/NH_4^+) (Weiner and Verlander, 2017).

It has been demonstrated that a rapid increase in ammonia leads to an increase in pH in different cell types (intracellular alkalization) (Marcaggi and Coles, 2001; Rose et al., 2005). The intracellular pH recovery depends on various elements such as entry rate of $NH_4^+$and function of transporters and membrane channels. Changes in the pH can disturb the homeostasis, alter the function of pH sensitive channels and impair metabolic communications between cells (Duffy et al., 2002). Potassium homeostasis can also be influenced by an increase in ammonia since NH_4^+ and K^+ compete for numerous transporters and co-transporters including H^+/K^+

ATPase and Na^+/K^+ transporters/exchangers. Also, resting membrane potential can be affected by ammonia associated K^+ changes. These changes can lead to depolarization in astrocytes and neurons; although 10 mM ammonium chloride has been shown not to trigger an action potential in neurons (Lazarenko et al., 2017). Additionally, ammonia can affect metabolism since it is an important substrate for multiple enzymatic reactions in several tissues including the brain. It has been previously shown that elevated levels of ammonia increase activity of glutamine synthetase (GS) in astrocytes leading glutamine-induced hypertonicity and consequently astrocyte swelling (Jayakumar et al., 2006). Moreover, ATP depletion can occur as a consequence of mitochondrial dysfunction and impaired respiratory chain enzymes as well as elevated activity of Na^+/K^+-ATPase in response to acute ammonia elevation (Heidari, 2019). Ammonia can also decrease the generation of cAMP which can subsequently lead to cellular dysfunction and energy impairment in astrocytes and neurons (Liskowsky et al., 1986). Furthermore, the role of hyperammonemia in oxygen homeostasis has been investigated, demonstrating decreased cortical oxygenation which could be responsible for disruption in the brain energy metabolism (Hadjihambi et al., 2022). Hyperammonemia can affect neurotransmission by dysregulating NMDA receptors, altering glutamatergic GABAergic neurotransmission, impacting cGMP pathway and resulting in ATP shortage and mitochondrial dysfunction (Monfort et al., 2005) which concomitantly could be contributing to neurological dysfunction and neurocognitive impairments in patients with HE (Jones, 2002). Although the levels of ammonia required to induce toxicity in either astrocytes or neurons remains undefined, the duration merits to be further investigated (Ochoa-Sanchez et al., 2021).

Oxidative stress

The phenomenon of oxidative stress occurs naturally in the body; in physiological conditions, reactive oxygen species (ROS) are maintained within the normal levels by antioxidant systems. It is known that in CLD, the balance between production and neutralization of ROS is impaired (Simicic et al., 2022). The liver is one of the main organs that produce antioxidants in the body therefore liver insufficiency leads to lower synthesis of proteins with antioxidant properties such as albumin and glutathione, resulting in decreased systemic antioxidant capacity. Moreover, hepatic necrosis can also lead to an increased release of oxidant enzymes such as liver-derived xanthine oxidase and aldehyde oxidase, which contribute to the formation of H_2O_2 and ROS production (Bosoi and Rose, 2013a). Additionally, liver dysfunction can trigger neutrophil dysfunction via ammonia toxicity. Neutrophil dysfunction can lead to the oxidative

burst and the release of ROS, which further contributes to higher levels of systemic oxidative stress (Shawcross et al., 2010).

Inflammation

The systemic release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and IL-12 is defined as an inflammatory response. It is known that stress, infection, and chronic disease can lead to a pro-inflammatory state. In CLD, intestinal barrier impairment and increased permeability are often reported, which result from oxidative damage and intestinal vascular congestion due to portal hypertension (Aguirre Valadez et al., 2016). These barrier impairments are associated with gut microbiota disturbance and small intestinal overgrowth (Aguirre Valadez et al., 2016). Moreover, gut microbiota dysbiosis is commonly observed in patient with CLD, especially in decompensated stages. Increased gut permeability and leakage of gut-related products including endotoxins is also contributing to systemic inflammatory response in patients with cirrhosis (Albillos et al., 2014; Dirchwolf et al., 2016; Hadjihambi et al., 2018). Additionally, ammonia directly influences neutrophils and impairs phagocytosis, which may worsen the immune response (Shawcross et al., 2008). It has been reported that 60% of the cirrhotic patients with HE present with systemic inflammation, diagnosed either by positive bacterial culture of by pro-inflammatory cytokine levels (Shawcross et al., 2011). Moreover, endotoxins, molecular complexes located in the outer membrane of gram-negative bacteria, can also enter the systemic circulation, causing endotoxemia, which further leads to activation of immune cells (Bellot et al., 2013). Elevated levels of circulating pro-inflammatory cytokines in decompensated cirrhotic patients, including TNF-α, and interleukins (ILs) have been previously reported (Albillos et al., 2014) and these levels were positively correlated with the severity of OHE in decompensated CLD patients (Odeh et al., 2004). Moreover, the liver is an organ with predominant innate immunity, defined as the first line of defense against pathogens, which plays a crucial role in systemic response to prevent infection and maintain homeostasis. Cirrhosis results in impaired local and systemic immune functions within the liver, giving rise to immune dysfunction. This, in turn, disrupts both innate and adaptive immunity, elevating the susceptibility to opportunistic infections and consequently exacerbating inflammation. (Noor and Manoria, 2017).

Other pathogenic factors

The role of several other pathogenic factors, such as blood bile acids, in the pathogenesis of HE has been investigated previously. It has been shown that bile acid levels are elevated in the

serum of patients who died of ALF and high levels of serum and brain bile acids have been found in BDL rats (Woolbright et al., 2014; McMillin and DeMorrow, 2016). While the exact role of bile acids in the pathogenesis of HE has not been fully identified, their effect on BBB permeability, neuronal dysfunction, and neuroinflammation have been reported previously (DeMorrow, 2019). Moreover, it has been known that lactate and manganese accumulation in the brain may also play a role in pathogenesis of HE (Rose et al., 1999a; Butterworth, 2013).

Precipitating factors of HE

Several precipitating events can trigger OHE. These events include but not limited to constipation, dehydration, gastrointestinal bleeding, infection, alcohol intoxication, renal failure, and medication (Rose et al., 2020). Constipation and GI bleeding can further lead to an increase in blood ammonia levels. Therefore, identification and correction of the precipitating events and subsequent treatment are important management approaches.

Episodic to recurrent HE

Episodic HE defines as presence of an episode of OHE while ≥ 2 overt HE episodes within 6 months is defined as recurrent HE (Vilstrup et al., 2014). Recurrent episodes of HE are associated with a poor prognosis, increased mortality, greater health burden, and reduced quality of life. Therefore, preventing recurrence of OHE is mainstay management for these patients (Sharma and Maharshi, 2015).

The impact of liver disease etiological factors on the brain

Etiological factors involved in the progression of CLD may contribute to brain impairment or possibly sensitivity. To date the role of several etiologies including NAFLD, HCV and alcohol have been investigated.

Diet / obesity

In NAFLD patients, several cognitive dysfunctions such as impairment in memory, attention, and concentration have been reported. These impairments were associated with negative impact in quality of life and everyday living (Kjærgaard et al., 2021). Additionally, it has been reported that hepatic steatosis is associated with a smaller total cerebral volume and premature aging (Weinstein et al., 2018). Clinical features of NAFLD may contribute to these cognitive dysfunctions, such as systemic and neuroinflammation, hyperammonemia, and disturbed gut microbiota (Kjærgaard et al., 2021).

Hepatitis C

HCV RNA has been detected in the post-mortem brain samples and cerebrospinal fluid (Laskus et al., 2002; Radkowski et al., 2002). However, the presence of HCV RNA in the brain has been found at a significantly lower magnitude, ranging from 1000 to 10,000 times less, in comparison to the liver. This discrepancy suggests that the brain serves as subtle site for HCV replication (Radkowski et al., 2002; Adinolfi et al., 2015). Regardless, patients with HCV infection represent various neurological and cognitive impairments. It has been shown that HCV infection results in sensory and motor peripheral neuropathy and may directly induce neurotoxicity. It has been also reported that cognitive function improves after successful HCV clearance (Kraus et al., 2013). The exact mechanism of extrahepatic complications of HCV infection is not fully understood; however, activation of numerous signaling pathways in response to hepatic and systemic inflammation leading to release of inflammatory cytokines and oxidative stress could play a major role (Mathew et al., 2016). Additionally, higher risk in development of neurodegenerative diseases such as Parkinson's and Alzheimer's disease has been documented in patients with chronic HCV infection (Sochocka et al., 2017; Wijarnpreecha et al., 2018).

Alcohol

Alcohol is a well-known neurotoxin, and the neurotoxic effects vary with dose, duration of exposure, brain region, age, and developmental stage (Squeglia et al., 2014). Heavy chronic or binge alcohol exposures in the mature brain can lead to severe, debilitating disorders of the central and peripheral nervous systems (de la Monte and Kril, 2014). Also, long-term heavy alcohol abuse results in loss of cerebral white matter and impairments in executive function (Sullivan et al., 2010; Bernardin et al., 2014). Some of the most important targets of chronic alcohol-related metabolic injuries include cortical-limbic circuits, the cerebellum, peripheral nerves, and skeletal muscle. The degenerative, metabolic, and toxic effects of alcohol target most of the cells in the CNS, such as neurons, oligodendrocytes, and astrocytes. Moreover, alcohol toxicity can cause neuronal inflammation, white matter atrophy, and impairments in synaptogenesis (de la Monte and Kril, 2014). The liver is the main organ for alcohol metabolism, which is mediated by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) oxidative pathways. Alcohol metabolites, including acetaldehyde, can be distributed systemically and reach the brain, entering the endothelial cells of the BBB. In addition, ADH converts acetaldehyde to acetate in the brain. Acetate, by increasing lipid peroxidation and free

radicals' production has further effects on the brain. Alcohol, by producing acetate, indirectly contributes to decreasing antioxidant activity by increasing the oxidative stress response (Pervin and Stephen, 2021). Alcohol-induced ROS production leads to the formation of H_2O_2, superoxide, and free radicals which in turn activate the Rho kinase (ROCK/JNK) signaling pathway, leading to the release of inflammatory cytokines in brain endothelial cells, including IL-6. In addition, increased production of ROS results in enhanced peroxidation of lipids, proteins, and phosphorylation of mitochondria, leading to decreased ATP production (Haorah et al., 2008). Moreover, alcohol metabolites may alter the function of astrocytes and oligodendrocytes, resulting in impaired cell-cell communication (Pervin and Stephen, 2021). Also, formation and maintenance of the myelin sheath are essential for signal transmission and cell interaction, which are usually disrupted by alcohol metabolites (Crews and Nixon, 2009).

HE treatments and therapeutics

Most of HE treatments targeting blood ammonia either by reducing the absorption of ammonia or increasing ammonia removal. Ammonia is produced in the gut and in CLD, the reduced function of the liver is not able to detoxify ammonia effectively. Thus, reducing ammonia production and absorption in the gut is one of the potential targets for HE treatments (Rose et al., 2020). Therapeutics used in clinic that target the gut to reduce production and absorption of ammonia are osmotic laxatives (lactulose and lactitol) and non-absorbable antibiotics (rifaximin) (Bajaj et al., 2012, 2013). In addition to the gut, another potential treatment target for hyperammonemia could be the stimulation of enzymes with the ability to remove ammonia, such as glutamine synthase (GS) which can detoxify ammonia by converting glutamate into glutamine. Also, other prospective treatments such as L-Ornithine L-Aspartate (LOLA) and Ornithine phenylacetate (OP) which act through substrate activation of GS results in ammonia removal (Rose et al., 2020).

Liver transplantation (LT)

Cirrhosis is the end stage of CLD caused by a variety of etiological factors; however, even after eliminating the hepatic insults, cirrhosis is primarily irreversible and therefore patients will continue to suffer from cirrhosis and eventually transition to the decompensated state. To date, the only curative option for these patients is LT. In the year 2022, 2936 patients underwent an LT, which constituted 20% of the total number of organ transplantations performed in Canada (Canadian Institute for Health Information).

Neurological complications after LT

Improvement in pre-transplant management, surgical techniques (perioperative conditions), and immunosuppressant regimen post-LT over the past decades resulted in a high success rate (85% survival at 1 year, 75% at 5 years) and improvements in short-term survival. Therefore, LT is no longer considered a high-risk procedure, and as such focus has shifted from survival to quality-of-life outcomes, addressing long-term complications (Roberts et al., 2004; Lim and Schiano, 2012). Historically, HE is defined as a metabolic syndrome that is believed to be completely resolved after LT; however, persistent neurological complications have been reported in patients following LT (Stracciari and Guarino, 2001; You et al., 2017; Weiss and Thabut, 2019). It is known that post-LT neurological complications negatively affect the quality of life in LT recipients, longer hospitalizations causing a financial burden on the healthcare system (Bhutiani et al., 2018). These neurological complications include but not limited to osmotic demyelination syndrome, seizures, cerebrovascular complications, ischemic stroke, CNS infection, brain hemorrhage, cerebral embolism, and posterior reversible encephalopathy syndrome (Estol et al., 1991; Lescot et al., 2013; Weiss and Thabut, 2019). It has been reported that previous history of OHE episodes play an influential role in the development and persistence of these neurological complications observed following LT (Sotil et al., 2009). However, exposure to intra-operative factors during transplant surgery, and post-transplant immunosuppression and medications have also been considered to impact neurological outcomes (Ochoa-Sanchez et al., 2021). Although these negative outcomes are well-documented, the exact underlying pathogenic mechanisms merits further investigation. Additional research is required in order to ascertain whether the occurrence of multiple episodes of OHE results in neuronal loss or injury, ultimately leading to persisting neurological impairments.

Pre-operative existing neurological impairments and neurological complications after LT

According to several studies, there is a strong association between pre-LT HE and neurological complications post-LT (Sotil et al., 2009). It has been reported by multiple studies that CHE pre-LT also impacts cognitive function post LT (Mechtcheriakov et al., 2004; Dhar et al., 2008; Ahluwalia et al., 2015; Nardelli et al., 2017). Moreover, a study by Sotil et al. shows that patients with the history of OHE have poor performance in diagnostic tests such as PHES and CFF post-LT compared to the patients without the history of OHE (Sotil et al., 2009). These

findings suggest that a history of and pre-existing OHE may cause permanent cell damage, which is less likely to be reversible following LT.

Intra-operative factors and neurological complications after LT

Liver transplantation is an invasive surgery with several unstable peri-operative conditions, with a risk for blood loss and hypovolemia, hypotension, ischemia, gas embolism and hyponatremia (Rabinstein and Keegan, 2013; Campagna et al., 2014; Derle et al., 2015). Furthermore, general anesthesia can also have an impact on the brain (Wu et al., 2019). Moreover, in a recent study by our group, we demonstrated a brain with CHE is sensitive to hypotensive insults resulting in neuronal cell loss/injury in BDL rats. The same hypotensive insult did not lead to cell loss or injury in SHAM rats or BDL rats treated for CHE with ornithine phenylacetate (OP). These results suggest patients with CHE at time of LT might be more susceptible to cerebral damage following intraoperative insults (Clément et al., 2021).

Post-operative factors and neurological complications after LT

Organ transplanted patients receive immunosuppression treatments to reduce the risk of acute cellular rejection after transplantation (Moini et al., 2015). The association of immunosuppressants with severe side effects such as malignancies, opportunistic infections and metabolic disease is well-known (Pillai and Levitsky, 2009). Also, the use of calcineurin inhibitors such as cyclosporine and tacrolimus at higher doses has been documented to be associated with neurotoxicity (Nelson et al., 2022). Other immunosuppressants such as corticosteroids may increase the risk developing mood disorders or psychosis (Zivković, 2013). Long-term immunosuppression may increase the risk of opportunistic infections, which can increase the risk of inflammation, impacting brain function (Linden, 2009). Although the exact mechanism behind post-LT neurological complications is not yet clear but the contribution of immunosuppressant treatments and medication is undeniable.

Evidence for permanent brain cell injury in CLD; Clinical findings

Several studies have indicated HE does not fully reverse and that overt HE episode(s) could lead to irreversible brain cell injury. A study by Bajaj et al showed that residual effects of HE episode(s) remains on cognitive functions such as executive function and working memory despite attainment of normal mental status and adequate treatment. Additionally, the severity of cognitive impairments was associated with the number of OHE episodes (Bajaj et al., 2010). Another study by using MRI showed that functional connectivity is impaired in patients with

CHE and who have a history of OHE. Furthermore, they also demonstrated that functional connectivity is gradually reduced in CHE however after a previous OHE this impairment is greater (Chen et al., 2013). A study by Ishihara et al, reported that patients with CHE and without history of OHE showed an improvement in functional brain connectivity using brain functional MRI (Ishihara et al., 2013). Also, cirrhotic patients with CHE but without OHE showed improvement and possible rebalancing in different major functional brain networks after LT (Lin et al., 2014). Prasad et al, showed that lactulose treatment for cirrhotic patients with CHE, without OHE, improved health related quality of life and cognitive function (Prasad et al., 2007). Other than cognitive dysfunction, there is evidence of irreversible effects induced by OHE on the brain such as brain atrophy (Chavarria and Cordoba, 2015). Zeneroli et al, reported the association of brain atrophy with persistent HE and unresponsiveness to treatment (Zeneroli et al., 1987). By using MRI analysis after LT, García Martínez et al., showed a decreased in brain size and detectable brain atrophy despite improvement in neurological impairment (García Martínez et al., 2010). Another study by Garcia-Martinez et al, showed that history of OHE affects the cognitive function after LT caused by neuronal and brain volume loss (Garcia-Martinez et al., 2011). These findings demonstrate the significant contribution of OHE in persisting neurological impairments in patients with cirrhosis. However, this has not been properly investigated.

Translational findings

Translational studies and animal models are important tools to investigate the underlying cause of diseases. However, animal or *in vitro* models of episodic OHE are lacking. Several *in vitro* and *in vivo* studies have shown that precipitating factors of HE including hyperammonemia can lead to neurological dysfunction, cell death/injury, oxidative stress and neuroinflammation in the brain (Fan et al., 2014; García-Lezana et al., 2017; Angelova et al., 2022); however, none of them explored the role of multiple episodes of OHE on the brain integrity.

Chronic liver disease, hepatic encephalopathy, and brain cell loss/injury

The link between CLD and brain health has been investigated for several years. Historically, HE has been known as a reversible syndrome. It defines decompensated cirrhosis and the pathogenic mechanisms mainly involve astrocytes (dysfunction, swelling and Alzheimer type II astrocytes). However, recent studies have demonstrated liver dysfunction can impact the brain beyond astrocytes and can predict neurodegeneration (Kelty et al., 2023). Several clinical

studies have explored the role of HE, OHE and history of HE in cognitive function and brain integrity using psychometric tests and MRI in patients before and after LT.

Neuronal cell loss/injury and neurodegeneration

Neurodegeneration or neurodegenerative disorders, defined as serious medical conditions which affect the neurons in human brain resulting in progressive loss of neuronal cells and neural tissue (Agrawal, 2020). Neuronal cell death/injury can occur during development or pathogenic conditions. Neuronal loss had a significant importance since adult neurons have limited capacity to proliferate or be replaced. Several mechanisms are involved in neuronal cell death including the role of non-neuronal cells in this process. It is known that several programmed cell death mechanisms (such as apoptosis, necroptosis, ferroptosis, pyroptosis, etc.), as well as aggregated and unfolded protein responses, inflammation, loss of connection between neuronal and non-neuronal cells, oxidants and oxidative stress, excitotoxicity, and microglia can contribute to neuronal cell loss (Fricker et al., 2018).

Mechanisms of cell death

Any irreversible degeneration of vital cellular function leading to loss of cellular integrity, such as cellular fragmentation and plasma permeability, is considered cell death. Cell death can be categorized into two types: passive and active. In the passive cell death process, there is no endogenous cellular contribution, an example of passive cell death is unregulated necrosis; However, in the active cell death process, cellular contribution is necessary (Stefanis, 2005).

Apoptosis

Apoptosis is a complex signaling pathway, and caspases play an essential role in this mechanism. Caspases are synthesized within the cell catalytically inactive zymogens and, based on their position in the apoptotic signaling pathway, are categorized into initiators, such as caspase-2, 8, 9, and 10, and effectors, such as caspase-3, 6, and 7. Initiator caspases autoactivate under apoptotic conditions. However, the activation of effector caspases is performed by initiator caspases through an internal cleavage to separate the large and small subunits. Upon cleavage of effector caspases, activation of important proteins responsible for maintaining cell integrity and various enzymes happens, which results in apoptotic morphology, including cytoplasmic shrinkage and DNA fragmentation (Riedl and Salvesen, 2007). There are two suggested pathways for activation of caspases; the intrinsic pathway, which initiates within the cell through endoplasmic reticulum (ER) stress, DNA damage, or

mitochondrial dysfunction, and the extrinsic pathway, which activates in response to an external signal through death receptors.

Intrinsic pathway of apoptosis

Activation of intrinsic apoptosis pathways involves non-receptor stimuli that act intracellularly either directly or through mitochondrial events (Fig. 7). Stimuli such as radiation, hypoxia, hyperthermia, viral infections, toxins, free radicals, or the absence of a growth factor, hormones, and cytokines can lead to changes in the mitochondrial inner membrane and the opening of the mitochondrial permeability transition pore, which results in loss of membrane potential and the release of the first group of pro-apoptotic proteins such as Cytochrome C, Smac/DIABLO, and HtrA2/Omi in the cytosol (Saelens et al., 2004). Cytochrome C binds and activates Apaf-1 as well as procaspase-9, leading to the activation of caspase-9 and the formation of apoptosomes. Upon activation, caspase-9 can directly cleave and activate caspase-3 and caspase-7. When the cell commits to die, the second group of pro-apoptotic proteins is released from the mitochondria. These proteins include AIF (apoptosis-inducing factor), endonuclease G, and caspase-activated DNAse (CAD) which are translocated to the nucleus, resulting DNA fragmentation and chromatin condensation (Enari et al., 1998; Joza et al., 2001; Li et al., 2001). Bcl2 family of proteins regulates these apoptotic mitochondrial events by controlling the permeability of the mitochondrial membrane. The members of this family could be either pro- or anti-apoptotic. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. The role of these proteins is essential since they can determine if the cell aborts apoptosis or commits to it (Elmore, 2007).

Extrinsic pathway of apoptosis

The extrinsic apoptotic signaling pathways involve transmembrane receptor-mediated interactions. The best-characterized death receptors that initiate caspase dependent apoptotic signaling cascades include Fas and tumor necrosis factor receptor (TNFR). Following the ligand binding, the death-induced signaling complex (DISC) is formed, which results in the activation of initiator caspases such as caspase-8 and 10 (Kischkel et al., 1995). Caspase-8 activation triggers the apoptotic signal by cleaving BID, a Bcl2-interacting protein, as well as activation of other caspases such as caspase-3 (Cavalcante et al., 2019).

Execution pathway

The execution phase is the end point of both intrinsic and extrinsic apoptosis pathways. Effector or executing caspases activate cytoplasmic endonuclease and other proteases, which can subsequently degrade nuclear material as well as nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 are known effector/executioner caspases, which cleave various substrates such as poly (ADP-ribose) polymerase (PARP), the plasma membrane cytoskeletal and nuclear proteins, and cytokeratins, leading to morphological and biochemical changes seen in apoptotic cells (Elmore, 2007).

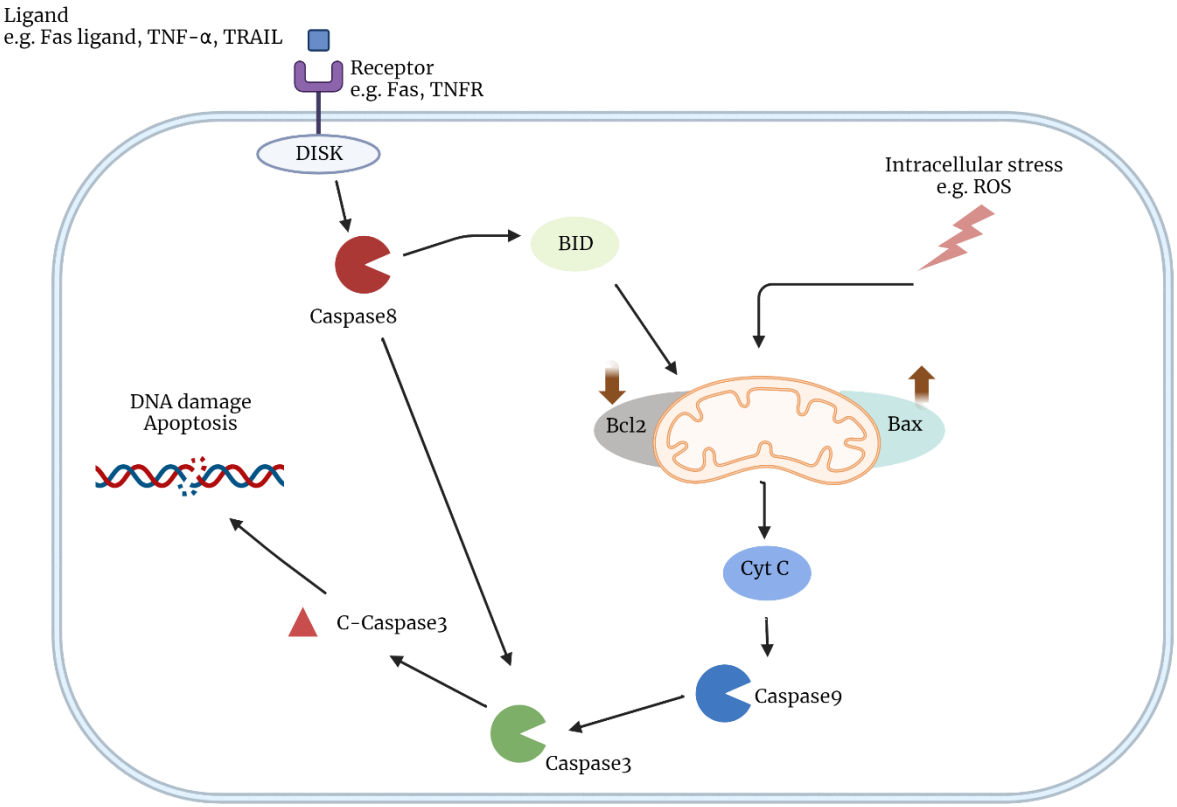

Figure 7. Apoptosis (intrinsic and extrinsic pathways). DISK: death-induced signaling complex, BID: BH3-interacting domain death agonist, Bcl2: apoptosis regulator Bcl-2, BAX: apoptosis regulator BAX, ROS: reactive oxygen species, Cyt C: cytochrome C, C-caspase3: cleaved caspase 3.

Necroptosis

Necroptosis is a regulated caspase independent form of cell death that is morphologically similar to necrosis, demonstrating swelling and plasma membrane degradation (Fig. 8). When caspases are inhibited and cell death is triggered through death receptors, receptor interacting kinase1 (RIP1) interacts with RIP3 and forms ripoptosomes, which initiates necroptosis. The RIP1/RIP3 complex phosphorylates mixed lineage kinase domain-like protein (MLKL) and form a necrosome. MLKL compromises the cellular membrane directly by forming pores in the plasma membrane or through the constitution of a platform at the plasma membrane for the opening of calcium or sodium ion channels, leading to ion influx, cell swelling, and rupture (Bertheloot et al., 2021). Several other stimuli, including ROS accumulation (Zhang et al., 2017; Barati et al., 2021) activation of mitochondrial antiviral signaling proteins, activation of death receptors (TNFR, Fas, and TRAILR), stimulation of pathogen and Toll-like receptors (TLR2, 3, 4, 5, or 9), activation of the T-cell receptor and anti-cancer drugs, and interferon or DNA damage, also result in necroptosis (Grootjans et al., 2017).

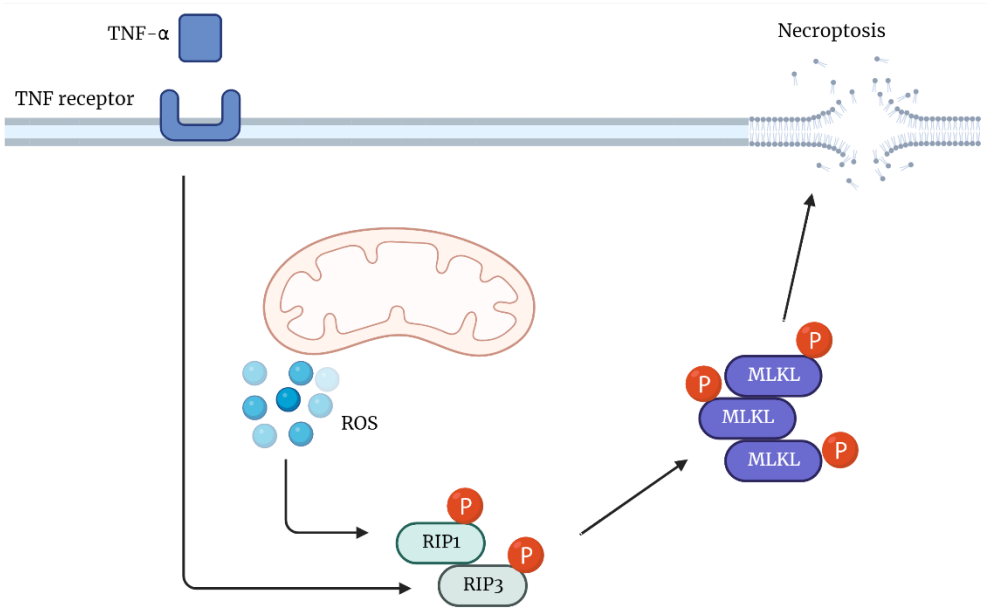

Figure 8. Necroptosis pathway. RIP: receptor-interacting serine/threonine-protein kinase, MLKL: mixed lineage kinase domain-like protein**.**

Other types of active cell death mechanisms

It is important to note that other than apoptosis and necroptosis, there are several active cell death types identified to date, including pyroptosis, autophagic cell death, autolysis, parthanatos, ferroptosis, mitopore, paraptosis, phagoptosis, and oncosis. Cell death mechanisms may differ depending on the cell type, death stimuli, and physiological condition (Stefanis, 2005).

Animal models of HE

Type A HE

To achieve an animal model of type A HE, two different approaches could be utilized: surgical manipulation or hepatotoxic substance administration. In the surgical method, in rodents or pigs, liver is excluded from circulation by anastomosis of the portal vein to the vena cava, followed by the ligation of the hepatic artery, thus obstructing all blood flow to the liver. This procedure leads to a rise in systemic ammonia levels, brain edema, and ultimately hepatic coma. Additionally, partial, or total hepatectomy is an alternative approach that leads to similar characteristics in an animal model and results in type A HE (Rahman and Hodgson, 2000; DeMorrow et al., 2021). Administration of hepatotoxic substances such as galactosamine, thioacetamide (TAA), carbon tetrachloride (CCL_4), azoxymethane, and acetaminophen also leads to severe hepatic injury, followed by neurological impairments, brain edema, and coma (DeMorrow et al., 2021).

Type B HE

Total or partial portal shunting results in type B HE. A surgical procedure of portocaval anastomosis (PCA) can be used in rodents or pigs to induce type B HE. Shunt, by diverting the portal blood from liver, induces hyperammonemia and neurological impairment without underlying liver dysfunction (DeMorrow et al., 2021).

Type C CHE

To date, several animal models of CLD have been characterized and utilized to investigate Type C HE, including liver toxin models (CCl_4 and TAA) and surgical models such as BDL. It is imperative for models of HE to develop liver disease/failure with hepatic damage (increased liver failure markers (ALT, AST, and bilirubin)) and hyperammonemia (DeMorrow et al., 2021). In hepatotoxic Type C HE animal models, chronic administration of lower doses (compared to ALF models) of TAA and CCL_4 leads to liver injury, hyperammonemia and

impaired neurological function (Yang et al., 2015; Hajipour et al., 2021). In the surgical approach, by ligating the common bile duct, progressive liver injury and severe fibrosis occur after four weeks. This procedure is the most commonly used approach for developing an animal model of type C HE, which is known as the bile duct ligated (BDL) model (Fig. 9). In this model, within 4-6 weeks after surgery, animals develop CHE and hyperammonemia with cognitive impairments such as motor coordination, memory deficits, and decreased activity. BDL rats also develop increased systemic inflammation, systemic oxidative stress, portal hypertension, GI bleeding, jaundice, and loss of muscle mass (Bosoi and Rose, 2012; Bosoi et al., 2017; DeMorrow et al., 2021; Ochoa-Sanchez et al., 2021).

Type C OHE

Administration of acute insults to animal models of type B and C HE triggers an OHE episode. Since the main player of the majority of the HE precipitating factors is ammonia, injection or administration of ammonia through diet can be used to induce OHE episodes (DeMorrow et al., 2021).

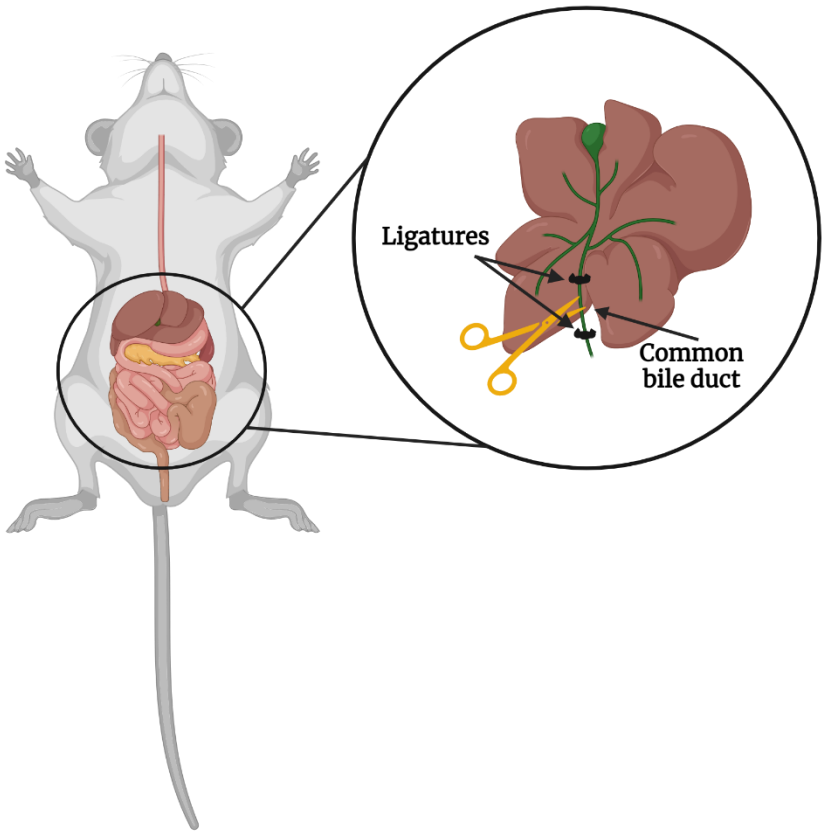

Figure 9: Bile duct ligation surgical model.

Hypotheses and aim

Study 1:

LT is the only curative treatment for cirrhosis which should resolve its associated complications, including HE. HE, by definition is a reversible metabolic syndrome, and therefore is believed to be resolved upon LT. However, several retrospective studies have demonstrated an association between a history of HE episodes and poor neurological outcomes following LT (Stracciari and Guarino, 2001; You et al., 2017; Weiss and Thabut, 2019). To date, the impact of HE episodes on the brain and their role in the irreversibility of HE remains unknown. We hypothesized that multiple episodes of OHE lead to permanent brain cell injury and exacerbate neurological dysfunction, hence reducing the likelihood of resolution following LT. To investigate the effect of multiple episodes of OHE on CHE development and brain cell loss/injury, we developed a novel animal model using BDL rats.

Aim 1: To characterize an animal model of episodic OHE and evaluate the impact of multiple ammonia induced OHE episodes on neurological status in rats with CLD.

Study 2:

It has been previously reported that alcohol-cirrhosis patients who died while in a hepatic coma, demonstrate greater cerebellar degeneration compared to those who died without alcohol-induced cirrhosis (Kril and Butterworth, 1997). It is known that constant alcohol consumption, independent of CLD, is often associated with global loss of brain and tissue, white matter shrinkage, and significant cortical volume loss, which results in impairment of brain function (Harper, 2009). Furthermore, patients with ALD demonstrate poor cognition and brain reserve compared to non-alcoholics (Ahluwalia et al., 2015). Additionally, it has been shown that global cognition function was poorer in cirrhotic patients with alcohol etiology after LT (Garcia-Martinez et al., 2011). However, the effect of alcohol consumption on the development of HE and brain integrity in the CLD setting remains unknown. Here, we hypothesized that continuous alcohol consumption worsens HE and leads to brain cell loss/injury, and hence irreversibility. To investigate the effect continuous alcohol consumption on HE development and brain cell loss/injury, we developed a novel animal model by administering alcohol (via gavage) in BDL rats.

Aim 2: To examine the effects of alcohol consumption on the development of HE, neurological decline, and brain integrity in rats with chronic liver disease.

Article 1

This article has been published in the JHEP Reports (JHEP Rep).

DOI: 10.1016/j.jhepr.2023.100904

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

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Keywords: Episodic hepatic encephalopathy, Ammonia toxicity, Neurodegeneration, Apoptosis, Neurological complications, Proteomics

Author contribution: F.T.: study concept and design, acquisition of data, analysis and interpretation of data, writing of the manuscript. R.O.: study concept and design, acquisition of data. K.D.: acquisition of data. M.O., C.B.: BDL surgery. C.L., M.L., L.S., Proteomics analysis. M.T., study concept and design, analysis and interpretation of data, critical revision of the manuscript. C.F.R., study concept and design, interpretation of data, drafting of the manuscript, critical revision of the manuscript, study supervision, obtained funding and approval of the final version of manuscript.

Data availability: The data that support the findings of this study are available from the corresponding author, C.F.R., upon request.

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.

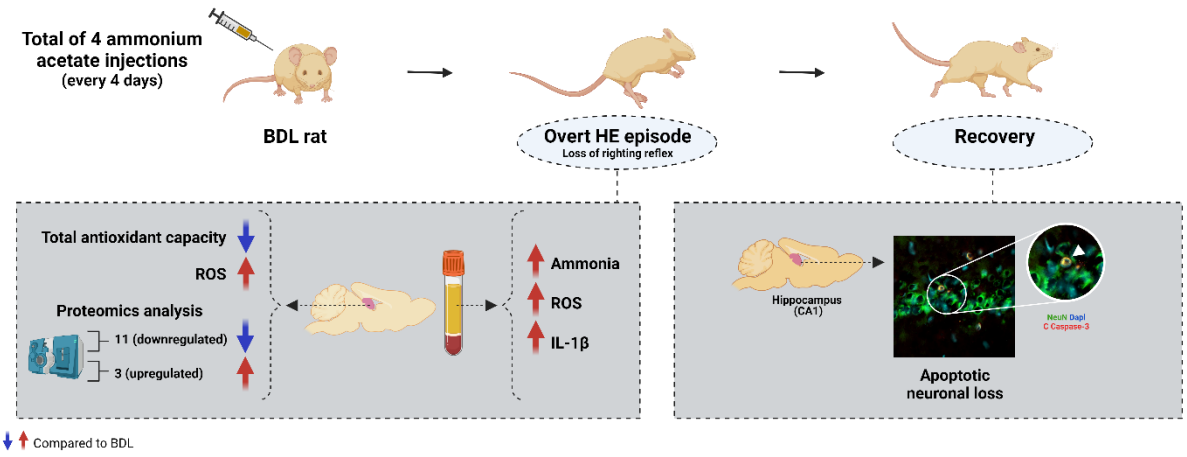
• Multiple ammonia-induced episodes of HE lead to permanent cell damage and therefore irreversibility.

• History of episodes of HE may justify persisting neurological complications observed in patients following liver transplantation.

Impact and implications

Hepatic encephalopathy (HE) defined as a reversible neuropsychiatric syndrome resolving following liver transplantation (LT); however, around 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 OHE episodes is critical for reducing the risk of irreversible neuronal injury in patients with cirrhosis.

Graphical abstract



Abstract

Background and 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 hypothesize 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 (CLD).

Methods: Episodes of overt HE (loss of righting reflex) were induced following injection of ammonium acetate in BDL rats (BDL-Ammonia) every 4 days starting at week 3 post-BDL. Neurobehavior was evaluated after last episode. Upon sacrifice, plasma ammonia, systemic oxidative stress, and inflammation markers were assessed. Neuronal markers including NeuN and SMI311 and apoptotic markers (cleaved caspase-3, Bax, Bcl2) were measured. Total antioxidant capacity (TAC), oxidative stress marker (4-HNE) and proinflammatory cytokines (TNF-α and IL-1β) were measured in the brain (hippocampus, frontal cortex, and cerebellum). Proteomic analysis was conducted in 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 to 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 TAC.

Conclusion: 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.

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 hospitalizations of patients with cirrhosis [2].

An increase in blood ammonia plays a major role in the pathogenesis of HE [1]. Hyperammonemia leads to an increase in blood-borne ammonia in the brain, since 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 and neurological damage. Therefore, the impact and the underlying mechanisms of multiple episodes of OHE on neurological integrity merits to be assessed. In order to do so, we first developed and characterized an animal model of OHE to subsequently evaluate the effect of repeated bouts of ammonia-induced OHE on neuronal integrity. We hypothesize multiple ammonia-induced episodes of OHE cause neuronal damage and permanent cell loss in rats with CLD.

Material 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 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 (total 4 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 behavioral 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 (OCT) compound and stored at -80°C for immunofluorescence analysis, and liver samples were fixed in 10% formalin for hematoxylin and eosin (H&E) staining.

Statistics

Data are expressed as mean ± standard error of the mean (SEM). One-way ANOVA and Two-Way ANOVA analyses with Tukey's multiple comparisons test post-hoc were performed, and p values < 0.05 were considered statistically significant. Statistical analysis was done using GraphPad Prism 8 (La Jolla, 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 to 40 minutes, and the duration of pre-coma was ranging from 5 to 35 minutes. The mean time to and duration of pre-coma did not significantly differ between subsequent episodes (Fig.1F-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 to episode 1 (5.44 ± 0.12 mmol/kg) ($p<0.05$ and $p<0.001$ respectively) (Fig.1C). Levels of blood ammonia preceding the 1st injection were significant in BDL rats (Ammonia and Vehicle) whereas blood ammonia levels were found significantly higher in BDL-Ammonia before 4th injection (S.Fig.1I). 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 (4th 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 last ammonia-injection, blood ammonia levels remained significantly higher in the BDL-Ammonia group compared to both the BDL-Vehicle and SHAM-Ammonia groups ($p<0.05$) (Fig.1E).

Liver disease assessment

Plasmatic liver markers including ALT, AST, ALP, GGT and bilirubin were significantly higher in both BDL-Vehicle and BDL-Ammonia groups compared to respective SHAM groups ($p<0.01$), with no significant difference found between 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 (S.Fig.1A-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 to respective SHAMs (Fig.2A-B). Using EchoMRI, less fat mass was found in both BDL-Vehicle and BDL-Ammonia groups compared to 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 to respective controls ($p<0.01$ and $p<0.001$ respectively) with no significant differences found in the BDL-Ammonia group compared to BDL-Vehicle ($p<0.05$) (Fig.2D-E). In addition, a significant decrease in lean mass was solely observed in the BDL-Ammonia group compared to other groups ($p<0.01$) (Fig.2F). However, the gastrocnemius muscle weighed significantly less in both BDL-Ammonia and BDL-Vehicle groups compared to 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 4th (last) ammonia-induced episode of HE. Plasmatic ROS levels were increased in both BDL-Vehicle and BDL-Ammonia groups compared to 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). Plasmatic levels of inflammatory marker IL-1β were found increased in BDL-Vehicle and BDL-Ammonia groups compared to respective SHAMs ($p<0.01$ and $p<0.001$ respectively). However, IL-1β levels were significantly higher in BDL-Ammonia compared to BDL-Vehicle ($p<0.001$). Plasmatic TNF-α levels also increased in both BDL-Vehicle and BDL-Ammonia groups compared to respective controls ($p<0.01$ and $p<0.001$, respectively) (Table.1).

In the brain, antioxidant capacity (TAC levels) was decreased in the hippocampus of both BDL-Vehicle and BDL-Ammonia groups compared to 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 to the BDL-Vehicle group ($p<0.05$). In the frontal cortex and cerebellum, TAC levels remained unchanged across all 4 groups (Fig.3B). In addition, 4-HNE, a marker of oxidative stress, was found to be increased in the hippocampus in the BDL-Ammonia group compared to all other groups. No significant difference was found between all groups in 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. However, brain TNF-α levels were increased only in the hippocampus of both BDL-Vehicle and BDL-ammonia groups compared to respective controls ($p<0.01$). Whereas in the cerebellum, a significant increase in TNF-α levels was only found in the BDL-Ammonia compared to BDL-Vehicle ($p<0.001$) (Table.1).

Neurological assessment

Novel object recognition test demonstrated significant decrease in performance in both short- and long-term memory in the BDL-Vehicle and BDL-Ammonia groups compared to respective controls ($p<0.05$ and $p<0.001$, respectively). However, LTM impairment was significantly different in the BDL-Ammonia group when compared to BDL-Vehicle ($p<0.05$) (Fig.4A-B).
Rota-rod motor coordination assessment showed a significant decrease in latency to fall at day two in the BDL-Ammonia group compared to BDL-Vehicle and respective control group ($p<0.01$) whereas on day three of the assessment, both BDL-Vehicle and BDL-Ammonia groups showed poor performance compared to respective controls ($p<0.01$ and $p<0.001$, respectively) (Fig.4C). The longitudinal comparison showed a significantly longer latency to fall in all groups at day three compared to their performance at day one (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 traveled remained unchanged between groups (S.Fig.1H).
Forelimb and hindlimb maximum muscle strength was significantly lower in BDL-Vehicle and BDL-Ammonia groups when compared to 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 to BDL-Vehicle ($p<0.05$) (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 to respective SHAMs. However, in the BDL-Ammonia group, a significantly increase of these apoptotic markers was found in the hippocampus compared to the BDL-Vehicle group (cleaved/pro-caspase-3; $p<0.001$ and Bax/Bcl2 ratio; $p<0.05$) (Fig.5A-B). Additionally, the hippocampus of BDL-Ammonia displayed a significant decrease in both neuronal markers NeuN and SMI311 ($p<0.001$ and $p<0.01$ respectively) as well as increased levels of the astrocytic marker, glial fibrillary acidic protein (GFAP) ($p<0.001$) compared to respective SHAM and BDL-Vehicle groups (Fig.5C-E, representative immunohistochemistry images from all brain regions are shown in S.Fig.3).These significant differences observed in the hippocampus of the BDL rats following 4 episodes were not demonstrated in BDL rats following 1 episode (S.Fig.2).

In order 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). Additionally, the colocalization of GFAP (astrocytic marker) and cleaved caspase-3 was found in all 3 studied brain regions (frontal cortex, hippocampus, and cerebellum) of both BDL-Vehicle and BDL-Ammonia groups (Fig.6E-G).

Proteomic analysis

In order to explore protein alterations occurring during an overt episode in the affected brain region (hippocampus), proteomic analysis identified 1202 proteins from which 438 proteins were significantly altered between all 4 groups. Among 438 proteins, 14 proteins were significantly changed between BDL-Vehicle and BDL-Ammonia, 275 and 73 proteins were significantly altered in BDL-Vehicle and BDL-Ammonia respectively when compared to respective SHAM groups. Additionally, 199 proteins were changed in SHAM-Ammonia versus SHAM-Vehicle. Two proteins were identified that were specifically altered in BDL-Ammonia when compared to BDL-Vehicle and SHAM-Ammonia (Fig.7).

Discussion

In this study, for the first time, we developed and characterized 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 in order 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 to patients without a history of OHE [13]. Furthermore, brain atrophy as well as the reduction in the neuronal marker N-acetylaspartate (NAA) (indicating neuronal loss), have been reported in patients who have endured from 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 due to elevated degree of hyperammonemia preceding each episode or equally suggesting the brain becomes sensitized 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 function (battery of behavioral tests) and neuronal integrity was assessed one week following 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, due to 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 episodes of OHE. However, both BDL rats (with or without ammonia injections) remained affected compared to 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 hyperammonemia (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 to 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 and ATP content have been reported in cases of hyperammonemia [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 signaling cascade, are widely used as apoptosis biomarkers in evaluating neurodegeneration [25, 26]. Interestingly, an increase in apoptotic markers was found in all 3 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 to 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., 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. 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 (PCA) model of hyperammonemia [33, 34] as well as 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 hyperammonemia 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, hemorrhage, 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 to 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] since it has been previously reported that hyperammonemia 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 due to a decrease in plasma antioxidants including albumin, catalase (CAT), glutathione reductase (GR), glutathione (GSH) and glutathione/oxidized glutathione ratio (GSH/GSSG) 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 damaging 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 to 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 overt HE. However, these results are limited to cerebral cortex and the history of OHE episodes in these patients was not reported [49].

Plasmatic 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 plasmatic levels of IL-1β and TNF-α have been previously reported in patients with HE [50] with plasmatic 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 to respective controls. Also, elevated levels of IL-1β were found in the frontal cortex both BDL-Vehicle and BDL-Ammonia groups compared to 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 to BDL-Vehicle rats. These results suggest an independent response

of pro-inflammatory cytokines arises in different brain regions following acute hyperammonemia.

Proteomic analysis in the hippocampus revealed alterations of multiple proteins. Out of 1202 analyzed, when controlling for liver disease (BDL) and ammonia-injections, 2 proteins were identified to be significantly altered in BDL-Ammonia when compared to BDL-Vehicle and respective SHAM. A significant downregulation of VILIP-2 (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 (VSNLs), 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 postmortem brains of Alzheimer's disease patients, 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 TN-R (Tenascin-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 (**S.Fig.**2). 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 since clinically, the

duration, frequency of episodes as well as number of episodes will vary considerably between patients. These clinical details remain to be explored and merit to be investigated.

Conclusion

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, as well as 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
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

Acknowledgements

The authors thank the behavior phenotyping core facilities of CRCHUM for behavior instruments. The authors gratefully acknowledge the financial support of Canadian Institutes of Health Research (CIHR). Some figures and graphical abstract are created with BioRender.com

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Table 1.

	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 ***,#

Legends

Table 1. Inflammatory markers

Higher levels of inflammatory cytokines (IL-1β and TNF-α) were found in BDL rats' plasma. However, IL-1β levels were even significantly higher in episodic rats when compared to BDL controls. Both cytokines' levels 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.

Figure 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 (1st and 4th 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 to SHAM-Vehicle. 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.

Figure 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.

Figure 3. Multiple ammonia induced OHE episodes and oxidative stress. (A) Plasmatic ROS was significantly higher in the BDL-Ammonia group when compared to 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.

Figure 4. Behavioral assessment. (A-B) STM and LTM performance was decreased in all BDL 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.

Figure 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 to 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

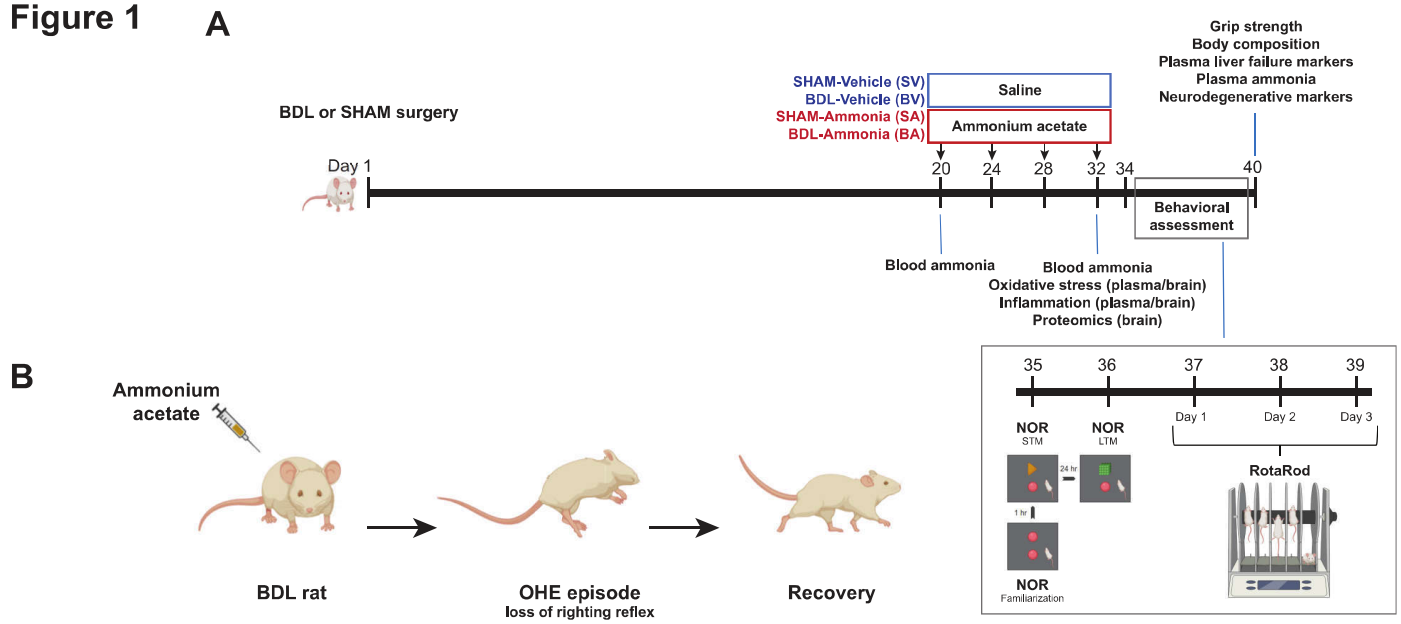
hippocampus of the BDL-Ammonia group. Two-Way ANOVA with Tukey's multiple comparisons. *p<0.05, **p<0.01 and ***p<0.001 vs respective controls #p<0.05 and ###p<0.001 BDL-Vehicle vs BDL-Ammonia.

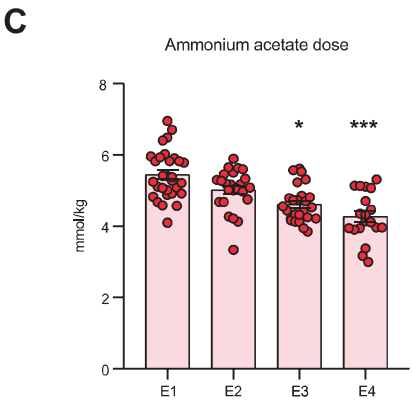
Figure 6. Immunohistochemistry. (A-C) Co-staining of cleaved caspase-3 (a marker of apoptosis) and NeuN (neuronal marker) in the frontal cortex and granular layer (GL) of cerebellum did not show any colocalization of apoptotic and neuronal markers in BDL-Vehicle and BDL-Ammonia; however, immunofluorescence 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 (a marker of apoptosis) and calbindin (a marker of Purkinje neurons) was observed in the Purkinje layer (PL) of 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.

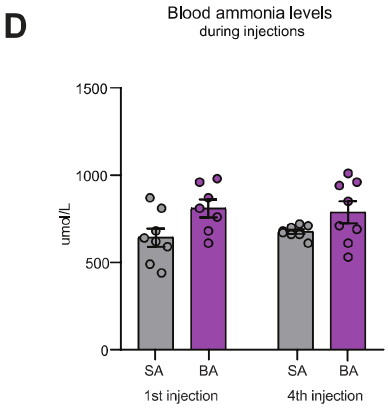
Figure 7. Proteomic analysis

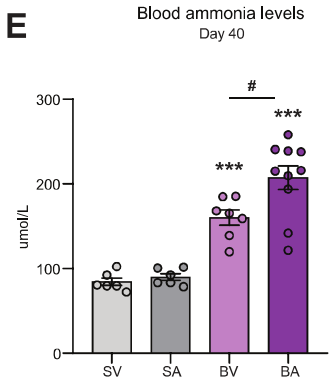
In the hippocampus, A) The Venn diagram used to compare and determine shared proteins with significant alteration among four comparison groups. B) The volcano plots demonstrating no significant change as well as up- and down- regulation of the expression of proteins between two groups.

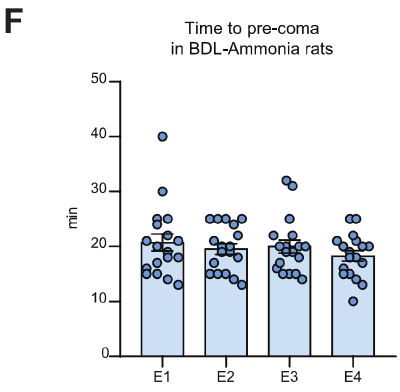
Figure 1


A
Grip strength
Body composition
Plasma liver failure markers
Plasma ammonia
Neurodegenerative markers
SHAM-Vehicle (SV)
BDL-Vehicle (BV)
SHAM-Ammonia (SA)
BDL-Ammonia (BA)
Saline
Ammonium acetate
BDL or SHAM surgery
Day 1
20
24
28
32
34
40
Behavioral assessment
Blood ammonia
Blood ammonia
Oxidative stress (plasma/brain)
Inflammation (plasma/brain)
Proteomics (brain)
B
Ammonium acetate
BDL rat
OHE episode
loss of righting reflex
Recovery
35
36
37
38
39
Day 1
Day 2
Day 3
NOR
STM
NOR
LTM
24 hr
1 hr
NOR
Familiarization
RotaRod


C
Ammonium acetate dose
mmol/kg
8
6
4
2
0
*
***
E1
E2
E3
E4


D
Blood ammonia levels
during injections
umol/L
1500
1000
500
0
SA
BA
SA
BA
1st injection
4th injection


E
Blood ammonia levels
Day 40
umol/L
300
200
100
0
#
***
***
SV
SA
BV
BA


F
Time to pre-coma
in BDL-Ammonia rats
min
50
40
30
20
10
0
E1
E2
E3
E4

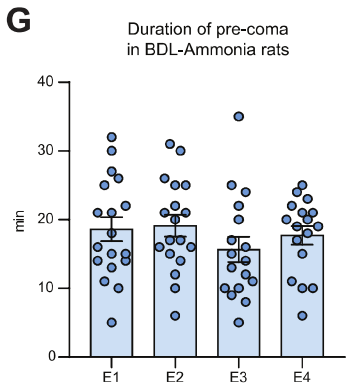

G
Duration of pre-coma
in BDL-Ammonia rats
min
40
30
20
10
0
E1
E2
E3
E4

Figure 2

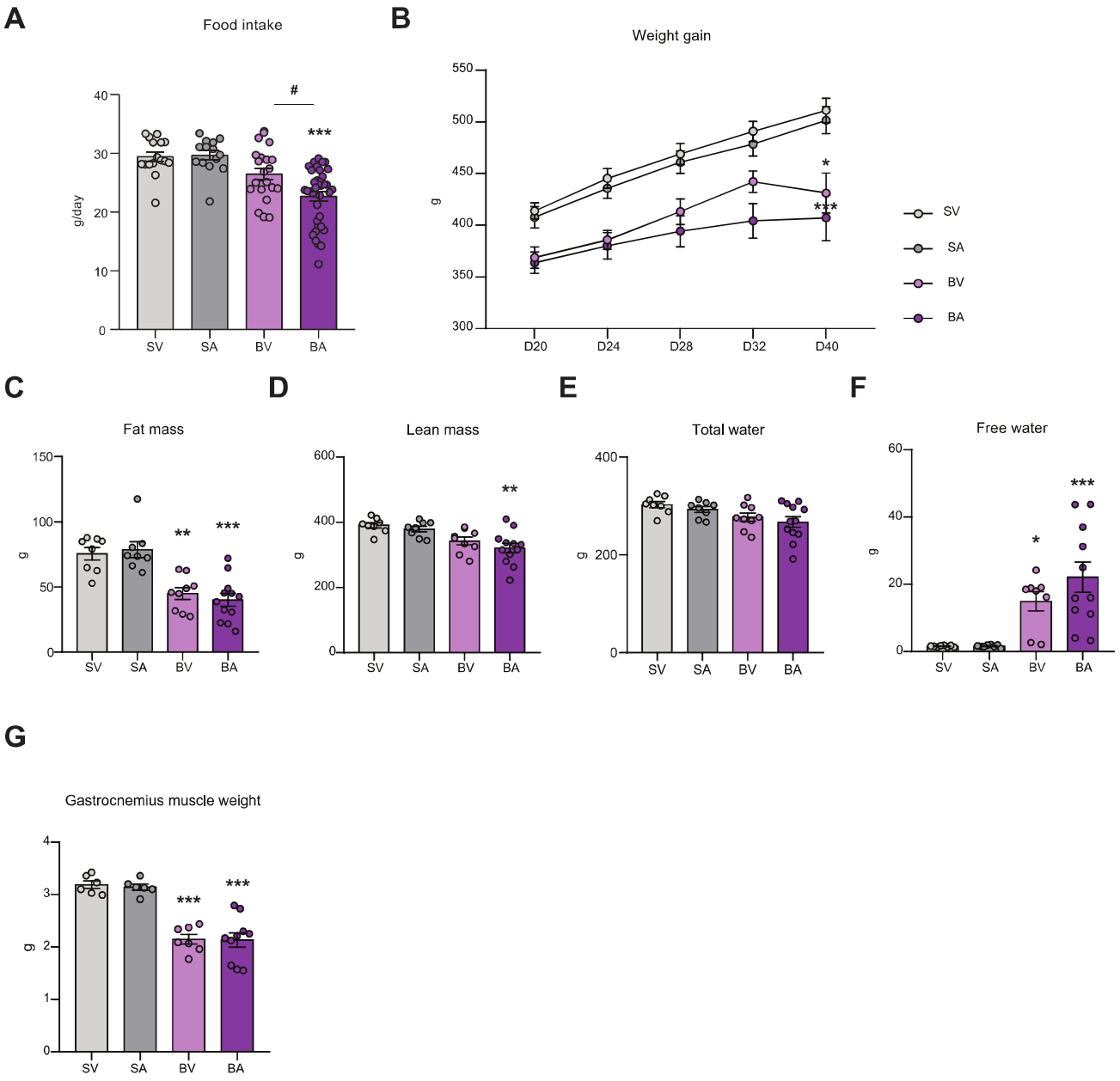
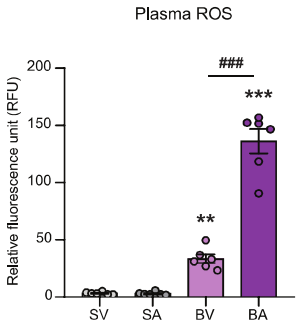
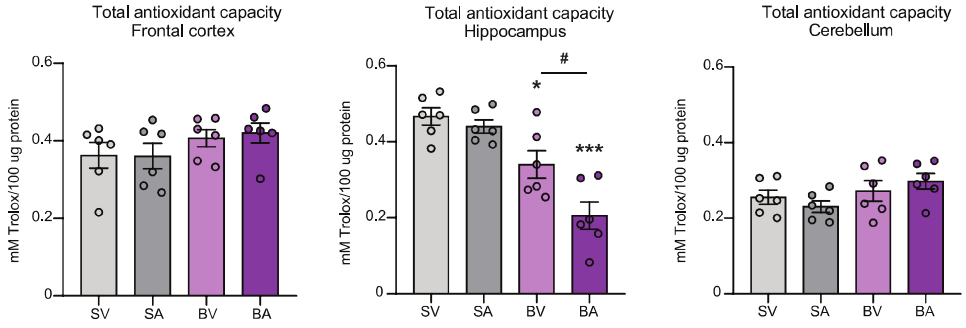

A
Food intake
g/day
40
30
20
10
0
#
***
SV
SA
BV
BA
B
Weight gain
g
550
500
450
400
350
300
*
***
D20
D24
D28
D32
D40
SV
SA
BV
BA
C
Fat mass
g
150
100
50
0
**
***
SV
SA
BV
BA
D
Lean mass
g
600
400
200
0
**
SV
SA
BV
BA
E
Total water
g
400
200
0
SV
SA
BV
BA
F
Free water
g
60
40
20
0
*
***
SV
SA
BV
BA
G
Gastrocnemius muscle weight
g
4
3
2
1
0
***
***
SV
SA
BV
BA

Figure 3

A


Plasma ROS
Relative fluorescence unit (RFU)
200
150
100
50
0
###
***
**
SV
SA
BV
BA

B


Total antioxidant capacity
Frontal cortex
mM Trolox/100 ug protein
0.6
0.4
0.2
0
SV
SA
BV
BA
Total antioxidant capacity
Hippocampus
mM Trolox/100 ug protein
#
*
***
Total antioxidant capacity
Cerebellum
mM Trolox/100 ug protein

C

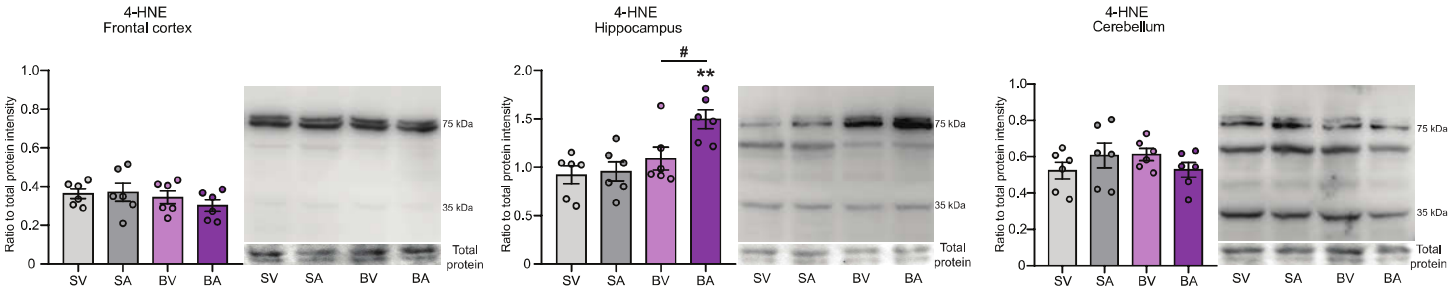

4-HNE
Frontal cortex
Ratio to total protein intensity
1.0
0.8
0.6
0.4
0.2
0
SV
SA
BV
BA
75 kDa
35 kDa
Total protein
4-HNE
Hippocampus
2.0
1.5
1.0
0.5
#
**
4-HNE
Cerebellum

Figure 4

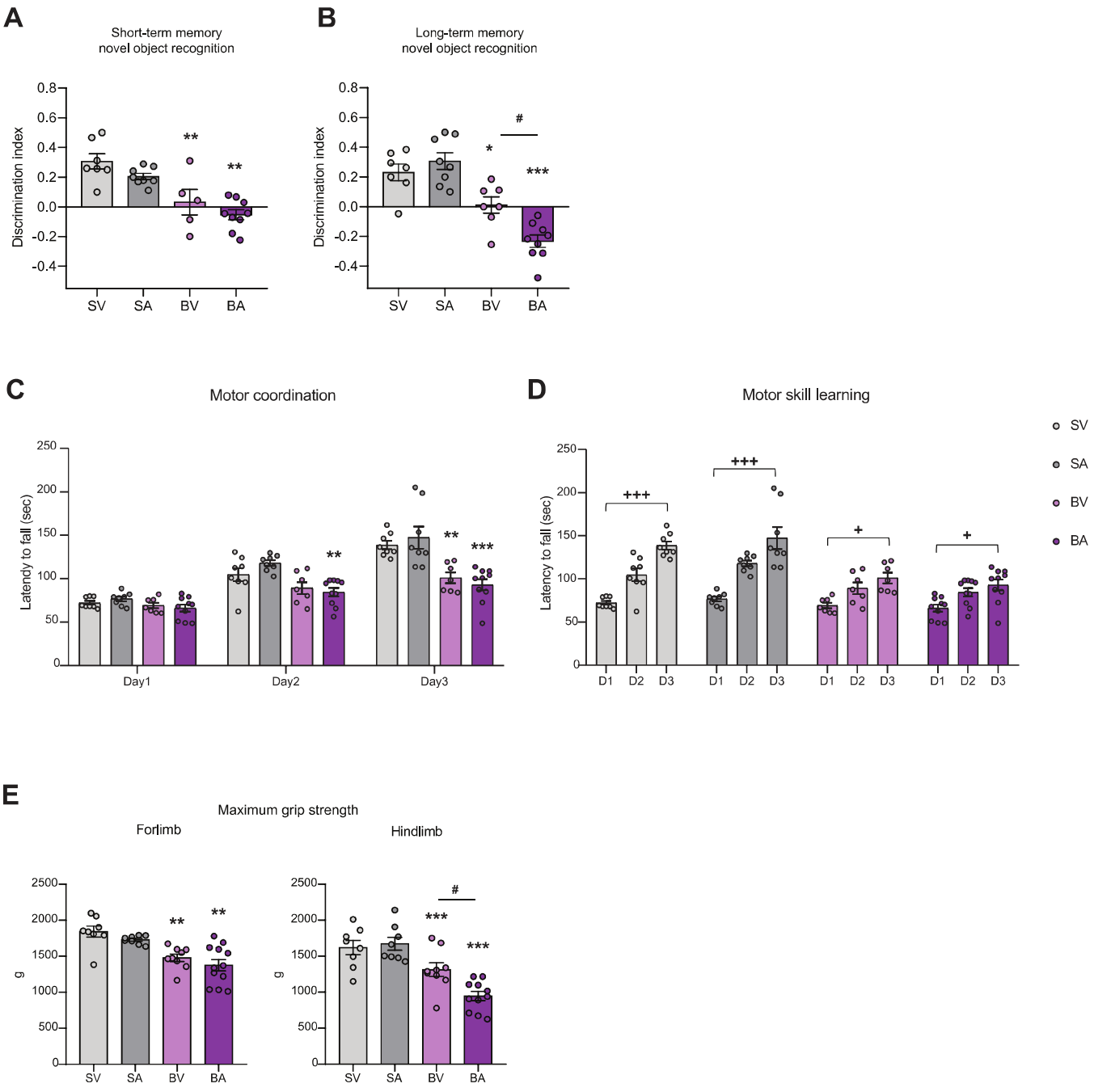

A
Short-term memory
novel object recognition
Discrimination index
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
**
**
SV
SA
BV
BA
B
Long-term memory
novel object recognition
Discrimination index
#
*
***
SV
SA
BV
BA
C
Motor coordination
Latency to fall (sec)
250
200
150
100
50
0
**
**
***
Day1
Day2
Day3
D
Motor skill learning
Latency to fall (sec)
+++
+++
+
+
D1
D2
D3
SV
SA
BV
BA
E
Maximum grip strength
Forlimb
Hindlimb
g
2500
2000
1500
1000
500
0
**
**
#
***
***
SV
SA
BV
BA

Figure 5

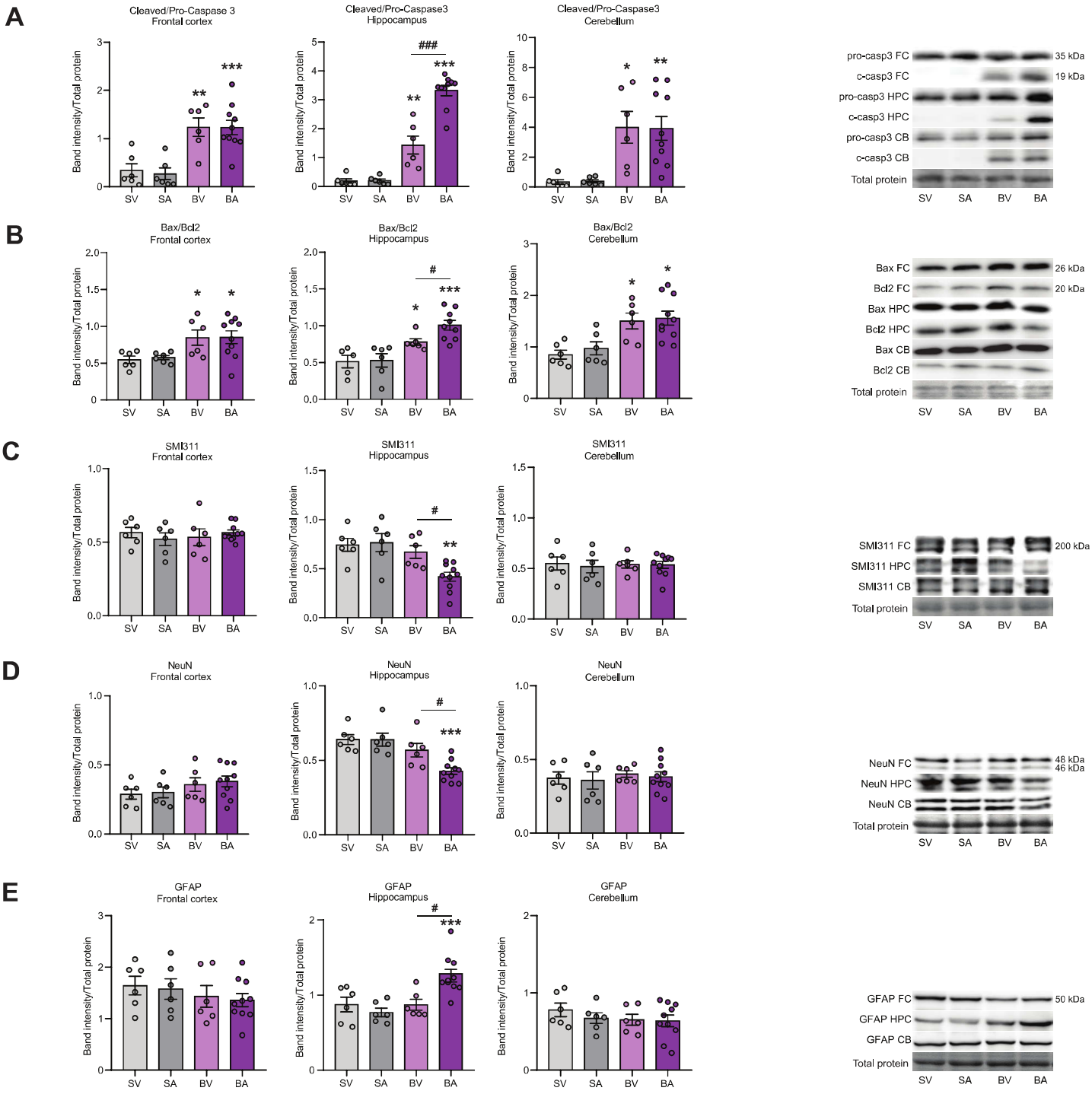

A
Cleaved/Pro-Caspase 3
Frontal cortex
Cleaved/Pro-Caspase3
Hippocampus
Cleaved/Pro-Caspase3
Cerebellum
Band intensity/Total protein
SV SA BV BA
pro-casp3 FC
c-casp3 FC
pro-casp3 HPC
c-casp3 HPC
pro-casp3 CB
c-casp3 CB
Total protein
35 kDa
19 kDa
B
Bax/Bcl2
Frontal cortex
Bax/Bcl2
Hippocampus
Bax/Bcl2
Cerebellum
Bax FC
Bcl2 FC
Bax HPC
Bcl2 HPC
Bax CB
Bcl2 CB
Total protein
26 kDa
20 kDa
C
SMI311
Frontal cortex
SMI311
Hippocampus
SMI311
Cerebellum
SMI311 FC
SMI311 HPC
SMI311 CB
Total protein
200 kDa
D
NeuN
Frontal cortex
NeuN
Hippocampus
NeuN
Cerebellum
NeuN FC
NeuN HPC
NeuN CB
Total protein
48 kDa
46 kDa
E
GFAP
Frontal cortex
GFAP
Hippocampus
GFAP
Cerebellum
GFAP FC
GFAP HPC
GFAP CB
Total protein
50 kDa

Figure 6

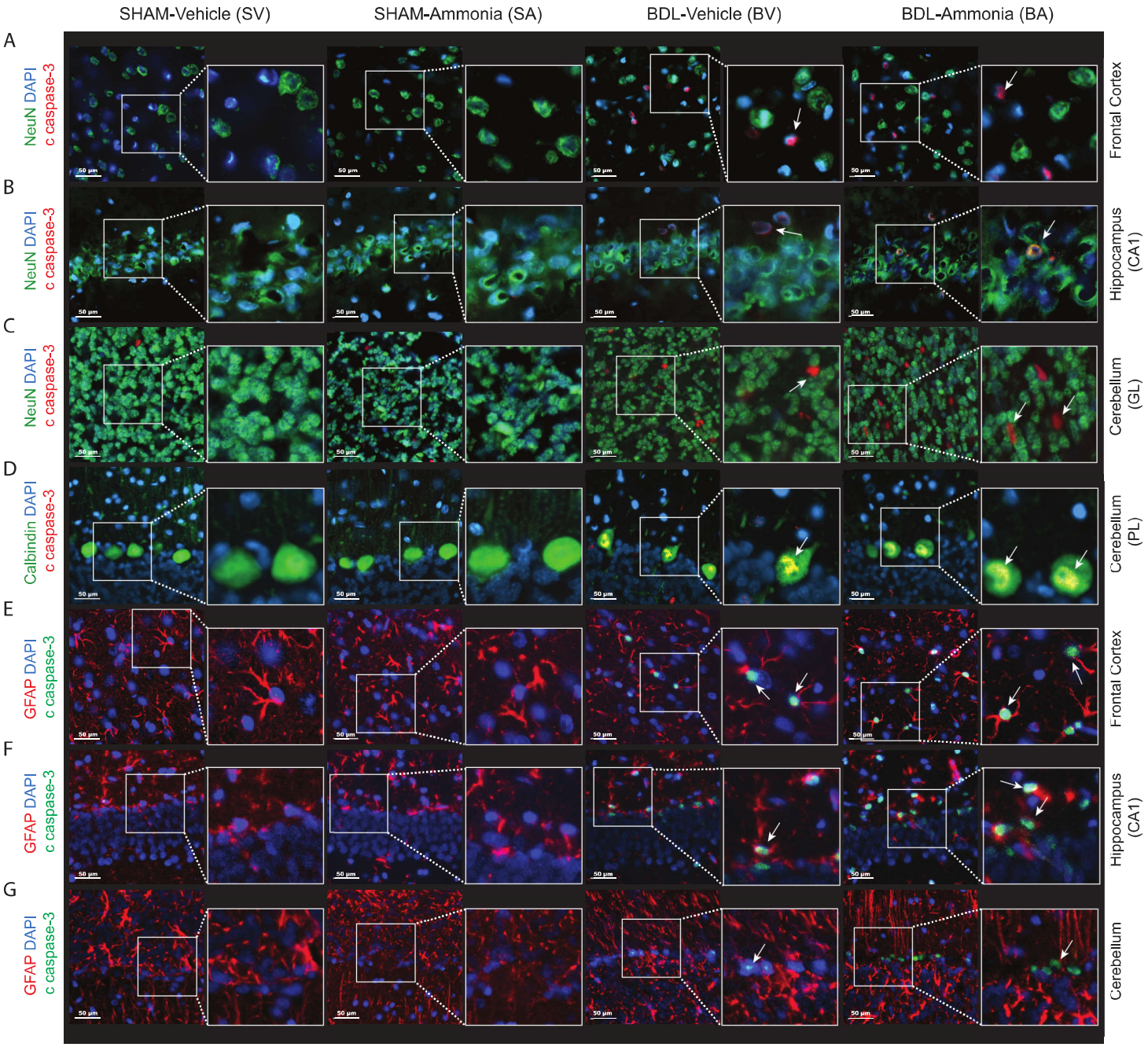
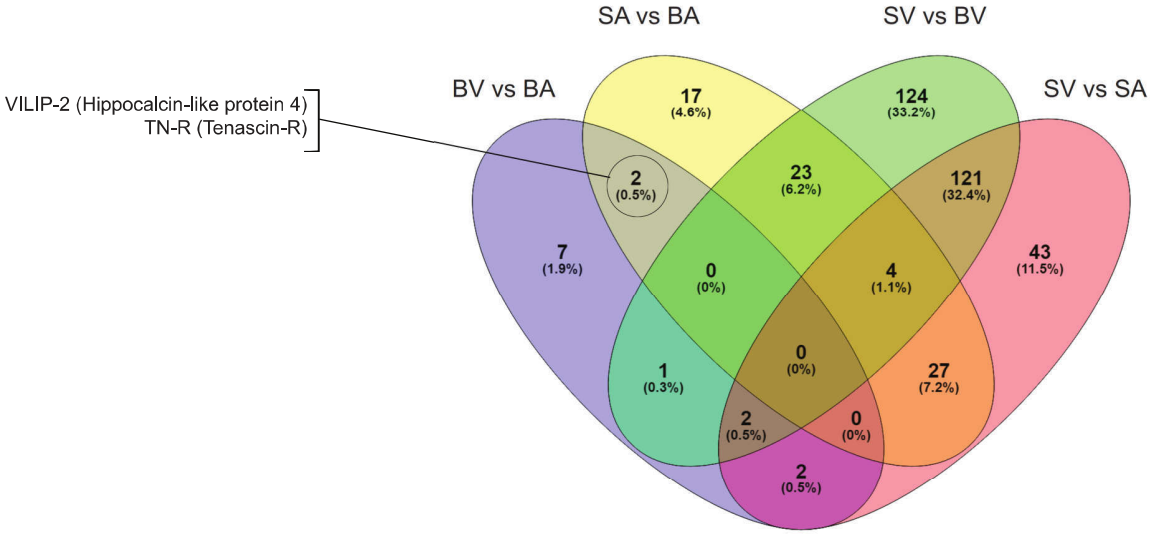
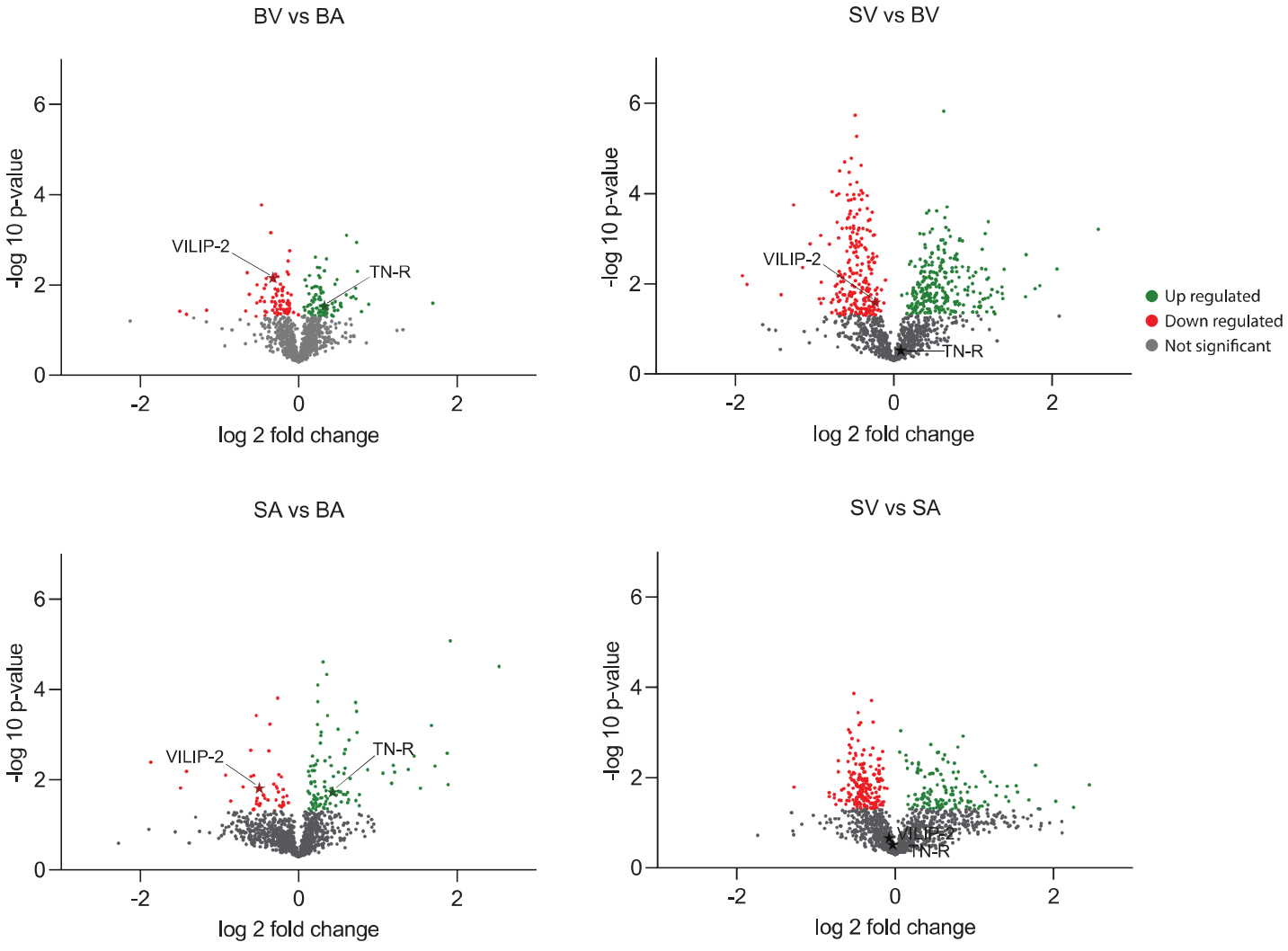

SHAM-Vehicle (SV)
SHAM-Ammonia (SA)
BDL-Vehicle (BV)
BDL-Ammonia (BA)
A
NeuN DAPI c caspase-3
Frontal Cortex
B
NeuN DAPI c caspase-3
Hippocampus (CA1)
C
NeuN DAPI c caspase-3
Cerebellum (GL)
D
Calbindin DAPI c caspase-3
Cerebellum (PL)
E
GFAP DAPI c caspase-3
Frontal Cortex
F
GFAP DAPI c caspase-3
Hippocampus (CA1)
G
GFAP DAPI c caspase-3
Cerebellum

Figure 7

A


VILIP-2 (Hippocalcin-like protein 4)
TN-R (Tenascin-R)
SA vs BA
SV vs BV
BV vs BA
SV vs SA
17 (4.6%)
124 (33.2%)
2 (0.5%)
23 (6.2%)
121 (32.4%)
7 (1.9%)
0 (0%)
4 (1.1%)
43 (11.5%)
0 (0%)
1 (0.3%)
27 (7.2%)
2 (0.5%)
0 (0%)
2 (0.5%)

B


BV vs BA
SV vs BV
SA vs BA
SV vs SA
-log 10 p-value
log 2 fold change
VILIP-2
TN-R
Up regulated
Down regulated
Not significant

Supplementary material

Results

One ammonia-induced episode of OHE is not enough for causing neurodegeneration

Following one OHE episode, no significant differences were found in neither neuronal marker of NeuN and SMI311 nor apoptotic marker of cleaved/pro-caspase-3 compared with the BDL-Vehicle group. Similarly, no significant changes were detected in GFAP expression levels in all brain regions of BDL rats with one episode compared to the BDL-Vehicle group. In support of the results mentioned above, 4 OHE episodes was significantly different than both no episodes (BDL-Vehicle) and 1 OHE episode (**S.Fig.**2B-F).

Supplementary figure legends

Supplementary Fig. 1. (A) Alanine aminotransferase, (B) Aspartate transaminase, (C) Alkaline phosphatase, (D) Gamma-glutamyl transferase and (E) Bilirubin were significantly higher in BDL-Vehicle and BDL-Ammonia groups compared to respective SHAM groups. (F) Albumin levels were significantly lower in all BDL rats. Two-Way ANOVA with Tukey's multiple comparisons. **p<0.01 and ***p<0.001 vs respective controls. (G) Hematoxylin and eosin staining in livers of BDL-Vehicle, and BDL-Ammonia show the same level of liver damage. (H) Total distance traveled remained unchanged between groups. (I) Blood ammonia levels in different time points. Two-Way ANOVA with Tukey's multiple comparisons. **p<0.01 and ***p<0.001 vs respective controls and ###p<0.001 BDL-Vehicle vs BDL-Ammonia.

Supplementary Fig. 2. Impact of one ammonia induced episode of OHE. (A) Experimental design. (B-F) Protein expression levels of the neurodegenerative markers were compared between BDL-Vehicle, BDL-Ammonia with 1 induced episode and BDL-Ammonia with 4 induced episodes. Neurodegeneration was not found in BDL-Vehicle or in BDL-Ammonia rats with 1 episode. The only group to demonstrate significant markers of neurodegeneration was BDL-Ammonia with 4 episodes. One-Way ANOVA with Tukey's multiple comparisons. *p<0.05, **p<0.01 and ***p<0.001 vs BDL-Vehicle.

Supplementary Fig. 3. Immunohistochemistry. (A-C) Co-staining of NeuN (neuronal marker) and GFAP (astrocytic marker) in frontal cortex, hippocampus, and granular layer (GL) of cerebellum. (D) Co-staining of Calbindin (a marker of Purkinje neurons) with GFAP in the Purkinje layer (PL) of cerebellum.

Material and methods

Bile duct ligation

Male Sprague–Dawley rats (n=95, 175–200 g; Charles River) were housed 2–3/cage in standard conditions with free access to food and water. After 48 hours of acclimation, bile duct ligation (BDL) or SHAM surgery was performed as described [1]. After surgery, to avoid the bias of surgery and cirrhosis complication development, SHAM and BDL rats were randomly assigned to either vehicle or ammonia groups to receive saline or ammonia injection accordingly. Analgesic treatment administrated before and up to 72 hours after the surgery with buprenorphine (sc. 0.05 mg/kg, every 12 hours), carprofen (sc. 5mg/kg, every 24 hours), and gabapentin (orally, 30 mg/kg every 12 hours).

Ammonium acetate dose

For inducing episodes, BDL rats were first injected with 1.5 ml/kg of ammonia acetate (150 mg/ml) and increased the volume (within 3 minutes) until rats showed lethargic phenotype which subsequently lead to a loss of righting reflex. Injected dose of ammonia was then calculated. An average of the injected dose used to induce each episode was then calculated and used for injections into SHAM rats, while respective controls received same volume of saline.

One episode versus 4 episodes of OHE

An additional group of BDL rats (6 rats per group) was used to evaluate the effect of one ammonia-induced OHE episode. BDL rats were assigned to three groups. The first group received 4 injections of saline (BDL-Vehicle) on day 20, 24, 28 and 32 following the BDL surgery. The second group received 3 injections of saline on day 20, 24 and 28 followed by one injection of ammonium acetate (BDL-Ammonia-1E) on day 32 following the BDL surgery. The third group received 4 injections of ammonium acetate (BDL-Ammonia-4E) on day 20, 24, 28 and 32 after BDL surgery. Rats were sacrificed during the last injection, and brains were flash-frozen with methylbutane cooled with dry ice and stored at -80°C for western blot analysis (**S. Fig.** 2A).

Behavioral assessment

Behavioral tests were conducted at week 5 following the surgery. Short- and long-term memory assessed with novel object recognition test (NOR) and motor coordination assessment performed by Rota-rod test.

Total distance traveled (activity)

Rats were placed in a black square box (90 cm^2) and the ambulatory movements were recorded for 5 minutes and analyzed with the SMART video tracking system (Panlab SMART v3.0, Harvard Apparatus, USA).

Novel object recognition test (NOR)

The novel object recognition test was used to evaluate short-memory (STM) and long-term memory (LTM) performance (8 rats per group). Rats were acclimated for 1 hour before the test was performed. Then, rats were placed in an empty box (60×45×33 cm) for 5 minutes for habituation. Ten minutes later, rats were placed in the same box with two identical objects (A+A) for 5 minutes for familiarization. One hour later, one of the objects was replaced with a new one (A+B), and the rats were returned to the box for STM assessment. 24 hours later, rats were placed in the same arena with a familiar and new object (A+C) for long-term memory assessment. The STM and LTM assessments were recorded for 5 minutes by a camera and reported as discrimination index ([Time (sec) spent exploring the novel object - Time (sec) spent exploring the familiar object])/ ([Total Time (sec) spent exploring]).

Rota-rod performance test

A Rota-rod test was conducted to assess motor coordination and motor skill learning (8-10 rats per group). On day 1, rats were placed on the non-rotating rod (7 cm in diameter), which was positioned 40 cm from the device floor for 5 minutes (ROTOR-ROD^{TM} System, San Diego Instruments). Then, the apparatus started, with a linear speed increase from 0 to 40 rpm over 5 minutes. The rat was forced to walk on the rod without falling, and the trial finished when the rat fell. Three trials per rat were performed with a 5-minute interval between each trial. Latency to fall recorded by apparatus and the best performance used for analysis. For motor skill learning, the same protocol was performed 24 hours and 48 hours after.

Skeletal muscle grip strength

Forelimb and hindlimb maximum muscle strength were measured with a grip strength digital meter (Chatillon®,USA) (8-10 rats per group). For forelimb, rats were allowed to grasp with both front paws the mesh pull bar. Immediately after horizontal position stabilization, the rat's tail was pulled backward by an operator. For hindlimb strength, the mesh was positioned at 25-degree angle and facing the rat away from the gauge. Rats were vertically placed on the gauge allowing to grasp the mesh with both hind paws, while the operator gently holds the rat's upper body and base of the tail. After vertical stabilization, the rat's tail was pulled backward until mesh release. All measurements were carried out by the same operator. Three trials per extremity were obtained and the maximum pick force (kg) was used for analysis.

Body weight, food intake and body composition

The body weight and food consumption of rats were measured before each injection and at the end of the model (8-12 rats per group). Water content, fat and lean mass were measured at the end of the model by nuclear magnetic resonance-EchoMRI-700® (EchoMRi LLC, USA). EchoMRI is a Nuclear Magnetic Resonance (NMR) instrument used for composition analysis. By exploiting the differences in relaxation times of the hydrogen proton spins in different environments, it measures contrast between soft tissues. The properties of the scanned materials are associated with the amplitude, duration, and spatial distribution of these signals and radio pulse sequences are applied to further amplify the high contrast between fat, lean tissue, and free water.

Liver markers and blood ammonia

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubin, and albumin were measured using the COBAS analyzer (c111, Roche, USA). Ammonia levels were measured in three points during the first and last injection and at the end of the model using PocketChem (BA PA-4140, Arkray, Japan) (6-10 samples per group).

Plasmatic oxidative stress

The 2',7'-dichlorofluorescein diacetate (Sigma-Aldrich) (DCFDA) (10 µM) was incubated with hydroxylamine (1 M, pH 8.5) for 30 minutes. Then, the mixture was added to 10 µl of plasma in triplicates (6 samples per group) in a 96-well plate. Later, fluorescence (λexc: 485 nm and λem: 528 nm) was measured with Synergy™ HT Multi-Detection Reader (Bio-Tek

Instruments, USA) every 2 minutes, up to 10 minutes, and the slope of time/readings was calculated, and the results were reported in relative fluorescence unit (RFU).

Cerebellar antioxidant capacity

Total antioxidant capacity (TAC) was measured with the antioxidant assay kit (Sigma-Aldrich, CS0790). The frontal cortex, hippocampus and cerebellum were dissected, and the protein lysate was prepared as described in the manual provided by the manufacturer, 100 µg of protein were used (6 samples per group).

Inflammatory cytokines

Brain samples of protein lysate (6 samples per group), including the frontal cortex, hippocampus, and cerebellum, were prepared in RIPA buffer. Brain and plasma cytokine levels were measured, using 200 µg of protein or 100 µl of plasma with ELISA kit for IL-1β (rat IL-1β/IL-1F2 DuoSet ELISA, R&D Biosystems, Canada; DY501) and TNF-α (Rat TNF alpha Uncoated ELISA, Invitrogen™ 88734088, USA).

Western blotting

The frontal cortex, hippocampus and cerebellum protein lysate (6-10 samples per group) were prepared in RIPA buffer supplemented with 1 mM PMSF and protease and phosphatase inhibitor cocktails (Sigma, P8340). The protein concentration was measured using DC protein assay (Bio-Rad Laboratories, 5000111, USA) and 40 µg of protein were loaded on stain-free 10% sodium dodecyl sulphate-polyacrylamide gel, and electrophoresed. After, the gels were activated with UV light for 45 sec using Chemidoc^{TM} imaging system (Bio-Rad Laboratories, 17001401, USA) and transferred on polyvinylidene difluoride membranes. Following the transfer, the membranes were imaged using ChemiDoc^{TM} for total protein quantification and subsequently blocked were blocked with 5% fat-free milk and PBS-T for one hour at room temperature (22°C). Membranes were then incubated in (1:1000) dilution of primary antibodies of NeuN (Millipore, MAB377), caspase-3 (Cell Signaling, 9662S), SMI311(Millipore, NE1017), Bax (Cell Signaling, 14796S), Bcl2 (Cell Signaling, 3498S), GFAP (Dako, Z0334) and 4-HNE (Millipore, 393207) overnight at 4°C. After, membranes were washed 5 times with PBS-T buffer and incubated with their corresponding secondary antibody coupled to horseradish peroxidase (1:10,000) for one hour at room temperature (22°C). After a few washes, membranes were exposed to chemiluminescence reagent (Clarity Max Western ECL Substrate, Bio-Rad Laboratories, 1705062, USA) and probed on Chemidoc^{TM}. Images were

quantified with Image Lab software (Bio-Rad Laboratories, version 6.0.1, USA), and the results were expressed as a ratio of the bands to the total protein.

Immunofluorescence and H&E staining

For visual assessments, brain slices (10 µm) were obtained using a cryostat (Leica, CM3050S, Germany) from 2 individual rats per group. Brain slides were blocked with PBS-0.5% Triton X–100 and 10% goat serum for 30 min. After, they were washed 3 times with PBS and incubated in (1:250) dilution of primary antibodies: cleaved caspase-3 (Cell Signaling, 9579), NeuN (Millipore, MAB377), GFAP (Dako, Z0334), GFAP (Sigma, G3893) and Calbindin (Invitrogen, MA5-24135) overnight at 4°C. The slides were then washed with PBS and incubated with corresponding secondary antibodies (donkey anti-mouse IgG coupled to Alexa488 fluorophore 1:200, Jackson Immuno Research, 715-545-150 or donkey anti-rabbit IgG coupled to Cyanine Cy™3 fluorophore, 1:200, Jackson Immuno Research, 711-165-152) for 1 hour, in the dark, at room temperature (22°C). Following washes, DAPI (4',6-diamidino-2-phenylindole) was used (1 µg/ml) and rinsed with PBS. After, the slides were covered with mounting media and coverslips, and images were acquired with ZEISS Axio Imager M2 ApoTome2 and ZEN 3.4 software (ZEISS, Germany). To evaluate liver histology, fixed liver tissue was blocked in paraffin, sliced (4µm) and stained with hematoxylin and eosin. Images were acquired with ZEISS Axio Imager M2 microscope (ZEISS, Germany).

Proteomic analysis

Sample preparation

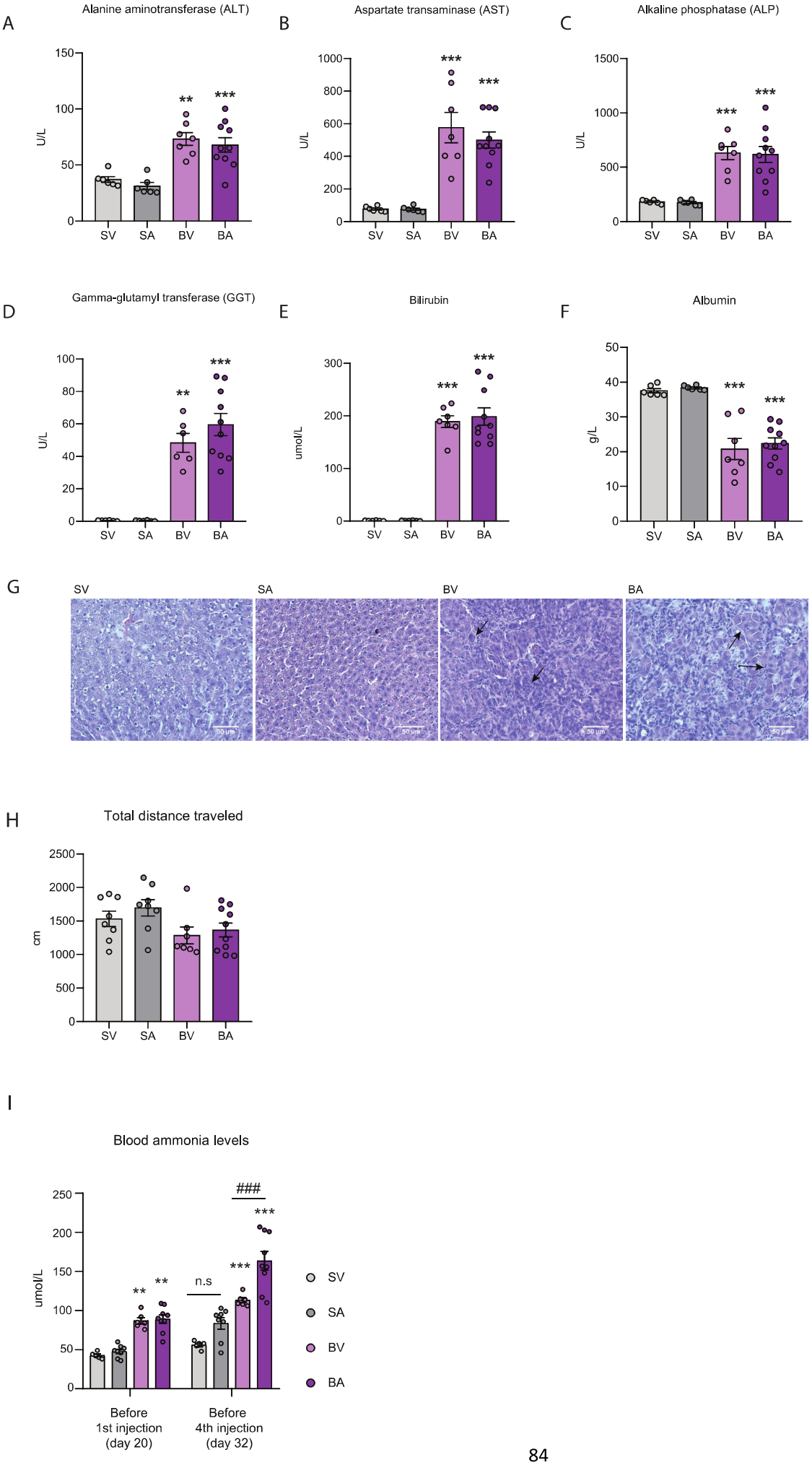
Hippocampal tissue samples (3 to 6 samples per group) were denatured with 200 µL solution containing 7 M urea and 2 M thiourea in water, followed by sonication for 15 min, and 400 µL of 100 mM ammonium bicarbonate (ABC) (pH 8.5) were then added. For each digestion, 50 µl of homogenate supernatant was combined with 200 µL ABC buffer. Disulfide reduction and alkylation was performed with 15 µL of 100 mM dithiothreitol (DTT) (15 min at 37°C) and 20 µL of 100 mM iodoacetamide (IAM) (30 min at 37°C in the dark). Proteins were digested with 10 µg trypsin for 4h at 37°C with gentle mixing. Solid-phase extraction (SPE) was performed on peptide digests using OASIS HLB 30 mg cartridges (Waters, Milford, MA). Cartridges were conditioned with methanol and water, samples were loaded, and washed with 1 mL of water before elution with 1 mL of methanol. Solvant was evaporated and samples reconstituted in 300 µL 10% acetonitrile (ACN) containing 0.2% formic acid (FA). Samples from each group were combined to also create pooled samples for protein identification.

LC-MS/MS analysis with IDA and SWATH

Samples were injected (25 µL) onto an Aeris™ PEPTIDE XB-C18 (1.7 µm, 100 × 2.1 mm) with a SecurityGuard ULTRA C18-peptide guard column (Phenomenex, Torrance, CA) using a Nexera UHPLC system (Shimadzu, Columbia, MD). Elution of peptides were performed using a gradient of water (A) and ACN (B), both containing 0.1% FA, at 40°C and a flow rate of 0.3 mL/min. The gradient of elution was as follows: 5% B from 0-2.5 min, increased to 30% in 37.5 min, up to 50% in 2 min, and 90% in 2 min, held for final 3 min. High-resolution mass spectrometry (HRMS) analysis was performed using a TripleTOF 5600+ (quadrupole–time-of-flight) mass spectrometer (Sciex, Concord, ON, Canada) in positive ion electrospray mode. The ion source parameters were set as follows: 30 psi curtain gas, 50 psi nebulizer (nitrogen) and drying (dry air) gases (GS1 and GS2), 500°C source temperature, 5000 V ionspray voltage and 80V declustering potential (DP). Information dependent acquisition (IDA) was acquired from the pooled samples to build an ion library for the quantification. Each cycle consisted of a survey TOF-MS from m/z 140 to 1250, followed by acquisition of MS/MS spectra between m/z 80 and 1500 of the 15 most intense ions, with collision-offset voltage (CE) set to 30 ± 10 V. The total cycle time was 1.05 s. Data was searched against the rat UniProtKB/Swiss-Prot database (February 2022). The search was performed using the ParagonTM Algorithm. The threshold for protein and peptide identifications was set to 1% global FDR. Each sample was analyzed for relative quantitative of proteins using data-independent SWATH acquisition. A single TOF-MS scan (m/z 140-1250) followed by 100 MS/MS experiments with variable Q1 windows acquiring spectra from m/z 80 to 1500, with a total cycle time of 2.7s and source and MS/MS conditions identical to the IDA method described above. Processing parameters were set at up to 4 peptides per protein and 3 transitions per peptide with XIC extraction width of 50 ppm and XIC extraction window of 2 min. Normalized proteins areas (based on total peak areas for each sample), resulting from the sum of all peptide transitions for a given protein, were used for subsequent statistical analyses.

1. Bosoi CR, Parent-Robitaille C, Anderson K, et al (2011) AST-120 (spherical carbon adsorbent) lowers ammonia levels and attenuates brain edema in bile duct-ligated rats. Hepatology 53:1995–2002. https://doi.org/10.1002/hep.24273

S Figure 1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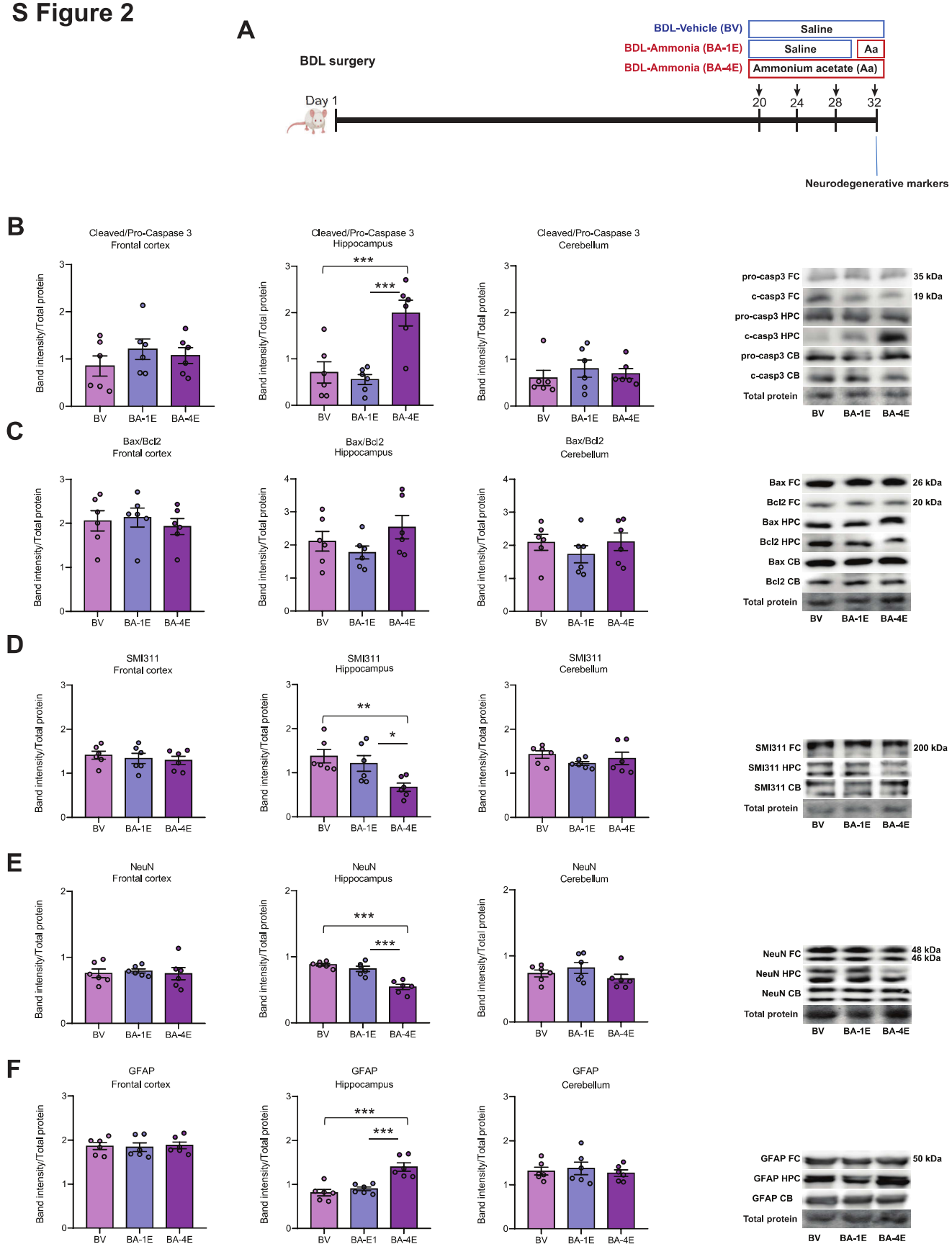
Alanine aminotransferase (ALT)
U/L
150
100
50
0
**
***
SV
SA
BV
BA
B
Aspartate transaminase (AST)
U/L
1000
800
600
400
200
0
***
***
SV
SA
BV
BA
C
Alkaline phosphatase (ALP)
U/L
1500
1000
500
0
***
***
SV
SA
BV
BA
D
Gamma-glutamyl transferase (GGT)
U/L
100
80
60
40
20
0
**
***
SV
SA
BV
BA
E
Bilirubin
umol/L
300
200
100
0
***
***
SV
SA
BV
BA
F
Albumin
g/L
50
40
30
20
10
0
***
***
SV
SA
BV
BA
G
SV
SA
BV
BA
50 μm
H
Total distance traveled
cm
2500
2000
1500
1000
500
0
SV
SA
BV
BA
I
Blood ammonia levels
umol/L
250
200
150
100
50
0
###
***
**
**
n.s
***
SV
SA
BV
BA
Before
1st injection
(day 20)
Before
4th injection
(day 32)

S Figure 2


A
BDL surgery
Day 1
BDL-Vehicle (BV)
BDL-Ammonia (BA-1E)
BDL-Ammonia (BA-4E)
Saline
Saline
Aa
Ammonium acetate (Aa)
20
24
28
32
Neurodegenerative markers
B
Cleaved/Pro-Caspase 3
Frontal cortex
Hippocampus
Cerebellum
Band intensity/Total protein
BV
BA-1E
BA-4E
BA-E1
pro-casp3 FC
c-casp3 FC
pro-casp3 HPC
c-casp3 HPC
pro-casp3 CB
c-casp3 CB
Total protein
35 kDa
19 kDa
C
Bax/Bcl2
Bax FC
Bcl2 FC
Bax HPC
Bcl2 HPC
Bax CB
Bcl2 CB
26 kDa
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D
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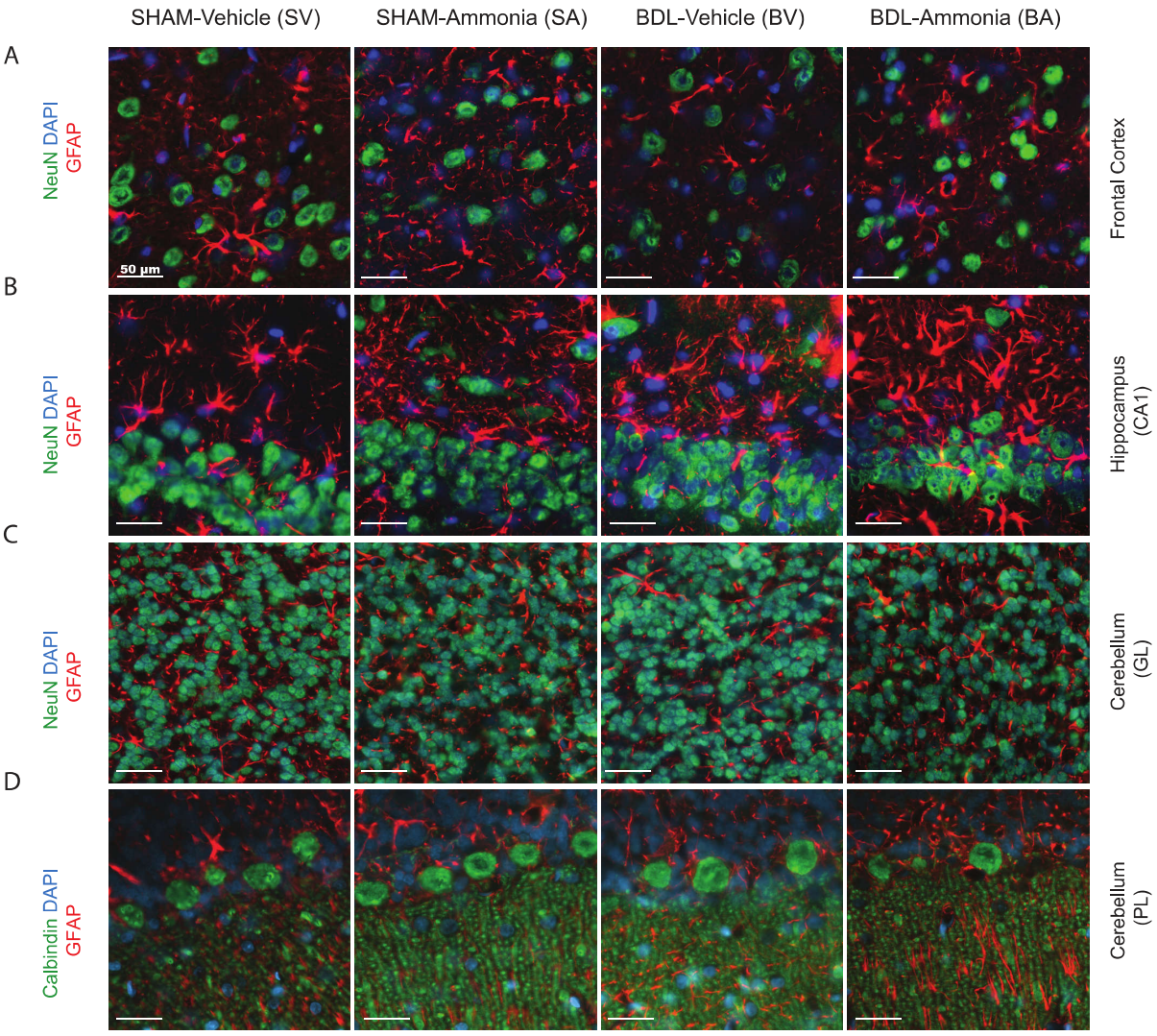
GFAP HPC
GFAP CB
50 kDa

S Figure 3


SHAM-Vehicle (SV)
SHAM-Ammonia (SA)
BDL-Vehicle (BV)
BDL-Ammonia (BA)
A
NeuN DAPI GFAP
50 µm
Frontal Cortex
B
NeuN DAPI GFAP
Hippocampus (CA1)
C
NeuN DAPI GFAP
Cerebellum (GL)
D
Calbindin DAPI GFAP
Cerebellum (PL)

Article 2

This article has been submitted to the Journal of Neuroscience Research (JNR).

Continuous alcohol consumption exacerbates hepatic encephalopathy and provokes neuronal cell death in rats with chronic liver disease

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Keywords: Alcohol, Oxidative stress, Hepatic encephalopathy, Neuronal cell loss, Chronic liver disease

Author contribution: F.T.: study concept and design, acquisition of data, analysis, and interpretation of data, drafting of the manuscript, statistical analysis. X.C.: study concept and design, acquisition of data, analysis, and interpretation of data. M.O.: acquisition of data. M.T.: study concept and design, analysis and interpretation of data, critical revision of the manuscript, technical support. C.F.R., study concept and design, interpretation of data, drafting of the manuscript, critical revision of the manuscript, study supervision, obtained funding.

Data availability: The data that support the findings of this study are available from the corresponding author, C.F.R., upon request.

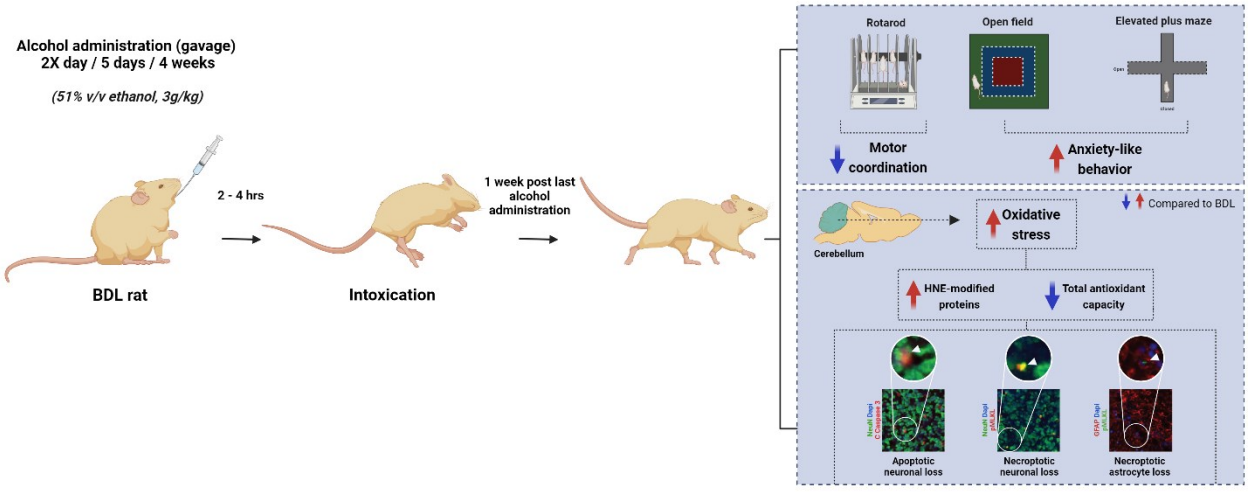
Highlights

- Continuous alcohol consumption in rats with chronic liver disease worsens hepatic encephalopathy.
- Alcohol ingestion during chronic liver disease is associated with a decrease in neuronal markers and an increase in apoptotic and necroptotic markers in the cerebellum of bile-duct ligated rats, indicating neuronal loss/injury.
- Reduced antioxidant capacity and increased 4-HNE modified proteins in the cerebellum suggest that alcohol triggers neuronal loss through oxidative stress pathways.
- These results indicate that alcohol consumption during chronic liver disease provokes neuronal cell loss/injury and exacerbates hepatic encephalopathy, rationalizing neurological dysfunction irreversibility.

Impact and implications

Hepatic encephalopathy (HE) is a common and debilitating cognitive and neurological complication of chronic liver disease (CLD). The pathogenesis of HE is commonly irrelated to the etiological factor of CLD. However, the adverse effect of excessive alcohol consumption on neurological integrity has been described and it has been documented that patients with alcohol-induced cirrhosis develop higher degrees of brain atrophy and lower levels of brain reserve versus patients with non-alcohol-induced cirrhosis. Additionally, patients with alcohol associated liver disease who are transplanted have a higher rate of poorer neurological outcome. Our study indicates alcohol consumption during CLD can sensitize the brain to HE or exacerbate HE, causing neuronal loss which may explain persisting neurological complications following liver transplantation (LT).

Graphical abstract



Abstract

Background and Aims: Hepatic encephalopathy (HE) is defined as decline in neurological function during chronic liver disease (CLD). Alcohol is a major etiological factor in the pathogenesis of fibrosis/cirrhosis and has also been documented to directly impact brain function. However, the role of alcohol in the development of HE in CLD remains unclear. Here, we investigated the impact of continuous alcohol consumption on neurological deterioration in rats with CLD.

Methods: Starting day 7 post-BDL surgery, rats were administered alcohol twice a day (51% v/v ethanol, dose of 3 g/kg, via gavage) for 4 weeks. Motor coordination was assessed weekly using rotarod and anxiety-like behavior was evaluated with open field and elevated plus maze at 5 weeks. Upon sacrifice, brains were collected for western blot and immunohistochemical analyses to investigate neuronal integrity and oxidative stress status in frontal cortex and cerebellum.

Results: Alcohol worsened motor coordination performance and increased anxiety-like behavior in BDL rats. These impairments were associated with decreased neuronal markers of NeuN and SMI311, increased apoptotic markers of cleaved/pro-caspase3 and Bax/Bcl2 ratio, increased necroptosis markers of pRIP3 and pMLKL, decreased total antioxidant capacity and increased 4-HNE (oxidative stress marker) in the cerebellum (not frontal cortex) of BDL-Alcohol rats when compared to respective controls. IHC confirmed the colocalization of cleaved capase-3 and pMLKL (apoptotic and necroptotic markers, respectively) in the granular neurons of the cerebellum of BDL-Alcohol rats.

Conclusion: Continuous alcohol consumption exacerbates HE which leads to associated apoptotic and necroptotic neuronal loss in the cerebellum of BDL-Alcohol rats. Additionally, higher levels of ROS marker of 4-HNE and decreased total antioxidant capacity in the cerebellum of BDL-Alcohol rats, suggest oxidative stress is the triggering factor of apoptotic and necroptotic neuronal loss/injury.

Introduction

Alcohol is a major etiological factor of chronic liver disease (CLD) where compulsive heavy alcohol consumption can consequently lead to progression of fibrosis and cirrhosis, accounting for 30% to 50% of cirrhosis-related deaths [1]. Hepatic encephalopathy (HE) is a neurological complication of cirrhosis, defined as brain dysfunction manifesting from subtle subclinical

alterations to coma [2]. HE develops irrelevant of the etiological factor of CLD and the pathogenesis of HE remains multifactorial with hyperammonemia playing an intricate role.

Alcohol is a neurotoxin and therefore the adverse effects of alcohol on the brain are noteworthy. Alcohol abuse, independent of CLD, can lead to brain atrophy, loss of gray and white matter volume, neuronal injury, and blood-brain barrier dysfunction [3–6]. In the context of CLD, it has been shown that global brain reserve is significantly impaired in alcohol cirrhosis patients compared to non-alcohol cirrhosis patients [7]. Furthermore, histological evaluation of brain samples from patients with alcohol cirrhosis who died in hepatic coma showed greater cerebral degeneration in comparison to non-alcohol induced cirrhosis [8]. It has been reported that alcohol consumption is common in patients living with CLD, with approximately 20%-69% of patients conceding to consuming various degrees of alcohol [9]. Appreciating alcohol's effect on the brain and the development of HE due to cirrhosis, the role and underlying mechanisms of alcohol consumption on the development and progress of HE is not well understood. Therefore, our goal was to the study the impact of continuous alcohol consumption, during the course of CLD, on the development of HE, neurological function, and brain integrity in the well-characterized bile-duct ligated (BDL) rat model of CLD and HE [10].

Material and method

Experimental design

Male Sprague–Dawley rats (n=80, 200–225 g; Charles River) were used. Rats were housed 2–3/cage with free access to food and water in standard conditions. After 48 hours of acclimation, bile duct ligation (BDL) or SHAM surgery was performed as described [11]. Analgesic treatment administrated before and up to 72 hours after the surgery with buprenorphine (s.c. 0.05 mg/kg, every 12 hours), carprofen (s.c. 5 mg/kg, every 24 hours), and gabapentin (p.o., 30 mg/kg every 12 hours). After surgery, to avoid the bias of surgery and cirrhosis complication development, SHAM and BDL rats were randomly assigned to either vehicle or alcohol groups to receive saline or alcohol accordingly. All experiments were approved by the Institutional Animal Care and Use Committee at the CRCHUM (4I15049CRr). Starting day 7 post-surgery, rats were gavaged with a dose of 3 g/kg (3h apart), twice a day, for 5 consecutive days per week, for 4 weeks with alcohol (51% v/v ethanol), controls received saline. Upon sacrifice, plasma and brains were collected and stored at -80°C until analysis. A separate set of animals were perfused with saline followed by 10% formalin before tissue collection; the brain samples

were placed in OCT compound and stored at -80°C for immunofluorescence analysis, and liver samples were stored in 10% formalin for Hematoxylin and Eosin (H&E) staining (Fig. 1).

Behavioral assessment

Intoxication score

Intoxication in animals (5-6 rats per group) was scored based on a 5-point scale (Table 1), modified from Majchrowicz [12], on week 3 (day 16), 4 (day 23) and 5 (day 30) 1- and 4-hours after last alcohol administration.

Rotarod performance test

Rotarod test was conducted to assess motor coordination and motor skill learning. On day 7 post-surgery (pre-alcohol administration), rats (8-12 per group) were placed on the non-rotating rode (7 cm in diameter), which was positioned 40 cm from the device floor for 5 min (ROTOR-ROD™ System, San Diego Instruments, USA). The apparatus was then started with a linear speed increase from 0 to 40 rpm over 5 min. The rat was forced to walk on the rod without falling, and the trial finished when the rat fell. Three trials per rat were performed with a 5-minute interval between each trial. Latency to fall recorded by apparatus and the best performance used for analysis. The same protocol was performed weekly (day 14, 21, 28 and 35; post two full days of wash out/pre-alcohol administration).

Elevated plus maze (EPM)

To assess anxiety like behavior, elevated plus maze (EPM) was used at the end of the model (on day 40). EPM arena is a cross-shaped maze with four 45 cm^2 arms, two open and two closed. The maze consists of three areas: open arms, closed arms, and center. Rats (8-12 per group) were placed in the center, and their ambulatory movements were recorded for 5 min and analyzed with the SMART video tracking system (Panlab SMART v3.0, Harvard Apparatus, USA). Total time (seconds) spent in the closed arms were used to assess anxiety.

Open field test (OF)

One hour after EPM test, the open field (OF) test was conducted to further assess the anxiety like behavior. The open field arena consists of a black square box (90 cm^2). The box was divided into three areas: walls, a center, and an intermediate area. Rats (8-12 per group) were placed in the corner and facing the wall, and their ambulatory movements were recorded for 5

min and analyzed with the SMART video tracking system (Panlab SMART v3.0, Harvard Apparatus, USA). Total time (seconds) spent in the center of the OF were used to assess anxiety.

Blood alcohol content (BAC)

Blood alcohol content were measured weekly (day 16, 23 and 30) 1-, 4-, and 24-hours after alcohol administration. Approximately, 60µl of saphenous vein blood was collected from each animal (4-5 per group) in pre-coated heparin tube. Samples were centrifuged at 4500g for 8 min and supernatant were stored at 4°C until assayed. BAC was assayed using a kit (Megazyme, K-ETOH) in accordance with the manufacturer's recommendations.

Plasma ammonia levels and liver failure markers

Plasma ammonia levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubin, and albumin were measured (6 rats per group) using the COBAS analyzer c111 (Roche, Switzerland).

Western blots

Frontal cortex and cerebellum tissue (6 rats per group) were dissected and homogenized with RIPA buffer supplemented with 1 mM PMSF and protease and phosphatase inhibitor cocktails (Sigma, P8340). The protein concentration was measured using DC protein assay (Bio-Rad Laboratories, 5000111) and 40 µg of protein were loaded on stain-free 10% sodium dodecyl sulphate-polyacrylamide gel and electrophoresed. After, the gels were activated with UV light for 45 sec using the Chemidoc™ imaging system (Bio-Rad Laboratories, 17001401, USA) and transferred on polyvinylidene difluoride membranes. After transfer, the membranes were imaged using ChemiDoc™ for total protein quantification and were blocked with 5% fat-free milk and PBS-T for one hour at room temperature (22°C). Membranes were then incubated in (1:1000) dilution of primary antibodies of NeuN (Millipore, MAB377), caspase-3 (Cell Signaling, 9662S), SMI311 (Millipore, NE1017), Bax (Cell Signaling, 14796S), Bcl2 (Cell Signaling, 3498S), GFAP (Dako, Z0334), 4-HNE (Millipore, 393207), RIP3 (Affinity Biosciences, AF7942), pRIP3 (Affinity Biosciences, AF7443), MLKL (Cell Signaling, 14993) and pMLKL (Affinity Biosciences, AF7420) overnight at 4°C. After, membranes were washed 5 times with PBS-T buffer and incubated with their corresponding secondary antibody coupled to horseradish peroxidase (1:10,000) for one hour at room temperature (22°C). Membranes were exposed to chemiluminescence reagent (Clarity Max Western ECL Substrate, Bio-Rad

Laboratories, 1705062) after 5 washes and probed on the Chemidoc™. Images were quantified with the Image Lab software (Bio-Rad Laboratories, version 6.0.1, USA), and the results were expressed as a ratio of the bands to the total protein.

Caspase-3 enzyme activity assay

Caspase-3 activity in the cerebellum tissue (6 rats per group) was measured in triplicate. Tissues were homogenized by sonication in lysis buffer (1% Triton X-100, 0.32 M Sucrose, 10 mM Tris (pH 8.0), 5 mM EDTA, 2 mM DTT, 1 mM PMSF) and protease and phosphatase inhibitor cocktails (Sigma, P8340). The tissue homogenates were centrifuged at 4°C for 20 min. Enzymatic reactions were undertaken in reaction buffer (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% CHAPS, 1 mM DTT) with 25 µg of protein and 40 µM fluorescent substrate of Ac-DEVD-AMC (Cayman chemicals, 14986). For the negative control caspase-3 inhibitor Ac-DEVD-CHO (Cayman, 10017) was used. Reactions were assessed after a 3-hour incubation (37°C) in the dark and stopped with the addition of 0.4 M NaOH and 0.4 M glycine buffer. Fluorescence was quantified by the Synergy™ HT Multi-Detection Reader (Bio-Tek Instruments, USA) at excitation wavelength 365 nm and emission wavelength 465 nm.

Cerebellar antioxidant capacity

The Antioxidant assay kit (Sigma-Aldrich, CS0790) was used to measure the total antioxidant capacity (TAC). Brain tissue (6 rats per group) was dissected, and the protein lysate was prepared as described in the manual provided by the manufacturer, 100µg of protein were used for the assay.

Immunofluorescence and H&E staining

Brain slices (10 µm) were obtained using a cryostat (Leica, CM3050S, Germany) and blocked with PBS-0.5% Triton X–100 and 10% goat serum for 30 min. After, they were washed 3 times with PBS and incubated in (1:250) dilution of primary antibodies: cleaved caspase-3 (Cell Signaling, 9579), NeuN (Millipore, MAB377), GFAP (Sigma, G3893), Calbindin (Invitrogen, MA5-24135) and pMLKL (Affinity Biosciences, AF7420) overnight at 4°C. Following 3 washes with PBS and incubated with corresponding secondary antibodies (donkey anti-mouse IgG coupled to Alexa488 fluorophore 1:200, Jackson Immuno Research, 715-545-150 or donkey anti-rabbit IgG coupled to Cyanine Cy3™ fluorophore, 1:200, Jackson Immuno Research, 711-165-152) for 1 hour, in the dark, at room temperature (22°C). After, slides were washed with PBS and DAPI (4',6-diamidino-2-phenylindole) was used (1 µg/ml) and rinsed

with PBS. After, the slides were covered with mounting media and coverslips. For assessing the liver histology, fixed liver tissue was blocked in paraffin, sliced (4μm) and stained with hematoxylin and eosin. The images were obtained with ZEISS Axio Imager M2 and ZEN 3.4 software (ZEISS, Germany).

Statistical analysis

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

Results

Weight gain

Both BDL-Vehicle and BDL-Alcohol rats demonstrated a significant reduction in weight starting from week 2 after BDL surgery compared to respective SHAMs ($p<0.001$). No significant differences in weight throughout 6 weeks were found between BDL-Vehicle and BDL-Alcohol (Fig. 2A).

Liver failure markers

Liver injury markers ALT, AST, ALP, GGT, and bilirubin were all significantly higher in both BDL-Vehicle and BDL-Alcohol rats when compared to respective SHAMs ($p<0.001$), with no significant difference found between BDL-Vehicle vs BDL-Alcohol and SHAM-Alcohol vs SHAM-Vehicle groups (Fig. 2B). Plasma albumin levels were found significantly lower in both BDL-Vehicle and BLD-Alcohol rats vs respective SHAMs ($p<0.001$), with no significant difference found between the 2 BDL experimental groups (Fig. 2C). Using hematoxylin and eosin staining, liver damage was observed in both BDL-Vehicle vs BDL-Alcohol groups vs respective SHAMs, but no additional differences were found in BDL-Alcohol vs BDL-Vehicle rats (Fig. 2E).

Plasma ammonia levels

Plasma ammonia levels were significantly increased in BDL-Vehicle and BDL-Alcohol rats compared to respective SHAMs ($p<0.05$ and $p<0.001$, respectively). However, there was no significant difference found between BDL-Vehicle and BDL-Alcohol rats (Fig. 2D).

Intoxication status

Acute alcohol administration led to delayed and/or loss of righting reflex, hypoactivity and ataxia symptoms in both BDL and SHAM rats. Intoxication status was scored 1- and 4-hours following alcohol administration at weeks 3 (day 16), 4 (day 23) and 5 (day 30). BDL rats overall developed more severe symptoms when compared to SHAM rats following alcohol administration. At week 4 and 5, greater than 20% of BDL rats lost their righting reflex (intoxication score of 4 based on scoring scale provided in Table 1) whereas loss of righting reflex was never observed in SHAM rats (Fig. 3A).

Blood alcohol content (BAC)

Blood alcohol content (BAC) measured on days 16, 23 and 30 at 1- and 4-hours following alcohol administration revealed similar levels (no significant difference) between SHAM-Alcohol and BDL-Alcohol rats. BAC levels in both BDL-Alcohol and SHAM-Alcohol were not detectable 24 hours after alcohol administration on all testing days (Fig. 3B).

Anxiety-like behavior and motor coordination

Anxiety-like behavior assessment (using EPM) showed a significant increase in the time spent in closed arms in both BDL-Vehicle and BDL-Alcohol rats when compared to respective SHAM rats ($p<0.05$), with a significant difference between BDL-Vehicle and BDL-Alcohol rats ($p<0.05$) (Fig. 3C).

In the OF test, the total distance traveled was not significantly different in both BDL-Vehicle and BDL-Alcohol when compared to respective SHAM rats. However, a significant decrease in time spent in the center was found for BDL-Alcohol rats compared to respective SHAM rats ($p<0.05$), which was not significantly different compared to BDL-Vehicle rats (Fig. 3D).

No significant differences were observed in motor coordination (rotarod) between all 4 groups one week after BDL/SHAM surgery (before alcohol/saline administration). Moreover, no significant differences in motor coordination were found in BDL-Vehicle rats through week 1 to 5 when compared to respective SHAMs. Alcohol did not significantly alter motor coordination performance in SHAM-Alcohol rats. However, alcohol administration impaired motor coordination (decrease in latency to fall) in BDL-Alcohol rats compared to respective SHAMs through week 2 to 5 ($p<0.001$) (Fig. 3E). Longitudinal analysis showed motor skill performance decline was solely observed in BDL-Alcohol rats at week 4 and 5 compared to week 1 ($p<0.05$ and $p<0.01$, respectively) (Fig. 3F).

Neuronal and astrocyte loss/injury

Western blot analysis revealed a significant decrease in expression levels of neuronal markers NeuN ($p<0.01$) and SMI311 ($p<0.05$) in the cerebellum of BDL-Alcohol rats compared to SHAM-Alcohol rats, with significant difference found between BDL-Vehicle and BDL-Alcohol ($p<0.05$). Similar differences were not found in frontal cortex. Expression levels of the astrocytic marker GFAP were decreased in BDL-Alcohol rats compared to SHAM-Alcohol and BDL-Vehicle rats in the cerebellum ($p<0.05$). No significant changes in GFAP protein expression were found between any groups in frontal cortex (Fig. 4A-B).

Cerebellar oxidative stress

Oxidative stress levels in the frontal cortex and cerebellum were analyzed with 4-HNE western blotting, showing a significant increase in 4-HNE levels in cerebellum of BDL-Alcohol rats when compared to both SHAM-Alcohol and BDL-Vehicle rats ($p<0.05$ and $p<0.05$, respectively). Similar changes were not observed in frontal cortex (Fig. 4C). In addition, total antioxidant capacity was significantly lower in the cerebellum of BDL-Alcohol rats when compared to SHAM-Alcohol and BDL-Vehicle rats ($p<0.001$ and $p<0.001$, respectively). In frontal cortex, total antioxidant capacity did not significantly differ between all groups (Fig. 4D).

Apoptosis and necroptosis

Additionally, an increase of caspase-3 enzyme activity was found in the cerebellum of BDL-Vehicle and BDL-Alcohol compared to respective SHAM rats ($p<0.01$) and a significantly higher difference in activity was found in BDL-Alcohol rats compared to BDL-Vehicle rats ($p<0.001$) (Fig. 4E). Moreover, higher protein levels of the apoptotic marker of cleaved/pro caspase-3 were found in the cerebellum of BDL-Vehicle and BDL-Alcohol rats compared to respective SHAM rats ($p<0.05$) with significantly higher levels found in BDL-Alcohol compared to BDL-Vehicle ($p<0.001$). Increased protein levels of Bax/Bcl2 ratio were found in the cerebellum in both BDL-Vehicle and BDL-Alcohol groups compared to respective SHAM rats ($p<0.01$), with no significant differences observed between vehicle and alcohol BDL groups (Fig. 4F).

In all 4 experimental groups, necroptosis markers of RIP-3 and MLKL were not significantly altered in the cerebellum. However, pRIP3 and pMLKL levels were significantly increased in the cerebellum of the BDL-Alcohol rats when compared to respective SHAM rats ($p<0.01$ and

$p<0.001$, respectively), which were significantly higher in BDL-Alcohol rats compared to BDL-Vehicle rats ($p<0.01$ and $p<0.001$, respectively) (Fig. 4G).

To further support our data, we performed immunohistochemistry to identify apoptotic and necroptotic cells using markers of cleaved caspase-3 and pMLKL respectively, along with the neuronal marker NeuN, Purkinje neuron marker calbindin, and astrocytic marker GFAP in the cerebellum. Our microscopic findings showed colocalization of cleaved caspase-3 with NeuN in the granular layer of the cerebellum in BDL-Alcohol rats, which was not found in BDL-Vehicle rats (Fig. 5A). Furthermore, no colocalization of cleaved caspase-3 with Purkinje neurons (calbindin) was found in the cerebellum of any of the experimental groups (Fig. 4B). Additionally, cleaved caspase-3 was found to be co-localized with GFAP in Purkinje layer in both BDL-Vehicle and BDL-Alcohol cerebellum (Fig. 5B-C). pMLKL was solely found in granular layer in the BDL-Alcohol group with a co-localizing with NeuN and GFAP (Fig. 5D-F).

Discussion

In this study, using the BDL rat, a well-established animal model of CLD and HE [10], we investigated, for the first time, the effect of continuous alcohol consumption on development of HE and brain integrity. Our results show that continuous alcohol administration aggravates HE with increased anxiety-like behavior and a further decline in motor coordination. We found neuronal death in the cerebellum in the BDL-Alcohol group which was not observed in the BDL-Vehicle group, whereas astrocyte loss was observed in both BDL experimental groups. The implicated apoptotic and necroptic cell death pathways were associated with cerebral oxidative stress with higher levels of 4-HNE and decreased levels of TAC. The results depict alcohol consumption during CLD via pathological oxidative stress, could sensitize the brain to HE and/or exacerbate HE and lead to cell death which could elucidate permanent cell injury observed in patients with alcohol-cirrhosis.

In our study, continuous alcohol administration did not worsen the progression of liver disease since no significant changes in plasma liver enzymes (ALT, ALP, AST, and GGT), albumin, and bilirubin levels were reported in BDL-Alcohol rats when compared to BDL-Vehicle rats. Plasma ammonia levels were also unchanged between the BDL experimental groups. In addition, liver histological analysis was similar in BDL-Alcohol and BDL-Vehicle. SHAM rats administered alcohol did not develop any signs of liver injury (no significant increase in liver enzymes or change in histology). This was expected since livers of rats are resistant to alcohol

toxicity [13] and only long-term alcohol administration (7 to 15 weeks) leads to steatosis with limited inflammation, fibrosis, or liver injury [14].

Each acute alcohol administration led to a similar spike in blood alcohol content (BAC) in both SHAM and BDL rats at 1 and 4 hours post-administration during weeks 3, 4, and 5. Blood alcohol was cleared from the blood in both groups at 24 hours. However, even though BAC similarly increased in both BDL and SHAM rats, BDL rats had a stronger reaction exhibiting loss of righting reflex, while SHAM rats only showed hypoactivity and delayed righting reflex. This confirms the higher intoxication score in BDL-Alcohol vs SHAM-Alcohol was not solely a result of elevated blood alcohol content. These results suggest that as disease progresses and HE develops in BDL rats, alcohol administration induces a more severe effect on the brains of BDL rats. This depicts alcohol as a potential contributor in the development and worsening of HE BDL rats.

To further investigate the effect of continuous alcohol consumption on development of HE, we performed a battery of behavioral tests to assess anxiety-like behavior and motor coordination one week following the final alcohol administration. Our group has previously shown that BDL rats demonstrate higher anxiety-like behavior [15] and impaired motor coordination [16]. In the present study, the open field and elevated plus maze tests demonstrated a significant increase in anxiety-like behavior in BDL-Alcohol rats when compared BDL-Vehicle rats and respective SHAM groups. Moreover, rotarod motor coordination assessment revealed decreased performance at week 4 in BDL-Alcohol rats compared to BDL-Vehicle and at week 2 to 5 compared to respective SHAM rats. The longitudinal analysis showed that motor coordination decreased solely in BDL-Alcohol rats at week 4 and 5 when compared to week 1. Collectively, these results suggest alcohol exacerbates HE, possibly sensitizing the brain in BDL rats. Interestingly, continuous alcohol administration did not result in behavioral alterations in SHAM rats compared to vehicle. Alcohol administration has been shown to impact the brain in naive rats as Teixeira et al., (2014), demonstrated that alcohol administration (gavage 6.5 g/kg/day, 22.5% w/v) for 55 days resulted in considerable impairments in their motor abilities, as well as reduced spontaneous movement and coordination problems [17]. Therefore, this suggests longer periods of alcohol administration may be required to induce a change in neurophenotype. Moreover, it is important to note that in our study, we assessed behavior 7 days following last alcohol administration whereas Teixeira et al., conducted their tests 24 hours following last alcohol consumption and so the alcohol washout period could play a major role in the difference in findings.

A study published by Kril and Butterworth showed a higher prevalence of neurodegeneration in alcoholic patients with cirrhosis who died while in hepatic coma compared to non-alcoholics [8]. Therefore, we decided to investigate the impact of alcohol on the brain in CLD, focusing on the frontal cortex and cerebellum; two regions related to the significant alterations found in the behavior assessments of BDL-Alcohol rats. The frontal cortex plays a crucial role in personality characteristics, decision-making, muscle control and movement and the cerebellum is involved in the coordination of voluntary muscle movements and in maintaining posture, balance, and equilibrium. Using two distinct neuronal markers (NeuN and SMI311) and astrocytic marker (GFAP), we demonstrated there was a decrease in protein levels of NeuN and SMI311 in the cerebellum (not in frontal cortex) solely in the BDL-Alcohol group compared to all other groups. In addition, in the cerebellum, GFAP expression was significantly decreased in BDL-Alcohol compared to respective SHAMs. Our results indicate alcohol consumption leads to cell loss in the cerebellum of BDL rats. This supports the observations where patients with alcohol liver disease exhibit reduced brain reserve, greater brain edema and cortical damage compared to nonalcoholic patients [7] and global brain atrophy is more frequently associated with alcoholic-related liver disease [18].

Alcohol is absorbed rapidly through digestive tract and uniformly diffused throughout all fluids and tissues [19]; however, the absorbed alcohol is primarily metabolized by the liver [20]. It has been well documented that alcohol provokes oxidative stress which is directly associated with the metabolism of alcohol. In the liver, alcohol is primarily metabolized via alcohol dehydrogenase (ADH), a cytosolic enzyme primarily expressed in the liver. ADH catalyzes alcohol and forms acetaldehyde by transferring H^+ from the OH group to the nicotinamide cofactor of nicotinamide adenine dinucleotide (NAD^+) reducing it to NADH [21]. The two minor additional pathways of alcohol metabolism by microsomal ethanol oxidizing system (cytochrome P450 2E1) and catalase also lead to acetaldehyde generation [22]. Acetaldehyde is a highly reactive molecule and is oxidized to acetate by mitochondrial acetaldehyde dehydrogenase (ALDH2) resulting in generation of NADH. Since alcohol can easily cross the BBB, it has been shown to directly affect numerous molecular targets in the brain, including synaptic function and transmission [23]. Moreover, in the brain, it has been shown that alcohol can suppress glucose metabolism and therefore acetate can be used as an energy source [24]. However, excess acetate can impact several metabolic processes, impacting brain function [25].

In this study we found higher levels of 4-HNE, the end-product of lipid peroxidation in the cerebellum of BDL-Alcohol rats. Similar results were not found in the frontal cortex. It is known that an increase in alcohol-induced NADH/NAD^+ ratio leads to ROS production [26]. As ROS increases, lipid peroxidation, a destructive process in which free radicals attack and damage the polyunsaturated fatty acids, occurs. Moreover, ROS regulation/elimination requires the presence of essential endogenous antioxidants, including superoxide dismutase, glutathione peroxidase, and reduced glutathione [27]. It has also been reported that alcohol-induced lipid peroxidation is associated with decrease in intracellular glutathione and antioxidant capacity [28]. We found a significant reduction in total antioxidant capacity in the cerebellum of BDL-Alcohol rats with similar results not found in the frontal cortex. Therefore, alcohol consumption not only leads to increased generation but also a reduced capacity to remove of ROS.

The impact of prolonged oxidative stress has been shown to be associated with neurodegeneration and neurodegenerative disease such as Parkinson's and Alzheimer diseases [29–31]. It is reported that lipid peroxidation (4-HNE) has been associated with a decrease in neuronal viability [32, 33]. Moreover, 4-HNE has been shown to contribute to mitochondrial permeability and cytochrome C release, as well as inducing apoptosis and necroptosis [34, 35]. Furthermore, *in vitro* studies have demonstrated that alcohol activates ROS production and induces oxidative damage [32, 36–38].

Caspase-3 as well as Bax/Bcl2 play a role in apoptotic signaling pathways and are commonly utilized as biomarkers to assess neurodegeneration [39, 40]. Moreover, RIP3 and MLKL serve as specific markers for studying necroptotic cell death [41]. In the cerebellum we measured significantly higher levels of caspase-3 enzyme activity in BDL-Vehicle and BDL-Alcohol rats (compared to respective SHAMs). However, these levels were further increased in BDL-Alcohol rats. Additionally, cleaved caspase-3 levels measured with western blot showed significantly higher increase in both BDL groups (compared to respective SHAMs). However, these levels were further increased in BDL-Alcohol rats, suggesting apoptosis in the cerebellum of both BDL-Vehicle and BDL-Alcohol rats. Phosphorylated RIP3 and MLKL both were found significantly increased only in the cerebellum of BDL-Alcohol rats. This indicates both necroptotic and apoptotic cell death mechanisms in the cerebellum of BDL-Alcohol rats.

To identify which cells were undergoing apoptosis and necroptosis, using immunofluorescence, we co-stained cleaved caspase-3 (apoptotic marker) and pMLKL

(indicator of necroptosis) with NeuN (neuronal marker). Our results showed colocalization of cleaved caspase-3 and pMLKL in the neurons located in the granular layer of BDL-Alcohol rats' cerebellum, suggesting alcohol induced apoptotic and necroptotic neuronal cell loss in the cerebellum of BDL rats. Overall, this suggests a potential link between elevated ROS levels and neuronal cell loss in the cerebellum of BDL-Alcohol rats. Our findings indicate that continuous alcohol consumption exacerbates HE and causes neuronal injury primarily in the cerebellum. Looking at cell death pathways in astrocytes, using immunofluorescence analysis, we observed the colocalization of pMLKL (necroptotic marker) with GFAP solely in the cerebellum of BDL-Alcohol rats, suggesting alcohol induces necroptotic cell death in astrocytes. Interestingly, cleaved caspase-3 (apoptotic marker) was localized with GFAP (astrocytic maker) in the cerebellum of both BDL-Vehicle and BDL-Alcohol. This combination of different cell death pathways remains to properly investigated. Historically, HE has been characterized with metabolic astrocyte dysfunction with astrocyte swelling, Alzheimer Type II astrocytosis and senescence [42–45]. However, under certain pathological conditions, cell loss/death can occur. Higher levels of cerebral degeneration, characterized by loss of Purkinje and granule cells, were reported in the cerebellum of patients who died during hepatic coma with end-stage alcohol liver disease vs non-alcohol liver disease [8]. Interestingly, a study in postmortem human brains found a statistically significant loss of astrocytes and oligodendrocytes in the hippocampus of alcoholics [46]. Another study showed that long-term *in vivo* chronic alcohol exposure decreases GFAP, in the cerebellum of rats [47]. Our results suggest that continuous alcohol consumption during CLD can lead to cell death, both astrocytes and neurons.

Historically, HE considered as a reversible syndrome in which neurological impairments should normalize following LT, a widely accepted and established standard of care for end-stage liver disease patients [48]. However, the assessment period pre-LT for those with end-stage alcoholic liver disease raises difficult questions. Usually, a period of abstinence lasting six months or longer is required for someone to be eligible for LT, although this criteria remains a topic of debate [49, 50]. Multiple studies have indicated that alcohol liver disease patients experience poor outcomes post-LT if they continue consuming alcohol [51–53]. Garcia-Martinez et al., found that a higher frequency of poor neurological outcomes following LT was observed in alcohol liver disease patients [54]. However, the impact of chronic alcohol consumption on the brain leading up to LT is not well understood. Our results demonstrate the negative impact of alcohol on the development of HE which is supported by the study of

Ahluwalia et al., who reported that alcohol liver disease patients have higher proportion of prior history of HE and worse cognition [7].

Conclusion

The results of our study suggest that continuous alcohol consumption in the context of CLD not only influences HE but also leads to irreversible neuronal injury/loss. This depicts that continuous alcohol consumption in patients with cirrhosis may cause cell death and therefore irreversibility which could consequently lead to poor neurological outcome following LT.

Table 1: Intoxication status

Score	Definition
1	Hypoactive
2	Ataxia
3	Ataxia with dragging abdomen, delayed righting reflex
4	Loss of righting reflex
5	Loss of righting reflex and loss of eye-blink reflex

Figure legends

Figure 1. Experimental design.

Figure 2. Weight gain, ammonia, and liver failure markers. (A) The weight gain remained unchanged in BDL-Alcohol compared to BDL-Vehicle (*p<0.05 and **p<0.01, and ***p<0.001 vs respective SHAM). (B) Alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, bilirubin, and alkaline phosphatase were significantly higher and (C) Albumin levels were significantly lower in all BDL rats (*p<0.05 and ***p<0.001 vs respective SHAM). (D) Ammonia levels increased in all BDL rats and remained unchanged compared to BDL-Vehicle (*p<0.05 and **p<0.01 vs respective SHAM). (F) Hematoxylin and eosin staining in livers of BDL-Vehicle, and BDL-Alcohol show the same level of liver damage.

Figure 3. Intoxication status, blood alcohol content, and behavioral assessment. (A) BDL-Alcohol rats showed more severe symptoms and received higher intoxication scores in 1- and

4 hours after alcohol administration during week 3, 4 and 5 after BDL surgery compared to SHAM-Alcohol rats. (B) Blood alcohol content (BAC) in week 3, 4 and 5 was not significantly different between SHAM-Alcohol and BDL-Alcohol 1- , 4- , and 24 hours after alcohol administration. (C) Time spent in closed arms of elevated plus maze was significantly higher in BDL-Alcohol rats (*p<0.05 vs respective SHAM, #p<0.05 vs BDL-Vehicle) (D) Time spent in the center of open field was significantly lower in BDL-Alcohol (*p<0.05 vs respective SHAM) (E) Latency to fall significantly decreased in BDL-Alcohol rats (**p<0.01 and ***p<0.001 vs respective SHAM, #p<0.05 vs BDL-Vehicle) (F) Latency to fall significantly decreased in BDL-Alcohol rats (*p<0.05 and **p<0.01 vs week 1).

Figure 4. Neuronal loss, oxidative stress, apoptosis, and necroptosis. Protein expression levels of NeuN, SMI311 and GFAP remained unchanged in (A) Frontal cortex and decreased significantly in (B) Cerebellum of BDL-Alcohol rats (*p<0.05 and **p<0.01 vs respective SHAM, #p<0.05 vs BDL-Vehicle). (C) 4-HNE levels showed a significantly higher expression only in the cerebellum of BDL-Alcohol rats (*p<0.05 vs respective SHAM, #p<0.05 vs BDL-Vehicle). (D) Total antioxidant capacity was significantly lower in the cerebellum of BDL-Alcohol rats (***p<0.001 vs respective SHAM, ###p<0.001 vs BDL-Vehicle). (E) Caspase-3 enzyme activity increased in the cerebellum of BDL rats; however, BDL-Alcohol rats had higher levels compare to BDL-Vehicle (**p<0.01 and ***p<0.001 vs respective SHAM, ###p<0.001 vs BDL-Vehicle). (F) Apoptotic markers of cleaved/pro-caspase-3 and Bax/Bcl2 ratio significantly increased in BDL-Alcohol cerebellum (*p<0.05 and **p<0.01, and ***p<0.001 vs respective SHAM, ###p<0.001 vs BDL-Vehicle). (G) Necroptotic markers of pRIP3 and pMLKL significantly increased in BDL-Alcohol cerebellum (**p<0.01 and ***p<0.001 vs respective SHAM, #p<0.05 and ###p<0.001 vs BDL-Vehicle).

Figure 5. Immunohistochemistry. (A) Co-staining of cleaved caspase-3 (apoptotic marker) with NeuN (neuronal marker) in the granular layer of cerebellum showed colocalization of apoptotic and neuronal markers only in BDL-Alcohol group. (B) Colocalization of cleaved caspase-3 and calbindin (a marker of Purkinji neurons) was not observed in the Purkinje layer. (C) Co-staining of cleaved caspase-3 and GFAP (astrocytic marker) in the cerebellum showed colocalization of apoptotic marker with astrocytes in both BDL-Vehicle and BDL-Alcohol groups. (D) Co-staining of pMLKL (marker of necroptosis) with NeuN in the granular layer of cerebellum showed colocalization of apoptotic and neuronal markers only in BDL-Alcohol group. (F) Colocalization of pMLKL and calbindin was not observed in the Purkinje layer. (G)

Co-staining of pMLKL and GFAP in the cerebellum showed colocalization of apoptotic marker with astrocytes only in BDL-Alcohol groups.

Abbreviations

HE: Hepatic encephalopathy

LT: Liver transplantation

CLD: Chronic liver disease

BDL: Bile duct ligation

BAC: Blood alcohol content

ROS: Reactive oxygen species

CNS: Central nervous system

TAC: Total antioxidant capacity

4-HNE: 4-Hydroxynonenal

SMI311: Anti-neurofilament marker

NeuN: Neuron-specific nuclear antigen

GFAP: Glial fibrillary acidic protein

RIP3: Receptor-interacting serine/threonine-protein kinase 3

MLKL: Mixed lineage kinase domain-like protein

ALT: Alanine transfernase

AST: Aspartate transferase

ALP: Alkaline phosphatase

GGT: Gamma-glutamyl transferase

Acknowledgements

The authors thank the behavior phenotyping core facilities of CRCHUM for behavior instruments. The authors gratefully acknowledge the financial support of Canadian Institutes of Health Research (CIHR). Some figures and graphical abstract are created with BioRender.com.

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Figure 1

A

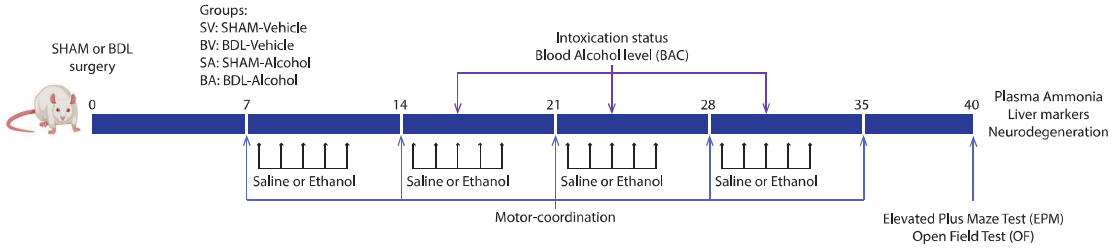

SHAM or BDL surgery
Groups:
SV: SHAM-Vehicle
BV: BDL-Vehicle
SA: SHAM-Alcohol
BA: BDL-Alcohol
Intoxication status
Blood Alcohol level (BAC)
0
7
14
21
28
35
40
Plasma Ammonia
Liver markers
Neurodegeneration
Saline or Ethanol
Saline or Ethanol
Saline or Ethanol
Saline or Ethanol
Motor-coordination
Elevated Plus Maze Test (EPM)
Open Field Test (OF)

Figure 2

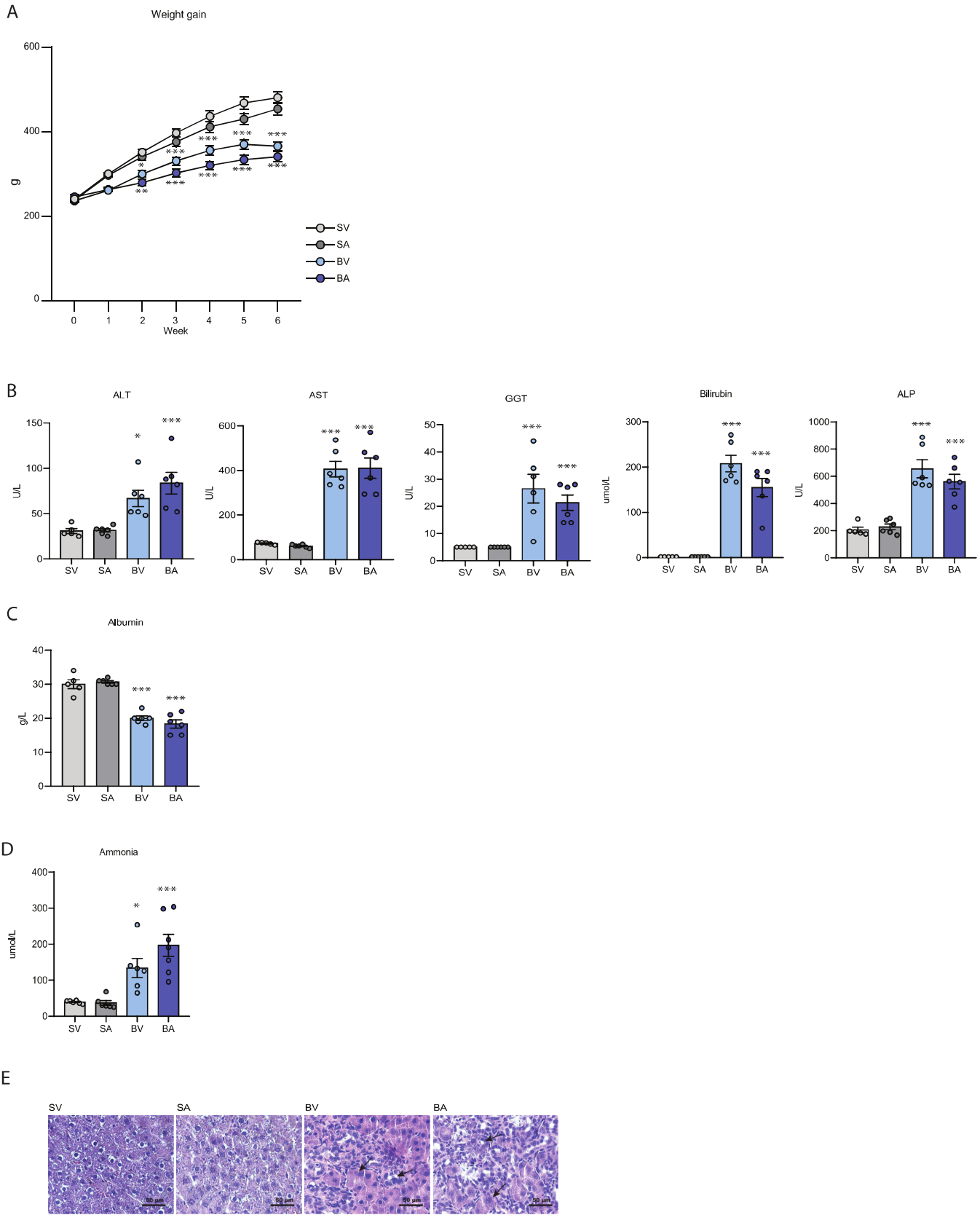
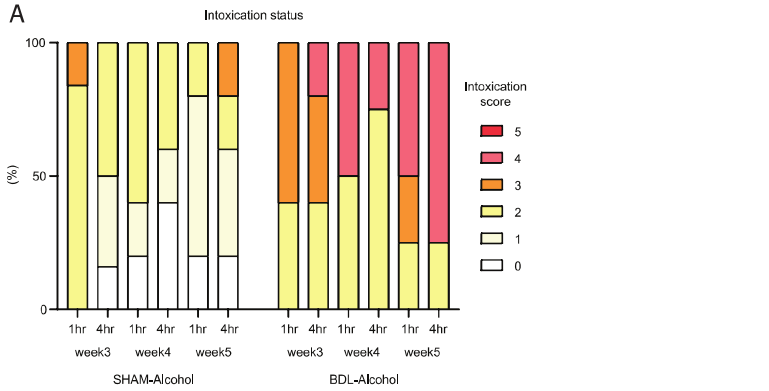
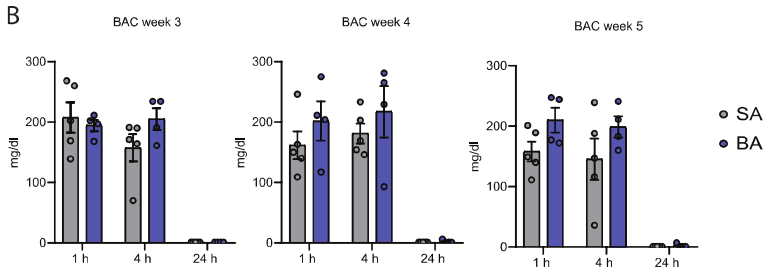
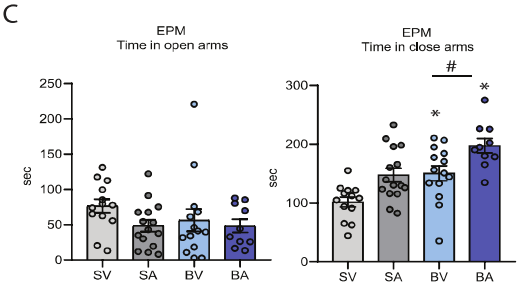
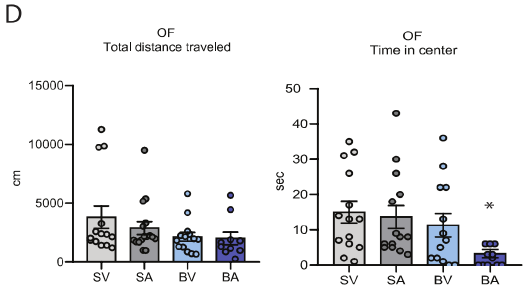

A
Weight gain
g
Week
SV
SA
BV
BA
B
ALT
AST
GGT
Bilirubin
ALP
U/L
umol/L
C
Albumin
g/L
D
Ammonia
umol/L
E
SV
SA
BV
BA

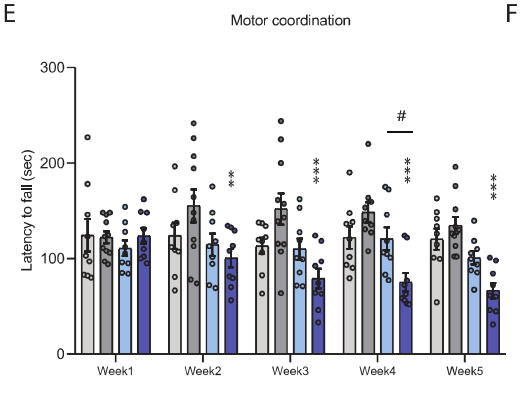
Figure 3


A
Intoxication status
100
50
0
(%)
1hr 4hr 1hr 4hr 1hr 4hr
week3 week4 week5
SHAM-Alcohol
1hr 4hr 1hr 4hr 1hr 4hr
week3 week4 week5
BDL-Alcohol
Intoxication score
5
4
3
2
1
0


B
BAC week 3
BAC week 4
BAC week 5
mg/dl
300
200
100
0
1 h
4 h
24 h
SA
BA


C
EPM
Time in open arms
sec
250
200
150
100
50
0
SV SA BV BA
EPM
Time in close arms
#
*
*
300
200
100
0
SV SA BV BA


D
OF
Total distance traveled
cm
15000
10000
5000
0
SV SA BV BA
OF
Time in center
sec
50
40
30
20
10
0
*
SV SA BV BA


E
Motor coordination
Latency to fall (sec)
300
200
100
0
#
**
***
***
***
Week1 Week2 Week3 Week4 Week5

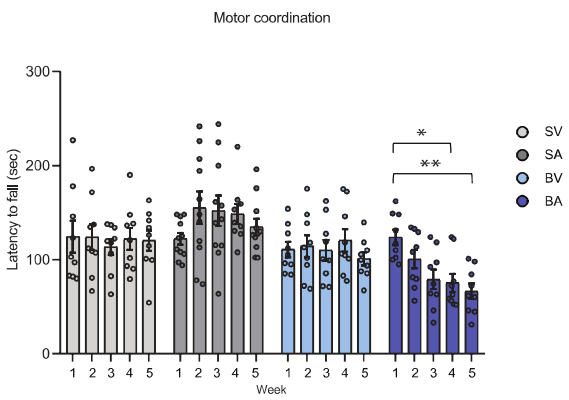

F
Motor coordination
Latency to fall (sec)
300
200
100
0
*
**
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Week
SV
SA
BV
BA

Figure 4

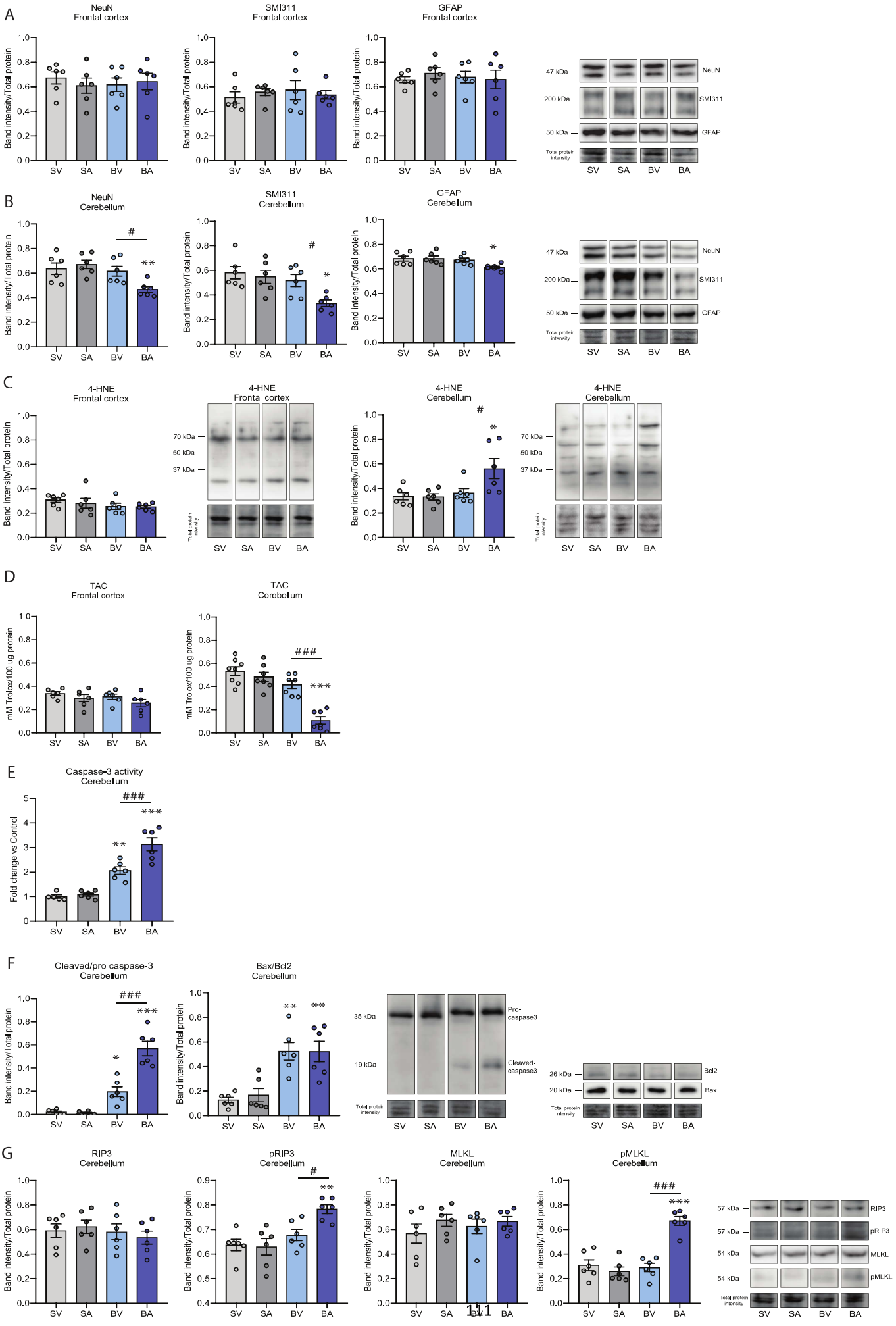
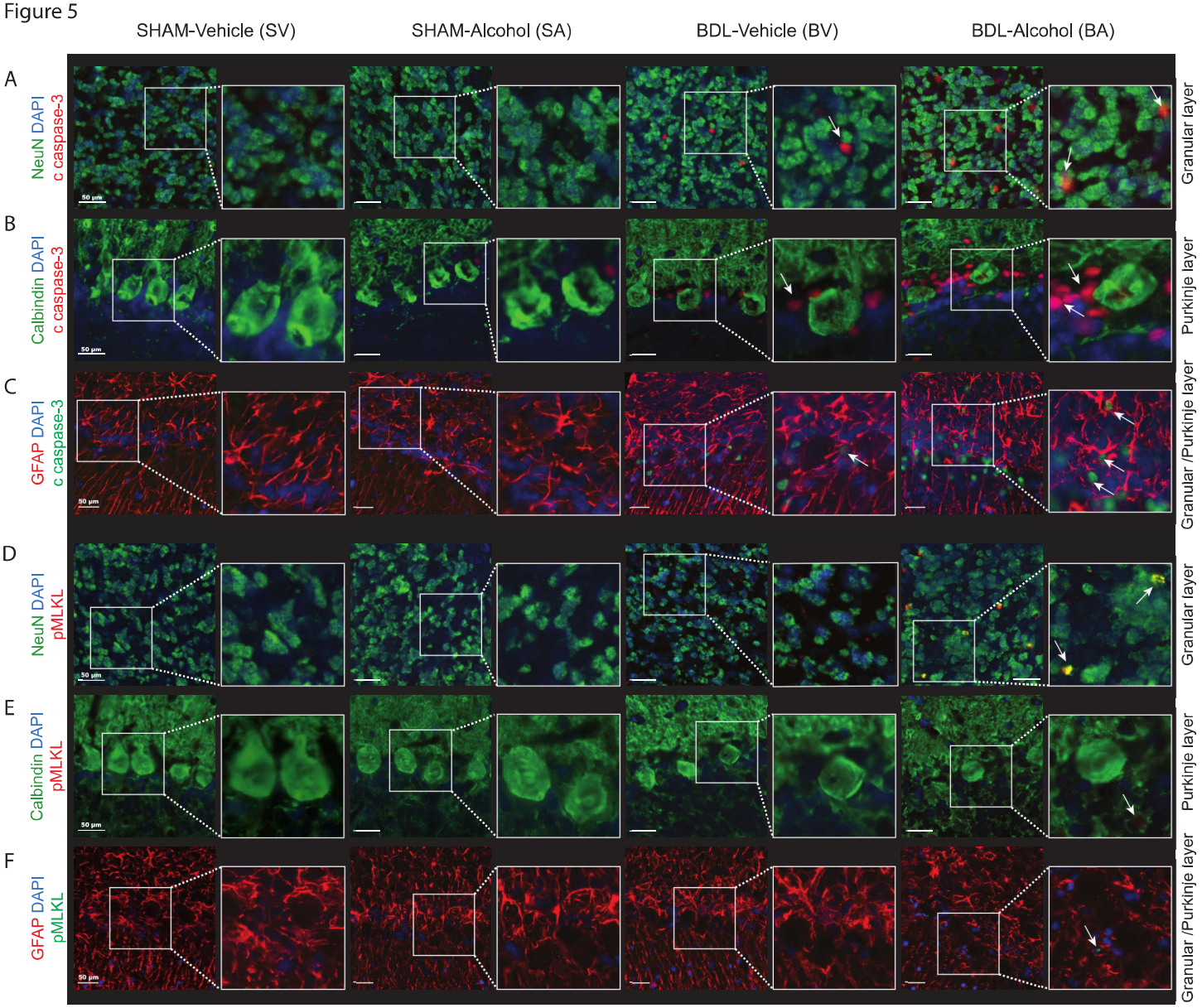

A
NeuN
Frontal cortex
SMI311
Frontal cortex
GFAP
Frontal cortex
Band intensity/Total protein
SV
SA
BV
BA
47 kDa
200 kDa
50 kDa
Total protein intensity
NeuN
SMI311
GFAP
B
NeuN
Cerebellum
SMI311
Cerebellum
GFAP
Cerebellum
#
*
**
C
4-HNE
Frontal cortex
4-HNE
Cerebellum
70 kDa
50 kDa
37 kDa
D
TAC
Frontal cortex
TAC
Cerebellum
mM Trolox/100 ug protein
###
***
E
Caspase-3 activity
Cerebellum
Fold change vs Control
F
Cleaved/pro caspase-3
Cerebellum
Bax/Bcl2
Cerebellum
35 kDa
19 kDa
Pro-caspase3
Cleaved-caspase3
26 kDa
20 kDa
Bcl2
Bax
G
RIP3
Cerebellum
pRIP3
Cerebellum
MLKL
Cerebellum
pMLKL
Cerebellum
57 kDa
54 kDa
RIP3
pRIP3
MLKL
pMLKL

Figure 5


SHAM-Vehicle (SV)
SHAM-Alcohol (SA)
BDL-Vehicle (BV)
BDL-Alcohol (BA)
A
NeuN DAPI
c caspase-3
Granular layer
B
Calbindin DAPI
c caspase-3
Purkinje layer
C
GFAP DAPI
c caspase-3
Granular /Purkinje layer
D
NeuN DAPI
pMLKL
Granular layer
E
Calbindin DAPI
pMLKL
Purkinje layer
F
GFAP DAPI
pMLKL
Granular /Purkinje layer

Highlights of the study

- Multiple episodes of OHE as well as continuous alcohol consumption in BDL rats leads to cerebral cell loss.
- Multiple episodes of OHE provoke neuronal loss in the hippocampus whereas constant alcohol consumption resulted in worsening of HE and neuronal and astrocyte loss in the cerebellum of BDL rats.
- In both studies higher levels of oxidative stress markers and decreased levels of total antioxidant capacity in the specific brain region (hippocampus in episodic OHE model and cerebellum in alcohol model) were found which suggests oxidative stress-related cell death.
- These results suggest that certain conditions, multiple episodes of OHE and constant alcohol consumption, can result in permanent brain cell damage (in different regions respectively) and therefore less likely to be reversible following treatment or LT.

Discussion

Multiple ammonia-induced episodes of OHE leads to neuronal loss in BDL rats

In our study, we utilized an animal model of CLD (BDL) in combination with multiple ammonia injections (as the most common precipitating factor of OHE) to examine the effect of multiple ammonia-induced episodes of OHE on neurological status and neuronal integrity. Our results showed that these multiple episodes of OHE contribute to cell loss/injury and therefore irreversible injury to the brain. Interestingly, our results demonstrated that one episode of OHE does not lead to any permanent neuronal cell loss/injury in BDL rats.

In this study we used the well-known precipitating factor of HE, ammonia, to induce multiple HE episodes in BDL rats. Our group previously shown that ammonia injection can lead to OHE, manifesting as loss of righting reflex (Macedo de Oliveira et al., 2022). We showed that injecting a reversible dose of ammonium acetate to BDL and SHAM rats led to the similar levels of blood ammonia in both BDL and SHAM. This can be explained since the acute rise in ammonia exceeds the metabolic capacity to clear ammonia in both BDL and SHAM rats. However, a few days after ammonia injections, ammonia returned to normal levels in SHAMs which was not observed in BDL, due to the decreased metabolic capacity to reduce ammonia

in BDL rats. However, this rise in blood ammonia levels induced episodic symptoms only in BDL rats and not in SHAM controls. This can explain the importance of a pathological environment in the toxicity of ammonia and our group has previous demonstrated the synergistic effect of oxidative stress and ammonia on brain dysfunction in BDL rats (Bosoi et al., 2012).

Our results demonstrated that lower doses of ammonia were required to induce a subsequent episode. It is known that BDL rats have a reduced capacity to metabolize ammonia due to CLD, which declines even further with a significant loss of muscle mass (Bosoi et al., 2017). We believe that multiple injections of ammonia may lead to an accumulation of blood ammonia leading to a higher baseline following each episode. Whether this increased baseline of hyperammonemia preceding each episode impacts the sensitivity of ammonia on the brain remains to be investigated.

We demonstrated that multiple OHE episodes lead to neuronal cell death only in the hippocampus of BDL rats. However, our results show that hyperammonemia (induced by BDL surgery) in the context of CLD, also leads to apoptotic astrocyte cell death. Interestingly, in BDL rats without OHE episodes, higher levels of apoptotic markers of cleaved caspase-3 and Bax/Bcl2 ratio were found in three different brain regions (frontal cortex, hippocampus, and cerebellum) when compared to respective SHAMs. Our immunofluorescence imaging confirmed the co-localization of cleaved caspase-3 with GFAP (astrocytic marker). This finding is aligned with previous findings demonstrating the association between hyperammonemia and astrocytic death (Hiba et al., 2016). However, in the BDL-Ammonia group, western blot analysis showed a significant increase of GFAP only in the hippocampus. GFAP is a cytoskeletal and structural protein which expressed in the astrocyte and being extensively used in astrocyte research; however, GFAP expression can increase both in proliferation and activation state of astrocytes. In this study we did not quantify the astrocyte population and astrocyte proliferation. We hypothesize that increased GFAP expression in the hippocampus of BDL-Ammonia rats represents astrogliosis due to loss of neurons in this region.

Moreover, immunofluorescence imaging of cerebellum revealed the co-localization of cleaved caspase-3 with Purkinje neurons in the cerebellum of both BDL groups. These results suggest apoptosis in Purkinje neurons. Loss of Purkinje neurons and astrocytes in the cerebellum have been reported in two other studies using animal model of hyperammonemia (García-Lezana et al., 2017; López-Cervantes et al., 2021). However, due to lack of quantification of Purkinje neurons' population, we could not conclude if a higher amount of Purkinje neurons were

subjected to apoptosis in BDL-Ammonia rats. Expression of cleaved caspase-3 in Bergman glia astrocytes of both BDL groups suggests apoptotic cell death in these astrocytes. This finding suggests impairment and cell death in Bergman glia astrocytes may results Purkinje neurons apoptosis which could indicate the important role of these supporting astrocyte in maintenance of Purkinje neurons.

Oxidative stress

To identify the potential triggering factors of brain cell loss/injury, we measured systemic and brain oxidative stress. Our findings revealed higher levels of systemic (plasma) oxidative stress in BDL-Ammonia rats when compared to respective SHAMs and BDL-Vehicle rats. Moreover, measuring total antioxidant capacity (TAC) and 4-HNE (end-product of lipid peroxidation) revealed a higher level of these oxidative stress markers in BDL-Ammonia hippocampus when compared to respective SHAMs and BDL-Vehicle rats. Our results suggest that oxidative stress could play a crucial role in inducing brain cell injury/loss in BDL-Ammonia rats.

Inflammation

Moreover, cytokine plasma levels of IL-1β and TNF-α were both significantly increased systemically in BDL and BDL-Ammonia rats; however, IL-1β levels were further increased in the BDL-Ammonia rats when compared to BDL-Vehicle rats. In the brain, cytokine levels were not equally increased in all three regions of the brain (frontal cortex, hippocampus, and cerebellum), suggesting each region creates different inflammatory responses. Both IL-1β and TNF-α levels were significantly elevated in BDL-Vehicle and BDL-Ammonia hippocampus; however, in the frontal cortex only IL-1β levels were significantly increased in BDL-Vehicle and BDL-Ammonia rats whereas in the cerebellum, only higher levels of TNF-α were detected in the BDL-Ammonia rats.

Proteomics

After demonstrating neuronal cell loss and injury in the hippocampus, proteomic analysis of the hippocampal samples conducted and revealed alteration in expression levels of several proteins. Out of total 1202 analyzed proteins, 438 proteins were significantly altered between all 4 groups. Among 438 proteins, 14 proteins were significantly changed between BDL-Vehicle and BDL-Ammonia, 275 and 73 proteins were significantly altered in BDL-Vehicle and BDL-Ammonia respectively when compared to respective SHAM groups. Additionally,

199 proteins were changed in SHAM-Ammonia versus SHAM-Vehicle, and finally only two proteins were solely changed in BDL-Ammonia samples when controlling for surgery and ammonia (respective SHAM and BDL rats). Downregulation of VILIP-2 (visinin-like protein 2/hippocalcin-like protein 4) and upregulation of TN-R (Tenascin-R) were identified. To date, very little has been published on VILIP-2 protein; however, this protein belongs to the subfamily of visinin-like proteins (VSNLs), shown to play neuroprotective and neurotoxic roles, which have been implicated in several neurodegenerative diseases (Braunewell and Klein-Szanto, 2009). On the other hand, TN-R is one of the major extracellular matrix components of the perineuronal nets (Morawski et al., 2014). The protein function is dependent on its presented physical form, which can be either promoting or inhibiting the extra cellular matrix. CNS injury could result in a decrease or increase in TN-R levels, influencing microglial and astrocytic reactivity and contributing to neurodegeneration or alternatively, neuroprotection. The role of TN-R in astroglial scar formation has been reported previously (Anlar and Gunel-Ozcan, 2012).

In conclusion, multiple ammonia-induced episodes of overt HE led to apoptotic neuronal cell loss/injury in the hippocampus of BDL rats. One ammonia-induced episode of OHE was not enough to induce any neuronal loss/injury in BDL rats. Our results suggest oxidative stress could be one of the main contributors in inducing apoptotic brain cell death. Moreover, elevated levels of pro-inflammatory cytokines, as well as alternation in brain protein expression may also play a role in this condition.

Animal models of episodic HE

Infection and hyperammonemia are common pathogenic factors with arise from precipitating events that cause OHE. Therefore, to precipitate an OHE episode in an animal model of CLD, insults such as ammonia (hyperammonemia) through diet or injection or endotoxins such as lipopolysaccharides (LPS) have been implicated. Several studies have shown that injection of ammonia could induce overt HE in PCA rats with type B HE (Bergqvist et al., 1996; Therrien et al., 1997). Moreover, Garcia-Lezana et al., characterized an animal model of episodic OHE by inducing episodes using hyperammonemia and/or LPS (continuous perfusion of 20µL/min for 3 hours once every two weeks with the dose of 55 µmol/kg/min and 3 mg/kg, respectively) in PCA rats with type B HE (García-Lezana et al., 2017). Considering the lack of adequate animal model to study the pathogenesis of multiple episodes of OHE in the CLD setting, we developed a novel model inducing multiple overt OHE episodes in BDL rats using a reversible dose of ammonium acetate. With this novel animal model, for the first time, we evaluated the

impact one and multiple (in total 4) episodes of OHE on brain integrity. In addition, for better understanding the pathophysiological mechanisms of episodic overt HE.

The impact of liver disease etiological factors on the brain

Studies have shown that the etiological factors of liver disease may contribute to neurological impairments and brain damage in patients with CLD. Since the common etiological factors such as NAFLD, hepatitis C infection and alcohol do not lead to severe fibrosis or cirrhosis in rodents, in our second study, we investigated the role alcohol in development of OHE and its effect on brain integrity in rats with CLD.

Role of alcohol in pathogenesis of liver disease

Alcohol is one of the main etiologies in development of CLD and cirrhosis and the role of alcohol in pathogenesis of liver disease been studied extensively. Hepatocytes express high levels of alcohol dehydrogenase (ADH), an alcohol-oxidizing enzyme. In addition to ADH, hepatocytes express high levels of catalase which can contribute in oxidizing alcohol to acetaldehyde (Osna et al., 2017). Although the catalase pathway of alcohol oxidation in the liver is minor, it has a more significant function in alcohol oxidation in the brain (Aragon et al., 1992). Oxidation of alcohol by ADH, uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor and generates NADH and acetaldehyde. Acetaldehyde is a highly reactive and toxic compound which can later binds to proteins, lipids and nucleic acids and form acetaldehyde abducts which can impair the function and structure of these macromolecules (Kenney, 1982; Donohue et al., 1983; Mauch et al., 1986; Brooks and Zakhari, 2014). To reduce acetaldehyde toxicity, hepatocytes rapidly oxidizing acetaldehyde by their mitochondrial aldehyde dehydrogenase 2 (ALDH2). ALDH2 further generates NADH and acetate, the latter released into circulation and utilized in multiple pathways. The elevated levels of NADH generate through ADH and ALDH2 leads to reduced NAD^+/NADH ration in hepatocytes which consequently lead to decreased cellular redox potential. These alterations result in significant metabolic changes from oxidative metabolism to reductive synthesis which promotes fatty acid formation and development of liver steatosis (Donohue, 2007). The other major hepatic enzyme which catalyzes ethanol to acetaldehyde is CYP2E1 which is associated with generation of significant amount of ROS molecules including hydroxyethyl radicals, superoxide anions and hydroxyl radicals (Osna et al., 2017).

Heavy alcohol consumption can lead to a spectrum of hepatic lesions including steatosis, hepatitis, fibrosis, and cirrhosis. Steatosis is the most common hepatic response in alcohol

drinkers, which if not stopped, can lead to a high risk of fibrosis and cirrhosis (Teli et al., 1995). Several mechanisms are involved in alcohol-induced steatosis. Alcohol metabolites such as acetaldehyde enhancing hepatic lipogenesis which caused by higher expression of lipogenic factors including sterol regulatory element-binding protein 1 (SREBP-1) which is involved in cholesterol biosynthesis and lipid homeostasis (Donohue, 2007). On the other hand, alcohol reduces hepatic lipid breakdown which is a consequence of decreased autophagy. It is believed that alcohol consumption leads to lower rates of autophagy since ethanol causes impaired lysosome biogenesis which can slow down the breakdown of lipid droplets in a steatotic liver. Additionally, hepatic lipid export is disrupted by alcohol. In physiological state liver exports cholesterol and triglycerides in from of very low-density proteins and any impairment in the synthesis or export of these molecules leads to hepatic accumulation. The exact mechanism contributing to this condition is not known; however, impairments in the synthesis of essential constituents of very low-density proteins could play an important role in alcohol-induced defective hepatic lipid export (Kharbanda et al., 1995).

Alcohol-induced steatosis can later lead to steatohepatitis. Resident (Kupffer cells) and infiltrating immune cells (macrophages) are playing an important role in liver inflammation, hence inducing steatohepatitis. One of the major factors of activation of immune cells are endotoxins. It has been shown that excess alcohol consumption can lead to gut microbiota imbalance and bacterial overgrowth and increase intestinal permeability (Bode and Bode, 2003). Endotoxins can be recognized by different types of receptors, activating a pro-inflammatory pathway and release of cytokines from Kupffer cells which attracts circulating immune cells. To resolve inflammation, Kupffer cells also release anti-inflammatory factors such as transforming growth factor-β1 (TGF-β1) which induces fibrogenesis. Release of proinflammatory cytokines and chemokines by immune cells results in a direct cytotoxic effect, promoting hepatic cell death and further steatohepatitis. Following hepatic injury, hepatic stellate cell (HSCs) become activated and contribute to excessive deposition of extracellular matrix components resulting in structural changes in the liver and deterioration of liver function. The active fibrogenesis and chronic inflammation may lead to formation of scar tissue and regenerative nodules which is the main pathological features of cirrhosis (Osna et al., 2017).

Role of alcohol in neurodegeneration and brain cell loss/injury

It is known that chronic alcohol abuse can lead to neurocognitive impairments, neuronal injury including synaptic degeneration and neuronal demyelination and BBB dysfunction (Sutherland

et al., 2014). Alcohol use disorder which is characterized by impaired ability to stop or control alcohol use despite adverse social, professional, or health consequences, is often associated with global loss of brain and tissue, white matter shrinkage, and significant cortical volume loss as a consequence of alcohol-induced oxidative stress, excitotoxicity or nutritional deficiency which may results in normal brain function impairment (Harper, 2009). It has been reported that the combine effect of alcohol neurotoxicity and nutritional deficiency may have a role in severe long-term effects and aggravating the clinical manifestation of neurological impairments (Crews and Nixon, 2009). Maintaining and regulating brain homeostasis requires different types of growth factors; however, elevated levels of alcohol metabolism such as acetaldehyde and ROS disrupt these regulatory factors and induce neuronal injury resulting in neurodegeneration (Przedborski et al., 2003). Additionally, alcohol promotes abnormal protein accumulation, DNA damage and lysosomal dysfunction accelerating neurodegeneration (Crews, 1999).

Alcohol-induced cerebral cell death has been investigated in numerous studies; however, the exact mechanisms still remain unknown. Disruption of BBB has been proposed since the interaction of alcohol metabolites and neurotoxins may lead to alteration of cytoskeletal structures and increase BBB permeability and neuroinflammation (Haorah et al., 2005; Liu et al., 2017). A potential pathway is oxidation of acetaldehyde in the brain. Acetaldehyde is one of the main metabolites of alcohol metabolism in the liver, and therefore circulating acetaldehyde can reach brain. ALDH is found in mitochondrial brain cells which can convert acetaldehyde to acetate. It is known that alcohol contributes to oxidative stress respond, and alcohol-induced ROS production via CYP2E1 leads to formation of H_2O_2, free radical and superoxide in neurons (Maffi et al., 2008). It's been reported that high levels of ROS activate lipid peroxidation in neurons leading to elevated levels of 4-HNE resulting in decreased neuronal cytoskeletal proteins, disruption of neurofilament and neuronal death (Anandatheerthavarada et al., 1993; Haorah et al., 2005; Maffi et al., 2008).

In addition, increased ROS due to alcohol exposure can affect other brain cell types such as astrocytes (de la Monte and Kril, 2014). Moreover, alcohol and its metabolites have the potential to alter the function of astrocytes which can further impair the cellular interaction and cell to cell communication. Astrocytes are necessary to maintain the BBB structure and neuronal homeostasis. Loss of astrocyte function can result in BBB disruption, changes in microvasculature and alteration of energy metabolism in the brain, which ultimately can contribute to neuronal cell loss/injury (Weber and Barros, 2015; Vasile et al., 2017). Additionally, numerous clinical studies reported cerebral atrophy, behavior and cognitive

deficits, long-term structural brain damage, and decrease brain metabolites including N-acetylaspartate (NAA) and choline-containing metabolites in chronic alcoholism (Chick et al., 1989; O'Neill et al., 2001; Parks et al., 2002; Topiwala and Ebmeier, 2018; Firbank et al., 2020).

Alcohol consumption and neuronal loss in chronic liver disease

Although the role of alcohol in the progression of CLD and cirrhosis is well-known, the effect of alcohol consumption in development of HE has not been properly investigated. Also, role of alcohol in neurodegeneration is widely investigated; however, the effect of alcohol on neuronal integrity and brain health in patient with cirrhosis is not well studied. The standard practice encourages abstinent for patients with cirrhosis, since prolonged abstinent can promote compensation of previous cirrhosis complications (Addolorato et al., 2013).

In a study by Krill and Butterworth, the histological evaluation of brain samples from patients who died during hepatic coma showed higher levels of cerebellar degeneration and Alzheimer type II astrocytes in alcoholic patients when compared to non-alcoholic. The cerebellar degeneration including gliosis and loss of Purkinje neurons was observed in 50% of alcoholic patients (Kril and Butterworth, 1997). These results suggest that alcohol might play a crucial role in neurodegeneration in patients with CLD which could explain the poor neurological outcome in these patients after LT. In another study by Zeneroli et al., role of HE and alcoholic liver cirrhosis in prevalence of brain atrophy by CT scan was investigated. Their results showed that both alcohol and HE can contribute to brain atrophy (Zeneroli et al., 1987). In a study by magnetization transfer imaging (MTR) showed a significant decrease in thalamus, pallidum, putamen, caudate nucleus, and occipital white matter of alcoholic cirrhosis; however, these alterations were not correlated with HE severity (Miese et al., 2006). Very few studies include alcoholic patients in their study cohort for investigating the improvement of neurological outcomes after LT. The exclusion criteria are justified since they believe that heavy alcohol consumption has toxic effect not only for the liver but also for the brain (Ishihara et al., 2013). However, this population requires to be properly evaluated in order to understand the impact of continuous alcohol consumption on brain integrity.

Although quality studies are lacking on the effect of continuous alcohol consumption on neurological outcomes after LT in patients with cirrhosis, there is anecdotal evidence showing poor neurological outcomes in patients post-LT (including patients with alcoholic cirrhosis) (Chavarria et al., 2011; Garcia-Martinez et al., 2011; Ahluwalia et al., 2015). Ahluwalia et al., showed that individuals with alcohol-cirrhosis have poorer cognition and a higher incidence of

past OHE than patients with non-alcoholic cirrhosis (Ahluwalia et al., 2015). Garcia-Martinez et al. reported that poorer neurological outcomes after LT was observed in patients with alcoholic etiology, diabetes mellitus and HE (Garcia-Martinez et al., 2011).

Animal models of alcoholic liver disease

There are several known animal models used to study alcoholic liver disease (ALD). In these experimental models, ALD induced by alcohol (ethanol) administration, chronic intragastric alcohol administration for several weeks or combination of alcohol administration with high fat diet, lipopolysaccharide, or genetic modifications. Depending on the aim of the study, alcohol can be administered chronically or acutely (binge alcohol consumption). However, alcohol feeding including long-term/chronic ethanol administration usually only leads to steatosis with limited inflammation, fibrosis, or liver injury (Ghosh Dastidar et al., 2018). One of the major challenges in developing animal models of ALD is the high rate (up to five times higher than human) of alcohol catabolism in rodents, which makes achieving the relevant blood alcohol levels and subsequent liver injury difficult (Holmes et al., 1986).

Continuous alcohol consumption worsens HE and leads to neuronal loss in BDL rats

In this study, we utilized our well-characterized BDL rat model to examine the effect of constant alcohol consumption on neurological status and neuronal integrity. Alcohol administration started as early as one-week post-surgery which allowed us to investigate the role of alcohol consumption during the development of HE in BDL model. Our results showed that constant alcohol consumption in the context of CLD and the presence of CHE can contribute to irreversible changes in the brain, and therefore increasing the risk of irreversible neurological complications post-LT.

In our study, we demonstrated that continuous alcohol consumption led to the worsening of HE (increased anxiety-like behavior and decline in motor coordination) and neuronal cell loss in the cerebellum of BDL rats. The same alcohol protocol did not lead to liver injury in SHAM rats. Moreover, BDL rats which received alcohol did not show any further liver injury when compared to BDL-Vehicle rats. Similar results with slightly elevated ALT and micro-steatosis were observed in rats with chronic (4 weeks) alcohol administration were posted previously (Aroor et al., 2012). However, in mild bile duct ligated rats which received 10% alcohol in drinking water for 13 weeks, higher levels of fibrosis were found when compared to SHAM and mild bile duct ligated rats (Giménez-Garzó et al., 2015). These findings suggest the

importance of the duration of alcohol administration in development and induction of fibrosis in rodent models of ALD. Moreover, our results showed a similar rise in blood alcohol levels in BDL and SHAM rats 1 hour and 4 hours after alcohol administration; however, BDL rats developed more severe intoxication symptoms compared to SHAM rats after alcohol administration, suggesting BDL brain is more sensible to alcohol compared to SHAM brain. Furthermore, to investigate the effect of continuous alcohol consumption on brain integrity we explored cell death pathways in the brain. Our results showed decreased levels of neuronal markers of SMI311 and NeuN and astrocytic marker of GFAP only in the cerebellum of BDL-Alcohol rats when compared to respective SHAMs and BDL-Vehicle rats. Since alcohol is a known triggering factor of oxidative stress which is one of the major precipitating elements of cell death, we investigate the oxidative stress by measuring two oxidative stress markers of total antioxidant capacity (TAC) and 4-HNE modified proteins in the brain. Our results showed higher levels of oxidative stress in the cerebellum of BDL-Alcohol rats when compared to respective SHAMs and BDL-Vehicle rats. It is known that oxidative stress can induce multiple types of cell death. Therefore, to identify the type of cell death and discover which cells are affected, we explored apoptotic and necroptotic cell death in the cerebellum of our experimental groups. Our results revealed apoptotic and necrotic neuronal loss in the granular layer neurons of the cerebellum of BDL-Alcohol rats. It worth mentioning that alcohol consumption did not alter the ammonia levels, suggesting the above-mentioned changes were solely associated with the continuous alcohol consumption in these rats. In summary, this evidence shows that alcohol neurotoxicity could play an additional role in the development and pathogenesis of HE in BDL rats, consequently, it can also contribute to permanent alterations in the brain.

Role of oxidative stress in brain cell loss/injury in chronic liver disease

Our both studies demonstrated that oxidative stress might be the main contributor to cell loss/injury in the brain. Our group previously showed that systemic oxidative stress is elevated in the BDL rats (Bosoi and Rose, 2012); however, oxidative stress was not demonstrated in the brain (frontal cortex) of BDL rats. Moreover, it has been reported that BDL rats demonstrate levels of systemic and CNS oxidative stress particularly in the hippocampus and cerebellum vs SHAM rats; however, a different strain of rats (Wistar) were used in this study and neuronal cell loss or injury was not investigated (Pierzchala et al., 2022). In our first study we showed that systemic oxidative stress is elevated in BDL rats and is further increased in the BDL-Ammonia rats. In the brain, first we measured the total antioxidant capacity in each region and

then relatively quantified the expression levels of oxidative stress biomarker of 4-HNE, a product of lipid peroxidation. In physiological state, lower levels of 4-HNE can be detoxified by cells through several mechanism including conjugation to reduced form of glutathione (GSH) but higher levels of 4-HNE in the oxidative stress state can disrupt signaling pathways, alter protein activity and function, induce inflammation, and trigger apoptotic cell death (Dalleau et al., 2013). Our results showed a significant decrease in total antioxidant capacity in the hippocampus of both BDL groups when compared to their respective SHAM controls; however, these levels where further decreased in BDL-Ammonia rats when compared to BDL-Vehicle rats. 4-HNE levels showed a significant increase only in the hippocampus of BDL-Ammonia rats compared to all other groups. The results of our second study demonstrated the elevated levels of 4-HNE and reduced levels of TAC in the cerebellum of BDL-Alcohol rats. In our both studies higher levels of oxidative stress were found in brain regions with neuronal loss. The association between higher oxidative stress levels and elevated 4-HNE with neuronal cell death has been reported in several neurodegenerative diseases (Kashyap et al., 2015). We hypothesized that increased oxidative stress may be the main triggering factor inducing neuronal loss in the hippocampus of BDL-Ammonia and the cerebellum of BDL-Alcohol rats (Fig. 10). Therefore, targeting oxidative stress, using antioxidants, might be a potential therapeutic approach in patients with CLD and HE. Our group has been previously shown that antioxidant treatment such as xanthine oxidase inhibitor, allopurinol, can reduce oxidative stress and brain edema in BDL rats (Bosoi and Rose, 2012).

CHE leads to OHE

It is known that in cirrhotic patients, CHE is a risk factor of developing OHE (Tapper, 2019). Therefore, patients with CHE are more susceptible to develop OHE (Hansen et al., 2022). Using animal models of BDL and PCA allows us to investigate this association more thoroughly. Moreover, our group previously shown that presence of both chronic hyperammonemia and systemic oxidative stress is necessary for development of brain edema (marker of HE) and motor locomotion deficit; and these two factors do independently lead to brain edema. It's been previously reported that CHE and brain edema in BDL model is associated with elevated systemic (not brain) oxidative stress and hyperammonemia. However, in the PCA rats, systemic oxidative stress is not present and hyperammonemia alone does not lead to brain edema. Treating BDL rats with allopurinol (a xanthine oxidase inhibitor) resulted in a decrease in systemic oxidative stress and improve in brain edema and locomotor activity (Bosoi et al., 2012). Additionally treating BDL rats with AST-120 (activated carbon

microspheres), an ammonia chelating treatment, led to a decrease in plasma ammonia, improvement in brain edema and locomotor activity, but no significant effect on systemic oxidative stress (Bosoi et al., 2011).

In our first study we showed that ammonia injection can induce an episode of OHE in BDL rats. However, even though acute injection of ammonia led to a similar rise in blood ammonia levels, SHAM rats did not develop an episode of OHE. This suggests hyperammonemia per se is not enough to induce an episode which may be explained by the fact SHAM rats do not have an increase in systemic oxidative stress nor CHE. Moreover, elevated brain oxidative stress was solely found in the BDL-Ammonia rats, which suggests ammonia injections not only elevate the systemic oxidative stress in these rats but also induces brain oxidative stress which is not found in BDL-Vehicle rats. It has been postulated that higher levels of ammonia (>500 μM) can induce cerebral oxidative stress (Bosoi et al., 2014).

Overall, chronic hyperammonemia and systemic oxidative stress as independent factors, synergistically induce CHE which is not associated with cerebral oxidative stress. However, higher levels of ammonia (acute insult of ammonia) can induce cerebral oxidative stress and lead to severe HE cellular loss.

Identification and treatment of precipitating factors of OHE

In our studies, we demonstrated that multiple episodes of overt HE and continuous alcohol consumption can result in irreversible brain cell loss or injury. Identifying the factors contributing to permanent neurological impairments may significantly enhance the quality of life for LT patients. Thus, adopting a strategy that involves abstaining from alcohol, implementing prophylaxis, and administering treatments to prevent OHE episodes could greatly improve patients' cognitive outcomes and overall quality of life post LT. Moreover, several precipitating factors can cause an OHE episode. The OHE episode must be treated, and the precipitating event must be identified and controlled. Some of common precipitating factors of HE episodes includes infections, dehydration, gastrointestinal bleeding, constipation, and electrolyte imbalance (Rose et al., 2020).

Treatment of HE

Treatment strategies for HE target lowering ammonia; either by reducing ammonia production or increasing ammonia removal. The gut is a target since the majority of ammonia is generated via protein digestion and amino acid deamination. In addition to the urea cycle found exclusively in the liver, two other enzymes play an important role in ammonia homeostasis

including glutamine synthetase (GS) which is mainly found in liver, muscle, and brain (astrocytes) and glutaminase which predominantly expressed in the gut, liver, kidneys, and brain (neurons).

Current available treatments for HE

Osmotic laxatives: Lactulose and lactitol are the first line of treatment for HE. They are non-absorbent disaccharides and work by increasing intestinal transit and acidifying the bowel. These simple sugars are not absorbed in the small intestine; therefore, they reach the colon. In the colon, they nourish the colonic lactobacilli which do not produce urease, ultimately leading to less ammonia production. Moreover, colonic lactobacilli use lactulose to produce lactic acid which further creates an environment to promote growth of lactobacilli, replacing other gut bacteria. Production of lactic acid also acidifies the colon and stool which known to be correlated with reduction of ammonia production (Wijdicks, 2018). Numerous randomized clinical trials and observational studies have shown the beneficial effect of lactulose on severity of HE, mortality, and serious adverse events such as variceal bleeding, liver failure, spontaneous bacterial peritonitis and hepatorenal syndrome (Gluud et al., 2016).

Non-absorbent antimicrobial agents: Rifaximin is a broad-spectrum antibiotic which acts against aerobic and anerobic, gram-positive and gram-negative enteric bacteria. By binding to the beta subunit of bacterial DNA dependent RNA-polymerase, it inhibits RNA synthesis in bacteria. Rifaximin is not systemically absorbed (<4%) which decreases the risk of bacterial resistance (Kogawa and Salgado, 2018). Rifaximin acts locally by reducing the effect of intestinal microbiota including ammonia-producing bacteria. It has been shown that use of rifaximin in combination with lactulose provides additional benefits such as increase in effectiveness of the treatment and decrease in mortality rate compared to lactulose alone in patients with cirrhosis and HE (Fu et al., 2022). Rifaximin was the first FDA approved treatment for the indication to prevent recurrent OHE (Bass et al., 2010).

Prospective treatments and therapeutics

Probiotics: Ammonia can be produced through urease containing bacteria in the gut. It is believed that probiotics can improve the gut dysbiosis and may lead to improvements in OHE development, lowering ammonia blood levels and patients' quality of life (Dalal et al., 2017).
Fecal microbiota transplantation (FMT): Transplantation of fecal microbiota from healthy individuals to patients with cirrhosis results in improvement of gut dysbiosis. It has been

reported that FMT improves cognition in HE patients which is associated with the enrichment of beneficial bacteria in the gut (Kao et al., 2016).

L-Ornithine L-Aspartate (LOLA): LOLA is a combination of two natural amino acids which acts through substrate activation mechanism of ammonia detoxification. LOLA induces the production of glutamine which results in ammonia detoxification. L-ornithine and L-aspartate are involved in the urea cycle, therefore could stimulate the urea cycle in residual hepatocytes. Also, it has been shown that LOLA stimulates GS in the muscle which results in glutamine synthesis and ammonia removal (Rose et al., 1999b). Both oral and intravenous administration of LOLA showed a significant beneficial effect on reducing HE grade and improvement of psychomotor function in patients with CHE (Sharma et al., 2014). Most importantly it has been reported that LOLA treatment can reduce the recurrence of OHE (Alvares-da-Silva et al., 2014), and it can improve grades and recovery time from OHE and decrease mortality (Jain et al., 2022).

Ornithine phenylacetate (OP): The mechanism of action of ornithine phenylacetate is similar to LOLA, inducing the production of glutamine which results in detoxifying ammonia. However, phenylacetate is provided to bind with glutamine to produce phenylacetylglutamine (PAGN) which can then not be re-metabolized to ammonia via glutaminase and PGGN is excreted by the kidneys. This further reduces the ammonia recycling by eliminating available glutamine derived from previously induced reaction. Several animal studies have shown that OP successfully reduced blood ammonia in cirrhosis and ALF (Jalan et al., 2007; Davies et al., 2009; Ytrebø et al., 2009; Balasubramaniyan et al., 2012; Oria et al., 2012; Wright et al., 2012). The Phase 2b study demonstrated OP was able to lower blood ammonia which correlated with improvement of HE grades in patients with cirrhosis (Safadi et al., 2022).

Sodium benzoate: Sodium benzoate (SB) is an ammonia lowering treatment which removes ammonia through non-urea cycle pathway. It is an FDA-approved treatment used for patients with hyperammonemia and urea cycle disorder (UCD) but has not been approved for the treatment of HE. SB is an active metabolic agent, benzoate forms benzoyl CoA through conjugation by coenzyme A, which later conjugates with glycine in kidneys and liver and forms hippurate, which is rapidly excreted through kidneys (Misel et al., 2013). Generation and formation of hippurate is unknown in patients with cirrhosis, especially in those with renal dysfunction. Even though non-FDA approved for the treatment of HE, few studies have reported the efficacy of this treatment in lowering ammonia (Campollo et al., 1994) and improving OHE episodes (Sushma et al., 1992).

Sodium phenylbutyrate / Glycerol phenylbutyrate: These two ammonia-lowering treatments are primarily used in the treatment for urea cycle disorder (UCD). The well tolerated treatments increase elimination of glutamine vis the generation of phenylacetylglutamine in the urine which subsequently reduce blood ammonia concentration (Misel et al., 2013). The conjugation of glutamine and phenylacetate reduce glutamine availability which disallows the glutamine to be metabolized to ammonia via glutaminase. The efficacy of this treatment has been reported in cirrhotic patients (Ghabril et al., 2013; Rockey et al., 2014; Weiss et al., 2018). However, there is an association between cirrhosis and hypervolemia resulting from renal sodium which limits this treatment due to the risk of higher sodium uptake in these patients (Hartleb and Gutkowski, 2012). Therefore, Glycerol phenylbutyrate, containing no sodium is being studied for treatment of hepatic HE (McGuire et al., 2010).

Antioxidants: Since oxidative stress is elevated in CLD patients and plays an important role in pathogenesis of HE, the impact of several antioxidant have been investigated as a potential HE treatment. It has been shown that antioxidants (vitamin C and E) in combination with zinc improved HE (Mousa et al., 2016). Also, the beneficial effect of Resveratrol, a polyphenol compound which acts like antioxidants, on HE has been reported (Kim and Song, 2021).

Embolization of portosystemic shunts: It has been shown that percutaneous embolization of large portosystemic shunts is a rescue treatment for patients with recurrent OHE episodes (Lynn et al., 2016). It has been reported that blood ammonia levels in these patients decreased post-procedure (Choudhary et al., 2017). Although this is a safe procedure, some studies have reported an increased risk of bleeding due to portal hypertension and poor clinical outcomes with increased risk of mortality (Zidi et al., 2007).

Nutrition and exercise: Malnutrition, sarcopenia, and muscle wasting are common observed patients with cirrhosis. Muscle is another important organ to detoxify ammonia, especially in patients with cirrhosis. Muscle GS can eliminate ammonia by amidating glutamate to glutamine (Dam et al., 2013). Also, it has been reported that the mortality and susceptibility of OHE episodes in patients with cirrhosis with sarcopenia is higher (Bhanji et al., 2018). Therefore, application of strategies to reverse or prevent the development of sarcopenia in these patients could have beneficial effect in increasing blood ammonia clearance. Sufficient nutritional intake of calories and protein along with regular exercise including aerobic and resistance training could improve sarcopenia which lead to better clinical outcome in these patients (Ebadi et al., 2019).

Extracorporeal albumin dialysis (ECAD): This procedure may improve OHE in patients with cirrhosis by eliminating of protein- and non-protein-bound toxins. It has been reported that

ECAD treatment may be associated with improvement of HE grades and a reduction in blood ammonia levels has been documented in patients (Hassanein et al., 2007).

Conclusion

In these two studies, we developed two novel animal models utilizing the BDL rat model to investigate the effects of multiple OHE episodes and continuous alcohol consumption on the development of HE and permanent brain injury/damage.

Our new animal model of episodic OHE allowed us to gain better insights into the role of multiple OHE episodes. The results showed that multiple OHE episodes lead to neuronal cell loss/injury, which is less likely to be reversible. These findings provide new insights on why neurological impairment in patients with a history of OHE is less likely to improve after LT. We also demonstrated that ammonia plays an active role in this process. Therefore, employing prophylaxis, such as ammonia-lowering strategies to reduce the risk of developing OHE and subsequent episodes will lead to a reduced risk of brain cell injury and better neurological outcome following LT patients.

Moreover, our research revealed that alcohol consumption in the context of cirrhosis could worsen HE and cause neuronal injury/loss. Understanding alcohol's neurotoxicity and educating patients living with cirrhosis about the effect of alcohol on their brain could be a powerful tool to improve neurological impairment and prevent permanent neuronal injury/loss in these patients, ultimately leading to a better quality of life.

Future directions

How many episodes of ammonia induced overt HE leads to neuronal loss/injury? One episode is not enough.

In our study we explored the role of one OHE episodes and 4 OHE episodes. Our results showed neuronal loss following multiple (4 OHE episodes), which was not observed following one OHE episode. However, further investigation (2 and 3 OHE episodes) could provide us with more clarity regarding the effect of ammonia-induced episodes on neuronal loss in the BDL model.

Role of ammonia lowering treatment in prevention of overt HE episodes

The novel animal model of episodic OHE provides a valuable platform to investigate various ammonia-lowering treatments in the prevention of OHE. It would be interesting to determine whether treating with ammonia-lowering agents will prevent ammonia-induced OHE episodes,

using same concentration of ammonia as described, and investigate whether neuronal loss could be avoided.

Role of exercise and muscle mass in prevention of overt HE episodes

It is known that BDL rats have lower muscle mass, and the role of muscle in detoxifying ammonia is well-understood. Due to liver dysfunction and lower muscle mass, BDLs have a reduced capacity for ammonia detoxification. Investigating the role of exercise and the potential benefits of improving muscle mass in the prevention of ammonia-induced OHE episodes could be a valuable area for further investigation.

Role of antioxidants in prevention of overt HE episodes

In our study, higher oxidative stress was associated with neuronal cell loss in episodic HE. Exploring the role of antioxidants in preventing neuronal cell loss would be interesting. Additionally, investigating the impact of episodes on the antioxidant properties of astrocytes and neurons would enhance our understanding.

The role of other precipitating factors of overt HE episodes such as inflammation in neuronal cell loss/injury

In our study we investigated the role of ammonia induced OHE episodes in neuronal cell loss/injury in rats with CLD. However, it would be interesting to explore the role of other precipitating factors of OHE episodes such as systemic inflammation induced by lipopolysaccharides (LPS).

The effect of the duration and concentration of factors

In our first study we showed that ammonia injections (a reversible dose) led to loss of righting reflex in BDL rats but not in SHAM rats. Our results demonstrated that multiple (total of 4) OHE episodes caused oxidative stress-induced neuronal injury/loss in the hippocampus of BDL rats. However, it would be interesting to know whether a lower dose of ammonium acetate (without inducing OHE episode) but given longer (ammonium acetate injections everyday post-BDL surgery) will lead to neuronal cell death or similar findings.

Moreover, in our second study, we showed that alcohol administration led to significant neurological alterations (higher intoxication scores) in BDL rats compared to SHAM rats. Our results demonstrated that alcohol administration worsened HE and caused oxidative stress-induced neuronal injury/loss in the cerebellum of BDL rats. However, it is worth investigating whether a lower dose of alcohol (without any significant intoxication symptoms) but given

longer (alcohol in drinking water starting one week before BDL surgery) will lead to neuronal cell death or similar incomes.

The mechanism of multiple overt HE episodes and alcohol neurotoxicity in female BDL rats

In our study, only male rats were used. In a recent study conducted by our group, we demonstrated that the same dose of ammonia injected to female BDL rats does not lead to an OHE episode (Macedo de Oliveira et al., 2022). We speculate higher levels of muscle mass may protect from the onset of OHE. However, in female rats, it is worth investigating to find the reversible ammonia dose that leads to OHE episodes and then exploring the role of multiple episodes of OHE.

Additionally, in the same recent study conducted by our group, we demonstrated that female BDL rats have a higher antioxidant capacity (Macedo de Oliveira et al., 2022). Therefore, it would be interesting to investigate the role of continuous alcohol administration in the development of OHE and brain cell loss/injury in female BDL rats with higher antioxidant capacity.

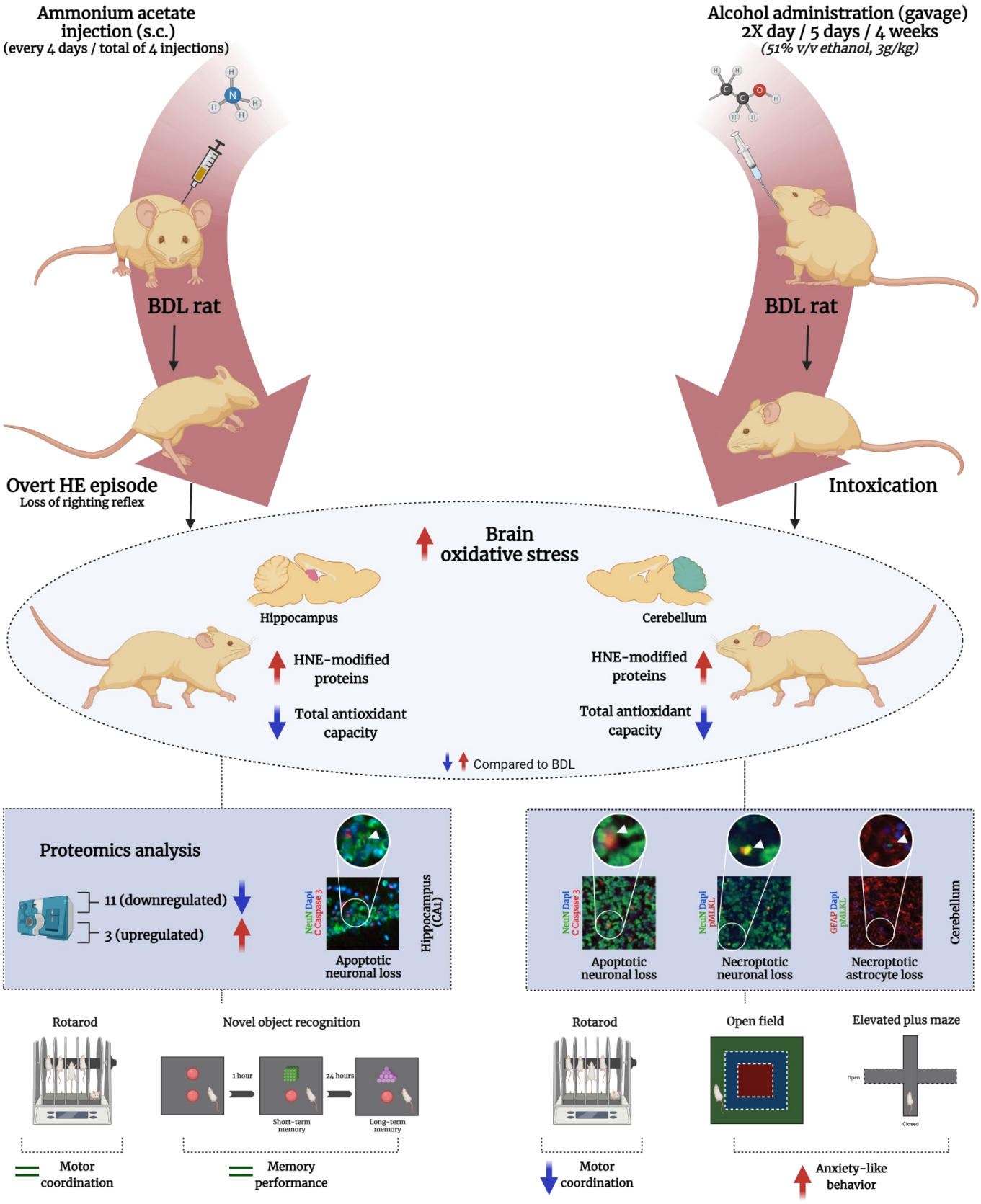


Figure 10: Oxidative stress may be the main triggering factor inducing neuronal loss in the hippocampus of BDL-Ammonia and the cerebellum of BDL-Alcohol rats

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Annex: Review paper

This article has been published in the journal of Neurochemical Research.
DOI: 10.1007/s11064-021-03372-4

Hepatic Encephalopathy: From Metabolic to Neurodegenerative. Neurochemical Research.

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Keywords: Hepatic encephalopathy, Liver transplantation, Neurological complications, Ammonia toxicity, Astrocytes, Neuronal cell loss

Neurochemical Research

Hepatic Encephalopathy: From Metabolic to Neurodegenerative

--Manuscript Draft--

Manuscript Number:	NERE-D-21-00271R1
Full Title:	Hepatic Encephalopathy: From Metabolic to Neurodegenerative
Article Type:	S.I. : In Honor of Vladimir Parpura
Keywords:	Hepatic encephalopathy; liver transplantation; neurological complications; ammonia toxicity; astrocytes; neuronal cell loss
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Funding Information:	Canadian Institutes of Health Research — Dr Christopher Rose
Abstract:	Hepatic encephalopathy (HE) is a neuropsychiatric syndrome of both acute and chronic liver disease. As a metabolic disorder, HE is considered to be reversible and therefore is expected to resolve following the replacement of the diseased liver with a healthy liver. However, persisting neurological complications are observed in up to 47% of transplanted patients. Several retrospective studies have shown that patients with a history of HE, particularly overt-HE, had persistent neurological complications even after liver transplantation (LT). These enduring neurological conditions significantly affect patient's quality of life and continue to add to the economic burden of chronic liver disease on health care systems. This review discusses the journey of the brain through the progression of liver disease, entering the invasive surgical procedure of LT and the conditions associated with the post-transplant period. In particular, it will discuss the vulnerability of the HE brain to peri-operative factors and post-LT conditions which may explain non-resolved neurological impairment following LT. In addition, the review will provide evidence; (i) supporting overt-HE impacts on neurological complications post-LT; (ii) that overt-HE leads to permanent neuronal injury and (iii) the pathophysiological role of ammonia toxicity on astrocyte and neuronal injury/damage. Together, these findings will provide new insights on the underlying mechanisms leading to neurological complications post-LT.

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Abbreviations:

AT2A, Alzheimer type II astrocytes
BBB, blood-brain barrier
BDL, bile duct ligation
BDNF, brain-derived neurotrophic factor
CCl4, carbon tetrachloride
cGMP, cyclic guanosine monophosphate
CLD, chronic liver disease
CNS, central nervous system
EEG, electroencephalogram
GS, glutamine synthetase
HE, hepatic encephalopathy
LT, liver transplantation
LTP, long-term potentiation
mHE, minimal HE
MRI, magnetic resonance imaging
NMDA, N-Methyl-D-aspartate
PCA, portocaval anastomosis
TAA, thioacetamide

Abstract

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome of both acute and chronic liver disease. As a metabolic disorder, HE is considered to be reversible and therefore is expected to resolve following the replacement of the diseased liver with a healthy liver. However, persisting neurological complications are observed in up to 47% of transplanted patients. Several retrospective studies have shown that patients with a history of HE, particularly overt-HE, had persistent neurological complications even after liver transplantation (LT). These enduring neurological conditions significantly affect patient's quality of life and continue to add to the economic burden of chronic liver disease on health care systems. This review discusses the journey of the brain through the progression of liver disease, entering the invasive surgical procedure of LT and the conditions associated with the post-transplant period. In particular, it will discuss the vulnerability of the HE brain to peri-operative factors and post-LT conditions which may explain non-resolved neurological impairment following LT. In addition, the review will provide evidence; (i) supporting overt-HE impacts on neurological complications post-LT; (ii) that overt-HE leads to permanent neuronal injury and (iii) the pathophysiological role of ammonia toxicity on astrocyte and neuronal injury/damage. Together, these findings will provide new insights on the underlying mechanisms leading to neurological complications post-LT.

Keywords: Hepatic encephalopathy, liver transplantation, neurological complications, ammonia toxicity, astrocytes, neuronal cell loss

Hepatic encephalopathy (HE)

Hepatic encephalopathy (HE) is a common and debilitating neuropsychiatric complication of chronic liver disease (CLD). HE is classified into two primary forms: covert/minimal HE (mHE) and overt-HE, encompassing sub-clinical and clinical signs, respectively. Characterized by impaired concentration, poor memory and cognition, lower speed of information processing, increased reaction time, mood disorders and disturbance in sleep-wake rhythms, mHE impacts the patients' health-related quality of life [1]. Moreover, mHE is associated with poor performance in daily activities, including the patient's ability to work and the proper capability to drive a car which in turn result in reduced work productivity and lost wages [2]. The prognostic value of mHE has been demonstrated to predict an episode of overt-HE which manifests with clinically evident symptoms such as lethargy, gross disorientation, asterixis, stupor and coma [3]. Overt-HE is associated with a poorer prognosis and higher mortality compared to other complications of cirrhosis [4] and is an independent factor of mortality regardless the degree of liver disease [5]. Between 53% and 83% of patients with CLD develop mHE and the incidence of overt-HE among patients with end-stage liver disease is 25-43%, with a 20% annual risk of developing further episodes of overt-HE [6–8]. HE is further divided into episodic HE defined by one episode of overt-HE occurring during a 6-month period, recurrent HE defined when 2 episodes occur within a 6-month period and persistent HE, defined by a pattern of behavioral alterations that are difficult to treat and never resolve [3].

An incidence of overt-HE is the most common cause for first-time hospitalization as well as for hospital readmissions in patients with CLD, with the mean annual hospital costs outweighing the outpatient costs [9, 10]. The burden of HE is multidimensional, imposing a significant economic burden to the patient, patients' caregivers, healthcare systems, and society. As one of the most frequent complications observed in CLD, the economic burden for HE is heavy, generating costs exceeding $11.9 billion per year (US statistics) [11].

Pathogenesis of HE

The pathogenesis of HE is multifactorial, and to date is not completely understood. Nevertheless, it is well accepted that ammonia plays a major role in disease progression [8]. The gut is the major source of systemic ammonia due to both dietary protein catabolism via amino acid deamination, and through urease containing bacteria which metabolize urea to ammonia [12]. In healthy conditions, the liver metabolizes and regulates circulating levels of ammonia via the urea cycle; a family of enzymes found exclusively in the liver that metabolize ammonia to less toxic urea. Therefore, during CLD, a reduced capacity to clear ammonia leads

to the development of hyperammonemia. Consequently, since ammonia easily cross the blood-brain barrier (BBB), high levels of blood ammonia cause deleterious effects to the brain [13]. It has been well documented that high brain ammonia is associated with dysregulation in metabolic pathways in astrocytes and neurons, microglia activation, and onset of cerebral edema, all believed to be implicated in the pathogenesis of HE [14]. In addition to hyperammonemia, other pathogenic factors such as systemic inflammation, oxidative stress, elevated bile acids, elevated manganese, and zinc deficiency may also synergistically contribute to the development and severity of HE [8].

Neurological complications following liver transplantation

Liver transplantation (LT) is the only curative treatment for end-stage liver disease. By definition, HE is a metabolic disorder and therefore is expected to completely resolve following the correction of the disease with LT. With advances and improvements in the LT surgical procedure and survival being less of a concern (85% at 1 year, 75% at 5 years) [15] quality of outcome, including neurological status and quality of life, has been increasingly supervised [16, 17]. In this context, even though most patients do improve following LT, numerous clinical studies have revealed that up to 47% of liver transplant recipients report neurological complications and enduring symptoms [17, 18]. These neurological outcomes appear to be specific to LT recipients since fewer neurological complications are observed in kidney or cardiac transplant recipients [19, 20]. Clinical neurological complications following LT include seizures, cerebrovascular complications, ischemic stroke, CNS infection, brain hemorrhage (hemorrhagic stroke), osmotic demyelination syndrome, cerebral embolism, central pontine myelinolysis and posterior reversible encephalopathy syndrome [17, 21, 22]. Moreover, some neurological complications after LT such as post-transplant encephalopathy, alteration consciousness, disorientation, confusion, memory impairment, headaches, difficulty concentrating, focal motor deficits, fatigue, sleep impairment, and mood disorders could last for years [16, 23–26] with neuropathological abnormalities found in up to 72% of patients at autopsy [27]. In some cases, global cognitive impairments were reported to be present 9-12 months following LT [28].

Brain cell connectivity assessments by functional magnetic resonance imaging (MRI) showed that cell connectivity density improved in some but not in all brain regions in patients following LT [29]. In accord, cerebral positron emission tomography (PET) showed persistent reduced cerebral function and metabolism in frontal regions of the brain in 20% of patients with cirrhosis after LT, with no improvement up to 10 years later [30]. In addition,

neuropsychological and electroencephalogram (EEG) analysis after LT showed that EEG activity normalized but global cognition remained impaired [28]. It is reported that neurological complications post-LT continue to weigh severely on the patient's quality of life and lead to longer stays in the hospital, thus causing further financial burden on the healthcare system [31]. Even though post-LT neurological complications can occur at any given time, improved survival of transplant recipients has led to increased detection of chronic neurological complications, which themselves impinge on morbidity and mortality [32]. Therefore, it is important to accurately identify the underlying cause(s) responsible for either persistent (HE sequelae) or de novo neurological complications observed following LT. Whether caused by (i) pre-existing HE (prior to LT), (ii) perioperative-induced neurological insults during LT or (iii) post-LT factors that remain to be determined.

Post-LT factors and neurological complications

Following insertion of a new liver, patients are placed on immunosuppressant agents (commonly calcineurin inhibitors) to reduce the risk of graft rejection, increase survival, and improve the longevity of the transplanted liver [33]. However, immunosuppression therapy is frequently associated with side effects, including malignancies, opportunistic infections, metabolic disorders, and organ toxicities infections [34]. Neurologic complications such as seizure, stroke, brain hemorrhage, and encephalopathy are observed in 15-30% of liver allograft which are regularly associated with opportunistic infections and immunosuppressant neurotoxicity [16]. Calcineurin dose, intravenous administration, as well as BBB permeability are believed to be responsible for the various neurological complications post-LT. The risk of infection is higher in immunosuppressed patients, and it is well documented that systemic infection/inflammation impacts brain function [35, 36]. Moreover, CNS infections in post-LT are not rare and chronic immunosuppression leads to a higher risk of developing primary CNS lymphoma [37]. The impact of post-LT complications is significant as they may negatively affect compliance with immunosuppression regimens and complicate post-LT management [38]. The precise underlying factors behind post-LT complications, including infections and chronic immunosuppression, may cause the development of neurological deficits but the association remains unclear (Fig. 1).

Intra-operative factors and neurological complications

During the surgical procedure of removing an ailing liver and inserting a healthy one, there is a risk for hypotension, blood loss (hypovolemia), cerebral hypoperfusion, blood transfusion,

ischemia, gas embolism and electrolyte correction (hyponatremia) [28, 32, 39]. These intra-operative factors could impact the brain, causing alterations of cerebrovascular system, seizures, ischemic or hemorrhagic stroke, osmotic demyelination syndrome, and encephalopathy [16, 17, 39, 40]. In addition, general anesthesia during surgery can precipitate post-operative long-term cognitive dysfunction via oxidative stress and neuronal apoptosis, [41] which may contribute to neurological complications after LT. Neurological complications are frequently (75% of cases) observed within the first month after LT. In particular, transplanted patients develop a delirium state, characterized by acute confusion, inattention, disorganized thinking and altered level of consciousness that can last days to weeks [22]. The underlying causes of delirium remain unidentified. However, intrinsic surgical aspects (including duration of surgery/anesthesia) per se may be responsible [26]. This reasoning is reinforced since the prevalence of neurological complications is particularly high in patients having received a LT (47%) compared to patients having undergone cardiac or kidney transplantation (1.7% and 1.6% respectively) [18–20]. The frequent number of neurological complications observed specifically in LT patients suggests that an acquired factor associated with liver disease (e.g., metabolic disorders or HE) or its combination with transplantation-associated insults may be responsible for complications rather than intra-operative transplantation insults. For instance, brain MRI analysis identified alterations in cerebral metabolites in patients with post-transplant encephalopathy vs patients without post- transplant encephalopathy. Moreover, both groups had similar alterations in cerebral metabolites pre-LT, suggesting that intra-operative factors associated with LT (surgery duration, complications, time under anesthesia, and others) may influence cerebral osmolytes and neurological performance after LT [24]. Nevertheless, further studies are needed to precisely understand to what extent intra-operative factors/insults impact brain function and contribute to neurological complications post-LT.

History of HE before LT and neurological complications post-LT

There is an extensive amount of studies demonstrating the strong association between HE pre-LT and neurological complications post-LT. Campagna et al., demonstrated that patients with a history of overt-HE fared worse on both paper and pencil and computerized psychometric assessments as well as electroencephalographic evaluations post-LT when compared to patients without a history of overt-HE [28]. Sotil et al., demonstrated that patients with a history of overt- HE had a worse performance on neuropsychological testing using the Psychometric Hepatic Encephalopathy Score (PHES) and critical flicker frequency compared to transplanted

patients without a history of HE [42]. Garcia-Martinez et al., elegantly demonstrated that the existence of mHE or episodic HE highly impacts cognitive function following LT [43]. In addition, patients with a history of mHE have demonstrated to continue experiencing neurological complications following LT [44–46]. Mechtcheriakov et al., showed that the prior history of mHE leads to incomplete improvement close to 3 years following LT [47]. Together, these discoveries indicate that history of HE is associated with neurological complications and brain structural impairments after LT.

HE: the vulnerable brain

The journey of the brain from the onset of liver disease to post-LT involves multiple encounters with a variety of insults such as etiological factors of liver disease, comorbidities, metabolic alterations, perioperative challenges, post-LT immunosuppression and other complications. Poor brain reserve, characterized by brain structural changes and inability to adapt or tolerate changes, has been shown to modulate the impact of brain disease or neurocognitive insults such as HE [48, 49]. A destabilized HE brain may be more sensitive to intra-operative factors as well as post-LT immunosuppression and complications, including infection. However, which components of HE lead to increased susceptibility is not clear. BBB alterations (increased permeability) in patients suffering from HE may lead to vulnerability [50], particularly in regard to immunosuppression neurotoxicity during the early days following LT [51]. In acute liver failure (ALF), intracranial hypertension impairs cerebral blood flow [52] increasing the risk of brain injury during LT [53]. Independent of HE, alterations in cerebral osmolytes have been shown to be associated with post-transplant encephalopathy [24]. Different detrimental factors in end-stage liver disease including inflammation, malnutrition/sarcopenia, altered glucose metabolism, and systemic hemodynamics alterations (hyperdynamic state, hypovolemia) are associated in some degree with increased risk of post-transplant neurological complications [40, 54–56].

Dhar et al., wisely demonstrated that HE *at the tim*e of LT leads to a higher prevalence of neurological complications post-LT [46]. More precisely, patients with severe HE had a higher risk of poorer neurological outcome than patients with mild HE. This study is of great value as it describes the neurological status of patients *at the time* of LT compared to other studies which document the events of HE that occurred in the months leading up to LT. This strongly suggests HE while the surgery renders the brain more susceptible to the perioperative insults of LT. In agreement, we recently demonstrated that in a rat model of CLD with mHE (BDL, bile-duct ligated model), exposition to hypotension leads to neurodegeneration; a finding which was not

replicated in naïve rats [57]. This permanent brain injury is primarily due to the hypotension stress which compromises energy supply to the brain; an organ with high energy demand to properly function [58]. This reinforces the proposition that, in the setting of mHE, the compromised brain becomes predisposed to what would normally be an innocuous hypotensive insult, resulting in cell injury and death. This could explain the anticipated susceptibility of patients with mHE to cerebral damage following intraoperative stress (i.e a hypotensive insult) and subsequently the enduring neurocognitive dysfunction following LT. Although the impact of overt-HE on the susceptibility of the brain to intra-operative or post-LT factors is less known, patients with severe HE receiving a new liver had a poorer neurological outcome than patients with mild HE [46]. However, it remains to be determined whether an overt-HE brain is more susceptible to perioperative insults and post-LT factors when compared to a mHE brain. Etiological factors involved in the progression of liver disease may render the brain vulnerable to insults. Liver cirrhosis associated to alcoholism has been related to neurological complications after LT with a higher rate of encephalopathy and seizures post-LT in alcoholic cirrhosis [59, 60]. Patients with alcohol-induced cirrhosis show poor brain reserve and are more susceptive to insults leading to neurological harm [48]. Patients with viral hepatitis (hepatitis C infection), have been associated with an increased risk of neurodegenerative disorders [61] such as Alzheimer's and Parkinson's disease. Particularly, hepatitis C infection impairs metabolic pathways of infected cells, autoimmune disorders, systemic or cerebral inflammation and alterations in neurotransmitter circuits [62] which may contribute to brain sensibility to perioperative LT factors. Furthermore, non-alcoholic liver disease has been documented to lead to premature brain aging [63]. During the progression of liver disease, the brain is exposed to many different factors and conditions which may or may not manifest as HE but could render the brain fragile and vulnerable to insults which are normally innocuous to a healthy brain (Fig. 1).

HE and brain damage

For over half a century, accumulating evidence strongly defined HE as "gliopathy" since significant alterations in glial cells are consistently identified. Reactive astrogliosis, described as astrocyte responses or remodeling to abnormal events in the CNS, including neurodegenerative events and diseases, as well as exposure to toxic substances that specifically damage astrocytes (e.g., ammonium in HE). Thus, in response to pathological stimuli astrocytes experience transcriptional, biochemical, morphological, metabolic, and physiological alterations. In turn, diseases astrocytes can initiate or contribute to the disease

progression [64]. For instance, astrocyte swelling, including Alzheimer type II astrogliosis (AT2A) are cardinal features of HE in CLD [65]. Morphological changes to neurons have not been well studied or documented, primarily due to the fact that HE is characterized as a metabolic disorder and therefore considered reversible following correction of the disease. However, it is becoming quite evident with studies carefully demonstrating that HE (or certain parameters of HE) do not fully reverse since residual HE (sequelae of HE) exists. This irreversible component of HE suggests permanent cell injury that may be an underlying cause for the observed neurological complications following LT. Moreover, there are multiple studies suggesting that irreversible cell injury occurs following episode(s) of overt-HE. Bajaj et al., demonstrated in cirrhotic patients experiencing their first episode of overt-HE that despite treatment and resolution of altered mental status, cognitive function (learning capacity, working memory) is persistently impaired (defined as persistent HE). In addition, the number of overt-HE episodes correlated with the severity of the persistent cognitive impairment, including a wider spectrum of impairments [66]. Moreover, brain functional MRI analysis in patients with mHE and history of overt-HE demonstrated that brain connectivity in different regions was further impaired compared to patients with current mHE without history of overt-HE [67]. Brain MRI studies in cirrhotic patients with a history of mHE, without past episodes of overt-HE, demonstrate increased mean diffusivity (MD) of water molecules in frontal and temporal lobes which improves following LT along with improved neurological performance [68]. Furthermore, brain functional MRI in cirrhotic patients without overt-HE showed that functional brain network (connectivity) was normalized along with cognition improvement after LT [69]. Patients with cirrhosis and mHE, without a history of overt-HE, have demonstrated improved cognition with treatment [70]. This suggests mHE with a history of overt-HE may have underlying permanent damage leading to persistent HE compared to mHE without episodes of overt-HE.

There is evidence insinuating overt-HE leads to irreversible brain damage. Brain atrophy (thinning of the cortex) has been documented in patients who have recovered from overt-HE [71] and is associated with persistent HE [72]. Moreover, brain MRI analysis after LT demonstrated that despite cognitive improvement following LT in patients with history of HE, white matter brain atrophy was still detectable short- (6-12 months) and long-term (6-9 years) following transplantation [73]. Furthermore, these findings were associated with a reduction in the neuronal marker NAA (N-acetyl-aspartate), indicating neuronal loss [43].

Independent of HE, etiological factors with chronic exposure have shown to provoke neuronal cell loss/damage. There is evidence to suggest that liver failure contributes to the severity of

neuronal loss in Wernicke's encephalopathy (acute neurological condition) [74]. Specifically, Wernicke's encephalopathy is associated with neuronal death in the mammillary bodies, thalamus, hypothalamus, and cerebellum brain regions [75]. Post-mortem brain analysis showed that patients (alcoholic and non-alcoholic) dying from severe HE (hepatic coma) had cerebellar degeneration and thalamic lesions, the latter being a characteristic of Wernicke's encephalopathy [76]. Further neuropathological evidence for loss of neurons in cirrhosis have been described in acquired (non-Wilsonian) hepatocerebral degeneration and post-shunt myelopathy [74].

Overall, numerous factors during the progression of liver disease, including the underlying causes of end-stage liver disease, as well as episode(s) of overt-HE and persistent HE can lead to permanent cell injury, justifying lasting neurological complications following LT (Fig. 1). However, prospective studies investigating the impact of HE, particularly in identifying which pathogenic conditions or risk factors provoke neuronal cell damage, are warranted.

Ammonia neurotoxicity

Hyperammonemia plays a major role in the pathogenesis of HE. Particularly, hyperammonemia leads to cerebral dysfunction associated with neuropsychiatric and neurological complications including impaired memory, shortened attention span, sleep-wake inversions, brain edema, intracranial hypertension, seizures, ataxia, and coma. Increased brain ammonia leads to abnormalities in intracellular pH, membrane potential and cell metabolism that contribute to a cascade of secondary neurotoxic effects and encephalopathy [13]. Furthermore, even if treatment of the acute episode of overt-HE improves mental status, there is evidence that the metabolic insult associated with overt-HE may cause irreversible neuronal injury. Montoliu et al., found that blood ammonia was higher in patients with mHE who experienced cortical thinning (superior cortex) compared to patients without mHE [77]. While historically it has been thoroughly demonstrated that astrocytes represent the principal target of ammonia toxicity, neurons have also been shown to be directly affected by ammonia [78].

Neurons

Neurons are responsible for communicating information in both chemical and electrical forms. These highly specialized cells transmit and receive signals to allow the regulation of all body functions. Therefore, alteration in their structure, function, connectivity, as well as their death, has been associated with different neurological disorders. There is vast evidence supporting that ammonia toxicity leads to brain cell alterations which are associated with cell structure,

morphology, proliferation/density, excitotoxicity, and ultimately cell death [14, 79]. Clinical studies using brain MRI demonstrated that neonatal hyperammonemic encephalopathy resulting from urea cycle disorders (UCD) leads to severe shrinkage and collapse of the brain [79, 80]. The neurotoxicity of ammonia is associated with the activation of different apoptotic substrates/pathways leading to neuronal death [81]. 28-day hyperammonemic cirrhotic-BDL rats showed that neurological impairments were associated with synaptic loss, apoptosis, and neuronal cell death in the hippocampus and substantia nigra compacta [82, 83] via intracellular Ca^{2+} overloading and continuous mPTP opening (mitochondrial permeability transition pore) [83]. Experiments in thioacetamide (TAA) rats, a model of liver injury and hyperammonemia, revealed that Bcl2/Bax apoptotic markers ratio was impaired in brain cortex and cerebellum suggesting a neurodegenerative proapoptotic process in those regions [84]. In addition, the modulation of neurodegeneration-related genes was found to be impaired in hyperammonemic 5- month portacaval anastomosis (PCA) rats, an effect associated with apoptosis in Bergmann glia of the cerebellum. Moreover, the same study showed a reduction of Purkinje neuron population, increased astrocyte size, and activated microglia in the cerebellum [85]. Hyperammonemic rats (4-week carbon tetrachloride (CCl_4) model) demonstrated neurological impairment together with hippocampal neuronal cell loss which was prevented through attenuation of hyperammonemia [86]. Furthermore, acute ammonia neurotoxicity in rats has been associated with major disturbances in mitochondrial function and eventual cellular apoptosis by increasing cytoplasmic protein p53, an essential apoptotic pathway/marker [87]. *In vitro* experiments with cultured primary cortical neurons from newborn rats exposed to different concentrations of ammonia (1-10 mM ammonium chloride for 24 or 48 h) decreased neuronal survival in a dose dependent manner, in which apoptosis was the dominant type of cell death with the highest dose of ammonia (10 mM) [78]. Aside from cell apoptosis, cultured neurons exposed to ammonia (5 mM NH_4Cl for 48 h) showed neurodegenerative changes such as free radical production (oxidative stress), and impaired mitochondrial inner membrane potential [88]. These ammonia-associated apoptotic effects are related to p53 activation, mitochondrial apoptotic pathway activation, and neurite degeneration, along with an increased expression of apoptotic markers such as Bax, caspase 8, caspase 9, and caspase 3 [89, 90]. Together, these findings indicate that the neurotoxicity of ammonia leads to the activation of apoptotic pathways particularly in neurons, a mechanism that contributes to cell death.

Neuroplasticity

Neuroplasticity plays a significant role in brain function including memory, cognition, locomotion, motor-skill learning, and mood regulation [91]. The functional and structural changes of dendrites and dendritic spines are relevant for brain connectivity and long-term synaptic plasticity associated with cognitive processes. Thus, neurodegenerative diseases stem from alterations in neuroplasticity that affect the axons, dendrites, and synapses which proceed to neuronal death and permanent neurological complications [92, 93]. There is evidence showing that neuroplasticity and neurogenesis are impaired in liver disease and HE, particularly in association with hyperammonemia [86, 94]. Experiments in BDL and CCl4 rats with mHE showed that hyperammonemia modified the structure of neurons, which was associated with motor coordination and cognitive impairments. Particularly, hyperammonemia reduced dendritic spine density in cortical and hippocampal neurons [86, 94]. The role of ammonia is fundamental since attenuation of ammonia improves cognitive function by preserving neuroplasticity and attenuating cell death [86]. Furthermore, hyperammonemia as a result of altered ammonia metabolism (transgenic mice with hyperammonemia from conditional knockout of hepatic glutamine synthetase) is associated with cognitive impairments and disturbed synaptic plasticity in cortico-striatal and hippocampal brain regions [95].

In the adult brain, the hippocampus is highly involved in neurogenesis and neuronal plasticity through a neurophysiological phenomenon named long-term potentiation (LTP), which is considered the hallmark of memory and learning [96]. Experiments in hippocampal brain slices (CA1 region) from hyperammonemic rats revealed that the degree of LTP was reduced [97]. In accord, hippocampal pyramidal neurons directly exposed to ammonia (100 μ M acutely) inhibited LTP [98]. These results indicate that neurocognitive decline associated with ammonia toxicity in the brain results from a reduction of LTP in the hippocampus which reduces neuronal synaptic strength.

Brain-derived neurotrophic factor (BDNF) plays a major role in brain plasticity, homeostasis and possesses neuroprotective effects including anti-apoptosis, anti-oxidation, and suppression of autophagy [99]. BDNF protects against mitochondrial dysfunction by reducing toxic NMDA receptor signaling, a major cause of excitotoxicity [99, 100]. Therefore, BDNF disruption has been involved in different neurodegenerative disorders such as Alzheimer's disease, in which total BDNF reduction is associated with impaired function on structural (spine density) and functionality (synaptic potentiation) [101]. In liver disease, BDNF's neuroprotective actions against NMDA toxicity [100] may be compromised in hyperammonemia and HE conditions, which are associated with reduced BDNF and overactivation of NMDA receptors. In HE,

serum BDNF is reduced in cirrhotic patients [102], while in cirrhotic-BDL rats with mHE, BDNF was found reduced in the hippocampus [103]. In hyperammonemic rats, hippocampal BDNF depletion is associated with a reduction of astrocyte BDNF production [104]. Moreover, cultured astrocytes exposed to ammonia showed that impaired glutamate uptake and glutamate neurotransmission were associated with a reduction of extracellular BDNF [104]. Together, these findings demonstrated that ammonia neurotoxicity impacts neuroplasticity via a reduction in BDNF expression leading to decrease in number of neurons.

Astrocytes

Astrocytes are subtype of glial cells in the CNS a subtype of glial cells involved in regulation of ions, neurotransmitters, metabolism or neuronal synaptic networks that maintain the homeostasis of the brain, whereas loss of homeostasis represents the underlying cause of all brain disorders [105]. Astrocytes, also known collectively as astroglia, are part of the physical structure of the brain that maintains the BBB. Astrocytes provide physical and metabolic support to neurons, including energy metabolism regulation, electrical insulation, neurotransmitter regulation, network homeostasis, extracellular ion balance, and protection from neurotoxins (e.g., ammonia) [105, 106].

Swelling

Astrocytes express the enzyme glutamine synthetase (GS) that metabolizes ammonia. Nevertheless, it has been shown that high ammonia in the brain leads to excessive intracellular formation of glutamine, an osmolyte that results in ion disturbance (osmotic pressure), and astrocyte swelling in high concentrations [14, 107–110]. In addition, cultured astrocytes exposed to ammonia (5 mM for 24 h) showed increased S-100β protein release, a biomarker of astrocytic brain damage [104]. Brain edema is associated with HE, believed to be the consequence of astrocyte swelling. Therefore, ammonia-induced astrocyte swelling may leave the neuronal network unprotected and without astrocyte support, will lead to altered neuronal integrity and function [88]. Post-mortem brain tissue from patients who died in hepatic coma as well as in vivo and in vitro models of hyperammonemia, have shown that ammonia toxicity is associated with a reduction of brain glial fibrillary acidic protein (GFAP), a cytoskeletal protein in astrocytes that maintain cell communication and the functioning of the BBB [86, 111, 112]. Thus, ammonia can impair the structure and function of astrocytes, and consequently synaptic integrity, astrocytic-neuronal trafficking substrates, provoke neuronal loss and worsen neurological performance [113].

Alzheimer type II astrogliosis (AT2A)

AT2A are morphological features in astrocytes characterized by enlarged pale nuclei and lack of cytoplasm (unrelated to Alzheimer disease), first described in Wilson's disease by Alois Alzheimer. AT2A are a pathological reactive astrocyte seen in systemic metabolic disorders, particularly those associated with hyperammonemia, which contributes to the development of HE. For instance, AT2A is a distinctive morphologic alteration in brain of humans and experimental animals suffering HE characterized by enlarged pale nuclei and lack of cytoplasm [114]. Indeed, AT2A is deemed the histopathologic hallmark of HE. However, the role of AT2A on astrocyte function and associated brain consequences remain to be elucidated. Nevertheless, considering that astrocytes carry out key function relevant for neuronal functioning (e.g., neurotransmitter uptake, and ion regulation), AT2A may be associated to neuronal dysfunction [114]. Nevertheless, AT2A has also been reported in a small percentage of cirrhotic patients without HE [65], suggesting that hyperammonemia, regardless HE, promotes AT2A. In vitro and in vivo experimental models of hyperammonemia confirmed that AT2A is associated with high ammonia levels [109, 114, 115]. For instance, cirrhotic-BDL rats on hyperammonemic diet displayed further increased brain ammonia levels and developed AT2A along with exacerbated motor incoordination as compared to BDL rats on regular diet [115]. Nonetheless, the exact neurological consequences of ammonia induced AT2A remains unknown, but it is possible that AT2A not only contributes to irreversible changes i astrocytes but also impacts neuronal function.

Senescence

Cellular senescence (arrest in synthesis phase of the cell cycle) in astrocytes has been associated in the progression of neurodegenerative and cognitive decline. For instance, post-mortem brain tissue analysis showed an up-regulation of oxidative stress and senescence markers (senescence- associated-β-D-galactosidase) in tissue from cirrhotic patients with history of HE but not in those without HE. In accord, elevated oxidative stress and astrocyte senescence markers along with reduced astrocyte proliferation were found in cultured rat astrocytes (but not in cultured neurons) when exposed to ammonia (5 mM NH_4Cl for 24, 48 and 72 h), a mechanism associated with the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) inflammatory pathway and up- regulation of heme oxygenase (HO-1; protective response to oxidative stress) [116, 117]. Thus, the inhibition of oxidative stress (reactive oxygen species, ROS formation) associated to ammonia toxicity in cultured astrocytes prevented senescence marker elevation, which may restore astrocyte proliferation [117, 118].

Moreover, ammonia-induced senescence in astrocytes is associated with mitochondrial metabolism dysfunction including ROS formation, and membrane potential impairment, two factors that lead to astrocyte degeneration, and swelling, but it does not affect astrocyte viability. Therefore, impaired astrocyte network (quantity and quality) may affect synaptic connectivity (strength and number), astrocyte-neuronal communication, neurotransmission, and neuronal survival [116].

Healthy astrocyte = healthy neuron

As mentioned above, astrocytes are strategic specialized cells that support and control neuronal activity via expression of ion channels, neurotransmitter receptors, and subcellular calcium dynamics, which critically contribute to neuronal transmission. Indeed, the communication between neurons and astrocytes is vital in achieving coordinated activity among neuronal ensembles. For instance, one astrocyte in the hippocampus is involved in approximately 120,000 synapses from either excitatory and inhibitory neurons, indicating that astrocyte activity regulates glutamatergic and GABAergic neurotransmission [119], while human astrocytes can cover from 270,000 to 2 million synapses [120]. Therefore, altered balance of astrocyte–neuronal signaling could cause different neuropathological states including HE [119]. Thus, astrocyte impairment (cell swelling, AT2A) due to ammonia toxicity will significantly tamper synaptic transmission and cause dysfunction (Fig. 2).

Glutamate is the major excitatory neurotransmitter involved in most of the brain functions including memory, learning, cognitive, emotional, and endocrine regulation. However, glutamate levels elevated above normal lead to uncontrolled continuous depolarization of neurons, a toxic process called excitotoxicity that results in neuronal death [121, 122]. Astrocytes play a vital role in clearing the synaptic cleft of glutamate since they contain high affinity glutamate transporters [14]. Intracellular glutamate within the astrocyte is metabolized to glutamine via the enzyme GS, aminating glutamate to glutamine and removing ammonia. The newly generated glutamine is shuttled back to the neuron allowing it to be metabolized to glutamate and ammonia via glutaminase. This will allow the neuron to replenish the releasable glutamate pool. However, ammonia-induced astrocyte swelling or AT2A may impair this communication between astrocytes and neurons. Inhibition of glutamate transporters in astrocytes will lead to excitotoxicity and possibly cell death [123]. It has been clearly demonstrated that cultured neurons die when exposed to NMDA-induced excitotoxicity [100]. The mechanism underlying glutamate excitotoxicity is multifactorial but excessive intracellular calcium concentration plays a major role via activation of ionotropic (NMDA

and AMPA receptors) and metabotropic glutamate receptors (mGluR), precipitating mitochondrial dysfunction, oxidative stress, and activation of apoptotic pathways [122]. Ammonia toxicity in the astrocyte can lead to astrocytic glutamate release which could be dependent or independent of astrocyte swelling [124]. Studies have shown NMDA antagonism increases survival in rats with acute liver failure [125]. Chronic hyperammonemia has shown to upregulate the gene expression of NMDA (GluN1 subunit) in the hippocampus of cirrhotic-BDL rats with HE [126]. In addition, HE and ammonia toxicity are associated with enhanced glutamate-nitric oxide-cGMP pathway associated with the NMDA receptors [127, 128]. In fact, increased intracellular cGMP has been shown to correlate with neurotoxicity, neuronal degeneration, and neuronal death [129, 130]. Additionally, ammonia toxicity has been shown to impact on glutamate levels leading to a depletion of intracellular glutamate and eventually neuronal death (glutamatergic cells) [81]. For instance, cirrhotic patients who died from hepatic coma had reduced glutamate concentration in brain regions associated with HE [123, 131]. Similarly, studies in cirrhotic-BDL rats have shown that glutamate concentration depletion in the hippocampus was correlated with systemic ammonia levels [132]. Overall, disrupted glutamate homeostasis has been well documented in HE, however the implications of glutamate excitotoxicity in neuronal cell death remains poorly defined. However, it is clear that healthy astrocytes are vital for optimal neuronal functioning. In vitro experiments show that co-incubating astrocytes with neurons prevents neurodegenerative effects (cell death, free radical production, and mitochondria membrane impairment) when exposed to ammonia (5 mM NH_4Cl for 48 h). This suggests that astrocytes protect neurons from ammonia oxicity [88]. Experiments in hyperammonemic TAA rats showed that ammonia toxicity on astrocytes is linked to neuronal integrity impairment, particularly affecting synaptic and neuronal function [113]. Furthermore, Kril and Butterworth, clearly demonstrated in brains from patients who died in grade 3-4 hepatic coma that cerebellar degeneration, characterized by loss of Purkinje cells was accompanied with AT2A in the Bergmann glia layer [76]. As mentioned above, it has been clearly demonstrated that brain edema (possibly low-grade edema) is due to astrocyte swelling [133, 134] which can affect function. Therefore, astrocyte swelling/dysfunction significantly impairs astrocyte-neuron communication, resulting in neuronal dysfunction, neurological decline and eventually neurodegeneration (Fig. 2).

Conclusions

In as much as HE is defined as a reversible syndrome, there are increasing studies demonstrating that this may not be the case. Most evidence stems from the incidences of

neurological complications detected in patients after LT. Whether the observed neurological complications and cognitive dysfunction post-LT are residual of HE (present before LT) and/or develop during the intra-operative procedures and/or post-LT, is difficult to determine and causes remains unclear. The brain during peri-operative conditions and post-LT management confronts a number of insults and factors that could be detrimental to proper brain function. However, since the majority of patients following LT succeed with positive neurological outcome, it is suggested that the status of the brain leading up to LT could impact neurological outcome post-LT. There is increasing evidence showing that an episode (or multiple episodes) of overt-HE induces permanent cell damage (persistent HE) in which patients are unresponsive to treatment. In addition, HE may render the brain sensitive to intra-operative and/or post-LT insults and conditions which in turn could cause newly or additional neurological impairments. Further studies are warranted investigating the impact of not only the number of episodes but also the frequency and intensity of episodes on neurological function and brain integrity. Duration of episodes should also be considered since it was reported that longer times spent in an overt-HE episode (>48 h) increases mortality [135].

It is clear that ammonia neurotoxicity not only impinges on astrocytes but can directly impact neurons as well. Ammonia toxicity affects astrocyte structure (AT2A, astrocyte swelling, senescence), altering function leading to astrocyte-neuron miscommunication and neurological impairment. Ammonia has also been shown to precipitate neuronal cell death. However, more studies are needed to thoroughly evaluate the extent of ammonia neurotoxicity while taking into consideration the concentration as well as the duration of ammonia exposure. Elevated blood ammonia has been shown to have prognostic value in patients with CLD [136, 137] and has shown to predict HE-related hospitalizations [138]. In addition, neuronal degeneration has been documented in animal models with chronic hyperammonemia [82–86]. Since the correlation between severity of HE and degree of hyperammonemia is weak, chronic elevated levels of blood ammonia could play an integral part in provoking neuronal injury and cell loss. Elevated blood ammonia has also been shown to be toxic to other organs [139] therefore monitoring ammonia is critical to help improve management in patients with CLD [8]. Considering the goal of LT is not only to extend the life span of patients with end-stage liver disease but also to improve the patients' quality of life, HE should be considered during transplant criteria. Specifically, the Model for End-Stage Liver Disease (MELD) score system should include HE for an improving organ allocation [140]. An interesting line of research is understanding the role of neurodegeneration in the onset of HE since the metabolic component

of HE frequently reverses after LT, whereas the structural component, underlying neurodegeneration, may persist [141].

In conclusion, the impact of HE not only leads to permanent cell damage under certain conditions but also renders the brain susceptible to intra-operative insults and post-LT management representing an increased risk of neurological complications post-LT. 30-45% of patients develop an episode of overt-HE while on the waiting list for a LT [43] and this highlights the importance of treating and managing HE with the goal in reducing the number of episodes of overt-HE. Development of preventive strategies that could improve the patient's neurological prognosis after LT remain to be considered.

Acknowledgements

The authors gratefully acknowledge the financial support of Canadian Institutes of Health Research (CIHR). Figures are created with BioRender.com.

Funding

Canadian Institutes of Health Research (CIHR)

Author contribution

R.O., F.T., and C.F.R., contributed to the review concept and wrote the manuscript review.

Conflict of interest

The authors declare that there is no conflict of interest.

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Legends

Fig. 1. The journey of the brain during the progression of liver disease until liver transplantation demonstrating its exposure to numerous factors and insults. The continuum of HE spans the range from normal cognitive function to minimal/covert HE (mHE) to overt HE (OHE). (A) History of HE (episodic, recurrent, permanent) may lead to permanent cell injury and neurodegeneration which will be irreversible and hence lead to residual HE following LT. In addition, an HE brain (B) entering in LT, exposed to intra-operative factors and insults, and/or (C) subjected to post-LT immunosuppression may also lead to irreversible components of brain injury and neurological complications.

Fig. 2. Ammonia concentration and duration leads to Astrocyte-Neuron miscommunication and neurodegeneration. Ammonia toxicity can affect astrocytes causing swelling, Alzheimer type II astrogliosis and/or senescence which in turn affects function and impairs astrocyte-neuron communication which may provoke neuronal cell loss. Ammonia toxicity has also shown to directly impact neurons causing apoptotic cell death. The level of ammonia required to induce cell damage or injury on either astrocytes or neurons remains undefined, but toxicity increases with duration and concentration of ammonia.

Figure 1

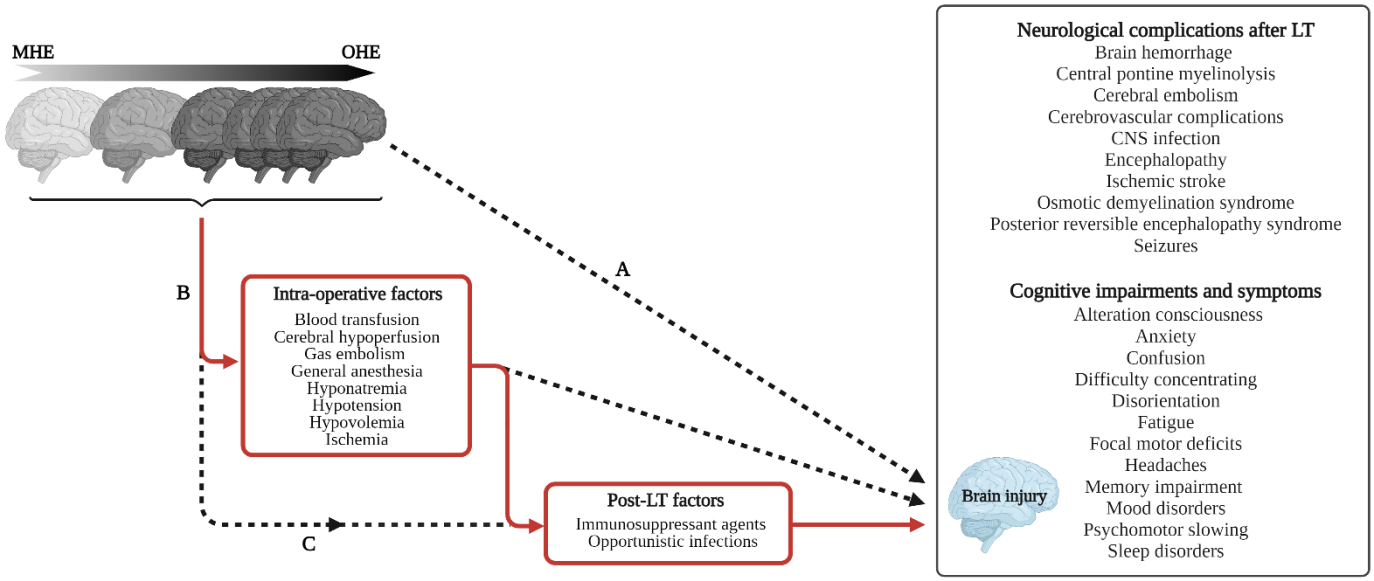
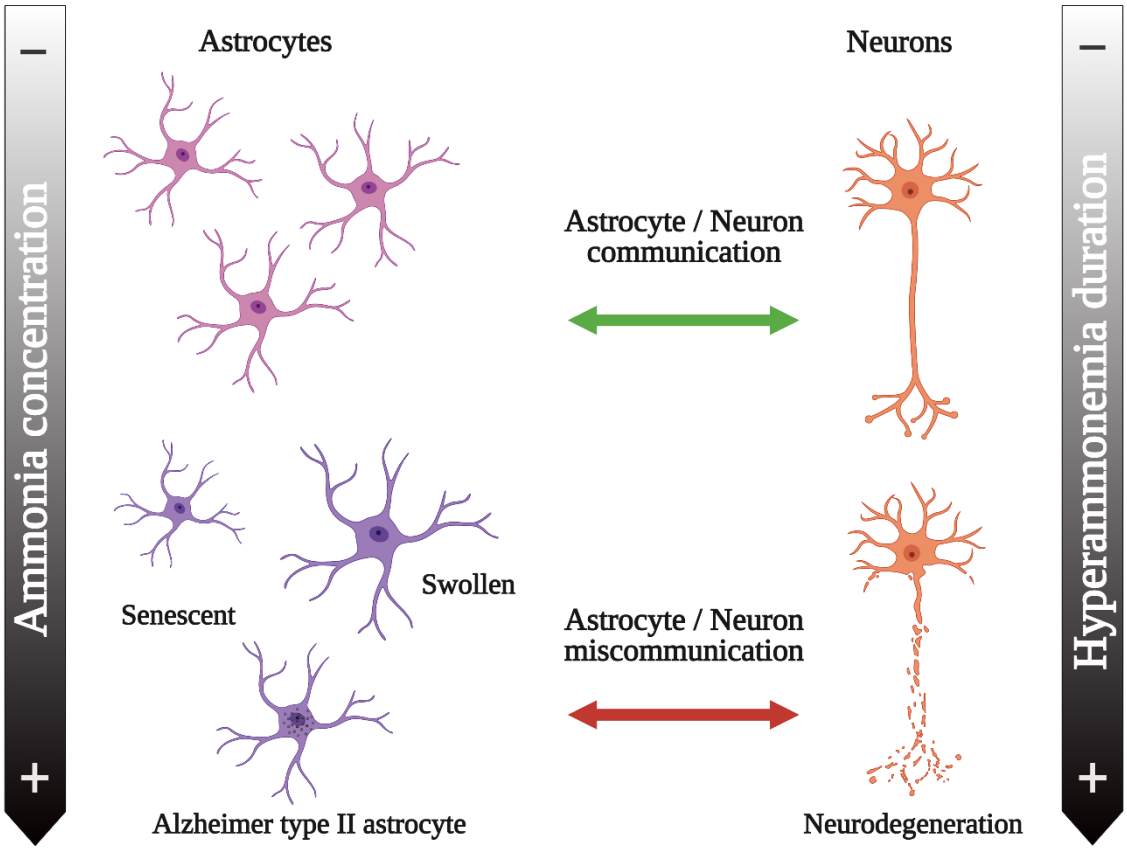

MHE
OHE
A
B
C
Intra-operative factors
Blood transfusion
Cerebral hypoperfusion
Gas embolism
General anesthesia
Hyponatremia
Hypotension
Hypovolemia
Ischemia
Post-LT factors
Immunosuppressant agents
Opportunistic infections
Brain injury
Neurological complications after LT
Brain hemorrhage
Central pontine myelinolysis
Cerebral embolism
Cerebrovascular complications
CNS infection
Encephalopathy
Ischemic stroke
Osmotic demyelination syndrome
Posterior reversible encephalopathy syndrome
Seizures
Cognitive impairments and symptoms
Alteration consciousness
Anxiety
Confusion
Difficulty concentrating
Disorientation
Fatigue
Focal motor deficits
Headaches
Memory impairment
Mood disorders
Psychomotor slowing
Sleep disorders

Figure 2


–
Ammonia concentration
+
Astrocytes
Neurons
Astrocyte / Neuron communication
Senescent
Swollen
Astrocyte / Neuron miscommunication
Alzheimer type II astrocyte
Neurodegeneration
–
Hyperammonemia duration
+