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EDITORIAL

Changing face of hepatic encephalopathy: Role of inflammation and oxidative stress

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Abstract

The face of hepatic encephalopathy (HE) is changing. This review explores how this neurocognitive disorder, which is associated with both acute and chronic liver injury, has grown to become a dynamic syndrome that spans a spectrum of neuropsychological impairment, from normal performance to coma. The central role of ammonia in the pathogenesis of HE remains incontrovertible. However, over the past 10 years, the HE community has begun to characterise the key roles of inflammation, infection, and oxidative/nitrosative stress in modulating the pathophysiological effects of ammonia on the astrocyte. This review explores the current thoughts and evidence base in this area and discusses the potential role of existing and novel therapies that might abrogate the oxidative and nitrosative stresses inflicted on the brain in patients with, or at risk of developing, HE.

Key words: Hepatic encephalopathy; Ammonia; Inflammation; Oxidative stress; Astrocyte

INTRODUCTION

Hepatic encephalopathy (HE) is a neurocognitive disorder in which brain function is impaired and is associated with both acute and chronic liver dysfunction. HE occurs in the presence of liver injury or when the liver is bypassed in the presence of a portosystemic shunt. In acute liver failure, patients may develop cerebral oedema and increased intracranial pressure. However, recent studies suggest that intracranial hypertension is less frequent than previously described, complicating 25% of acute cases and only 9% of those with sub-acute liver failure[1]. In cirrhosis, it causes a range of neuropsychiatric and motor disturbances spanning a spectrum of abnormalities, which encompass short-term memory impairment, slowing of reaction time, poor concentration, psychomotor retardation, and sensory dysfunction, through to more clinically apparent neurological signs and symptoms. In its most severe form, patients can develop confusion, stupor, and coma[2]. However, abnormalities can be subtle and only become apparent on formal psychometric testing (minimal HE). Minimal HE is thought to be a disorder of executive functioning, primarily leading to impairments in selective atten-

tion, response inhibition, and working memory. This frequently impacts on quality of life[3] and specifically impairs navigation skills[4], which can be demonstrated utilising a driving simulator that correlates impairment with response inhibition and attention[5]. HE has generally been considered to be a reversible process following liver transplantation, although recent studies have suggested that this may not always be the case[6].

The "World Congress of Gastroenterology" in 2002 developed a set of consensus definitions, which has led to the classification of HE into three different types, A-C. (Table 1)[2]. In addition, the clinical presentation of HE was categorised into four main subtypes (Table 2). The heterogeneous nature of the presentation of HE has been the cause of great consternation, and has made the interpretation of comparative studies problematic. The staging of overt HE remains an imprecise art, which is often hampered by its fluctuant course. Thus, more objective methods using electroencephalographic techniques have been developed to assess HE. The effectiveness of using the bispectral index to grade and monitor the course of HE has high discriminative power in patients with both low and high grades of HE, and can be utilised as a simple and objective method of grading HE[7]. It has recently been suggested that we should consider HE as a spectrum of neurocognitive impairment in patients with cirrhosis; the spectrum spanning normal performance to coma[8].

Table 1 Classification of hepatic encephalopathy[2]

Type	Definition
A	Acute and hyperacute liver failure
B	Portosystemic bypass without intrinsic hepatocellular disease
C	Cirrhosis and portal hypertension with portosystemic shunts

A: Acute; B: Bypass; C: Cirrhosis.

Table 2 Clinical presentation of hepatic encephalopathy[2]

Encephalopathy	Definition
Acute	Acute liver dysfunction
Recurrent or episodic	Episodes of mental alteration in a patient with cirrhosis, even in the absence of a known precipitating factor
Persistent	Neurological deficit that persists despite the reversal of liver injury, such as following liver transplantation or the removal of a precipitating factor
Minimal (previously known as subclinical)	No evidence of overt encephalopathy, but subtle cognitive deficits might be detected with a neuropsychological function test battery

THE AMMONIA HYPOTHESIS

Ammonia was first thought to play a major role in the development of HE when studies by Hahn *et al*[9], Nencki *et al*[10] and Nencki *et al*[11] in the 1890s described the "meat intoxification syndrome". By diverting blood away from the liver utilising a surgical shunt from the portal vein into the vena cava of dogs, within 6 wk of the portocaval shunt being constructed, it was observed that the dogs developed symptoms such as aggression, irritability, and convulsions, similar to the symptoms exhibited by patients with cirrhosis and overt HE. The portocaval shunt allows blood to bypass the liver, resulting in a lack of urea metabolism, and arterial ammonia levels were found to be increased. When ammonium salts were administered to the dogs, they rapidly fell into a coma and died[9]. Ammonia was later confirmed as the main causative factor of the "meat intoxification syndrome" in portocaval shunted dogs in 1922[12]. The role of ammonia became increasingly recognised as being important when Gabuzda *et al*[13] and Phillips *et al*[14] attempted to treat patients with ascites with cation exchange resins that absorbed sodium but released ammonium ions, leading to the adverse effect of significant reversible neurological dysfunction, which was indistinguishable from the syndrome we now know as HE. Blood ammonia concentration was subsequently noted to be elevated in patients with liver disease and hepatic coma[15]; the highest values being found in those patients who were comatose[16].

Subsequently, other investigators have shown that ammonia plays a definitive role in the development of HE. Bessman *et al*[17] demonstrated a positive arteriovenous difference in ammonia levels in patients with cirrhosis, suggesting an uptake of free ammonia into the brain. More recently, Ehrlich *et al*[18] demonstrated that by constructing an end-to-side portocaval anastomosis in rats and injecting them with ammonium acetate, the rats demonstrated typical characteristics of HE, such as drowsiness, seizures, and coma in association with elevated blood and brain ammonia concentrations, compared to control rats. Lockwood *et al*[19] were then able to demonstrate the first evidence linking ammonia to HE in humans, using positron emission tomography (PET). A ^{13}N tracer demonstrated that the rate of uptake of ammonia in the brains of patients was greater in those with HE than without. It was postulated that an increased ammonia uptake in the brain was linked to an increased permeability of the blood-brain barrier to ammonia[20]. In acute liver failure, arterial ammonia concentrations of > 150 μmol/L predict a greater likelihood of dying from brain herniation[21], and intracranial hypertension develops in 55% of cases with an arterial ammonia concentration > 200 μmol/L[1]. In cirrhosis, there is no doubt that blood ammonia concentrations are elevated, but there is conflicting evidence regarding the relationship between ammonia concentration and HE severity. Moreover, it is not unusual in clinical practice to see patients with cirrhosis presenting with symptoms of overt HE who have normal or only mildly elevated arterial ammonia concentrations. Indeed, numerous studies have shown that a single test for blood ammonia concentration is a poor method for assessing HE[22]. Furthermore, Ong *et al*[23] studied the blood ammonia levels of patients with chronic liver disease and compared these to their mental states. In patients considered not to have any sign of HE, 60% had ammonia levels higher than normal, whereas there was a high proportion of those with grade 3 or 4

HE with normal or only mildly elevated blood ammonia levels. Whilst there is no denying the involvement of ammonia in the pathogenesis of HE, it seems that there might be other factors involved which are as, if not more, important.

THE ASTROCYTE IN HE

Astrocytes are a type of glial cell found within the central nervous system (CNS), which are involved in maintaining cells within the CNS, including providing nutrients for neurones. Astrocytes are particularly vulnerable to the effects of ammonia in the brain. One reason for this is that the enzyme glutamine synthetase is mainly located within astrocytes. Norenberg *et al*[24] found glutamine synthetase exclusively within astrocytes in rat brains, and none within neurones or other glial cells. It is also important to note that the end-processes of astrocytes surround the capillaries in the CNS. Theoretically, this would ensure that any toxin entering the brain, such as ammonia, is immediately metabolised, protecting other CNS cells from its damaging effects[25]. This theory was tested by Rao *et al*[26], who investigated the effects of ammonia exposure on purely neuronal cultures and co-cultures of neurons and astrocytes. The cultures containing neurons alone showed significant increases in cell death, apoptotic cells, degeneration of neuronal processes, and free radical levels. However, these changes were not detected in the co-cultures, indicating a protective function of astrocytes.

The blood brain barrier remains anatomically intact in HE[27]; however, PET studies have demonstrated an increased permeability-surface area to ammonia with increasing severity of disease[20].

HE in patients with chronic liver disease is characterised neuropathologically by Alzheimer type Ⅱ astrocytosis. This describes morphological changes to astrocytes, which include a large swollen nucleus, prominent nucleolus, and margination of the chromatin pattern. These neuropathological findings have been replicated in the brains of patients with congenital abnormalities of urea cycle enzymes[28], in experimental animal models[29,30], and astrocyte cultures exposed chronically to ammonia[31]. Therefore, it is likely that ammonia taken up into the brain interacts with astrocytes, eventually leading to these characteristic changes.

In acute liver failure, an increased brain ammonia concentration causes astrocyte swelling and patients develop cytotoxic brain oedema[32]. Kato *et al*[32] used electron microscopy to study the cells of patients who died of fulminant hepatic failure. They found brain oedema to be present, with pronounced swelling of astrocytes. Glutamine synthetase catalyses the conversion of ammonia and glutamate to glutamine. As a result, hyperammonemia can lead to excessive levels of glutamine within astrocytes, causing the cells to swell, and therefore explaining the oedema and intracranial hypertension seen with fulminant hepatic failure. Willard-Mack *et al*[33] used rats to investigate whether inducing an acute onset of hyperammonemia caused astrocytes to swell and if inhibiting the action of glutamine synthetase prevented these astrocytic changes. The study found that 8 h after inducing plasma hyperammonemia, changes in astrocyte morphology could be identified. These changes included an increased number of organelles, increased cytoplasmic volume, and an increased nuclear volume. They also found that inhibiting glutamine synthetase attenuated the enlargement of the nuclei and prevented the increase in astrocyte water content seen with hyperammonemia. The results of this study suggest that the production of glutamine by ammonia detoxification, results in water being drawn into astrocytes through osmotic pressure.

There might also be a potential role for vasogenic brain oedema in acute liver failure. This is believed to result from damage to the blood-brain barrier, leading to uncontrolled movement of plasma components and water to extracellular areas of the brain[34]. Consistent with this, animal studies have shown an increased permeability of the blood brain barrier to substances that are normally unable to cross it[35,36]. It has been suggested that perhaps in the early stages of HE, cytotoxic brain oedema predominates, and is enhanced in the later stages by vasogenic brain oedema following damage to the blood brain barrier[37]; however, the ability of mannitol to reduce intracranial hypertension in patients with fulminant hepatic failure indicates that the blood brain barrier remains largely intact[38].

The presence of low-grade astrocyte swelling has been further investigated in human patients with cirrhosis. Córdoba *et al*[39] used magnetic resonance spectroscopy and the magnetisation transfer ratio (a measure of free water in the brain) to assess cirrhotic patients before and after liver transplantation. The results showed a high level of free water in the brain before liver transplantation, which then reduced after transplantation. This correlated with changes in neuropsychological function, suggesting that brain oedema plays a direct role in the changes observed in HE. A further finding of the study was that brain glutamine levels also correlated with the changes in brain water and neuropsychological function, providing further evidence to the theory that hyperammonemia plays an important role in the pathophysiology of HE. Balata *et al*[40] showed that inducing hyperammonemia in patients with cirrhosis leads to an increase in brain glutamine, which results in an increase in brain water, and deterioration in neuropsychological function.

Interestingly, brain oedema and the consequent risk of intracranial hypertension are rarely complications of chronic liver failure, and are more often associated with fulminant hepatic failure. One possible suggestion for this is that in chronic liver disease, cells have more time to use compensatory mechanisms to adapt to the osmotic changes taking place[41].

Ammonia is directly toxic to the brain, and in acute liver failure causes disarray of inhibitory and excitatory neurotransmission[42], impairs brain energy metabolism[43-46], alters expression of several genes that code for important proteins involved in brain function[47,48], and impairs autoregulation of cerebral blood flow[49]. In patients with cirrhosis, there appears to be a shift in the balance between

inhibitory and excitatory neurotransmission towards a net increase in inhibitory neurotransmission.

THE CHANGING FACE OF HE

Although it is widely accepted that ammonia has a key role to play in the pathophysiology of HE, the clinical picture is not always so straightforward. Frequently, the arterial concentration of ammonia can be elevated in the absence of symptoms of HE, and the correlation between the severity of HE and ammonia concentration in patients with cirrhosis can be poor. The theory that several factors could contribute together to the clinical picture of HE was first suggested by Zieve *et al*[50] in 1974, who described the possible synergistic effects of several toxins, with ammonia. Since this first suggestion, it has become increasingly apparent that aspects of the inflammatory response (such as elevation of pro-inflammatory cytokines) in response to infection and/or systemic inflammation, and oxidative stress, participate in a synergistic relationship with ammonia in the pathogenesis of HE[51-53].

THE ROLE OF INFECTION AND INFLAMMATION IN HE

Acute liver failure

Studies in patients with acute liver failure have shown a more rapid progression to severe HE in those patients with evidence of a systemic inflammatory response, supporting a link between inflammation and HE[51]. In addition, in patients with acetaminophen-induced acute liver failure, infection and/or the resulting systemic inflammatory response were shown to be important factors contributing to an increase in the severity of HE[52]. Furthermore, in the advanced stages of HE in acute liver failure, the brain produces a number of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6[54,55]. This relationship is supported by evidence derived from therapeutic interventions, such as moderate hypothermia, that reduce cerebral oedema by reducing cerebral blood flow and inflammatory responses[56,57].

Cirrhosis

In patients with cirrhosis, there is mounting evidence for the role of inflammation in exacerbating the symptoms of HE, thus reinforcing the potential synergistic effects of ammonia and inflammation. Studies have shown this to be the case in patients with minimal HE, and across the whole spectrum of patients with varying degrees of overt HE (Westhaven grades 0-4)[53,58,59]. A recent study confirmed that the presence and severity of minimal HE in cirrhosis is independent of the severity of liver disease and plasma ammonia concentration, but markers of inflammation are significantly higher in those with minimal HE compared to those without[59]. In a further study, significant deterioration of neuropsychological test scores in patients with cirrhosis following induced hyperammonemia during the inflammatory state, but not after its resolution, suggested that inflammation might be important in modulating the cerebral effect of ammonia in liver disease, supporting an inflammatory hypothesis[53].

Synergy with ammonia

As inflammation, infection, and ammonia have been shown to be important in the pathogenesis of HE in cirrhosis, the question has to be raised as to whether infection and inflammation have a synergistic relationship with ammonia[60]. Marini and Broussard used mice with a deficiency in a critical urea cycle enzyme conferring chronic hyperammonemia, to demonstrate an increased sensitivity to inflammation. Furthermore, the hyperammonemic mice developed longer lasting and stronger cognitive defects when exposed to an inflammatory stimulus[61]. In a bile duct ligated (BDL) rat model, Jover *et al*[62] fed an ammonia-containing diet for 2 wk following ligation and compared animals sacrificed 7 d later to those fed a normal chow diet. Ammonia-fed BDL rats had increased cerebral ammonia and demonstrated the presence of type II Alzheimer astrocytosis analogous to patients with cirrhosis presenting with episodic HE. Both BDL groups had evidence of systemic inflammation, but the ammonia-fed BDL rats had increased brain glutamine, decreased brain myoinositol, and a significant increase in brain water compared to BDL controls, alluding to a potential synergistic relationship between ammonia and systemic inflammation. Wright *et al*[27] went on to explore the hypothesis that the inflammatory response induced by lipopolysaccharide (LPS) exacerbates brain oedema in BDL rats. LPS administration increased brain water in ammonia-fed, BDL, and sham-operated animals significantly, but this was associated with the progression to pre-coma only in the BDL animals. LPS induced cytotoxic brain swelling, but the anatomical integrity of the blood brain barrier was maintained. There was evidence of brain and systemic inflammation in BDL rats, which was significantly increased in LPS-treated animals. Nitrosation of proteins in the frontal cortex of BDL and LPS-treated animals was demonstrated. These data provide further evidence that in a background of cirrhosis and hyperammonemia, superimposed inflammation has an important role in the development of HE.

The ammonia-induced nitrosation of astrocytic proteins shown by Wright *et al*[27] has also been demonstrated in isolated astrocytes and astroglial tissue in brain sections of portocaval shunted rats[63]. However, ammonia alone cannot be responsible, because protein nitrosation was not demonstrated in ammonia fed sham-operated and ammonia-fed BDL rats in the absence of an inflammatory stimulus. Therefore, both ammonia and an additional inflammatory insult might need to be present for nitrosation of brain proteins to occur in animals with "subliminal" inflammation, such as that which has been observed in the BDL model[27]. This is further supported by recent work that demonstrated the presence of tyrosine nitration in astrocyte cultures in the presence of concentrations of TNF-α typically observed in patients with acute liver failure[64].

Inflammation and the brain

During an episode of infection, cytokines cannot directly cross the blood brain barrier and are unable to have a direct effect. Nevertheless, the peripheral immune system can still signal the brain to elicit a response during infection and inflammation through the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, both in the periphery and in the brain. Brain signalling may occur by direct transport of the cytokine across the blood brain barrier *via* an active transport mechanism, the interaction of the cytokine with circumventricular organs and activation of afferent neurons of the vagus nerve[65]. Endothelial cells, along with the astrocyte, are major constituents of the blood brain barrier. Endothelial cells are activated during infection, resulting in the release of various mediators into the brain. Activated microglial cells and astrocytes have the ability to produce a full repertoire of cytokines in response to inflammation and injury. One such cytokine is IL-1β, which has been shown *in vitro* to compromise the integrity of the blood brain barrier. This is mediated through the cyclo-oxygenase (COX) pathway within the endothelial cell[66]. In a portocaval shunted rat model that is more akin to a model of minimal HE, Cauli *et al*[67] demonstrated an improved learning ability following the administration of supra-therapeutic doses of the non-steroidal anti-inflammatory drug (NSAID), ibuprofen. This was accompanied by normalisation of COX and inducible NO activity within the cerebral cortex but interestingly also an increase in TNF-α. It is unclear however, how this NSAID specifically interacts with the glutamate-nitric oxide-cGMP pathway and how COX plays a role in the pathogenesis of minimal HE without identification of the specific COX isoform involved and in the absence of neuroanatomical, proteomic and genomic data. Nevertheless, the therapeutic use of NSAID in HE is not novel. Indomethacin (non-selective COX inhibitor) has been shown in patients with acute liver failure[68], and in a portocaval shunted rat model[69], to improve intracranial hypertension and cerebral oedema. Unfortunately, NSAID use is associated with a number of systemic complications, including cardiovascular/renal compromise and cellular prostaglandin metabolism, which impact greatly on not only astroglial function, but also on the development of organ dysfunction, particularly in the context of patients with longstanding liver disease.

TNF-α is released early during infection and can also influence the permeability of the blood brain barrier[70]. Moreover, an association between circulating TNF-α levels in patients with acute[71] and chronic liver failure[72] and the severity of HE, regardless of aetiology, has been recognised. Endothelial cells have receptors for IL-1β and TNF-α which can transduce signals which ultimately culminate in the intracerebral synthesis of NO and prostanoids[73]. Bémeur *et al*[74] investigated the effect of *IL-1β*, *TNF-α* and interferon-α (*IFN-α*) gene deletions on the onset of HE. Deletion of the *IFN-γ* gene had no effect on brain water levels or neuropsychiatric status. On the other hand, *IL-1β* and *TNF-α* gene deletions significantly delayed the onset of HE and brain oedema.

The relationship between the brain and inflammation is not one way. Molecular and neurophysiological studies during the past decade have suggested that pro-inflammatory responses are controlled by evolutionary neural circuits that operate reflexively[75,76]. The afferent arc of the reflex consists of nerves that sense injury and infection. This activates efferent neural circuits including the cholinergic anti-inflammatory pathway, which modulate immune responses and the progression of inflammatory disease. It might therefore be possible to target neural networks for the treatment of inflammation. This novel and fascinating body of work has recently been reviewed by Tracey[77].

Innate immune dysfunction

Innate immune dysfunction occurs in both acute and chronic liver failure, and up to 50% of admissions to hospital in patients with cirrhosis are likely to be related to the development of infection. In response to infection, the body initiates the innate immune response with phagocytic cells, such as monocytes and neutrophils. This response is particularly relevant to the liver, as the liver is the first organ to encounter bacteria or other toxins absorbed in the gut from the portal vein. Bacterial translocation of organisms from the gut in patients with cirrhosis and portal hypertension results in chronic endotoxemia. This culminates in a local milieu of pro-inflammatory cytokines/chemokines which can upregulate adhesion receptors and activate neutrophils[78]. There is significant literature on the immune response to infection in liver disease, which involves an important role of phagocytes and release of inflammatory cytokines. Patients with cirrhosis are functionally immunosuppressed and have impairment of several host defence mechanisms. The hemodynamic derangement of cirrhosis resembles that produced by endotoxin, and bacteremia can greatly exacerbate this state[79].

Neutrophils are a key component of the innate immune response. Ammonia has been shown to induce neutrophil dysfunction by inducing cell swelling, impaired phagocytosis, and increased oxidative burst in normal neutrophils *ex vivo*, in ammonia-fed rats and in patients with cirrhosis given an ammonia load[80]. Not only does this make patients potentially vulnerable to developing bacterial and fungal infections, but induces oxidative stress, and may ultimately culminate in a "sepsis-like" immune paralysis[81] and a reduction in monocyte HLA-DR expression[82].

OXIDATIVE STRESS

The evidence for the role of oxidative stress in the pathogenesis of HE is incontrovertible. Animal studies have shown significant reductions in the activities of glutathione peroxidase and superoxide dismutase enzymes, both in the liver and brain of rats exposed to ammonium acetate. Superoxide levels, in submitochondrial particles, were found to be elevated in ammonia-exposed rats[83] and lipid peroxidation has been shown to be increased, further demonstrating that hyperammonemia induces oxidative stress[84].

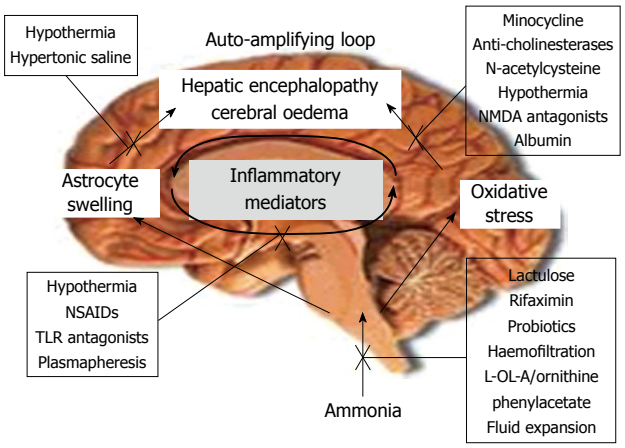

Figure 1 The "Two-hit" hypothesis. In a background of liver injury and hyperammonemia, a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia can drive further astrocyte swelling, oxidative stress and lead to a rapid deterioration in neurocognitive function. The close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop". The sites of action of potential therapies are indicated on the Figure. L-OL-A: L-ornithine L aspartate; NMDA: N-methyl D-aspartate; NSAID: Non-steroidal anti-inflammatory; TLR: Toll-like receptor.

N-methyl D-aspartate (NMDA) receptors play a key role in the production of free radicals and an NMDA antagonist can prevent the calcium-mediated increase in oxidative stress[85]. *In vivo* excessive ammonia-induced NMDA receptor activation reduces antioxidant enzyme activity and results in increased production of superoxide anions[86]. It is, however, extremely difficult to differentiate whether it is oxidative stress that influences astrocyte swelling or whether astrocyte swelling itself induces oxidative stress through NMDA receptor and calcium-dependent mechanisms[87]. Either way, whether one considers that "the chicken came before the egg or vice versa", it would imply that the close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop" which promotes the development of HE[88] (Figure 1).

The production of reactive oxygen species (ROS) can arise in a number of different ways. Aside from ROS arising from neutrophil activation[80] and local and systemic inflammation/infection, ammonia and hypo-osmotic swelling-induced nitric oxide synthesis, the activation of NADPH oxidase[89], and mitochondrial glutamine uptake all generate ROS[90-92]. From these data we can propose a "two-hit hypothesis" in the pathogenesis of HE. Liver dysfunction leads invariably to hyperammonemia, which leads to astrocyte swelling, and in the longer term, structural changes to astrocytes (Alzheimer's type II astrocytosis). After this initial "hit", a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia in a patient with cirrhosis can drive further astrocyte swelling, oxidative stress, and lead to a rapid deterioration in neuropsychological function (Figure 1 and Table 3).

Table 3 Factors precipitating hepatic encephalopathy[2]

Precipitating factors	Ammonia load e.g. upper gastrointestinal bleed or portocaval shunt
	Inflammation/oxidative stress
	Infection
	Dehydration
	Hyponatremia
	Sedative drugs e.g. benzodiazepines

Uptake of ammonia by astrocytes leads to the production of glutamine through the action of glutamine synthetase. Glutamine exposure in cultured astrocytes increases oxidative stress[91]. Mitochondrial glutamine uptake and subsequent cleavage of glutamine by phosphate-activated glutaminase elevates mitochondrial ammonia, which stimulates ROS production *via* induction of the mitochondrial permeability transition (MPT)[93]. However, cultured astrocytes exposed to ammonia produce ROS and begin swelling almost immediately, whereas MPT induction and glutamine accumulation occur thereafter.

Although astrocytes are relatively resistant to oxidative and nitrosative stress, neighbouring neurones are vulnerable to free radical attack. This can compromise brain energy metabolism and neurotransmission in patients with HE. Furthermore, ammonia, TNF-α, benzodiazepines, and hyponatremia can all trigger nitric oxide-dependent mobilisation of zinc which can augment GABAergic neurotransmission[94].

The mechanism through which free radical production is increased is currently not fully understood. One suggestion is based on findings that link an increase in calcium release to hyperammonemia. Rose *et al*[95] exposed cultured mice astrocytes to ammonium chloride. They observed a transient increase in the concentration of calcium ions from intracellular stores. The use of a calcium chelator (BAPTA) prevented the ammonia-induced production of free radicals[25]. Another possibility is that ROS are produced through activation of NMDA receptors[96].

One other area of research interest involves oxidation of RNA. It has been shown that in patients with Alzheimer's disease, there is significant RNA oxidation, which might result in impairments in protein synthesis and, consequently, cognitive function in patients[97]. Görg *et al*[98] reported the effects on cultured rat astrocytes and rat brain *in vivo* of ammonia exposure. Ammonia exposure was associated with a rapid, reversible oxidation of RNA (thought to involve NMDA receptor activation and calcium release). Consistent with this theory is the fact that some substrates required for learning and memory require protein synthesis[96]. Disruption of this protein synthesis *via* RNA oxidation might therefore interfere with cognitive function.

THERAPEUTIC STRATEGIES IN HE

To date, most therapeutic strategies in HE have been focused on lowering arterial concentrations of ammonia

and modulating inter-organ ammonia metabolism, but these remain largely ineffective. Treatments based on the hypothesis that the colon is the primary organ responsible for the generation of ammonia have ranged from dietary protein restriction, to the use of non-absorbable disaccharides, non-absorbable antibiotics, and colectomy[99]. However, Córdoba *et al*[100] showed that diets with normal protein content can be administered safely to patients with cirrhosis with episodic HE and that protein restriction does not have any beneficial effect for cirrhotic patients during an episode of HE and indeed, might even be detrimental in a patient with an underlying catabolic state.

It has been demonstrated that lactulose administered to patients with minimal HE in an unblinded open label study[101] might be of benefit and another open label randomised placebo controlled study in patients with a previous history of overt HE suggested that lactulose might delay the onset of a recurrent episode of HE[102]. However, in a recently published systematic review[103], which had very few high quality studies to base its findings on, lactulose was not found to have any impact on mortality in patients with cirrhosis presenting acutely with overt HE.

The use of non-absorbable antibiotics had been largely abandoned after concern that long-term administration of neomycin might lead to problems with nephrotoxicity and ototoxicity, and with metronidazole might lead to peripheral neuropathy. However, support for this strategy has been recently reinvigorated with the publication of the largest double blind placebo controlled study (n = 299) by Bass *et al*[104], which compared rifaximin (which has no known long term toxicity) favourably with placebo for the secondary prophylaxis of HE.

Benzodiazepine antagonists such as flumazenil also emerged as a potential therapy for HE patients. An analysis of six randomised controlled trials showed that 27% patients treated with flumazenil showed a clinical improvement, whilst 19% of treated patients showed an electroencephalographic improvement[105].

In the sickest cohorts, direct ammonia removal by hemofiltration in the intensive care unit is effective, but unfortunately by this stage multiorgan dysfunction and bacteremia might have superseded. Likewise, albumin dialysis in patients with acute-on-chronic liver failure improves HE grade[106], but the improvement is independent of changes in ammonia or cytokines[107] and remains controversial[108].

To address the issue of inter-organ ammonia metabolism, recent studies in patients with cirrhosis have shown that other than the gut, kidneys and muscle might be important targets[99]. Volume expansion produces significant increases in renal ammonia excretion resulting in a reduction in plasma ammonia concentration. This was shown to improve mental state, supporting the notion that the kidneys can be manipulated favourably[109]. During the hyperammonemic state, muscle detoxifies ammonia through conversion to glutamine[110,111]. L-ornithine L-aspartate (LOLA), which is a mixture of two amino acids, provides intermediates that increase glutamate availability for synthesis of glutamine and illustrates the concept that muscle can detoxify ammonia. Administration to animals with acute liver failure resulted in reduced brain water[112], but a recent study in patients with acute liver failure did not have any impact on brain dysfunction or survival[113]. When given to patients with cirrhosis and HE, administration of LOLA resulted in an improvement in HE compared with placebo-treated controls[114], although a recent meta-analysis concluded that it had little effect in patients with minimal HE[115]. Jalan *et al*[116] have hypothesised that this inefficacy might result from an accumulation of glutamine resulting in a rebound rise in circulating ammonia. By utilising a strategy which enables the excretion of glutamine, Davies *et al*[117] have demonstrated a synergy between L-ornithine and phenylacetate in reducing arterial ammonia in BDL rats.

However, in this review we have already convincingly demonstrated that ammonia, although central in the development of HE, is not solely responsible for its development. Infection/inflammation and oxidative stress are key determinants and indeed act synergistically with ammonia. Although ammonia could potentially be responsible for the development of neutrophil dysfunction, a patient with cirrhosis presents independently as a model of chronic endotoxemia that has direct implications on the innate and adaptive immune systems. We must therefore also look to therapies that directly or indirectly target the proinflammatory milieu.

Potential therapeutic strategies might include NMDA antagonists[84], leukodepletion[118], antagonism of pro-inflammatory cytokines[119], antioxidants [N-acetylcysteine (NAC)[120] and albumin[107,121]], anti-inflammatories (COX inhibitors[67] and minocycline[122,123]), probiotics[124] and hypothermia[125]. Excitement surrounds the prospect of small molecules that modulate toll-like receptor (TLR)-4 signalling, which can potentially down regulate neutrophil activation and other cellular responses. Early data indicate that TLR-4 antagonists can reduce LPS-stimulated cytokine release in healthy volunteers and results from phase 3 clinical trials are awaited. Inhibition of TLR-2, 4, and 9 prevented the increase in neutrophil oxidative burst induced from plasma from patients with alcoholic hepatitis. Furthermore, albumin, an endotoxin scavenger, prevented the deleterious effect of patients' plasma on neutrophil phagocytosis, spontaneous oxidative burst, and TLR expression[121]. This might also explain the beneficial role of albumin dialysis on HE[107,126].

When administered early after an overdose of acetaminophen, intravenous NAC prevents hepatic necrosis by replenishing stores of glutathione[127]. In patients with acute liver failure secondary to an overdose of acetaminophen, and in patients with acute liver failure secondary to other causes, NAC has been shown to increase oxygen delivery and consumption associated with increases in mean arterial pressure, cardiac index[128], and cerebral perfusion pressure[120]. These beneficial hemodynamic effects have been shown to be mediated by enhanced activity of the nitric oxide/soluble cGMP system[129] and suggest that NAC could have a beneficial role in the treatment of patients with cirrhosis who have developed overt HE.

As the role of central pro-inflammatory mechanisms are believed to be important in the pathogenesis of HE, then another novel therapeutic candidate drug to be considered is minocycline, which has been shown in two very recent studies by Jiang *et al*[122,123] to have anti-inflammatory effects in rats with acute liver failure. Minocyline treatment prevented both microglial activation [CD11b/c (OX-42) expression on immunohistochemistry] as well as the upregulation of IL-1β, IL-6, TNF-α, heme-oxygenase-1, eNOS, iNOS mRNA and protein expression with a concomitant attenuation of the progression of HE and brain edema, and at least in part, by reduction of oxidative/nitrosative stress. Thus, minocycline might also have promise in patients with acute and chronic liver failure cirrhosis and HE, and could be taken forward into randomised placebo controlled trials.

Modulation of intestinal microbiota is an emerging strategy to reduce the bacterial translocation of LPS and other bacterial activators of TLRs. Probiotics have been shown to reduce bacterial translocation and were shown to improve liver function and prevent the development of infection and HE in patients with cirrhosis[124]. Furthermore, probiotics have been shown to restore neutrophil phagocytic capacity in patients with alcoholic cirrhosis, possibly by reducing endogenous levels of IL-10 and TLR-4 expression[130].

Recent studies show that hypothermia is efficacious in patients with uncontrolled intracranial hypertension that are undergoing liver transplantation[56,125]. Hypothermia displays many beneficial effects on brain water and intracranial hypertension relating to decreased brain ammonia, cerebral blood flow, mediators of inflammation, and oxidative stress[131]. The sites of action of potential therapies for HE is shown in Figure 1.

CONCLUSION

HE is a dynamic neuropsychological spectral disorder that develops after liver injury. The pathophysiological mechanisms behind the development of HE are still not fully understood, but ammonia and the downstream consequences of ammonia uptake by astrocytes remain fundamental to the process. Ammonia not only leads to astrocyte swelling, but also alters neurotransmission, mitochondrial function, and induces oxidative stress. Astrocyte swelling and oxidative stress are closely related and result in "an auto-amplifying" loop. The presence of local and systemic inflammation and the release of ROS further exacerbate the cerebral effects of ammonia. Anti-inflammatory and anti-oxidative strategies may abrogate these effects and offer real treatment options to patients with HE in the future.

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