Université de Montréal

Encéphalopathie hépatique: Physiopathologie et nouvelles approches thérapeutiques

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Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiae Doctor (Ph.D)

en Sciences Biomédicales

Décembre 1999

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée:

Encéphalopathie hépatique: Physiopathologie et nouvelles approches thérapeutiques

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Thèse acceptée le: 14 avril 2000

ABSTRACT

Hepatic encephalopathy (HE) is a neuropsychiatric disorder occurring in both acute and chronic liver diseases. Depending on the duration and degree of hepatic dysfunction, HE may be present as one of two major types; portal-systemic encephalopathy (PSE) (chronic liver failure) and fulminant hepatic failure (FHF) (acute liver failure).

Hyperammonemia is a key feature of both PSE and FHF and it is strongly suggested that ammonia toxicity is implicated directly or indirectly in the pathogenesis of both forms of HE.

The present thesis comprises 5 articles demonstrating various aspects of the pathophysiology and new approaches to the treatment of HE.

In chapter 2.1; article 1, using *in vivo* microdialysis, brain extracellular glutamate levels were found to be increased in correlation with arterial ammonia levels and the degree of neurological impairment in rats with FHF due to liver devascularization. *N*-methyl-D-aspartate (NMDA) receptor binding was found to be unchanged in rats with liver devascularization compared to control rats.

Treatments for both forms of HE continue to focus on ammonia-lowering strategies. When administered to portacaval shunted (PCS) rats, L-ornithine-L-aspartate (OA), two substrates of the urea cycle, was observed to result in a lowering of plasma ammonia and increased plasma urea levels as well as protection against ammonia-induced coma (chapter 2.2; article 2). In acute liver failure, peripheral ammonia is removed via muscle glutamine synthetase (GS). This was confirmed in the study (chapter 2.3; article 3) in rats with liver devascularization where OA also lowered plasma ammonia and protected rats against coma and brain edema. GS activity in muscle was increased following OA treatment. Mild hypothermia was shown to be protective against coma and brain edema in rats with liver devascularization (chapter 2.5; article 5). In mildly hypothermic rats, plasma ammonia levels were unaffected whereas cerebrospinal fluid (CSF) ammonia levels

were lowered suggesting that hypothermia prevents increased ammonia uptake into brain. This protective effect was associated with a decrease in extracellular brain glutamate levels, supporting the proposal that glutamate may be implicated in the pathogenesis of brain edema in FHF.

Although the precise pathophysiologic mechanisms responsible for HE in FHF are not completely understood, an increased glutamatergic neurotransmission could contribute to this phenomenon.

Another potential neurotoxin, manganese, is believed to be implicated in the pathogenesis of PSE. Manganese levels were found to be increased in both autopsied brain tissue from patients and in brain tissue from experimental animal models of PSE (chapter 2.4; article 4). It is suggested that manganese deposition is responsible for the signal hyperintensities on T_1-weighted magnetic resonance (MR) images and the extrapyramidal symptoms found in PSE.

RÉSUMÉ

L'encéphalopathie hépatique (EH) est un désordre neuropsychiatrique que l'on retrouve soit dans la période aiguë ou la phase chronique d'une maladie du foie. Ainsi, selon la durée de l'atteinte hépatique, l'EH peut se présenter de 2 façons: la première étant l'encéphalopathie porto-systémique et la seconde l'encéphalopathie rencontrée au cours des hépatites fulminantes.

L'encéphalopathie porto-systémique est secondaire à la dérivation porto-systémique du sang veineux tel que rencontré spontanément lors de l'hypertension portale ou soit suite à une anastomose portocave chirurgicale ou radiologique (shunt intra-hépatique portosystémique transjugulaire ou TIPS). Cliniquement, l'EH porto-systémique est un syndrome neurologique qui se développe lentement; le stade précoce est souvent peu apparent et se caractérise par des modifications du cycle du sommeil ainsi que des changements mineurs de personnalité. Une baisse du niveau d'attention ainsi qu'une incoordination musculaire apparaissent ensuite, progressant lentement vers la léthargie, la stupeur et le coma. Du point de vue anatomopathologique, l'EH porto-systémique est caractérisée par une astrocytose sans évidence d'altérations neuronales structurelles.

L'hyperammonémie est une caractéristique importante de l'EH porto-systémique et de l'encéphalopathie aiguë des hépatites fulminantes. Il est admis que l'ammoniaque est impliqué directement et/ou indirectement dans la pathogénèse dans ces deux types d'EH. À forte concentration, l'ammoniaque a le potentiel d'affecter le système nerveux central de divers façons. Il y a d'abord un effet direct de l'ion ammonium sur la neurotransmission inhibitrice ou excitatrice ainsi qu'une inhibition de l'enzyme α-cétoglutarate déshydrogénase dans le cycle de Krebs, ce qui a comme conséquence directe d'altérer le métabolisme énergétique du cerveau. Cependant, le métabolisme énergétique du cerveau ne semble affecté que dans les stades très avancés de l'EH porto-systémique ou d'encéphalopathie aiguë des hépatites fulminantes. L'insuffisance hépatique chronique se traduit par une augmentation des

concentrations de manganèse dans le sang et le cerveau. Une sélectivité des dépôts de manganèse est l'hypothèse la plus probable afin d'expliquer les signaux hyperintenses localisés dans le pallidum tel que démontré par l'imagerie par résonance magnétique chez les patients cirrhotiques. La section 2.5 démontre que les dépôts de manganèse sont augmentés dans les globus pallidus prélevés à partir d'autopsie du tissu cérébral chez des patients cirrhotiques. La concentration de manganèse est aussi élevée dans le globus pallidus dans deux modèles animaux d'insuffisance hépatique chronique. De plus, une corrélation a été établie entre le degré de dérivation porto-systémique et la quantité de dépôts de manganèse. Au contraire, le manganèse cérébral n'est pas augmenté dans un modèle animal d'hépatite fulminante, ce qui suggère que l'accumulation découle de l'insuffisance hépatique chronique, plus particulièrement suite à une dérivation porto-systémique.

L'EH est caractérisée par des perturbations de plusieurs systèmes de neurotransmission cérébrale. Le système glutamatergique est celui qui a été le plus étudié et on croit qu'il est impliqué dans la pathogénèse de l'EH.

Des nouveaux traitements sont requis pour traiter ou stabiliser l'EH chez les patients atteints d'EH porto-systémique ou d'encéphalopathie aiguë afin d'augmenter la période de temps nécessaire pour pouvoir effectuer une transplantation. Les traitements actuels sont soit inefficaces ou comportent des effets secondaires très néfastes. Afin de traiter l'EH porto-systémique, on préconise comme thérapie des stratégies axées sur la diminution de l'ammoniaque sérique.

La L-ornithine-L-aspartate (OA) est composée de deux substrats du cycle de l'urée qui se sont avérés efficaces pour réduire l'ammoniaque et améliorer les symptômes cliniques chez des patients hyperammonémiques ayant une EH porto-systémique. Nous avons démontré un effet protecteur de l'OA sur le coma précipité par une infusion d'ammoniaque chez des rats ayant une dérivation porto-cave. L'effet protecteur s'accompagne d'une réduction significative de l'ammoniaque plasmatique ainsi que d'une augmentation significative de l'urée plasmatique, ce qui suggère que la réduction de la concentration plasmatique de l'ammoniaque est en partie le résultat

d'une augmentation de la synthèse d'urée par le foie. Nous croyons aussi que l'OA peut, par l'intermédiaire des transaminases, mener à la production de trois molécules de glutamate. Ce substrat (le glutamate) peut ensuite stimuler l'activité de la glutamine synthétase dans les muscles, le foie et le cerveau pour ainsi former de la glutamine. Cette possibilité est soutenue par l'augmentation du glutamate et de la glutamine dans le plasma et le liquide céphalo-rachidien (LCR).

Contrairement à l'EH porto-systémique, l'insuffisance hépatique fulminante (HF) progresse très rapidement en quelques heures ou jours seulement, vers un état mental altéré, la stupeur et finalement le coma. Les convulsions sont rares mais des myoclonies sont souvent rencontrées avant le coma. Dans cette condition, le taux de mortalité est élevé et la mort est souvent causée par une hernie du tronc cérébral secondaire à une hypertension intracrânienne causée par un oedème cérébral massif. L'oedème cellulaire des astrocytes est fréquemment observé mais l'astrocytose Alzheimer de Type II (voir insuffisance hépatique chronique) n'est pas une caractéristique neuropathologique de l'hépatite fulminante. L'ammoniaque est aussi incriminé dans la physiopathologie de ce type d'encéphalopathie.

Afin d'élucider davantage la pathophysiologie de l'encéphalopathie des HF, nous avons mesuré, par le biais d'une microdialyse cérébrale *in vivo*, les concentrations extracellulaires des acides aminés dans le cortex frontal de rats atteints d'HF induite par dévascularisation hépatique afin d'établir une relation avec le degré d'atteinte neurologique. Dans ce modèle, on retrouve un oedème cérébral accompagné d'une augmentation de l'eau, tel que mesurée dans le cortex frontal. Les concentrations extracellulaires de glutamate sont significativement élevées trois heures avant le début du stade précoma et continuent à augmenter jusqu'à l'état comateux. Ces données suggèrent que l'HF mène à une augmentation de la libération de glutamate et/ou une diminution de la recapture du glutamate de l'espace extracellulaire. Récemment, des études sur le tissu cérébral de rats encéphalopathiques suite à une HF ont révélé une diminution de la concentration protéique et de l'expression génique de GLT-1, un transporteur astrocytaire du

glutamate. L'augmentation de la production de la glutamine via la glutamine synthétase est aussi possiblement impliquée dans la pathogénèse de ce type d'encéphalopathie. Cependant, l'augmentation constante des concentrations de glutamine extracellulaire n'est pas corrélée avec la sévérité de l'encéphalopathie. Ceci suggère que la glutamine joue une rôle plutôt mineur dans la pathogénèse de l'oedème cérébral.

Les traitements de l'encéphalopathie des HF sont axés sur le contrôle de l'hypertension intracrânienne. La transplantation hépatique demeure le traitement ultime mais d'autres traitements sont nécessaires afin de prolonger la vie des patients en attente de transplantation. Des stratégies visant à diminuer les taux d'ammoniaque ont été développées depuis qu'une étude récente a démontré que la survenue d'hernie cérébrale chez les patients souffrant d'HF était corrélée à la concentration artérielle d'ammoniaque. Nous avons démontré que l'infusion d'OA chez des rats ayant subi une dévascularisation hépatique entraînait un délai significatif avant l'apparition du coma, et une diminution significative de l'ammoniaque du plasma et du LCR comparativement aux contrôles. Cette diminution d'ammoniaque était accompagnée d'une réduction du contenu cérébral en eau. Le glutamate et la glutamine plasmatiques furent aussi significativement augmentés et puisque le cycle de l'urée est non-fonctionnel dans un foie dévascularisé, la réduction d'ammoniaque ne pouvait donc être induite que par la stimulation de la glutamine synthétase des muscles squelettiques. Le glutamate présent dans le LCR diminue parallèlement à la réduction du contenu cérébral en eau.

Un autre des traitements qui fut récemment développé pour contrôler l'encéphalopathie des HF est l'hypothermie modérée. En réduisant la température corporelle de rats ayant subi une dévascularisation hépatique à 34°C, nous avons démontré un effet protecteur de cette procédure sur l'apparition du coma et de l'oedème cérébral. Les niveaux des acides aminés furent aussi mesurés en utilisant la méthode de microdialyse cérébrale *in vivo* et nous avons démontré une diminution du glutamate extracellulaire chez les rats protégés par l'hypothermie. Les

mécanismes possiblement impliqués dans l'action bénéfique de l'hypothermie modérée incluent la diminution du transfert de l'ammoniaque sanguin vers le cerveau et la diminution des concentrations extracellulaires cérébrales d'acides aminés excitateurs tels que le glutamate. Ces résultats ajoutent davantage de crédibilité à la notion que la disponibilité élevée du glutamate dans l'espace extracellulaire et donc, une neurotransmission glutamatergique élevée, est impliquée dans la pathogénèse de certaines des complications cérébrales rencontrées au cours de l'HF. Ces résultats appuient aussi l'hypothèse que l'hypothermie modérée peut s'avérer une méthode efficace de prévention de l'encéphalopathie et de l'oedème cérébral, deux complications cérébrales très sévères de l'HF.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
AAT	Aspartate aminotransferase
ALF	Acute liver failure
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CCl_4	Carbon tetrachloride
Cl^-	Chloride
CMR_A	Cerebral metabolic rate for ammonia
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DBI	Diazepine binding inhibitor
EEG	Electroencephalogram
FHF	Fulminant hepatic failure
GABA	Gamma-aminobutyric acid
GS	Glutamine synthetase
GSADH	Glutamate semialdehyde dehydrogenase
H^+	Hydrogen
HAL	Hepatic artery ligation
HE	Hepatic encephalopathy
HVA	Homovanillic acid
ICP	Intracranial pressure

K^+	Potassium
KA	Kainate
Mn^{2+}	Manganese
MR	Magnetic resonance
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSO	Methionine sulfoximine
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	L-ornithine-L-aspartate
OAT	Ornithine aminotransferase
ODN	Octadecaneuropeptide
OLT	Orthotopic liver transplantation
PCA	Portacaval anastomosis
PCS	Portacaval shunted
PET	Positron emission tomography
PRE	Presynaptic neuron
POST	Postsynaptic neuron
PS	Permeability surface
PSE	Portal-systemic encephalopathy
PTBR	Peripheral-type benzodiazepine receptor
TIPS	Transjugular intrahepatic portal-systemic shunt

ACKNOWLEDGMENTS

First, I would like to express my heartfelt thanks to Dr. Roger F. Butterworth (Directeur de l'Unité de Recherche en Sciences Neurologiques, Hôpital Saint-Luc, Montréal, Québec, Canada), my mentor, my supervisor and my friend for having inspired me to fulfill this goal of mine. Without your direction, your enthusiasm and your patience this goal would not have been scored. "It is like a soccer game, right Rog!?"

I would like to thank Dominique and "les deux Manons" for helping me when help was needed. Also technical support from François, André, Bernard and Christian.

Je tiens à remercier tous les membres du Centre du Recherche de m'avoir permis de travailler dans un environnement si agréable et sympathique.

I would like to thank my colleagues/friends with whom I worked with everyday, for making my Ph.D successful and enjoyable.

To all my friends who have tried to understand what I have been doing all these years, now you can read it!

To my family; Mum, Dad and my brother Jason. If it wasn't for you guys I would probably be playing in the NHL...but hey!

Merci à la Faculté des Études Supérieures de l'Université de Montréal.

It is the desire to succeed,

and not the fear of failing.....

CHAPTER 1

REVIEW OF THE LITERATURE

1. HEPATIC ENCEPHALOPATHY

1.1 Definitions and classification

HE is a complex neuropsychiatric syndrome occurring in acute or chronic liver disease. It is present in one of two major forms depending on the speed of onset, the extent of liver damage, the extent of portal-systemic shunting, and the presence of a precipitating factor.

A relationship between the functional status of the liver and that of the brain has been known for centuries (Frerichs, 1860) and a healthy liver is necessary to maintain normal brain function. Neurological impairment may occur in such diverse clinical situations as acute, subacute and chronic liver diseases, inherited errors of the urea cycle and spontaneous or iatrogenic portal-systemic venous shunting including, surgical portacaval anastomosis (PCA) or transjugular intrahepatic portal-systemic shunts (TIPS) aimed at surgically treating and reducing portal hypertension. PSE is associated with increased portal-systemic shunting of gut-derived constituents of portal venous blood due to their impaired extraction by the failing liver and, in most instances, their passage through intrahepatic and/or extrahepatic portal-systemic venous collateral channels. These gut-derived substances gain access to the brain and accumulate to toxic levels, possibly resulting in neurological impairment. The clinical manifestations of HE range from subtle abnormalities detectable only by psychometric testing, to deep coma. HE may be present in 50-70% of all patients with cirrhosis, including those with abnormalities identified only by psychometric testing (Gitlin et al., 1986). It has been conventional to classify HE as a reversible metabolic encephalopathy in which most manifestations are reversible with medical treatment.

Subclinical HE is the term applied to chronic liver disease patients with portal hypertension and portal-systemic shunting and who do not display overt behavioural, neurological, or electroencephalogram (EEG) changes but who have abnormal scores

in psychometric tests. These changes are reversed by effective treatment (Rikkers et al., 1978).

FHF is an acute, frequently fatal illness that results from severe inflammatory or necrotic liver disease of rapid onset in healthy individuals as opposed to progressive liver failure from underlying cirrhosis (McCaul et al., 1986). Acute HE typically progresses through altered mental status with abrupt onset of delirium, to stupor and coma within hours or days and a mortality rate of about 90% without liver transplantation. The principle cause of death in FHF is brain herniation caused by increased intracranial pressure (ICP) that results from cytotoxic brain edema. The many causes of FHF include viral diseases, such as hepatitis A, B, C, idiosyncratic drug reactions, toxins and certain mushrooms and medicinal herbs. Although viral hepatitis is the most common cause of FHF worldwide, regional variations in etiology are observed. For example, intentional acetaminophen (paracetamol) overdose is the most common cause of FHF in the United Kingdom.

Prerenal azotemia, acute tubular necrosis, hepatorenal syndrome, and drug-induced nephrotoxicity frequently complicate FHF and have an adverse effect on prognosis (Moore et al., 1991). Renal failure occurs in approximately 75% of patients after acetominophen overdose and in 30% to 40% of patients with other causes of FHF (O'Grady et al., 1988). Hypoglycemia induced by FHF may result in a rapid deterioration of mental status. Signs of hypoglycemia may be masked, therefore blood glucose levels are measured frequently and supplemental intravenous glucose is administered as needed. Abnormal coagulation is common in FHF and predisposes patients to bleeding at venous access and arterial puncture sites as well as bleeding from the gastrointestinal tract.

1.2 Clinical manifestations

Neurologically, PSE progresses slowly in contrast to FHF which progresses rapidly. The earliest clinical signs are often subtle psychiatric and behavioural

changes that may be more apparent to the patient's family and close friends than to the neurologist (Gitlin et al., 1986; Pappas and Jones, 1983). These changes are primarily due to mild impairment of intellectual function that reflects predominantly bilateral forebrain, parietal, and temporal dysfunction. Encephalopathy is graded on a scale of I to IV (Table 1) with prognosis inversely correlated with the degree of encephalopathy. In early stages the presence of pronounced intellectual impairment may be masked by relatively well preserved verbal ability (Gilberstadt et al., 1980; Schomerus et al., 1981). As encephalopathy progresses, intellectual ability deteriorates, motor function becomes impaired, and ultimately consciousness is impaired. Other behavioural complications of PSE include altered sleep patterns, anxiety, altered mood, behaviour and personality changes (Jones, 1995). With further progression of liver disease, asterixis, stupor and coma ensue. Hypertonia, hyperreflexia, and positive Babinski signs may be elicited and tend to precede the occurrence of hypotonia and diminished deep tendon reflexes in late stages. In contrast to most other metabolic encephalopathies, features of PSE may include manifestations of extrapyramidal dysfunction, muscular rigidity, bradykinesia, hypokinesia, Parkinsonian-like tremor, and dyskinesia.

FHF presents with a variety of clinical features which usually occur when ICP reaches 30 mm Hg. Nonspecific symptoms such as nausea or vomiting rapidly progress to the development of encephalopathy and/or coma which is often abrupt. In the early phases of encephalopathy, the degree of cognitive impairment may fluctuate. Unlike PSE, agitation, delusion, and hyperkinesis are short lived, and coma rapidly ensues. In most cases hyperventilation is marked, and pupils are dilated and react sluggishly to light.

Table 1: Clinical stages of hepatic encephalopathy. (Conn and Lieberthal, 1979)

Grade 0	No abnormality
Grade 1	Mild confusion
	Euphoria or depression
	Decreased attention
	Impaired performance in mental tasks (addition or subtraction)
	Slurred speech
	Irritability + anxiety
	Reversal of sleep rhythm
Grade 2	Drowsiness
	Lethargy
	Apathy
	Obvious personality changes
	Inappropriate behaviour
	Disorientation for time and place
Grade 3	Somnolence but rousable upon stimuli
	Amnesia
	Confusion
	Gross disorientation
	Stupor
	Delirium
Grade 4	Coma

2. ANIMAL MODELS OF HEPATIC ENCEPHALOPATHY

2.1 Portal-systemic encephalopathy

Clinically, PSE develops following a surgical treatment (PCA or TIPS) for reducing portal-hypertension. PCA is feasible in animals whereas TIPS has not been developed experimentally. PCA in rat is a model for PSE which has been well characterized for many years (Lee and Fisher, 1961).

2.1.1 Portacaval shunt

A portacaval shunt is an end-to-side anastomosis of the portal vein and the inferior vena cava. Rats are anesthesized with halothane for the entire surgery. Sham-operated controls undergo the same surgical procedure (along with similar clamping times) but without suturing. PCS rats are studied 4 weeks after PCA. Overall mortality for shunted rats is generally 5-10% (Lee and Fisher, 1961; Hindfelt et al., 1977; Butterworth et al., 1988a).

Following a 5-10 day period of postoperative weight loss, PCS rats show normal feeding patterns and, at the time of sacrifice (4 weeks), there are no significant differences in weight gain between shunted and sham-operated animals. Liver weight is reduced by >50% in shunted rats and blood ammonia levels rise from a mean value of 0.17 mM prior to shunting to 0.3 mM 4 weeks after the shunt (Butterworth and Giguere, 1986). Ammonia concentrations of brain tissue from rats 4 weeks after PCA are significantly increased; for example, in cerebral cortex, ammonia levels rise to 0.5 mM (Butterworth and Giguere, 1986).

Although PCA *per se* does not result in overt neurological abnormalities, detailed psychometric and neurobehavioural assessments have revealed discreet abnormalities in neurological function. Such abnormalities include changes in sleep patterns, decreased startled response to tactile and auditory stimuli, as well as

decreased spontaneous locomotor activity and exploratory behaviour and abnormal reflexes (Beaubernard et al., 1977; Giguere and Butterworth, 1984; Bengtsson et al., 1985). Such symptoms may be comparable to those observed in grade 1 PSE in humans with chronic liver disease. For this reason, the rat with PCA best represents an animal model of minimal or subclinical PSE.

2.1.1.1 Subacute portal-systemic encephalopathy

Subacute PSE is induced by intraperitoneal injections of ammonium acetate (3.8 mmol/kg) 4 weeks after end-to-side PCA. This protocol results in decreased locomotor activity, multifocal myoclonus, and abnormalities of righting and corneal reflexes (Hindfelt et al., 1977; Giguere and Butterworth, 1984). Within 30 minutes of ammonia administration, rats fall into a deep anesthetic coma lasting up to 1 hr. EEGs obtained from such animals show progressive slowing of brain wave activity to a predominant frequency of 4-5 Hz accompanied by triphasic waves similar to those observed in humans in hepatic coma (Hindfelt et al., 1977). The neurobehavioural response of PCS rats to ammonia loading is generally separated into two well defined stages of encephalopathy, namely the precoma stage at which rats first lose their ability to right themselves when placed on their sides but who have normal corneal reflex, and the coma stage at which rats have lost both righting and corneal reflexes (Butterworth et al., 1988a; Giguere and Butterworth, 1984). Control groups of animals used in these studies were PCS rats that received equimolar concentrations of sodium acetate and/or sham-operated control animals that received ammonium acetate at 3.8 mmol/kg.

2.2 Experimental cirrhosis

Contrary to PCS animals, experimental cirrhosis in animals is used to evaluate HE in relation to a necrotic liver and portal-systemic shunting. Spontaneous

PSE develops through the development of portal-systemic collaterals. Secondary biliary and carbon tetrachloride (CCl_4)-induced cirrhosis are the most commonly used animal models of experimental cirrhotics.

2.2.1 Secondary biliary cirrhosis

In this model, adult rats are anesthetized for the entire surgery with halothane. The common bile duct is dissected and cleaned. Preceding the ligature of the common bile duct is an intracholedochal injection of 10% formalin (Colombato et al., 1994). Sham-operated controls undergo the same surgical procedure except no ligations are done. Overall mortality for bile duct ligated rats is low. Vitamin K is given intramuscularly to compensate for the coagulopathy. Six weeks after the bile duct ligation, marked jaundice, ascites, portal hypertension, portal-systemic shunting and hyperammonemia develop (Kountouras et al., 1984).

2.2.2 CCl_4-induced cirrhosis

To induce liver cirrhosis using CCl_4, rats are given drinking water containing phenobarbitone for 10-14 days. Phenobarbitone induces enzymes that increase the sensitivity of the liver to the CCl_4. Rats are then given weekly doses of intragastric CCl_4(for 7-10 weeks) under light halothane/oxygen anesthesia, until ascites develops. Pathologically, the model is characterized by micronodular liver histology, a finely nodular liver surface, a shunted liver, splenomegaly and testicular atrophy, portal hypertension and decreased plasma albumin. CCl_4-induced cirrhotic rats become hyperammonemic and show varying degrees of encephalopathy (Bengtsson et al., 1987; Yamamoto and Sugihara, 1987).

2.3 Fulminant hepatic failure

Animal models of FHF are developed along two lines: the exclusion of the liver from the circulation or the administration of a specific hepatotoxin.

2.3.1 Anhepatic models

Anhepatic models can be accomplished by two different techniques; 1) by devascularizing the liver or 2) by removing the liver.

2.3.1.1 Hepatic Devascularization

In this model, following PCA (48 hrs), a rat is reanesthesized with halothane and the hepatic artery is ligated. Thus the liver is devascularized and left *in situ*. Its afferent circulation is both diverted and interrupted.

In experimental animal models of FHF, brain ammonia concentrations are in the 1-5 mM range. After surgery has been completed, animals recover consciousness but eventually fall into precoma and coma. These two main stages of encephalopathy are defined as 1) loss of righting reflex; pre-coma and 2) loss of corneal reflex; coma. Pre-coma usually occurs 8-12 h after hepatic artery ligation (HAL) with coma following at 12-15 h post HAL.

FHF rats become hypothermic, a state that has been shown to affect the neurological picture (Traber et al., 1989). Therefore, body temperature must be maintained at 37°C using a heat lamp or heating pad.

Liver devascularization results in severe hypoglycemia. This may occur in the first hours after the HAL and thus requires periodic surveillance. Dextrose (10%) in water may be administered subcutaneously with monitoring of serum glucose (Zieve et al., 1984).

2.3.1.2 Total hepatectomy

In total hepatectomy, the liver is removed and the splanchnic circulation is rerouted by a series of surgical interventions. The anhepatic model is used in experiments to eliminate the possibility of toxins arising from a necrotic liver.

2.3.2 Toxin models

Toxic models of FHF have generally utilized galactosamine, acetaminophen, or thioacetamide as experimental agents. Galactosamine is a direct hepatotoxin, whereas acetaminophen and thioacetamide must be converted to toxic metabolites within the liver via the microsomal cytochrome P450 system. The pathologic nature of the hepatic damage produced by each compound varies. Rats with thioacetamide-induced FHF develop hypothermia, hypoglycemia, hyperammonemia and brain edema (Zimmermann et al., 1989).

3. NEUROPATHOLOGY IN HEPATIC ENCEPHALOPATHY

Neuropathologically, HE is characterized by astrocytic rather than neuronal changes. Astrocytic abnormalities occur both in FHF (astrocytic swelling) and PSE (Alzheimer Type II astrocytosis).

3.1 Astrocytes

Astrocytes are the most numerous cells in the central nervous system (CNS), outnumbering neurons by as much as ten to one and occupying about one third of the volume in the cerebral cortex (Pope, 1978). Astrocytes form a "barrier" around capillaries, pia, and ependyma, thus anatomically segregating blood and CSF from

neurons and the external environment. Their cytoplasmic processes fill much of the interstices of the CNS and are intimately positioned at such critical sites such as around the synaptic complex and at the nodes of Ranvier. Astrocytes are connected to each other by gap junctions (Dermietzel et al., 1991; Fischer and Kettenmann, 1985), forming an anatomical syncytium and thus allowing functional responses in astrocytes to extend to distal sites (Enkvist and McCarthy, 1992). Astrocytes are dynamic and metabolically active cells possessing a rate of metabolic activity in the same range as neurons (Hertz, 1981; Hertz and Peng, 1991). These dynamic cells have critical metabolic supportive functions involved in the maintenance and regulation of the extracellular microenvironment. They are involved in potassium (K^+) buffering, in the homeostasis of other ions including hydrogen (H^+) and calcium (Ca^{2+}) (Walz, 1989; Ransom and Sontheimer, 1992), osmoregulation (Walz, 1989), development and regulation of the blood-brain barrier (BBB) (Janzer and Raff, 1987; Risau and Wolburg, 1990), provision of nutrients and neurotransmitter precursors to neurons (glutamine, alanine, α-ketoglutarate, malate, succinate, lactate and pyruvate) (Westergaard et al., 1995), detoxification of ammonia, drugs and hormones (Abramovitz et al., 1988), metabolism of CO_2 (Anderson et al., 1984), free radical scavenging (Makar et al., 1994), metal sequestration (Sawada et al., 1994), uptake and release of neurotransmitters and neuromodulators (Shousboe, 1981), inflammatory/immune responses (Hertz et al., 1990), and neurotrophism (Rutishauser, 1993). Increasing evidence strongly indicates that astrocytes significantly influence neuronal excitability and neurotransmission (Nedergaard, 1994).

Astrocytes are the main cells prominently involved in HE. No significant or consistent morphological changes have been identified in neurons or other cell types of the CNS. The significance of the astrocyte response in HE is still not completely clear, but it has been strongly advocated that HE represents a primary "gliopathy" (Norenberg, 1981) in which a disturbance in critical astrocytic functions (such as neurotransmitter uptake and ion homeostasis) can result in neuronal dysfunction lead

to CNS derangements.

3.2 The astrocyte in portal-systemic encephalopathy: Alzheimer type II astrocytosis

Neuropathological studies reveal that chronic hyperammonemia in the adult, resulting from liver cirrhosis or congenital urea cycle deficits, leads to a characteristic pattern of astrocytic changes known as Alzheimer Type II astrocytosis (Dolman et al., 1988; Harding et al., 1984; Von Hosselin and Alzheimer, 1912). Alzheimer Type II astrocytosis has been described in cases of severe hyperammonemic encephalopathy in the absence of liver disease or urea cycle deficits in patients following PCA as a part of a surgical treatment of pancreatic carcinoma (McDermott and Adams, 1954), or following bladder dome resection (Boogerd et al., 1990). Furthermore, ammonia-treated primary cultured astrocytes show Alzheimer Type II changes (Norenberg, 1989; Norenberg, 1993; Norenberg, 1995).

Increasing evidence that astrocytes are critically involved in the pathogenesis of PSE derives largely from pathological findings where a positive correlation is found between the severity of PSE and the degree of Alzheimer Type II astrocytosis (Martin et al., 1987). Alzheimer Type II changes occur in protoplasmic (grey matter) astrocytes, particularly in cerebral cortical and basal ganglia structures. It is characterized by a large pale nucleus, prominent nucleolus and margination of the chromatin pattern (Figure 1). Some animal models of PSE show this astroglial change (Norenberg, 1981).

No consistent histological changes to neurons have been described in the brain in chronic liver disease and so it has been proposed that PSE represents a "astrocytopathy" or a "gliopathy".

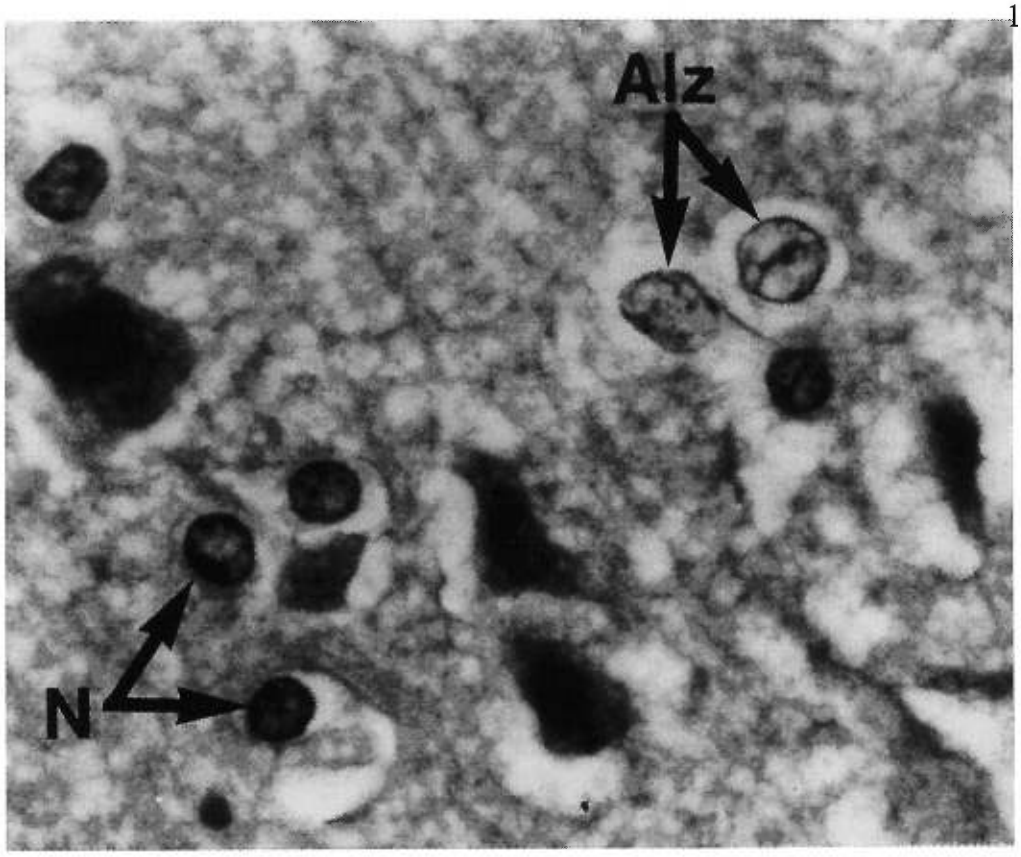

Figure 1: An Alzheimer Type II astrocyte (Alz) and a normal astrocyte (N) in frontal cortex of a cirrhotic patient who died in hepatic coma.

3.3 The astrocyte in fulminant hepatic failure: cytotoxic edema

The first reports of brain swelling in acute hepatic necrosis date back to the Second World War, where it was reported that edematous brains were found at autopsy in patients who died of hepatitis (Lucke, 1944). Later on, this correlation was confirmed that brain swelling was the cause of death in FHF (Ware et al., 1971; Gazzard et al., 1975). Cerebral edema occurs in 75% of FHF patients with grade IV encephalopathy (Anonymous, 1991; Williams and Gimson, 1991).

In contrast to the situation in chronic liver disease, HE in FHF results in cerebral edema and increased ICP. Cerebral edema and raised ICP are probably manifestations of the same pathological process. Cerebral edema leads to raised ICP once the volume capacity of the brain skull has been exceeded (Blei, 1991). It seems

that rapid, rather than slow loss of hepatocellular function favours the development of cerebral edema and raised ICP, possibly because an appreciable time interval is required for osmolar compensation to take place in response to changes in metabolites in the brain when the liver fails as could be the case in chronic HE (Cordoba and Blei, 1996). An acute or chronic increase in ICP in FHF may lead to brain ischemia due to compression of cerebral vasculature (Wendon et al., 1994) and/or brain stem herniation. Indeed, herniation of the cerebellum or uncinate process secondary to raised ICP is a common cause of death in patients with FHF (Gazzard et al., 1975). The neuropathologic picture therefore is one of cellular (astrocytic) swelling (Kato et al., 1992) rather than Alzheimer Type II astrocytosis which is characteristic of chronic liver disease. Astroglial swelling and/or cerebral edema is found at coma stages in most experimental animal models of FHF (Swain et al., 1991). Furthermore, cerebral edema in both experimental and human FHF is cytotoxic (rather than vasogenic) in nature with astrocytic swelling being consistently reported (Kato et al., 1992; Blei and Traber, 1989).

4. PATHOPHYSIOLOGY OF HEPATIC ENCEPHALOPATHY AND BRAIN EDEMA

4.1. Ammonia

Under normal conditions, ammonia is produced either endogenously through the gut or by the actions of catabolizing enzymes on exogenous compounds. Hyperammonemia, commonly occurring as a consequence of hepatic failure, leads to impairment of CNS function.

4.1.1 Physiology of ammonia

Under normal physiological conditions, 98-99% of ammonia is present in blood as ammonium ions (NH_4^+) and this is reflected by the dissociation constant of 9.13-9.15 at 37°C (Bromberg et al., 1960). Ammonia is lipid soluble, diffusing through plasma membranes rapidly whereas ammonium ions are hydrophilic and cross the plasma membrane through potassium channels (Raichle and Larson, 1981). Ammonium ions have a permeability 0.3 times that of potassium (Binstock and Lecar, 1969). Under normal conditions of blood and intracellular brain pH of 7.4 and 7.0 respectively, and by applying the Henderson-Hasselbach equation, the ratio of brain to blood ammonium is 2:1. However, when brain ammonia metabolism is disturbed acutely by the administration of ammonium salts, the brain/blood ammonia concentration ratio increases and may remain increased for a considerable time (Cooper and Lai, 1987).

4.1.2 Ammonia production

Normal healthy persons generate considerable amounts of ammonia in the intestine. Ammonia is derived from protein digestion of food and deamination of glutamine (Weber and Veach, 1979; Windmueller and Spaeth, 1974). The ammonia produced enters the bloodstream through the portal vein. In addition to gut sources, some interorgan transport of ammonia also takes place. Ammonia is generated as a normal product of many metabolic reactions throughout the body, the most important being the glutamate dehydrogenase, glutaminase and adenosine monophosphate (AMP) deaminase reactions. Some of this metabolically-generated ammonia is released to urine following deamination of glutamine by the kidneys however the kidney can also liberate ammonia to blood (Imler et al., 1984; Nash and Benedict, 1921). In addition, skeletal muscles release ammonia to blood, particularly during and after vigorous exercise (Babij et al., 1983a; 1983b).

4.1.3 Ammonia elimination

Ammonia is normally removed by the liver's urea cycle or via GS, localized in the liver, brain (specifically astrocytes) and skeletal muscle. The brain and skeletal muscle are devoid of a urea cycle.

4.1.3.1 Ammonia removal by the liver

Ammonia removal by liver is distinct from that of extrahepatic cells. Liver ammonia removal is accomplished through two different metabolic pathways that rely on an intact urea cycle and GS. These separate pathways are compartmentalized in two different types of liver cells; periportal and perivenous. Urea synthesis is localized in periportal cells and GS is situated in the perivenous cells (Haussinger, 1983), which represent 6-7% of total hepatocytes (Gebhardt and Mecke, 1983). The periportal system has a high affinity (but low capacity) for ammonia, whereas the perivenous system has a low affinity but a high capacity for ammonia (Szerb and Butterworth, 1992; Haussinger, 1983). The synthesis of urea represents approximately 70% of total ammonia detoxification by the liver, the remaining 30% occurs by the formation of glutamine via GS. Perivenous GS acts as a scavenger for ammonia that escapes urea synthesis.

4.1.3.2 Ammonia removal by the brain

Removal of ammonia by the brain is well controlled, yet is quantitatively much more limited than that removed by the liver. Brain ammonia concentrations are maintained at relatively low levels (40-50 μmol/L) (Butterworth, 1998) by a series of enzymatic reactions. In brain, ammonia removal occurs exclusively via glutamine synthesis. Astrocytes are the cells responsible for synthesizing glutamine through the actions of GS. (Norenberg and Martinez-Hernandez, 1979). Neurons do

not express GS. It has been shown that, under normal physiological conditions, GS operates at near maximal capacity (Cooper and Lai, 1987). In hyperammonemic conditions, GS activity does not increase in response to the increased ammonia and is thus unable to cope with potentially neurotoxic ammonia concentrations.

4.1.3.3 Ammonia removal by the muscle

Skeletal muscle, as with brain, is devoid of a urea cycle. However, skeletal muscle like brain expresses GS (Ruderman, 1975). GS activity in skeletal muscle is less than that in brain or liver but the quantity of muscle mass renders muscle important as a major organ for ammonia removal. Under normal physiological conditions, 50% of arterial ammonia is metabolized by skeletal muscle (Lockwood et al., 1979).

Contrary to the brain, GS in muscle is inducible. GS has been found to increase in activity (up to 8 fold) in hyperammonemic rats compared to controls rats (Hod et al., 1982), and this was accompanied by an increase in blood glutamine.

4.1.4 Ammonia and portal systemic encephalopathy

Evidence for an association between HE and ammonia dates back over a century when it was observed that feeding meat to dogs with PCA resulted in loss of coordination, stupor, and coma, leading to the suggestion that nitrogenous products were the causative factor in so called "meat intoxication" (Eck, 1877). In 1952, ascites was treated in cirrhotic patients with ion-exchange resins that absorbed sodium and released ammonium ions. This treatment resulted in significant reductions in ascitic volume but precipitated severe neurological symptoms that were indistinguishable from PSE (Gabuzda et al., 1952). The accumulation of ammonia, predominantly as a result of poor hepatic function and portal-systemic shunting, has been judged to be the most important factor in the pathogenesis of PSE. Factors

supporting the importance of ammonia include the production of Alzheimer Type II astrocytosis in hyperammonemia, and in urea-cycle enzyme deficiencies which occur in children with inborn errors of the urea cycle. Cirrhotic patients with PSE have elevated CSF and blood ammonia levels. Here, loss of hepatic function as well as portal-systemic shunting of blood leads to an accumulation of ammonia.

Arterial blood and brain ammonia concentrations are frequently elevated in patients with PSE. Also, portacaval shunting in animal models similarly results in a 2-3 fold increase in brain ammonia (Butterworth, 1991a). Further evidence consistent with accumulation of toxic levels of ammonia in human HE is provided by the results of recent studies using positron emission tomography (PET) and $^{13}NH_3$ (Lockwood et al., 1991). Such studies demonstrate an increase in the cerebral metabolic rate for ammonia (CMR_A) i.e. the rate at which ammonia is taken up and metabolized by brain. Furthermore, this increased rate was accompanied by an increase in the permeability-surface area product (Figure 2), a measure of BBB permeability, suggesting that in chronic liver failure the barrier is increasingly permeable to ammonia.

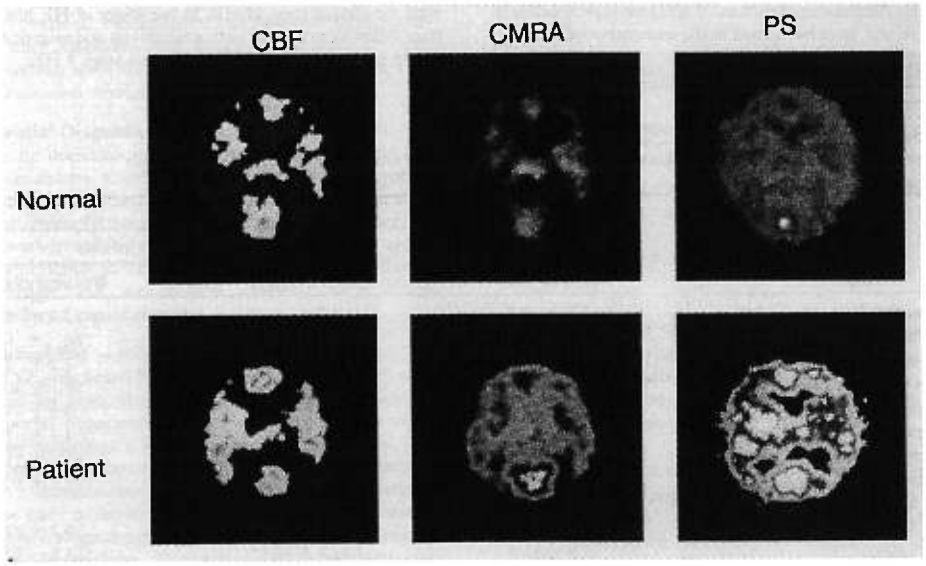

Figure 2: $^{13}NH_3$-PET images of a control subject compared to a cirrhotic patient with HE. Cerebral blood flow (CBF), cerebral metabolic rate for ammonia (CMR_A), and permeability surface area product (PS) are increased in HE patient.

Other evidence in support of a major role of ammonia in HE includes 1) therapies which reduce circulating ammonia concentrations and demonstrate an amelioration of PSE in cirrhotic patients (Conn and Lieberthal, 1979); 2) precipitation of encephalopathy in patients with cirrhosis (hypersensitivity) by administration of ammoniagenic substances such as high protein diet amino acids, urea and ammonia-releasing enzymes. However, evidence against ammonia as the sole factor in the development of HE is the poor correlation between serum and CSF concentrations of ammonia and the severity of HE (Stahl, 1963). Indeed, plasma ammonia levels may be normal in liver failure in the presence of elevated CNS levels of ammonia (Ehrlich et al., 1980; Lockwood et al., 1991).

Therefore, to date, the ammonia hypothesis remains the best explanation of the clinical pathological and neurochemical features of PSE (Butterworth, 1991a).

4.1.5 Ammonia and FHF

Ammonia-related mechanisms have also been proposed in the pathogenesis of brain edema in FHF. Brain ammonia levels are increased in experimental animals with FHF and these increases have been reported to be in the 0.5-5 mM range (Swain et al., 1992b). Increased brain water content has been described in dogs with urease-induced hyperammonemia (Levin et al., 1989) as well as in rats with ammonia infusions (Takahashi et al., 1991). Furthermore, treatment of cerebral cortical slices with ammonia in pathophysiological (millimolar) concentrations results in significant cell swelling (Ganz et al., 1989). Similar results have been described following the exposure of cultured astrocytes to ammonia (Norenberg et al., 1991). Cerebral edema is also commonly encountered in Reye's syndrome (hyperammonemia with no liver failure). Furthermore, in primates, an infusion of ammonia results in cortical astrocytic swelling and a subsequent increase in ICP (Voorhoies et al., 1983). A constant intravenous infusion of ammonia into normal rats causes brain edema (Takahashi et al., 1991), while a similar infusion in rats after PCA results in a faster

rise in brain water content and an elevation of ICP (Blei et al., 1994). Ammonia has been suggested to contribute to the pathogenesis of cerebral edema and raised ICP by promoting increased conversion of glutamate to the organic osmolyte glutamine (which accumulates in astrocytes) thereby impairing cellular osmoregulation (Cordoba and Blei, 1996; Blei, 1991; Brusilow, 1986). A series of observations in experimental animals and humans support a pathogenetic role for glutamine in ammonia-induced swelling. Ammonia is not primarily responsible for swelling, as brain edema could be prevented (Takahashi et al., 1991) or ameliorated (Blei et al., 1994) *in vivo* by co-administration of methionine-sulfoximine (MSO), an irreversible inhibitor of GS and whose effects result in reduced glutamine formation and a higher concentration of brain ammonia. In fact, observations made over three decades ago in mice had shown a protective effect of MSO on mortality associated with ammonia administration (Warren and Schenker, 1964). In *In vitro* studies, ammonia-induced swelling of primary astrocyte cultures could also be reduced by MSO (Norenberg and Bender, 1994). There is clear evidence that glutamine increases in the brain in FHF. In experimental models of FHF, where brain edema can be detected in several species and preparations (Blei et al., 1992), a 4-6 fold rise in brain glutamine can be detected (Swain et al., 1992b). In two rat models of FHF, the increase in glutamine preceded the rise in brain water (Olafsson et al., 1995). In human FHF, nuclear magnetic resonance spectroscopy (NMR) showed a clear increase in the glutamine peak (McConnell et al., 1995), though quantitation is imprecise with this technique. In post-mortem samples of human brain, glutamine exhibited a three fold rise in FHF patients over controls (Record et al., 1976).

Still, there are many unresolved questions before glutamine can be considered as a key factor in the pathogenesis in FHF. When MSO was administered to rats after hepatic devascularization, animals succumbed with a picture of generalized seizures, associated with very high ammonia levels arising from the combination of liver failure and GS inhibition (Olafsson, and Blei, unpublished results). Furthermore, administration of MSO also increases the concentration of brain

glutamate and excitotoxicity due to glutamate may play a role in the well described convulsant actions of this drug, preventing it from being used as a treatment against brain edema in FHF.

Perhaps the most prominent criticism is the fact that glutamine also increases in the brain of cirrhotic individuals in coma, where brain edema is seldom noted. The lack of time to develop an osmolar compensation may explain this discrepancy (Cordoba, 1996).

4.2 Manganese

Accumulation of another neurotoxic substance, manganese, has been suggested to contribute to the pathogenesis of HE. There is increasing evidence to suggest that manganese deposition occurs in the brain, more specifically in the basal ganglia of patients with chronic liver disease and also in patients with spontaneous or surgically-induced PCS.

Manganese is an essential metal for biological function, but excessive exposure is toxic. Elevated levels in adulthood occur in situations with irreversible brain disease characterized by prominent psychological and neurological disturbances (Cotzias et al., 1999; Barbeau et al., 1976).

4.2.1 Physiology of manganese

Manganese functions as an integral component of several enzymes important in the CNS such as GS (Wedler and Denman, 1984). GS contains eight Mn^{2+} per octamer (Wedler and Toms, 1986) and accounts for approximately 80% of the total manganese content in brain (Wedler and Denman, 1984).

4.2.2 Manganese elimination

Average daily oral manganese intake is 2.5-3 mg. Only 1-3% of this normally reaches the systemic circulation because it is rapidly cleared; 97-99% of manganese is cleared by the liver and excreted into the bile by the biliary tract (Papavasiliou et al., 1966).

4.2.3 Manganese and portal-systemic encephalopathy

Magnetic resonance imaging (MRI) consistently shows a characteristically abnormal signal hyperintensity in the globus pallidus on T_1-weighted MR images in over 80% of cirrhotic patients (Kulisevsky et al., 1992; Spahr et al., 1996; Weissenborn et al., 1995; Inoue et al., 1991) (Figure 3).

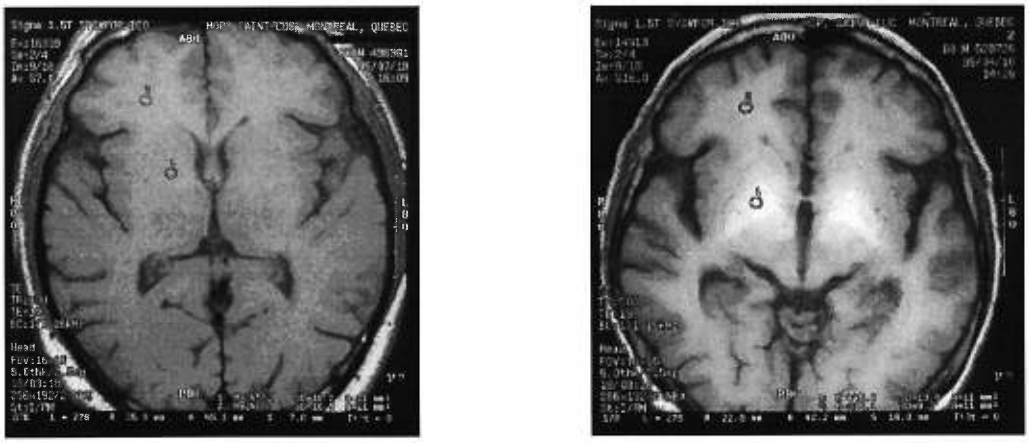

Figure 3: Axial T_1-weighted MRI in a normal (left panel) and in a cirrhotic patient (right panel). Note hyperintense signal in globus pallidus of cirrhotic patient.

Such images could theoretically be caused by products of lipid deposition, calcification (Dell et al., 1988), melanin (Gomori et al., 1986), products of hemoglobin breakdown (Gomori et al., 1985) (such as methemoglobin) or

manganese. Further evidence suggesting that MR images result from manganese deposition is provided by observations in patients receiving long term parenteral nutrition where typical pallidal images disappeared after cessation of manganese supplementation (Mirowitz et al., 1991; Mirowitz and Westrich, 1992). Prolonged inhalation of manganese dusts in miners produces similar MR images (Nelson et al., 1993). Furthermore, exposure of monkeys to manganese administered either by inhalation or intravenously results in selective pallidal hyperintensities on MRI (Shinotoh et al., 1995) similar to that observed in cirrhotic patients. Recent autopsy studies in cirrhotic patients demonstrated an increased content in the brain and more specifically in the pallidum (Pomier Layrargues et al., 1995; Krieger et al., 1995; Maeda et al., 1997; Pomier Layrargues et al., 1995b). This finding correlates strongly with pallidal T_1-weighted MR signal hyperintensities and blood manganese concentrations (Figure 4) (Spahr et al., 1996). Manganese toxicity produces similar changes in MRI of the basal ganglia, and it has been suggested that this deposition of manganese in the basal ganglia may contribute to the pathogenesis of PSE.

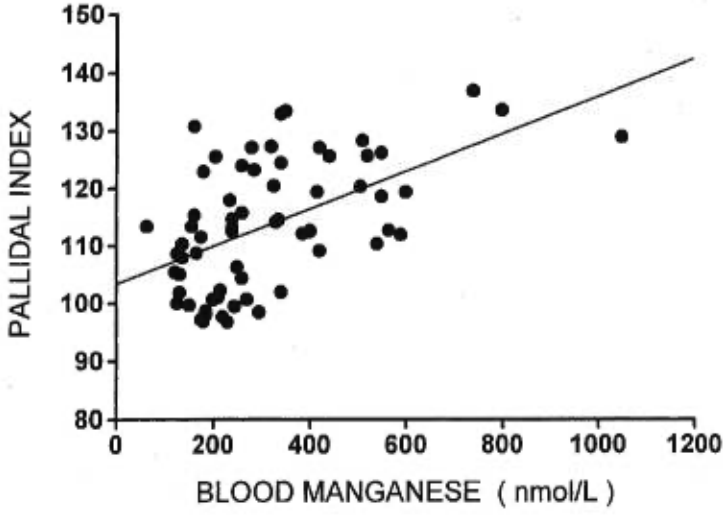

Figure 4: Correlation between pallidal index and blood manganese concentrations in 57 cirrhotic patients (r = 0.543).

4.2.4 Manganese and FHF

No studies of manganese have been performed in relation to FHF.

4.3 Blood-brain barrier

The BBB exists at the level of the endothelial cells lining the capillaries which supply the brain parenchyma. The BBB ensures protection of the biochemical environment of the brain. The BBB plays an important role in excluding unwanted toxic substances from the brain while at the same time conserving neurotransmitter substances within the brain. Molecules reaching the extracellular tissue space in the brain must either be lipid soluble or have specific membrane carrier systems.

4.3.1 The blood brain barrier and portal-systemic encephalopathy

Patients with PSE have a functional but not structural derangement in the BBB that results in alterations in transport of amino acids (James et al., 1978; Mans et al., 1982). The mechanism of this functional disturbance is believed to be due to increased blood ammonia.

4.3.2 The blood brain barrier and FHF

Findings in patients with FHF suggest that a gross breakdown of the BBB is unlikely. Tomographic clinical evidence shows no focal changes in BBB (Munoz et al., 1991) and brain biopsies taken at the time of death show intact tight junctions in the capillary endothelial cells (Kato et al., 1992). This is supported by the fact that plasma mannitol, which is used for treatment of brain edema, requires a grossly intact barrier to exert its osmotic effect. Therefore, it has been suggested that the cytotoxic

brain edema occurs due to alterations in brain energy metabolism which have been found to be a late onset of FHF.

4.4 Brain energy metabolism

4.4.1 Brain energy metabolism in portal-systemic encephalopathy

If present at sufficient concentrations, ammonia has the potential to ultimately cause cerebral energy failure. Ammonia inhibits both pyruvate dehydrogenase (McKhann and Tower, 1961) and α-ketoglutarate dehydrogenase (Lai and Cooper, 1986) thereby depleting the intermediates of the citric acid cycle in the CNS and potentially leading to ATP depletion. This is supported by the finding of increased lactate/pyruvate ratios in brains of experimental PSE (Hawkins et al., 1973). Increased lactate production most likely results from decreased entry of pyruvate into the tricarboxylic acid cycle following ammonia-induced inhibition of α-ketoglutarate dehydrogenase (i.e decreased pyruvate oxidation) (McKhann and Tower, 1961) which also leads to decreases in the cytoplasmic NAD^+/NADH ratio and a concomitant increase in the mitochondrial NAD^+/NADH ratio. It has been suggested that these alterations in redox states are the consequence of ammonia-induced inhibition of the malate-aspartate shuttle (Hindfelt et al., 1977). Consistent with these mechanisms, increased CSF and brain lactate concentrations have frequently been reported in hyperammonemia resulting from liver disease (Hindfelt et al., 1977; Therrien et al., 1991) and congenital urea cycle disorders (Ratnakumari et al., 1992). Increased CSF lactate has also been reported in patients with PSE (Yao et al., 1987). CSF lactate concentrations are increased in direct correlation with deterioration of neurological function in subacute PSE resulting from ammonia treatment of PCS rats (Therrien et al., 1991).

Patients with PSE have decreased CBF and decreased consumption of glucose and oxygen (i.e the cerebral metabolic rate (CMR)) (Lockwood et al., 1991). PET

has shown correlations between alterations in CBF and the severity of neuropsychological function (O'Carroll et al., 1999). Decreased brain glucose ultilization in early PSE is most probably the result of decreased energy demand (i.e. reduced neuronal activity in brain in PSE results in decreased energy needs and consequently reduced glucose consumption). Administration of ammonium salts to PCS rats ultimately results in abnormalities in cerebral energy metabolism (decreases in brain levels of high energy phosphates) but these changes occur after the onset of coma (Hindfelt et al., 1977). Also animals with severe encephalopathy prior to the coma stage do not manifest significant reductions in brain ATP content suggesting that in this experimental model of PSE, the cerebral energy deficit is a late stage phenomenon (i.e at coma stage). This also suggests that PSE is, at least in the late stages, the consequence of reduced neuronal activity as a result of neurotransmission failure rather than primary energy failure in brain. Extrapolation of this model to human beings is difficult, but changes in CBF and glucose metabolism may be an epiphenomenon, secondary to a global depression in the function of the CNS, rather than the cause of PSE. The issue of cerebral energy metabolism in relation to PSE is presently facilitated by the availability of NMR techniques using ^{31}P. Studies using ^{31}P-NMR have been undertaken in cirrhotic patients with mild PSE and as found in the animal studies, no significant alterations of cerebral high energy phosphates were observed (Taylor-Robinson et al., 1994). Decreased cerebral oxygen consumption and glucose metabolism may be consequences of PSE rather than primary pathogenetic factors (Basile et al., 1991).

In 1991, Vallance and Moncada proposed a hypothesis that the free radical nitric oxide was implicated in the hyperdynamic circulation associated with cirrhosis (Vallance and Moncada, 1991). More recently, evidence in support of an involvement of nitric oxide (NO) in HE has emerged. A generalized increase in the activity of nitric oxide synthase (NOS), the enzyme responsible for NO production, has been demonstrated in the brains of rats following PCA (Raghavendra Rao et al., 1995). Increased neuronal NOS protein and mRNA were subsequently reported in

the brains of rats following PCA (Raghavendra Rao et al., 1997). Increased NO production in brain could be responsible for oxidative stress as well as the alterations of cerebral perfusion reported in both humans and experimental animals with chronic liver failure.

4.4.2 Brain energy metabolism in FHF

Although ammonia in concentrations encountered in brain in experimental FHF inhibits the tricarboxylic acid cycle enzyme α-ketoglutarate dehydrogenase, FHF, at least at early stages, does not appear to result in impaired cerebral energy metabolism. Direct measurements of high energy phosphates in the brains of rats with FHF reveal no significant alterations (Cooper and Plum, 1987) and monitoring of high energy phosphates using ^{1}H-NMR also failed to reveal any changes (Bates et al., 1989).

It has been discussed that in FHF, the cerebral circulation is altered and that this may cause brain swelling . Larsen has proposed that an increase in CBF results in brain edema and ICP (Larsen, 1996, Schafer and Jones, 1982), this possibly being mediated by the production iNOS (Blei, personal communication). Alternatively, measurements of CBF and arterial-venous differences across the brain led one group to conclude that brain ischemia may be present in patients with FHF and brain edema (Wendon et al., 1994). This was based on findings of low CBF and increased arterial-venous difference of lactate. It was proposed that ischemia would inhibit the activity of the cellular Na^{+}/K^{+}-ATPase, impair neurotransmission and result in brain swelling (Wendon et al., 1994). More recent studies did not reveal the presence of brain ischemia at earlier stages of FHF (Larsen, 1996).

4.5 Alterations of neurotransmitter systems

4.5.1 Electrophysiological effects of ammonia

As the leading candidate in the etiology of HE, ammonia has been shown to cause deleterious effects on CNS function by both direct and indirect mechanisms. Direct effects of the ammonium ion (NH_4^+) on both inhibitory and excitatory neurotransmission have been reported (Raabe, 1987; Fan et al., 1990; Szerb and Butterworth, 1992).

Ammonium ions affect inhibitory synaptic transmission at concentrations in the 0.5-1.3 mM range (normal range; 40-50 μM), a range which is clearly within that observed in hyperammonemic conditions in HE. Ammonia at these concentrations has been found to impair postsynaptic inhibition in the cerebral cortex, thalamus, brain stem and spinal cord preparations by hyperpolarizing Cl^--dependent inhibitory postsynaptic potentials, an effect which results from the inactivation of the extrusion of Cl^- from neurons by NH_4^+ (Raabe, 1989). Ammonium ions also decrease the hyperpolarization action of Ca^{2+} and voltage-dependent Cl^- currents. These currents modify the inhibitory neurotransmitter synaptic input to neurons and increase neuronal excitability, a mechanism which would be responsible for the seizures encountered in acute hyperammonemic crises observed both in urea cycle enzyme deficiencies (Michalak and Butterworth, 1997a) and in FHF (Ellis et al., 1997).

Millimolar concentrations of ammonia also inhibit excitatory neurotransmission. Effects on both the presynaptic and postsynaptic neural membranes have been proposed. For example, glutamatergic synaptic transmission from Schaffer collaterals to CA1 neurons in hippocampus is reversibly depressed by 1 mM ammonia (Szerb and Butterworth, 1992). Furthermore, the firing of CA1 (glutamatergic) neurons evoked by iontophoretic application of glutamate is inhibited by 2 mM ammonia, leading to the proposal that ammonia decreases excitatory synaptic transmission by a direct postsynaptic action (Fan et al., 1990). Also

millimolar concentrations of ammonia reduce the degree of depolarization induced by both NMDA and AMPA receptors (Lombardi et al., 1994).

4.5.2 Glutamate

Glutamate is the major excitatory neurotransmitter of the mammalian brain. The essential steps involved in the synthesis, release and uptake of glutamate are shown schematically in figure 5.

In the brain, glutamate is synthesized in nerve terminals from two sources: from glucose via the Kreb's cycle, transamination of α-ketoglutarate and from glutamine that is synthesized in the glial cells, transported into the nerve terminals, and locally converted by glutaminase into glutamate. In glutamatergic nerve terminals, glutamate is stored in synaptic vesicles and is released upon depolarization by a Ca^{2+}-dependent exocytotic process. Following depolarization of the presynaptic neuron (PRE), glutamate is released into the synaptic cleft where it stimulates receptors situated either on the postsynaptic membrane (POST) or on the membrane of the adjacent perineuronal astrocyte. *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA) and metabotropic subclasses of glutamate receptors are distributed in a region-selective manner in the mammalian brain (Monaghan et al., 1989). (Postsynaptically), two ion-gated types of glutamate receptors are localized on neuronal membranes: the NMDA and the non-NMDA receptors (KA and AMPA), whereas only KA and AMPA receptors are localized on astrocytic membranes. Upon activation of these ion-gated receptors by glutamate, a Na^{+} and Ca^{2+} influx occurs and subsequently depolarization of the cell. Both astrocytic and neuronal membranes contain metabotropic receptors. This family of receptors is not ion-gated and stimulates Ca^{2+} second messenger pathways upon activation. Glutamate is removed from the extracellular space by a family of high affinity uptake carriers in the plasma membrane of neurons and glial cells. These transporters require energy to

accumulate glutamate inside cells from the transmembrane ion gradients (for Na^+, K^+ and H^+) which are ultimately established by the Na^+/K^+ pump. Several of these transporters have been cloned and sequenced and their cellular localization has been confirmed (Rothstein et al., 1994). From *in situ* hybridization (Torp et al., 1994) and immunohistochemical studies (Danbolt et al., 1992; Levy et al., 1993; Rothstein et al., 1994), GLT-1 and GLAST have been found to be astrocytic glutamate transporters whereas EAAC-1 a neuronal glutamate transporter. The rapid removal of glutamate from the synapse by high-affinity uptake not only serves to terminate the excitatory signal and recycle glutamate but also plays an important role in the maintenance of extracellular levels of glutamate below those that could induce excitotoxic damage. Astrocytes can accumulate glutamate intracellularly to levels up to 10,000 times greater than those in the extracellular space (Nicholls and Attwell, 1990). Following uptake by the astrocyte, GS catalyzes the amination of glutamate to glutamine in an ATP-consuming reaction. The resulting glutamine is released into the extracellular space from which it diffuses into the CSF or is retransported via a low-affinity process into the presynaptic nerve terminal as the immediate precursor of neurotransmitter glutamate. This "glutamate-glutamine cycle" reflects the compartmentation of ammonia metabolism in brain (Berl et al., 1962) and since the cycle is directly implicated in ammonia removal (via GS in the astrocyte), it has been proposed that hyperammonemic syndromes result in impaired neuron-astrocytic trafficking of glutamate (Butterworth, 1993).

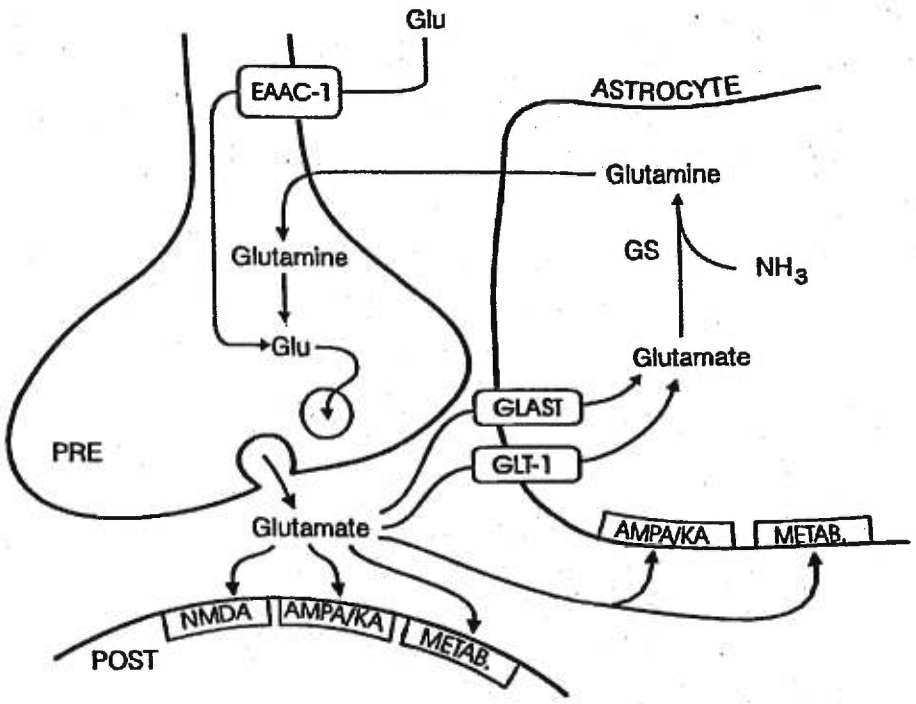

Figure 5: Simplified schematic diagram showing the glutamatergic neurotransmission system.

4.5.2.1 The glutamate system in portal-systemic encephalopathy

Rat hippocampal slices exposed to blood extracts from PSE patients demonstrate a dose-dependent inhibition in the high affinity uptake of the non-metabolizable glutamate analogue, D-aspartate. Moreover, the relative potency of D-aspartate uptake inhibition correlated significantly with ammonia concentrations in blood extracts from these patients (Schmidt et al., 1990). Previous studies using both *in vitro* and *in vivo* techniques have also demonstrated significant alterations of glutamate transport in brains of chronic liver failure. For example, electrically-stimulated Ca^{2+}-dependent release of glutamate (i.e. glutamate released from nerve terminals) in superfused hippocampal slices from PCS rats is significantly increased (Butterworth et al., 1991b) and *in vivo* release of glutamate from cerebral cortices of

rats using the "cortical cup" approach was also found to be increased following portacaval-shunting (Moroni et al., 1983). In addition, release of glutamate from the cerebral cortex was reportedly increased in PCS animals using *in vivo* cerebral microdialysis (Tossman et al., 1987). It has also been demonstrated that 5 mM ammonia blocks glutamate uptake in synaptosomal preparations (Mena and Cotman, 1985). Furthermore, treatment of cultured astrocytes with 2 mM ammonia for 4 days resulted in a 35% decrease in capacity for glutamate uptake by these cells (Norenberg et al., 1985). It is suggested that altered neuronal release and astrocyte uptake constitute impaired neuron-astrocytic "trafficking" of glutamate in PSE (Butterworth, 1992).

This was followed by a report of a selective loss of NMDA receptor sites in the brains of PCS rats (Peterson et al., 1990). A similar selective loss of NMDA receptor density has been reported in subacute hyperammonemia (Raghavendra Rao et al., 1991). On the other hand, non-NMDA receptor densities were found to be significantly reduced in the brains of dogs with congenital PSE (Maddison et al., 1991).

4.5.2.2 The glutamate system in FHF

Brain concentrations of the excitatory amino acids glutamate and aspartate are significantly reduced in FHF resulting from hepatic devascularization in the rat (Swain et al., 1992a; 1992b) as well as in thioacetamide-induced FHF in the same species. Reductions of brain glutamate parallel the deterioration of neurological status in these animals and CSF concentrations of excitatory amino acids are concomitantly increased (Swain et al., 1992a). Glutamate concentrations are also reduced in autopsied brain tissue from patients who died in FHF (Record et al., 1976). Using the technique of *in vivo* cerebral microdialysis, several reports have consistently described increased extracellular concentrations of glutamate in experimental ischemic liver failure in the rat (Bosman et al., 1992) and rabbit (de

Knegt et al., 1994).

Removal of glutamate released into the synaptic cleft by the presynaptic neuronis dependent upon high affinity uptake systems located both on neuronal and astrocytic membranes. The increased concentrations of glutamate in the extracellular fluid of the brain in FHF, therefore, could be the consequence of a failure in the capacity of one or more than one of these transporters. A recent study using synaptosomal (nerve ending) preparations from rats with FHF due to thioacetamide administration revealed a significant reduction in high affinity glutamate uptake (Oppong et al., 1995). Earlier studies demonstrated a significant inhibition of high-affinity glutamate uptake by synaptosomal preparations following exposure to 5 mM ammonia (Mena and Cotman, 1985). Also, studies have demonstrated an effect of millimolar concentrations of ammonia on glutamate uptake into rat synaptic vesicles (Naito and Ueda, 1985). Millimolar concentrations of ammonia inhibit the high-affinity uptake of D-aspartate by cultured astrocytes (Bender and Norenberg, 1996; Norenberg et al., 1985).

4.5.3 GABA

GABA is the major inhibitory neurotransmitter in the brain (Basile et al., 1991) and does not cross the BBB. Increased GABA-mediated neurotransmission is associated with impairments of motor function and decreased consciousness, two of the typical manifestations of HE (Anderson, 1984; Schafer et al., 1984; Basile et al., 1991; Jones et al., 1994).

4.5.3.1 GABA and portal-systemic encephalopathy

Many studies, predominantly in animal models, support the hypothesis that increased GABA-mediated neurotransmission contributes to the characteristic neuroinhibition (Jones et al., 1994). Plasma ammonia concentrations typically found

in patients with grade II-III HE (100-400 uM) can be associated with an ammonia induced enhancement of neuronal inhibition (Basile and Jones, 1997) (see section 4.5.1). Recently, ammonia in concentrations that commonly occur in plasma in liver failure has been shown to facilitate GABA-gated Cl^- currents in cultured cortical neurons (Basile and Jones, 1997) and to increase selectively the binding of agonist ligands (^{3}H-flunitrazepam) to the $GABA_A$/benzodiazepine receptor complex (Basile and Jones, 1997; Ha and Basile, 1997).

On the other hand, direct studies in human PSE yielded negative results; no significant alterations of GABA (Lavoie et al., 1987a) or GABA-related enzymes (Lavoie et al., 1987b) or benzodiazepine receptors (Butterworth et al., 1988b) were apparent in the brains of these patients. Subsequent studies in cirrhotic and PCS dogs likewise revealed no alterations in brain GABA levels and GABA uptake (Roy et al., 1988).

4.5.3.2 GABA and fulminant hepatic failure

It was first found that gut-derived GABA, by virtue of its decreased removal by liver, increased brain uptake levels of GABA in a rabbit model of FHF (Bassett et al., 1990; Schafer and Jones, 1982). However, no evidence for increased GABA transport was demonstrated in galactosamine-induced FHF (Knudsen et al., 1988). Furthermore, GABA levels in brains of patients in FHF have been shown to be unchanged compared to controls (Record et al., 1976). On the contrary, the thioacetamide-induced rat model demonstrated that brain GABA levels were decreased (Zimmermann et al., 1989). $GABA_A$ receptor forms a supramolecular receptor with a benzodiazepine binding site. "Natural" benzodiazepines have been proposed to play a role in the development of FHF, since beneficial effects have been demonstrated using different benzodiazepine antagonists in rats with FHF (Baraldi et al., 1984).

4.5.4 Monoamines

Monoamines have been suggested to be implicated in the development of HE in both acute and chronic liver failure. The serotonergic system is thought to be implicated in the neuropsychiatric symptoms, whereas alterations of the dopaminergic system may relate to the extrapyramidal symptoms found in HE. Tryptophan is metabolized by 3 different pathways; to serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), to kynurenine and quinolinic acid or to tryptamine. Tyrosine is metabolized to dopamine, noradrenaline (norepinephrine) and adrenaline (epinephrine). Oxidation of dopamine leads to the formation of homovanillic acid (HVA).

4.5.4.1 Monoamines and PSE

Many of the neuropsychiatric symptoms of early PSE such as altered sleep patterns are signs that have classically been attributed to modifications of (5-HT) neurotransmission. It has been demonstrated that the administration of large doses of tryptophan to patients affected by hepatic disorders or to dogs with a portacaval shunt may lead to coma (Rossi-Fanelli et al., 1982). Increased brain tissue concentrations of tryptophan were observed in PCS rats (Bengtsson et al., 1991) and in cirrhotic patients in hepatic coma (Bergeron et al., 1989; Young et al., 1975).

Also, increased concentrations of the (5-HT) metabolite, 5-HIAA, have consistently been reported in both CSF and brain tissue from patients (Bergeron et al., 1989; Young et al., 1975) and experimental animals (Bengtsson et al., 1991; Bergeron et al., 1990) with severe encephalopathy resulting from chronic liver failure. More recently, studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma reveal increased activities of the 5HT-metabolizing enzyme monoamine oxidase (MAO-A) (Raghavendra Rao et al., 1993a). Kynurenic and quinolinic acid have pronounced effects on neuronal activity, showing activity at

excitatory amino acid receptors on neurons in the CNS (Perkins and Stone, 1983). Tryptamine, a neuroactive metabolite of tryptophan which produces indoleacetic acid, has been found to be increased in patients in hepatic coma (Yonug and Lal, 1980). Furthermore, densities of high-affinity binding sites for [^{3}H]-tryptamine are concomitantly reduced in brain tissue from these patients (Mousseau et al., 1994). Postsynaptic $5HT_2$ binding sites are increased in hippocampi of cirrhotic patients who died in hepatic coma (Raghavendra Rao et al., 1993a) suggesting upregulation of these sites consistent with a (5-HT) synaptic deficit (Holm et al., 1988).

Neuromuscular abnormalities including extrapyramidal symptoms such as tremor and rigidity form part of the clinical syndrome of PSE. By analogy with extrapyramidal symptoms in Parkinson's disease, a neurodegenerative disorder characterized neurochemically by a nigrostriatal dopamine deficit, it has been suggested that the extrapyramidal signs and symptoms in PSE may be the consequence of abnormalities of dopamine neurotransmission. Studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma reveal increased brain concentrations of the dopamine metabolite HVA (Bergeron et al., 1989). Furthermore, HVA concentrations are increased in the brains of rats following PCA (Bergeron et al., 1995) suggesting increased dopamine turnover in the brains of these animals. Dopamine D_2 receptor densities are downregulated in the globus pallidus of patients who died in hepatic coma (Mousseau et al., 1993).

In human autopsy studies in material from patients dying with hepatic coma, brain noradrenaline and dopamine levels were increased (Bergeron et al., 1989; Cuilleret et al., 1980). On the basis of these findings, it was proposed that alterations of the noradrenaline system could be responsible for many of the neuropsychiatric symptoms encountered in PSE.

4.5.4.2 Monoamines and FHF

Thioacetamide-induced FHF as well as liver devascularization result in increases in extracellular brain concentrations of the serotonin metabolite 5-HIAA (Bergqvist et al., 1995; Kaneko et al., 1998; Michalak et al., in press). On the other hand, *in vivo* cerebral microdialysis studies in rats with FHF did not reveal any significant alterations of extracellular 5HT in brain (Michalak et al., 1998; Michalak et al., in press), suggesting a possibility of increased 5HT turnover. Extracellular levels of the serotonin precursor, L-tryptophan and changes in serotonergic receptors have also been described in the brains of animals with FHF resulting from thioacetamide-induced FHF (Kaneko et al., 1998), or with liver devascularization (Michalak et al., in press) as well as in models of acute hyperammonemia (Alexander et al., 1995). Recently, region-selective decreases of the 5-HT transporter sites have been demonstrated in rats with liver devascularization (Michalak et al., in press).

There is a growing body of evidence that central noradrenergic systems may also be implicated in the pathophysiology of FHF. Hepatectomy (Hadesman et al., 1995; Tyce and Owen, 1978; Herlin et al., 1983), liver devascularization (Murakami et al., 1992) as well as thioacetamide-induced toxic liver injury (Yurdaydin et al., 1990) in the rat, all result in decreased brain concentrations of noradrenaline. Hepatectomy results in increased concentrations of noradrenaline in ventriculocisternal perfusates (McKinzie et al., 1996) and *in vivo* cerebral microdialysis studies have revealed significant increases of extracellular brain concentrations of noradrenaline at precoma and coma stages of encephalopathy in rats with experimental FHF (Michalak et al., 1998). Additional studies demonstrate a loss of noradrenaline transporter sites in cortical and subcortical brain structures from these animals (Michalak et al., in press), at coma stages of encephalopathy in rats with experimental FHF. This loss of transporter sites was accompanied by a selective loss of $\alpha 1$ and $\beta 1$ subclasses of noradenaline receptor sites (Michalak et al., 1998).

4.5.5 Other neurotransmitters and hepatic encephalopathy

The endogenous opioid system of the brain may also be implicated in the mediation of some of the neuropsychiatric effects of chronic liver disease on CNS function. Cirrhotic patients are hypersensitive to morphine (Laidlaw et al., 1961) and portacaval-shunting in the rat results in increased pain sensitivity (Salomon et al., 1976), a phenomenon in which the endogenous opioid system is known to be involved (Van Ree et al., 1976). Increased plasma levels of the endogenous opioid met-enkephalin have been reported in patients with primary biliary cirrhosis (Thorton and Losowsky, 1988) and brain extracts from experimental animals with chronic liver failure contain modified concentrations of β-endorphin (Zeneroli et al., 1988; Panerai et al., 1982).

4.6 Peripheral-type benzodiazepine receptor

The mammalian brain contains two distinct types of benzodiazepine receptors: the type forming part of the $GABA_A$/benzodiazepine receptor complex, situated on the postsynaptic neuronal membrane; and the "peripheral-type" benzodiazepine receptor (PTBR), which is a hetero-oligomeric protein, so named because it is found in many peripheral tissues. In brain, PTBRs are concentrated on the outer mitochondrial membrane of astrocytes (Anholt et al., 1986; Itzhak et al., 1993) and appear to be implicated in cellular metabolism and cell proliferative processes.

4.6.1 Peripheral-type benzodiazepine receptor and portal-systemic encephalopathy

There is strong evidence to suggest that PTBRs and their endogenous ligands may play an important role in several tissues in the cellular response to chronic liver

disease and PSE. Densities of PTBRs are increased in autopsied brain tisssue from cirrhotic patients who died in hepatic coma (Lavoie et al., 1990), in the brains and kidneys of rats following PCA (Giguere et al., 1992; Raghavendra Rao et al., 1994) as well as in the brains and peripheral tissues of mice with chronic hyperammonemia resulting from deficiency of a urea cycle enzyme (Raghavendra Rao et al., 1993b). These observations suggest that the increased PTBRs in PSE are the consequence of exposure to increased concentrations of ammonia. Furthermore, concentrations of diazepam binding inhibitor (DBI) and octadecaneuropeptide (ODN), endogenous ligands for the PTBR, are increased in CSF of patients with PSE (Rothstein et al., 1989) and in brain of PCS rats (Butterworth et al., 1991c). PTBR, (mitochondrial receptor) activation may result in altered cellular energy metabolism (Anholt, 1986) or in neurosteroid synthesis. Some neurosteroids are potent positive allosteric modulators of the $GABA_A$ receptor in brain (Krueger and Papadopoulos, 1992)

4.6.2 Peripheral-type benzodiazepine receptor and fulminant hepatic failure

Benzodiazepine ligands and neurosteroids may contribute to swelling (Bender and Norenberg, 1998). Treatment with the PTBR agonist Ro5-4864 exacerbated ammonia-induced swelling whereas the use of a peripheral antagonist, PK-11195, diminished the extent of swelling in ammonia-treated cultured astrocytes (Norenberg and Bender, 1994). PTBR binding sites have also been shown to be increased in hyperammonemic mice and thioacetamide-treated mice (Itzhak et al., 1995).

5. MANAGEMENT AND TREATMENT OF PORTAL-SYSTEMIC ENCEPHALOPATHY

Patients with PSE fall into two groups. First are those patients who have episodic-encephalopathy generally associated with a precipitating factor, such as dietary-protein loading, gastrointestinal bleeding, exacerbation of the underlying liver disease, sepsis, dehydration, hypokalaemia, hypoxia, use of sedatives, or constipation. Secondly there are patients with subclinical HE.

Management of PSE therefore involves the detection and treatment of precipitating factors, the HE itself, and the underlying liver disease.

Although the level of ammonia in the arterial blood tends to be elevated in patients with more advanced hepatocellular dysfunction, especially when substantial portalsystemic shunting is present, it may not be well correlated with grade of HE. On the other hand, practical bedside tests, such as the number-connection and other trail-making tests are easily administered and provide useful information, especially in patients with subclinical HE.

The question of treatment for subclinical HE is unclear as is the diagnosis. There is some divergence of opinion as to whether treatment is warranted for patients with subclinical HE. However patients identified in this way should be thoroughly assessed for functional impairment, since intervention may enhance the capacity to perform practical tasks such as driving an automobile.

5.1 Ammonia-lowering strategies

Management of the precipitating factor, restriction of dietary protein, avoidance of constipation, and manipulation of the bowel flora are the mainstays of therapy (Ferenci et al., 1996). All of the above mentioned precipitating factors may lead to ammonia elevation both peripherally and centrally. Therefore, ammonia-lowering strategies are classically used to treat PSE.

5.1.1 Restriction of dietary protein

An excess of dietary protein is a well known ammonia-producing precipitant of PSE (Mullen and Weber, 1991). Restriction of dietary protein is effective (Ferenci et al., 1996) but should be used only in the short term to avoid harmful nutritional consequences (O'Keefe et al., 1980). Howeverit should be borne in mind that patients with cirrhosis often require minimal daily protein intakes of 0.8 to 1.0 g per kilogram to maintain nitrogen balance (Swart et al., 1989). A positive nitrogenous balance may have positive effects on encephalopathy (Morgan et al., 1995) by promoting hepatic regeneration and increasing the capacity of muscle to detoxify ammonia (Lockwood et al., 1979).

5.1.2 Bowel cleansing

Since ammonia is produced in the gut, it follows that bowel cleansing should be a mainstay of therapy. Colonic cleansing reduces bacterial counts (Wolpert et al., 1970) and therefore lowers ammonia in cirrhotics (Vince et al., 1973). Different laxatives may be used, but non-absorbable disaccharides are preferred as they result in additional effects that potentiate the elimination of nitrogenous compounds (Weber and Fresard, 1981; Uribe et al., 1981).

5.1.3 Non-absorbable disaccharides

The beneficial effects of lactulose and lactitol result from its conversion in the lower bowel to acid metabolites and the subsequent trapping of ammonia due to the pH gradient across the intestinal wall (Castell and Moore, 1971; Conn and Lieberthal, 1979). Also, the decrease in pH creates an environment that is hostile to the survival of urease-producing intestinal bacteria and may promote the growth of non-urease-producing lactobacilli, resulting in reduced production of ammonia in the colonic

lumen (Bircher and Ulrich, 1994). Lactulose and lactitol act by ensuring bowel movements and by affecting bacterial metabolism, including production and absorption of ammonia.

5.1.4 Antibiotics

Antibiotics with activity against urease-producing bacteria also reduce the production of intestinal ammonia, and are sometimes used. Neomycin is the most widely used (Orlandi et al., 1981; Atterbury et al., 1978; Orlandi et al., 1994) and is effective for modifying the bacterial flora (Fisher and Faloon, 1957) but it also causes many alterations in the intestinal mucosa metabolism and may decrease blood ammonia via a non-bacterial effect (van Berlo et al., 1988). However, neomycin has serious ototoxicity and renal toxicity and should not be used for long-term periods or in patients with renal dysfunction. Alternative antibiotics, such as tetracycline, metronidazole, and vancomycin also improve encephalopathy (Morgan et al., 1982; Tarao et al., 1990) by affecting other bacterial populations than neomycin but also have significant long-term toxic effects (Ferenci et al., 1996).

5.1.5 Carnitine

By a mechanism that has not yet been fully elucidated, L-carnitine has been shown to prevent acute ammonia toxicity and to enhance the efficacy of ammonia elimination as urea and glutamine. Administration of L-carnitine prevents death in mice given a lethal dose of ammonium acetate (O'Connor et al., 1984). Orally administered L-carnitine protected rats after PCA against intraperitoneally injected ammonium acetate (survival ranging from 35-100%). However, this protective effect did not correlate with brain carnitine levels. Blood ammonia levels did not differ significantly between PCA rats and rats receiving carnitine and those receiving regular drinking water (Hearn et al., 1989). Administration of L-carnitine protected

against the onset of ammonia-precipitated coma in PCA rats (Therrien et al., 1997). Treatment with L-carnitine reduced CSF ammonia at both precoma and coma stages of encephalopathy. The time course of this protective effect paralleled blood and CSF L-carnitine accumulation. CSF lactate increases following ammonia acetate administration to PCS rats were significantly attenuated following L-carnitine treatment. However, L-carnitine treatment did not lead to significant reductions in plasma ammonia nor CSF or brain glutamine in these animals (Therrien et al., 1997). This peripheral non-lowering effect of ammonia by carnitine was not found with others (O'Connor et al., 1984; Matsouka and Igisu, 1993). In cirrhotic patients subjected to a rectal ammonium overload test, intravenous L-carnitine improved psychometric tests significantly after 30 min, whereas circulating ammonium levels were not influenced. However, the increase in ammonia after rectal ammonia overload was significantly lower in L-carnitine-treated patients that manifest portal hypertension than in those without (Del Olmo et al., 1990).

5.1.6 Sodium benzoate

An entirely different approach to eliminate ammonia involves the use of benzoate, which has been successfully introduced for treatment of various inborn errors of the urea cycle. Benzoate reacts with glycine to form hippurate. For each molecule of benzoate, one mole of nitrogen is excreted into the urine. In a controlled study, benzoate improved HE in patients with cirrhosis (Mendenhall et al., 1986). Benzoate has been compared to lactulose for the treatement of acute HE, with similar results (Sushman et al., 1992). The cost of lactulose is 30 times that of sodium benzoate. The efficacy of benzoate may be limited in patients with poor liver function by the inability to carry out the conjugation step.

5.1.7 L-Ornithine-L-aspartate

Stimulation of urea cycle function has been long been proposed as a potentially therapeutic approach in PSE. Urea precursors such as arginine and ornithine have been tested and both appeared to lower ammonia levels in both experimental animals (Zieve et al., 1986) and in cirrhotic patients (Najarian and Harper, 1956; Tobe, 1961). However, a subsequent controlled study failed to confirm any therapeutic efficacy of arginine in cirrhotic patients with PSE (Reynolds et al., 1958). Enhancement of endogenous ornithine concentrations following inactivation of ornithine aminotransferase by 5-fluoromethylornithine protects against lethal ammonia acetate intoxication (Seiler et al., 1989). However, no ammonia lowering effect of 5-fluoromethylornithine was observed in PCS rats (Therrien et al., 1994).

OA provides critical substrates for both ureagenesis and glutamine synthesis. Ornithine is a specific activator of ornithine carbamyl transferase and of carbamylphosphate synthetase and in addition is also a substrate for ureagenesis (Zieve et al., 1986; Banko and Zollner, 1985).

Aspartate and ornithine, after conversion to α-ketoglutarate, are substrates for glutamine synthesis, which is performed exclusively by a small population of perivenous hepatocytes, the so-called perivenous scavenger cells (Gebhardt et al., 1991). The ammonia-lowering effect resulting from the stimulation of these two basic mechanisms of ammonia detoxification has been studied in animals (Vogels et al., 1995; Salvatore et al., 1964) and was confirmed in humans in clinical trials (Henglein-Ottermann, 1976; Lewelling et al., 1991).

Uncontrolled clinical studies supported the ammonia-lowering effects of OA (Kircheis et al., 1994; Kircheis et al., 1997). OA infusions at high doses prevented hyperammonemia after an oral protein load, but had no effect on fasting ammonia levels (Staedt et al., 1993). Furthermore, several controlled clinical trials in human cirrhotics have demonstrated both an ammonia-lowering effect of OA and a

concomitant improvement in mental test scores and number connection test scores (Henglein-Ottermann, 1976; Liehr et al., 1992).

5.2 Centrally-acting drugs

In contrast to the multiple strategies used successfully to lower blood ammonia and improve neurological status in PSE, centrally-acting drugs which act directly on neuronal excitability have not been widely used. One reason for this is that the precise neurotransmitter changes responsible for PSE are still under investigation. Flumazenil, a benzodiazepine antagonist, has been widely used (Pomier Layrargues et al., 1994; Gyr et al., 1996) and lately, NMDA antagonists are showing protective effects in several experimental animal models of PSE (Marcaida et al., 1992; Vogels et al., 1997).

5.2.1 Benzodiazepine receptor antagonists

In a controlled clinical trial studying the effects of the benzodiazepine receptor antagonist flumazenil in grade IV PSE patients, a subgroup of patients was found to manifest an amelioration (40-60%) of neurological symptoms (Pomier Layrargues and Butterworth, 1992). In two of the five patients who responded, reversal of encephalopathy appeared to be due to the action of flumazenil on the benzodiazepines present in the brain as a result of prior exposure to benzodiazepine medication. However, no clear correlation was acted between the clinical response to the benzodiazepine antagonist and the presence of benzodiazepine receptor ligands in the blood of these patients (Butterworth et al., 1995) suggesting that the beneficial effects of flumazenil were not the result of the inhibition of the action of blood-borne substances with benzodiazepine receptor agonist properties in these patients. In addition, the effect of flumazenil was transient and incomplete, suggesting that other factors may also contribute to the pathogenesis of HE.

5.2.2 NMDA receptor antagonists

Increases of intracellular brain glutamine concentration, as found in rats with FHF, may promote osmotic swelling by exhaustion of compensatory mechanisms of cell volume regulation (Norenberg and Bender, 1994) and may induce increased concentrations of extracellular excitatory amino acids, glutamate and aspartate, which in turn may be responsible for overstimulation of the NMDA receptor. Such an overactivity of the NMDA receptor might also contribute to the process of cell swelling due to an increased flux of ions (Na^+, Ca^+, Cl^-) (Rothman and Olney, 1987).

In a model of ammonia-infused encephalopathy in PCS rats, the NMDA receptor antagonist memantine was administered intravenously or intraperitoneally. It has been shown to attenuate the clinical manifestations of encephalopathy significantly, suggesting that over-stimulation of the NMDA receptor is implicated in the pathogenesis of hyperammonemia-induced encephalopathy. This protective effect was associated with a significant decrease of CSF glutamate and the prevention of the increase in ICP and brain water content (Vogels et al., 1997).

6. PREVENTION AND TREATMENT OF CEREBRAL EDEMA IN ACUTE LIVER FAILURE

6.1 Ammonia-lowering strategies

Ammonia has been demonstrated to be implicated in brain edema and increased ICP in experimental animal models of FHF (Swain et al., 1992b). Recently, arterial ammonia concentrations in patients with FHF have been found to correlate with cerebral herniation (Clemmesen et al., 1999). However, ammonia-lowering strategies have not been extensively used in FHF.

6.2 Mannitol

Intravenous infusions of hypertonic mannitol appear to be useful in decreasing ICP in patients with FHF. Decreases of ICP of approximately 20 to 25 mm Hg have been reported (Ede and Williams, 1996). For maximal reduction of ICP, mannitol is infused rapidly and urine output is monitored hourly. In patients with hepatic and renal failure, mannitol should only be used when hemodialysis or hemofiltration are in progress because mannitol infusions may cause hyperosmolarity and fluid overload, which could exacerbate cerebral edema (Ede and Williams, 1996).

6.3 Extracorporeal bioartificial liver support systems

A number of extracorporeal bioartificial liver support systems have been developed for clinical assessment in recent years. The hybrid bioartificial liver intermittently exposes separated plasma to a cartridge containing porcine hepatocytes attached to collagen-coated microcarriers after the plasma has been passed through a charcoal column designed to remove substances toxic to the hepatocytes (Sussman et al., 1994). The extracorporeal liver assist device on the other hand, consists of a mass of liver cells housed in a hollow bioreactor, and continuously exposes whole blood to cartridges containing well differentiated human hepatoblastoma cells (Demetriou et al., 1995). Pilot studies have demonstrated some neurological improvement and modest biochemical changes with the use of these devices, but without prolonging survival (Sussman et al., 1994; Demetriou et al., 1995; Ellis et al., 1996).

Further appraisal of these systems is needed in properly constructed trials that take note of the heterogeneity of FHF, and assess their impact on survival as well as their potential role as a bridge to transplantation.

6.4 Hypothermia

Hypothermia has recently been suggested as a "new treatment" for acute neurological disorders. Although, research in this area has been ongoing for more than 30 years, a renewed surge of interest in this phenomenon has emerged. Early studies of ischemia showed a neuroprotective action of deep systemic hypothermia (<32°C), however these results were confounded by a variety of systemic complications (Steen et al., 1979).

In 1987, Busto et al., first demonstrated that in transient global ischemia, a decrease of only 2°C in brain temperature was enough to significantly decrease the extent of histopathological injury in selectively vulnerable brain regions (Busto et al., 1987). Minamisawa et al., (1990), confirmed the protective effect of mild hypothermia using whole body cooling in ischemic rats.

Interest in the therapeutic use of mild hypothermia has led to clinical trials. Two randomized clinical trials in head trauma have shown benefits of body cooling to 34°C in the management of intracranial hypertension as well as in late recovery (Marion et al., 1997).

Survival of rats after hepatic devascularization is significantly prolonged and brain edema is shown to be absent in hypothermic rats with FHF, when the rectal temperature spontaneously dropped to 26.9°C (Traber et al., 1989). Further, no increase in ICP were reported in pigs with FHF when their body temperatures were maintained at 34°C. In patients with FHF, beneficial effects of moderate hypothermia have also been reported, however these results are somewhat inconclusive as the patients had received multiple pharmacotherapies.

7. LIVER TRANSPLANTATION

Orthotopic liver transplantation (OLT) has revolutionized the management of FHF. OLT has been shown to improve survival significantly. Survival rates after transplantation average 61-63%, but have reached 93% in some exceptional series (Mirza et al., 1995; Detre et al., 1994; Ascher et al., 1993). Excluding paracetamol induced FHF, 45-51% of patients admitted with FHF undergo transplantation (Emond et al., 1989; Castells et al., 1993; Munoz et al., 1993). The equivalent figures for paracetamol cases in the UK are only 7-9% (Mirza et al., 1995; O'Grady et al., 1991; Makin et al., 1995). Up to 27% of patients are considered to have contraindications to transplantation at the time of admission, and up to another 18% develop contraindications while they are waiting for a suitable graft to become available (Emond et al., 1989; Castells et al., 1993; Munoz et al., 1993). The principal contraindications to transplantation are uncontrolled intracranial hypertension, irreversible neurological damage, uncontrolled systemic hypotension, severe hemodynamic instability and sepsis.

CHAPTER 2

STUDIES ON THE PATHOPHYSIOLOGY AND NOVEL APPROACHES TO THERAPY OF HEPATIC ENCEPHALOPATHY

2.1 Article 1

Neuroactive amino acids and glutamate (NMDA) receptors in frontal cortex of rats with experimental acute liver failure

Hepatology, 24 (4), 1996

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ABSTRACT

It has been proposed that alterations of excitatory and inhibitory amino acids play a role in the pathogenesis of hepatic encephalopathy in acute liver failure. To evaluate this possibility, *in vivo* cerebral microdialysis was used to sample extracellular concentrations of amino acids in frontal cortex of unanesthetized rats at various times during the progression of encephalopathy resulting from acute liver failure. Liver failure was induced by portacaval anastomosis followed 24 hours later by hepatic artery ligation. Dialysate concentrations of amino acids were measured by high performance liquid chromatography (HPLC) with fluorescence detection. Deterioration of neurological status was accompanied by two- to four- fold increases in extracellular glutamate, glutamine and glycine; concentrations of α-aminobutyric acid (GABA) and taurine were unchanged. Densities of binding sites for the glutamate (*N*-methyl-D-aspartate [NMDA]) receptor ligand [^{3}H]-MK-801, assessed using quantitative receptor autoradiography, however, were unchanged in frontal cortex of rats at coma stages of ischemic liver failure. Increased extracellular glutamate concentrations were positively correlated with the severity of encephalopathy and with arterial ammonia concentrations. Such changes may result from an ammonia-induced reduction in the capacity for astrocytes to uptake glutamate. Increased extracellular glutamate in brain, together with increases in concentrations of glycine, a positive allosteric modulator of glutamate (NMDA) receptors, are consistent with increased NMDA-related glutamatergic neurotransmission in this model of acute liver failure. Increased extracellular glutamate, therefore, could contribute to the pathogenesis of hepatic encephalopathy and brain edema in acute liver failure.

INTRODUCTION

Hepatic encephalopathy in acute liver failure is a clinical syndrome resulting from severe inflammatory and/or necrotic liver disease. Death frequently results from brain herniation caused by raised intracranial pressure resulting from brain edema, which is caused by a progressive increase in brain water content (Blei, 1991). Abnormalities in brain and cerebrospinal fluid (CSF) amino acid concentrations suggest that amino acids may be implicated in the pathogenesis of hepatic encephalopathy in acute liver failure. For example, studies in experimental animals with either ischemic or thioacetamide-induced acute liver failure reveal decreased levels of excitatory amino acids (glutamate, aspartate) in brain (Swain et al., 1992a; Swain et al., 1992b); CSF concentrations of these amino acids were concomitantly increased. No changes were found in brain or CSF concentrations of γ-aminobutyric acid (GABA) in either ischemic or thioacetamide-induced acute liver failure (Swain et al., 1992b). Similar negative results were reported for GABA in autopsied brain tissue from patients with acute liver failure (Record et al., 1976).

The amino acids glutamine and taurine are involved in the regulation of water homeostasis in the brain. Brain and CSF concentrations of these amino acids are modified in acute liver failure. Increased glutamine was observed in the brains of animals following ammonia infusions (Takahashi et al., 1991) as well as in the brains of rats with ischemic liver failure (Mans et al., 1994). Taurine concentrations are increased in the CSF of rats with either ischemic or thioacetamide-induced liver failure (Swain et al., 1992b).

Whole-brain or CSF concentrations of amino acids do not necessarily reflect concentrations in the active (extracellular) compartment of brain. In order to address this issue, in the present study, using *in vivo* microdialysis, concentrations of glutamate and other neurotransmitter-related amino acids were measured in the extracellular space of the brains of rats with experimental ischemic liver failure at various times during the progression of encephalopathy. In addition, extracellular concentrations of glycine, an amino acid with allosteric modulatory properties on the

glutamate (N-methyl-D-aspartate [NMDA]) receptor complex (Johnson et al., 1987), were measured, as were densities of binding sites for the glutamate (NMDA) receptor ligand [^{3}H]-MK-801 using quantitative receptor autoradiography in frontal cortex of rats with ischemic liver failure.

MATERIALS AND METHODS

Materials

O-phthaldialdehyde (OPA) reagent solution, 2-mercaptoethanol, Tris-acetate, Ringer's solution constituents and amino acid standards were purchased from Sigma Chemical Co. (St.Louis, MO, U.S.A.). Sodium phosphate (monobasic), methanol (high-performance liquid chromatography [HPLC]-grade) and tetrahydrofuran (HPLC grade) were obtained from Anachemia (Montreal, Québec, Canada). Fresh, double-distilled, deionized water was used for preparation of standard amino acid solutions and buffers. The mobile phase used for HPLC was filtered through 0.45-mm-pore-size membrane filters (Millipore Corp., Bedford, MA, U.S.A.) and degassed under vacuum before use.

[^{3}H]MK-801 was obtained from Dupont-New England Nuclear (Boston, MA, USA) and nonradioactive MK-801 from Research Biochemicals International (Natick, MA, USA). [^{3}H]-sensitive film was purchased from Amersham (Arlington Heights, IL, USA).

Surgical techniques

Male Sprague-Dawley rats (weighing 175-200g) were anesthetized with halothane. An end-to-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (Lee et al., 1961) as previously reported (Rao et al., 1994). Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated. The inferior vena cava was partially clamped (anastomosis clamp; Roboz Instruments, Washington D.C.) and an elliptical piece of vein (1.5 times the portal vein diameter) was removed. The portal vein was ligated and cut. An

end-to-side portacaval anastomosis (shunt;[SH]) was performed under a dissecting microscope. Total surgery time was ~15 min. Sham-operated control rats (SM), matched for weight, were anesthetized with halothane and a laparotomy was performed. The inferior vena cava and portal vein were clamped for 15 min, then released. Following surgery, animals were allowed to recover in individual cages under constant conditions of temperature, humidity and light cycles, with free access to standard rat chow and water.

Twenty four hours after surgery, an intracerebral guide cannula was stereotactically implanted. For this, portacaval-shunted and sham-operated rats were anesthetized with halothane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, U.S.A.). A medial incision was made over the skull, and a burr hole was drilled to allow vertical access to the frontal cortex. A disposable plastic/siliconized intracerebral guide cannula was inserted through the burr hole to the required depth [coordinates: +3.2 mm (saggital), -1.5 mm (lateral) and -2.5 mm (depth) relative to the bregma and based on the atlas of Paxinos and Watson (Paxinos et al., 1982)]. The guide cannula was secured in place using dental acrylic and closed with a dummy probe. Total surgery time was ~15 min. Throughout all surgical procedures, a rectal temperature probe was inserted, and the temperature was maintained at 37°C using a CMA/150 temperature controller.

Following recovery from anesthesia, animals were maintained in the microdialysis chamber of the CMA/120 system for freely moving animals with free access to rat chow and water. Next morning (i.e. 24 hr later), animals were reanesthetized with halothane and subjected to hepatic artery ligation (HAL) or laparotomy (SM). Four groups of surgically-operated rats were created with four rats in each group, namely liver ischemia (SH+HAL) and the three necessary controls groups SH+SM, SM+HAL and SM+SM. Aortic and peritoneal catheters were implanted in all animals. After the operation, arterial blood glucose levels were monitored. Supplemental glucose was administered intraperitoneally as needed to maintain normal blood glucose levels. An equivalent volume of 0.9% saline was

administered to control animals.

Microdialysis

The microdialysis system (CMA/Microdialysis AB, Sweden, Bioanalytical Systems, West Lafayette, IN, U.S.A.) consisted of a microinjection pump (CMA/100), a syringe selector (CMA/111), a temperature controller (CMA/150), a microfraction controller (CMA/140) and a housing system for freely moving animals (CMA/120).

Following hepatic artery ligation or sham operation (ie: 48 hr after the portacaval shunt) the dummy probe was removed from the intracerebral guide cannula implanted on the previous day (as described above) and microdialysis probes (CMA/12; 2mm long, 500 mm o.d.) were inserted carefully into frontal cortex of freely moving animals. The probes were perfused by means of a microinjection pump (CMA/100) at a constant flow rate of 2 μl/min. The perfusion medium consisting of Ringer's solution (147 mM NaCl, 4mM KCl, 2.4 mM $CaCl_2$) (Benveniste et al., 1990) was prepared daily using deionized water, filtered through 0.45-mm pore size Millipore filters and degassed before use. Preliminary studies demonstrated that extracellular amino acid concentrations had stabilized within 3 hr of the start of dialysis. After this initial stabilization period, 20-min fractions were collected using a CMA/140 microfraction collector.

Animals were assessed neurologically each 30 min. during the progression of acute liver failure. Animals that could no longer right themselves after being placed on their back were considered to have loss of righting ability (precoma stage) and animals in which a corneal reflex could not be elicited were considered to be in coma (coma stage). Samples for amino acid analysis in extracellular fluid were taken hourly and amino acids measured at coma, precoma stages and at one hour intervals prior to the precoma stage. At the end of the experiment (1hr in coma stage), animals were killed by decapitation and brains were removed, frozen, sectioned and stained with cresyl violet for neuropathological verification of probe placement.

Microdialysis fractions were capped, frozen immediately after collection, and stored at -70°C until time of analysis.

All experiments were in accordance with the 1984 *Principles of the Guide for the Care and Use of Experimental Animals, Vol.2* of the Canadian Council on Animal Care, Ottawa and the *Guiding Principles for Research Involving Animals and Humans* (Recommendations from the Declaration of Helsinki) approved by the Council of the American Physiological Society.

Amino acid analysis by HPLC

Dialysate samples (coma stage, precoma stage, and at hourly intervals prior to precoma stage) were analyzed using a Perkin-Elmer reverse-phase HPLC system with fluorescence detection and precolumn *o*-phthalaldehyde derivatization (Lavoie et al., 1987a). The HPLC system consisted of a solvent delivery system (series 400; Perkin-Elmer) coupled to a filter fluorometer (LC-10 fluorescence detector; Perkin-Elmer; exicitation at 370 nm, emission at 418-700 nm). Sample injections were performed using a 50 µl loop of a CMA/200 autosampler with derivatization accessories. The column used was a reverse-phase Perkin-Elmer C18-5m fitted with a Vydec reverse-phase C18 guard column. The chromatography was performed with a gradient between methanol and sodium phosphate buffer [50 mM (pH 5.25) containing 2% tetrahydrofuran] (Hazell et al., 1993) at a constant flow rate of 1 ml/min; for a further 10 min. The gradient was then run from 25 to 50% methanol over 45 min, held at 50% methanol for a further 10 min, and returned to 25% methanol/75% buffer over 5 min, before reequilibration for reuse. Chromatograms were computed using a programmable recording integrator (LC-100; Perkin-Elmer). From standard chromatograms of amino acids including the internal standard (homoserine), detector response factors for the individual amino acids were calculated relative to the internal standard. Concentrations of amino acids in the extracellular liquid samples were calculated by reference to the internal standard (Lavoie et al., 1987a).

Quantitative receptor autoradiography

Separate groups of rats (n=6 per group: SH+HAL, SH+SM, SM+HAL, SM+SM) were used for autoradiographic studies. Anesthesia, surgery and treatment of the animals were as described in Microdialysis section. At coma stages of encephalopathy in SH+HAL rats and equivalent time points in the control groups, rats were decapitated. Brains were removed quickly and frozen in isopentane chilled on dry ice. Horizontal brain sections (20 μm thick) were cut using a cryostat and thaw-mounted onto gelatin-coated glass microscopic slides. Sections were stored at -20°C before use in binding experiments.

Brain sections were thawed at room temperature for 30 min and prewashed in 50 mM tris-acetate buffer (pH 7.4) for 30 min at 4°C and blow-dried under a stream of cool air. Tissue sections were then incubated for 2 h in 50 mM tris-acetate (pH 7.4) at room temperature containing 5 nM [^{3}H]MK-801 (total binding). Nonspecific binding was determined in the presence of 5 μM unlabeled MK-801. Following the incubation, sections were dipped quickly into 50 mM tris-acetate buffer (pH 7.4) at 4°C, rinsed in cold buffer for 80 min, and blow-dried under a stream of warm air (Sakurai et al., 1991).

Autoradiograms were prepared by apposing sections together with tissue-calibrated standards (Amersham microscales) to [^{3}H]sensitive Hyperfilm (Amersham) for 4 weeks. Films were developed and tissue concentrations of [^{3}H]MK-801 were measured by quantitative densitometry analysis using a MCID computer-based densitometer and image analysis system (Imaging Research, Ontario, Canada), inter-rater variability of the quantitative densitometric technique was 15%. Specific binding of [^{3}H]MK-801 was calculated as the difference between total binding and nonspecific binding. Brain regions of interest were identified according to an established rat brain atlas (Paxinos et al., 1982).

Statistical analysis

Microdialysis data presented are mean values ± SD from four animals. Differences between groups (SH+HAL, SH+SM, SM+HAL, SM+SM) at the same time point were compared by one-way ANOVA (Bonferroni's correction), and differences between time of analysis (coma, precoma, and 1 hr, 2 hr, 3 hr, 4 hr, 5 hr and 6 hr before precoma) were compared by means of one-way ANOVA with repeated measures (Bonferroni's correction). Autoradiography data are mean values ± SD from six animals per group. $P < 0.05$ was considered significant. Linear regression analysis was used to establish the correlation between extracellular brain amino acids and arterial ammonia.

RESULTS

Rats with liver ischemia (SH+HAL) exhibited a reproducible sequence of neurological symptoms of encephalopathy progressing through loss of activity, loss of righting ability (precoma) and loss of corneal reflex (coma). Animals in the three control groups (SH+SM, SM+HAL, SM+SM) showed no neurological abnormalities. Extracellular amino acids in frontal cortex of rats with ischemic acute liver failure are presented in figures 1 to 5.

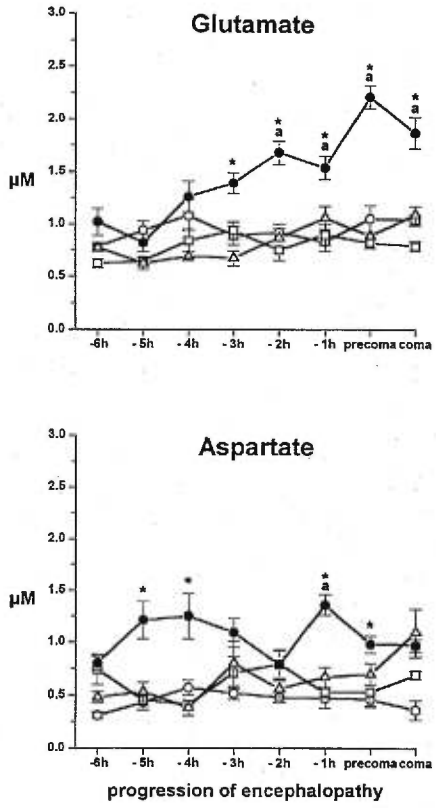

Figure 1: Extracellular concentrations of excitatory amino acids glutamate and aspartate as a function of the progression of encephalopathy in ischemic liver failure. Experimental groups ○ sham-sham, □ portacaval shunt-sham, △ sham-hepatic artery ligation, ● portacaval shunt-hepatic artery ligation. Data points represent means of duplicate determinations in 4 rats per treatment group. Vertical bars indicate S.D. Values in portacaval shunt-hepatic artery ligation significantly different from all control values at the same time point indicated by *$p<0.05$ by Analysis of Variance. Values significantly different from -6 h time point indicated by "a" $p<0.05$ by Analysis of Variance.

As shown in Figure 1, extracellular aspartate levels (Figure 1a) were increased at several time points during ischemic liver failure (SH+HAL). However, there was no correlation between increased aspartate concentrations and severity of neurological symptoms. In contrast, the concentrations of glutamate in extracellular fluid from ischemic rats were increased starting 3hr before the precoma stage compared to values from all control groups of animals. The magnitude of the increased extracellular glutamate showed a positive correlation with severity of encephalopathy (Figure 1b) as well as with arterial ammonia concentrations (Figure 6).

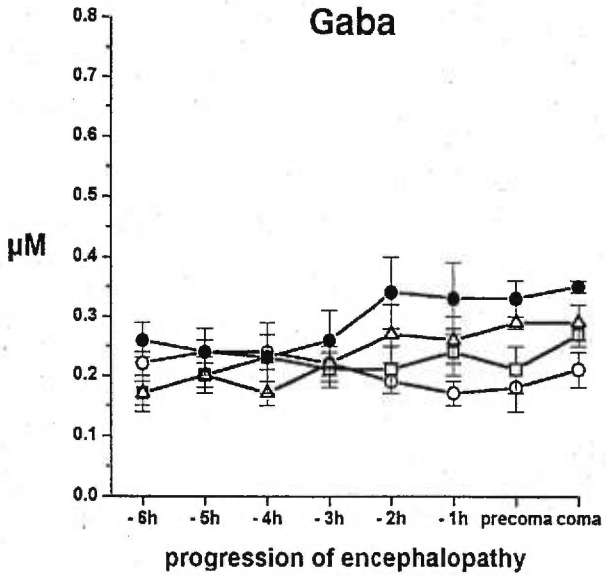

Figure 2: Extracellular concentrations of the inhibitory amino acid GABA as a function of the progression of encephalopathy in ischemic liver failure. Experimental groups and data analysis as summarized in legend to Figure 1. There were no significant differences between extracellular GABA concentrations in rats with ischemic liver failure and controls at any time point during progression of encephalopathy.

In contrast to glutamate, extracellular concentrations of GABA were unchanged at all time points during the development of severe encephalopathy due

to ischemic liver failure (Figure 2). Similar negative findings were observed for taurine (Figure 4). On the other hand, extracellular glycine concentrations in brains of rats with ischemic liver failure were increased 1 h before the precoma stage (Figure 5). At coma stage of encephalopathy (loss of corneal reflex) a second increase of glycine concentrations in the extracellular fluid was observed.

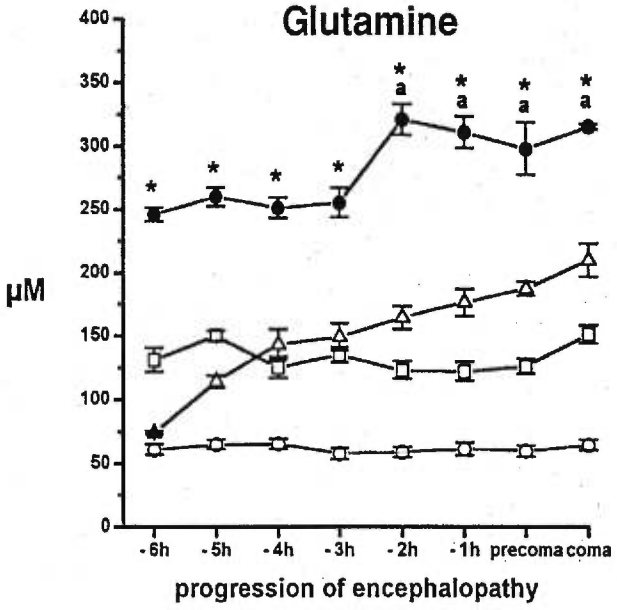

Figure 3: Extracellular concentrations of glutamine as a function of the progression of encephalopathy in ischemic liver failure. Experimental groups and data analysis as summarized in legend to Figure 1. Values significantly different from all control groups at the same time point indicated by *$p<0.05$; values significantly different from -6 h time point indicated by "a" $p<0.05$ by Analysis of Variance.

Brain glutamine concentrations were increased 2 to 3-fold in the extracellular space early during the progression of encephalopathy (Figure 3) in this model of ischemic liver failure. At 2 h before loss of righting reflex (precoma), glutamine concentrations manifested a further increase in extracellular fluid. However, onset of precoma or coma stages of encephalopathy were not associated with further increases of extracellular glutamine.

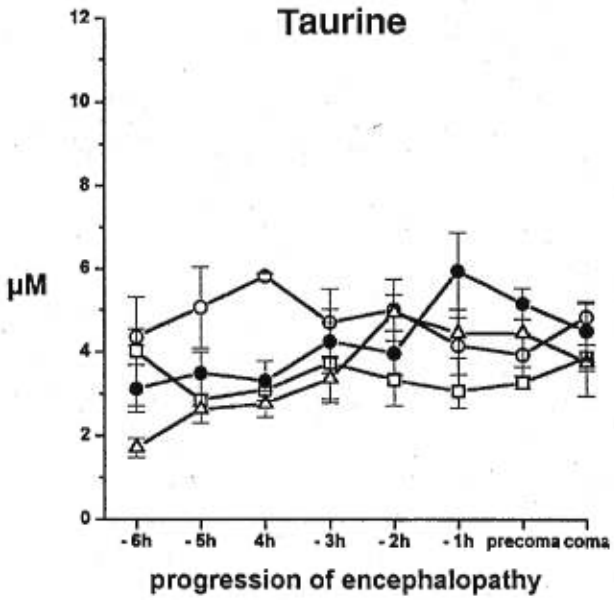

Figure 4: Extracellular concentrations of taurine as a function of the progression of encephalopathy in ischemic liver failure. Experimental groups and data analysis as summarized in legend to Figure 1. There were no significant differences between extracellular taurine concentrations in rats with ischemic liver failure and controls at any time point during progression of encephalopathy.

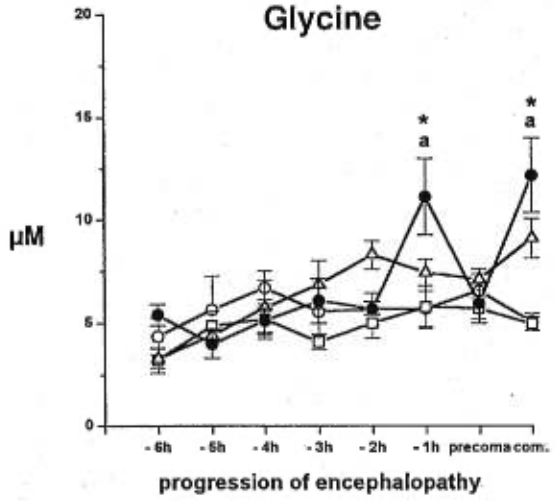

Figure 5: Extracellular concentrations of glycine as a function of the progression of encephalopathy in ischemic liver failure. Experimental groups and data analysis as summarized in legend to Figure 1. Values significantly different from control groups at a given time point indicated by *$p<0.05$; values significantly different from -6h time point indicated by "a" $p<0.05$ by Analysis of Variance.

Quantitative autoradiographic measurement of ^{3}H-MK-801 binding sites in frontal cortex of rats with ischemic liver failure at coma stages of encephalopathy revealed no significant differences from those in the three control groups (Table 1).

Table 1: Densities of binding sites for the glutamate (NMDA) receptor ligand ^{3}H-MK-801 in frontal cortex at coma stage in ischemic liver failure.

Treatment group	n	^{3}H-MK-801 binding site density (pmol/mg.tissue)
sham/sham	6	123.2 ± 10.4
shunt/sham	6	97.6 ± 10.3
sham/hal	6	122.3 ± 7.3
shunt/hal	6	122.0 ± 9.9

Data represent means of duplicate determinations of ^{3}H-MK-801 binding site densities obtained using quantitative receptor autoradiography. Sham: sham-operation; shunt: portacaval shunt; hal: hepatic artery ligation. There were no significant differences between binding site densities in any treatment group compared to sham/sham controls by Analysis of Variance.

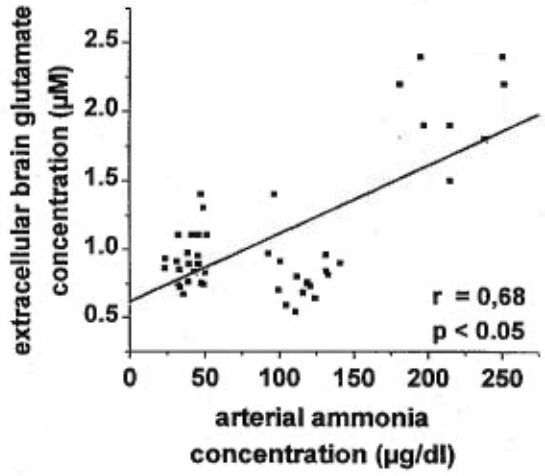

Figure 6: Correlation between extracellular brain concentrations of glutamate and arterial ammonia concentrations in rats from all treatment groups.

DISCUSSION

In vivo cerebral microdialysis has the advantage of facilitating the removal of small, low molecular weight substances from the extracellular space of brain in awake, freely moving animals, thus permitting the measurement of these substances in relation to the degree of neurological impairment. In the present study, selective changes in amino acids in the extracellular space of frontal cortex were observed following liver devascularization in the rat, a commonly used experimental animal model of acute liver failure (Blei et al., 1992). In the case of the excitatory amino acid glutamate, extracellular concentrations were significantly elevated 3 hours before the onset of severe signs of encephalopathy and continued to increase as neurological function deteriorated. In contrast, concentrations of the other excitatory dicarboxylic amino acid, aspartate, although increased at several time points during ischemic liver failure, showed no significant correlation with the progression of encephalopathy. The finding of 2 to 3-fold increases of glutamate in extracellular fluid during the progression of encephalopathy in acute liver failure confirms and extends the results of previous studies showing increases of glutamate in CSF at coma stages of encephalopathy in the same experimental animal model of acute liver failure (Swain et al., 1992b). Increased extracellular concentrations of glutamate have also been previously reported in other experimental animal models of acute liver failure in the rat (Bosman et al., 1992) and in the rabbit (de Knegt., 1994). Together with the results of the present study, these findings suggest that experimental acute liver failure results in increased glutamate release and/or diminished glutamate reuptake from the extracellular space in brain.

It should be noted that the order of magnitude of glutamate increase in the extracellular fluid of rats in ischemic liver failure is much less than that observed in cerebral ischemia or in brain trauma, two conditions resulting in neuronal death mediated by stimulation of the NMDA subclass of glutamate receptor followed by calcium-related toxic mechanisms. HE in ischemic liver failure, on the other hand, is not accompanied by neuronal cell death, despite increased extracellular glutamate

presumably due to the relatively modest quantities of increased glutamate release in this condition. Increased extracellular glutamate in acute liver failure is more likely due to decreased reuptake of the amino acid due to defective astrocytic glutamate transporters. Indeed, preliminary results demonstrate decreased gene expression for GLT-1, the astrocytic glutamate transporter in frontal cortex of rats with acute liver failure (Buu and Butterworth, unpublished results). Other evidence in favor of such a possibility is provided by the recent report of decreased glutamate uptake by synaptosomal preparations from rats with thioacetamide-induced acute liver failure (Oppong et al., 1995). A likely explanation for the diminished uptake of glutamate in acute liver failure may relate to the exposure of brain to high concentrations of ammonia generated in liver failure. Previous studies in the ischemic model of acute liver failure reveal brain ammonia concentrations in the 2 to 5 mM range (Swain et al., 1992a) and a significant inhibitory effect of 5 mM ammonia on glutamate uptake by synaptosomes has been described (Mena et al., 1985). High affinity uptake of glutamate into astrocytes is also inhibited by exposure to millimolar concentrations of ammonia (Norenberg et al., 1985). In the experimental model of ischemic liver failure used in the present study, brain ammonia concentrations are elevated to concentrations shown to inhibit glutamate uptake mechanisms *in vitro*. Furthermore, the magnitude of extracellular glutamate concentrations in ischemic liver failure observed in the present study are positively correlated with arterial blood ammonia concentrations. Assuming such mechanisms pertain also to the *in vivo* situation, the present findings of increased extracellular concentrations of glutamate *in vivo* are likely the consequence of ammonia-induced diminished removal of glutamate from the synaptic cleft. Similar mechanisms have previously been proposed to explain the findings of increased extracellular glutamate in cortical (Moroni et al., 1983) and hippocampal (Butterworth et al., 1991b) preparations and the concomitant loss of postsynaptic glutamate receptors (Peterson et al., 1990) from several brain structures following portacaval anastomosis leading to the suggestion that portal-systemic encephalopathy is, in part, the consequence of decreased trafficking of glutamate

between neurons and astrocytes (Butterworth et al., 1993)). Findings of the present study suggest that similar mechanisms may be implicated in the pathogenesis of hepatic encephalopathy in acute liver failure. However, in contrast to portacaval shunted rats, rats with ischemic liver failure, concentrations of the inhibitory amino acids GABA and taurine remained within the range of those of control groups at all time points during the development of encephalopathy. The findings of unchanged GABA extend the results of a previous study in the same animal model of acute liver failure in which CSF and brain GABA concentrations were likewise found to be unaltered (Swain et al., 1992b). Furthermore, neither brain (Record et al., 1976) nor CSF (Moroni et al., 1987) GABA concentrations are increased in human HE associated with acute liver failure. Thus, increased extracellular GABA concentrations and, by inference, GABA-mediated transmission does not appear to play a role in the pathogenesis of HE nor of brain edema in acute liver failure. The finding in the present study of unchanged taurine concentrations in cerebral cortical dialysates of rats with ischemic liver failure contrasts those of a previous study (Bosman et al., 1992) in which increased taurine concentrations were reported. Possible explanations for this discrepancy include different experimental paradigms, different rat strains and much slower flow rates used for dialysis in the present study.

Concentrations of glutamine in the extracellular compartment of brain were elevated two- to five fold early during the progression of encephalopathy in ischemic liver failure and this increase was maintained throughout the progression of the disorder. Increased glutamine is undoubtedly the consequence of increased ammonia removal by brain in ischemic liver failure. Brain, being devoid of a urea cycle, removes excess ammonia by glutamine synthesis and a previous report described three- to five fold increases in brain glutamine in this model of acute liver failure (Swain et al., 1992a). Interestingly, in line with the results of the present study, brain glutamine concentrations although elevated consistently, were not precisely correlated with the severity of encephalopathy. Increased brain glutamine content, rather than playing a role in the pathogenesis of encephalopathy in acute liver failure,

therefore, may be implicated in the phenomenon of brain edema (Blei, 1991; Blei et al., 1994). In favour of such a possibility, treatment of animals with methionine sulfoximine, an inhibitor of glutamine synthesis in brain, inhibits water accumulation and the subsequent increase in intracranial pressure in experimental liver failure (Blei et al., 1994).

In conclusion, results of the present study reveal a selective increase of extracellular glutamate in experimental ischemic liver failure. The magnitude of this increase was correlated with deterioration of neurological status and with arterial blood ammonia concentrations in these animals. In the light of previous reports of an inhibitory effect of ammonia on glutamate uptake from the extracellular space in brain, the findings of the present study suggest that exposure of brain to increased ammonia concentrations resulting from acute liver failure results in glutamate accumulation in the extracellular space of brain. Increased extracellular glutamate and consequently increased glutamatergic neurotransmission, could thus contribute to the phenomenon of HE in acute liver failure. Furthermore, exposure of perineuronal astrocytes to increased glutamate *in vivo* could contribute to the phenomenon of cytotoxic edema in this condition. Pharmacological manipulation of the glutamatergic system could therefore offer a potentially useful target for treatment strategies for these serious neurological complications of acute liver failure.

ACKNOWLEDGMENTS

The studies described were made possible by a grant from The Medical Research Council of Canada (PG11118). A.M. is recipient of a Canadian Liver Foundation Fellowship and G.T. was recipient of a Canadian Liver Foundation Studentship (1993-1995).

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2.2 Article 2

L-Ornithine-L-aspartate in experimental portal-systemic encephalopathy: therapeutic efficacy and mechanism of action

Metabolic Brain Disease, 13 (2), 1998

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ABSTRACT

Strategies aimed at the lowering of blood ammonia remain the treatment of choice in portal-systemic encephalopathy (PSE). L-ornithine-L-aspartate (OA) has recently been shown to be effective in the prevention of ammonia-precipitated coma in humans with PSE. These findings prompted the study of mechanisms of the protective effect of OA in portacaval-shunted rats in which reversible coma was precipiated by ammonium acetate administration (3.85 mmol/kg i.p.). OA infusions (300 mg/kg/h, i.v.) offered complete protection in 12/12 animals compared to 0/12 saline-infused controls. This protective effect was accompanied by significant reductions of blood ammonia, concomitant increases of urea production and significant increases in blood and cerebrospinal fluid (CSF) glutamate and glutamine. Increased CSF concentrations of leucine and alanine also accompanied the protective effect of OA. These findings demonstrate the therapeutic efficacy of OA in the prevention of ammonia-precipitated coma in portacaval-shunted rats and suggest that this protective effect is both peripherally-mediated (increased urea and glutamine synthesis) and centrally-mediated (increased glutamate synthesis).

Keywords: Portal-systemic Encephalopathy; Hepatic Encephalopathy; Ammonia; L-ornithine-L-aspartate; Glutamine synthesis

INTRODUCTION

Of the possible neurotoxins implicated in hepatic encephalopathy (HE), ammonia was the first to be incriminated (Eck, 1877) and finds itself still the leading candidate as a key factor in its pathogenesis (Butterworth et al., 1987, Butterworth, 1994; Lockwood et al., 1991). Recent studies using Positron Emission Tomography (PET) and $^{13}NH_3$ confirm that, in chronic liver failure, brain utilizes ammonia at increased rates and that the blood-brain barrier becomes more permeable to ammonia (Lockwood et al., 1991).

Portal-systemic encephalopathy (PSE) is a common neuropsychiatric disorder resulting from chronic liver disease. Clinical features of PSE include impaired mental function, neurological disturbances such as asterixis or flapping tremor, and altered states of consciousness progressing to stupor and coma. Multiple PSE episodes are common and are associated with precipitating factors such as gastrointestinal bleeding, constipation or sedative use (Sherlock, 1985). Symptoms of PSE are generally reversible suggesting a metabolic etiology.

Ammonia, once taken up by the brain, is transformed into glutamine by glutamine synthetase (GS). However, the ammonia-buffering capacity of GS is limited (Butterworth et al., 1988) in chronic liver failure so that brain ammonia concentrations rise to attain millimolar concentrations which are deleterious to brain function. Neurochemical mechanisms so far proposed to explain the neurotoxic effects of ammonia include direct effects on excitatory and inhibitory neurotransmission (Raabe, 1989) and on cerebral energy metabolism (Hindfelt et al., 1977). Furthermore, astrocytic uptake of glutamate has been found to be inhibited by high levels of ammonia (Bender and Norenberg, 1996) leading to the proposal that PSE may result from glutamatergic synaptic dysregulation (Butterworth, 1996).

Therapeutic strategies aimed at reducing blood and brain ammonia are the mainstay in the prevention and treatment of PSE. However, few treatments of hyperammonemic syndromes have so far been designed to counteract the molecular actions of ammonia. Clinical studies have shown that L-ornithine-L-aspartate (OA) reduces blood ammonia concentrations and improves the clinical symptoms of HE in chronic liver disease (Staedt et al., 1993; Kircheis et al., 1997). However, the mechanism responsible for OA's ammonia-lowering action has not been fully elucidated. The present study was undertaken to assess the efficacy of OA in the prevention of severe encephalopathy precipitated by ammonium acetate treatment of

portacaval-shunted rats. The protective effect was studied in relation to changes in plasma and CSF amino acids.

MATERIALS and METHODS

O-Phthalaldehyde (OPA) reagent solution, 2-mercaptoethanol and amino acid standards were purchased from Sigma Chemical Co., St-Louis, MO; Methanol (HPLC grade), sodium phosphate (monobasic) and tetrahydrofuran (THF, HPLC grade) from Anachemia, Montreal, Quebec, Canada. OA was provided by Merz + Co., Frankfurt, Germany. All reagents and solvents were of reagent-grade purity unless otherwise stated. Double-distilled deionized water was used for preparation of amino acid solutions and buffers. The mobile phase was filtered through 0.45 μm filters (Type GS, Millipore Corporation, Bedford, MA) and degassed under vacuum prior to use.

Portocaval anastomosis (PCA)

Adult male Sprague-Dawley rats weighing 175-200 g were anesthetized with halothane and an end-to-side PCA was performed according to the guidelines of Lee and Fisher (1961). Rats underwent a laparotomy, the inferior vena cava and portal vein were isolated allowing the inferior vena cava to be clamped (anastomosis clamp, Roboz Instruments Inc.), and the removal of an elliptical piece of vein 1.5 times the portal vein diameter. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was <15 minutes. Following surgery, all animals were housed individually with free access to standard laboratory chow and water under constant conditions of temperature, humidity, and light cycles. Overall perioperative mortality for shunted rats was less than 10%.

Four weeks following PCA, rats were pretreated with OA (0.3-1 g/kg body weight/hr, i.v) or isotonic saline 6 hours before ammonium acetate (3.85 mmoles/kg body weight, i.p.) to precipitate severe reversible neurological signs of

encephalopathy.

Venous and arterial catheters

Under halothane anesthesia, rats underwent a second laparotomy and two PE-50 catheters were inserted; a venous catheter in the vena cava and an arterial catheter in the aorta. Following recovery from anesthesia, a constant OA infusion was pumped through the venous catheter and blood samples were collected through the arterial catheter.

Cisterna magna catheters

Rats were maintained under halothane anesthesia for the duration of the catheter implantation. Cisterna magna catheters were installed as previously described (Swain et al., 1992). The animal's head was mounted with the skull in a horizontal position in a stereotaxic apparatus. A 3 cm incision was made in the skin from the back of the head and the overlying connective tissue was removed to expose the skull. A small hole was drilled in the skull using a dental burr (009) on the midline immediately rostral to the interparietal-occipital bone suture. The hole was drilled in such a way that the occipital bone could be used as a guideline while inserting the cannula (PE-10 tubing, Clay Adams, Parsippany, NJ). The catheter was slowly inserted into the cisterna magna. Correctness of placement of the CSF-inflow window was verified by clearing the CSF outway, at which time a spontaneous flow of CSF occurred. When successful implantation had been confirmed, the skull was cleaned and dried and a small amount of dental acrylic cement (Yates and Bird, Chicago, IL) was applied to cover part of the inserted cannula. The whole operation took approximately 20 minutes. Rats were housed individually and were allowed to recover from the operation. Twenty-four hours after this operation, Silastic tubing (Dow Corning, Midland, MI) was connected to the cannula for serial CSF sampling.

Neurological evaluation

Following ammonium acetate administration to rats with PCA, a rapidly progressive deterioration of neurological status was observed which started with lethargy, progressing to loss of righting reflex and ultimately loss of corneal reflex and deep coma, as previously described (Butterworth and Giguère, 1984; Therrien and Butterworth, 1991).

Measurement of plasma amino acids

400 µl samples of blood were withdrawn and collected in heparinized micro-tubes just before the start of infusion of OA, 1 hour after the start of OA infusion and just prior to the administration of ammonium acetate. A last sample was taken from animals from both treatment groups when the saline-treated rats became comatose. Blood samples were centrifuged and the plasma was removed, aliquoted and stored at -80°C.

Amino acids were derivatized with o-phthalaldehyde, separated and analysed using HPLC with fluorescence detection according to the method of Spink et al., 1986. Plasma samples were treated with acidified methanol (1:10), the mixture was left for 20 minutes at 4°C and centrifuged for 10 minutes at 15,400 g at 4°C. Twelve µl of the supernatant was then withdrawn and mixed with 3 µl of homoserine 0.01 mM as internal standard and reacted at room temperature with 20 µl OPA reagent for 42 seconds, in a total volume of 35 µl. Twenty five µl of this solution was then injected onto the HPLC column (C18 Pecosphere, Perkin Elmer) with a CMA/200 autosampler. The HPLC system consisted of a solvent delivery system (Perkin Elmer, Series 400) coupled to a fluorometer (Perkin Elmer, LC-10 Fluorescence Detector, excitation 370 nm, emission 418-700 nm). Samples were injected in a 50 µl loop of a Rheodyne 7125 injector system (Rheodyne, Cotati, CA, U.S.A). Peak area measurements were computed using a programmable recording integrator (Perkin-Elmer LC-100). The chromatograph was run with gradients of increasing organic solvent concentration generated by differential pumping rates of Solvents A

and B. Solvent A contained 30 mM disodium phosphate, 30 mM sodium acetate, 2% (v/v) methanol, and 0.05% (w/v) sodium azide adjusted to pH 6.2 with glacial acetic acid. Tetrahydrofuran 1% (v/v) was added just before running the system. Solvent B consisted of a mixture of water:methanol:acetonitrile:tetrahydrofuran in 19:15:15:1 ratio by volume. Each gradient program ended with a 10 min. hold at 100% solvent B followed by a 5-min reequilibration with 100% solvent A. Amino acid content of plasma was calculated by peak area analysis using standard curves and the internal standard.

Measurement of CSF amino acids

Amino acids were analyzed using a Perkin-Elmer reverse phase HPLC system with fluorescence detection and precolumn o-phthalaldehyde derivatization as previously described (Therrien and Butterworth, 1991). The HPLC system consisted of a solvent delivery system (series 400; Perkin-Elmer; Corwalk, CT) coupled to a filter fluorometer (LC-10 fluorescence detector; Perkin-Elmer; excitation at 370 nm, emission at 418-700 nm). Samples were injected using a 50 µl loop of a CMA/200 autosampler with derivatization accessories. The column used was a reverse-phase Perkin-Elmer C18.5 m fitted with a Vydec reverse-phase C18 guard column. The chromatogram was run with gradients of increasing organic solvent concentration generated by differential pumping rates of solvents A and B. Solvent A contained 30 mmol/L sodium phosphate, 30 mmol/L sodium acetate, 2% (vol/vol) methanol, and 0.05% (w/vol) sodium azide adjusted to pH 6.2 with glacial acetic acid. Tetrahydrofuran 1% (vol/vol) was added just before running the system. Solvent B consisted of a mixture of water:methanol:acetonitrile:tetrahydrofuran in 19:15:15:1 ratio by volume. Each gradient program ended with a 10-minute hold at 100% solvent B followed by a 10 minute reequilibration with solvent A. Amino acid content of CSF or brain was calculated by peak area analysis using standard curves and the internal standard.

Measurement of ammonia

Ammonia concentrations were estimated in plasma using a commercial ammonia test kit, which uses an ion-exchange method followed by colorimetric measurement of isolated ammonia nitrogen with the Berthelot phenate-hypochlorate reaction (Dienst, 1961). Ammonia was measured within 24 h of sampling.

Statistical analysis

Data are expressed as mean ± S.E.M. For comparison of data between OA and saline-treated groups, Student *t* tests with Bonferroni correction were used. P values of <0.05 were considered to be significant.

RESULTS

Protective effect of OA in portacaval-shunted rats administered ammonium acetate

OA (300 mg/kg/h, i.v for 6 hours) afforded complete protection against ammonia acetate-induced encephalopathy in PCS rats. The protective effect observed was highly significant; 12/12 saline-treated PCS controls showed deterioration of neurological status progressing to coma whereas none of 12 OA-treated PCS rats showed deterioration of neurological status. All rats in the saline-treated control group regained consciousness within 3h of ammonium acetate administration.

Effects of OA on plasma ammonia in ammonium acetate-treated portacaval-shunted rats

Following ammonium acetate administration, the increase of plasma ammonia was significantly reduced in OA-treated PCS rats compared to levels in saline-treated PCS rats at all time points (Figure 1).

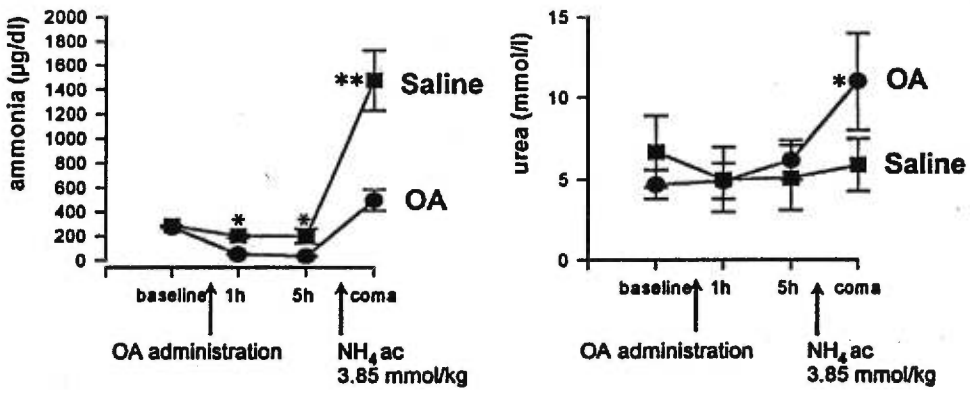

Figure 1: Protective effect of OA (300 mg/kg/h, i.v) in ammonium acetate-induced coma in portacaval-shunted rats in relation to plasma ammonia (left panel) and urea (right panel). Values significantly different from saline-treated controls indicated by *p<0.05, **p<0.01.

Effects of OA on plasma urea in ammonium acetate-treated portacaval-shunted rats

Administration of OA to ammonium acetate-treated PCS rats resulted in a significant increase in plasma urea (11.56±2.1 mM) compared to saline-treated PCS controls at the coma timepoint (5.91±1.6 mM, p<0.02) (Figure 1).

Effects of OA on plasma amino acids in ammonium acetate-treated portacaval-shunted rats

The effects of OA treatment (300 mg/Kg/h) on plasma amino acid concentrations measured at times equivalent to those at which saline-treated controls were comatose (ie: had lost their corneal reflexes) are shown in Table 1.

Table 1: Plasma amino acid concentrations in ammonium acetate-treated, portacaval-shunted rats: Effect of OA

	Amino acid concentration (μM)	
	Saline-treated	OA-treated
Amino Acid		
Glutamate	69.6±13.1	2102.1±187.3*
Aspartate	17.1±2.3	5585.5±367.6*
Glutamine	466.5±33.6	1216.8±65.1*
Arginine	150.7±17.6	395.8±51.4*
Tyrosine	111.2±15.3	112.1±11.4
Tryptophan	49.1±3.1	65.3±9.1
Phenylalanine	73.7±7.6	64.1±5.7
Leucine	54.2±5.1	142.8±9.4*
Isoleucine	63.0±10.7	134.1±15.8*
Valine	106.6±17.1	195.0±20.1*
Asparagine	30.4±3.4	74.8±5.7*
GABA	4.9±0.7	15.6±1.7*
Glycine	376.6±22.1	451.6±36.1
Taurine	112.9±12.5	211.2±19.8*
Alanine	188.8±18.3	1087.9±109.8*
Threonine	141.8±27.3	419.9±68.1*
Serine	85.6±17.1	244.5±20.1*

Values represent mean S.E. of duplicate determination from groups of six rats. *$p<0.05$ compared to saline-treated controls by Student t-test.

OA treatment prevented rats from becoming comatose and also resulted in 5-fold increases of alanine ($p<0.01$), 3-fold increases in glutamate, glutamine and GABA ($p<0.01$) and 2-fold increases of the branched chain amino acids leucine, isoleucine and valine ($p<0.01$). Two-fold increases of arginine, asparagine, taurine, threonine and serine were also noted after OA treatment.

Effects of OA on CSF amino acids in ammonium acetate-treated portacaval-shunted rats

In contrast to the effects in plasma, OA treatment resulted in less generalized alterations of amino acids in CSF of ammonium acetate-treated portacaval-shunted rats (Table 2).

Table 2: Cerebrospinal Fluid Amino Acid Concentrations in Ammonium Acetate-Treated, Portacaval-Shunted Rats: Effect of OA

	Amino acid concentration (μM)	
	Saline-treated	OA-treated
Amino acid		
Glutamate	6.2±1.1	17.5±3.1*
Aspartate	12.3±3.9	19.2±2.8
Glutamine	989.1±45.9	1227.3±61.5*
Arginine	34.6±2.7	35.6±5.5
Tyrosine	13.5±2.7	14.9±1.5
Tryptophan	7.6±0.7	7.4±1.3
Phenylalanine	15.4±2.4	15.5±1.7
Leucine	7.3±0.4	10.7±0.9*
Isoleucine	11.9±1.4	12.1±2.5
Valine	11.5±2.4	13.2±1.2
Asparagine	7.9±0.7	9.7±0.7
GABA	0.8±0.1	0.9±0.2
Glycine	20.3±2.2	22.2±3.6
Taurine	17.8±1.9	17.7±1.6
Alanine	44.4±0.7	54.1±3.1*
Threonine	67.1±3.9	63.1±4.5
Serine	72.3±2.9	87.7±6.2*

(Legend as in Table 1)

CSF glutamate was increased 3-fold ($p<0.01$) following OA treatment and significant 30-50% increases of glutamine, leucine, alanine and serine were also noted in the CSF of these animals. OA treatment did not result in significant alterations of other neuroactive amino acids (aspartate, glycine, GABA, taurine) nor of aromatic amino acids (tyrosine, phenylalanine, tryptophan).

DISCUSSION

Results of the present study demonstrate a significant protective effect of OA in ammonia-precipitated coma in rats following PCA. This finding is consistent with clinical studies in which administration of OA results in decreases in plasma ammonia levels and improvement of clinical symptoms in hyperammonemic patients with portal-systemic encephalopathy (Kircheis et al., 1997).

The protective effect of OA in ammonia-induced encephalopathy in shunted

rats was accompanied by significant reductions in plasma ammonia and a small but significant increase in plasma urea suggesting that the reduction in plasma ammonia levels by OA is therefore partly the result of increased urea synthesis in liver; both ornithine and aspartate are metabolic substrates for the urea cycle (Figure 2). Increasing the ornithine concentration may stimulate flux via ornithine transcarbamylase (OTC) whereas aspartate affords a second nitrogen donor for the synthesis of urea. Consistent with increased urea cycle flux are the findings of 2-3-fold increases of plasma arginine following OA administration in the present and a previous (Vogels et al., 1995) study .

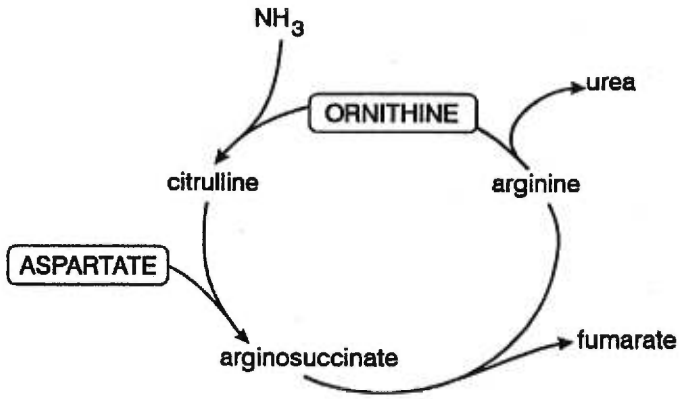

Figure 2: Simplified schematic representation of the urea cycle showing potential points of stimulation of urea cycle flux by L-ornithine and L-aspartate.

Increased urea synthesis by the liver following OA treatment despite the presence of an end-to-side portacaval anastomosis is not surprising. A previous study revealed enhanced urea synthesis and a lowering of blood ammonia following OA administration to cirrhotic rats (Gebhardt et al., 1997).

Other potentially beneficial actions of L-ornithine and L-aspartate probably involve their ability to cause increased glutamate concentrations following stimulation of transaminases (Figure 3).

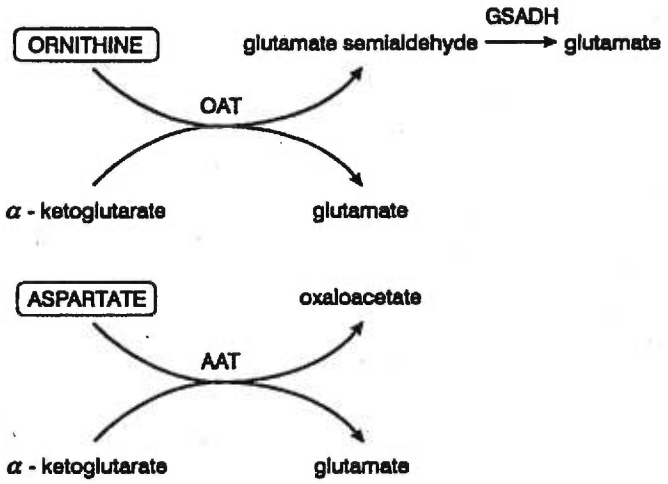

Figure 3. Displacement of transaminase equilibria by OA. OAT: ornithine aminotransferase; AAT: aspartate aminotransferase; GSADH: glutamate semialdehyde dehydrogenase.

Displacement by OA of the ornithine transaminase equilibrium towards glutamate and glutamate semialdehyde (and subsequently glutamate by the action of glutamate semialdehyde dehydrogenase, GSADH) and the aspartate aminotransferase equilibrium towards glutamate formation potentially affords 3 molecules of glutamate. That such mechanisms are operative following OA treatment of PCA shunted rats in the present study is supported by findings of 3-fold increases of glutamate in body fluids of treated animals (Tables 1,2). The finding of comparable increases of glutamate in both plasma and CSF following OA treatment suggests that the above mechanisms (Figure 3) are operative both in brain and in peripheral tissues. L-ornithine is a substrate for the γ^+ transporter at the blood-brain barrier (BBB), which is almost completely impermeable to glutamate or aspartate. Increased BBB uptake of L-ornithine would be expected to result in increased synthesis of glutamate in brain via the transamination reactions (Figure 3).

Increased tissue concentrations of glutamate would then have the potential to stimulate the synthesis of glutamine via glutamine synthesis (GS). GS is present in relatively high specific activities in liver, brain and skeletal muscle. Furthermore,

portacaval shunting results in induction of GS in muscle (Girard and Butterworth, 1992). Given the increased availability of enzyme substrate (glutamate) and the possibility of enzyme induction in muscle, it is therefore likely that an important mechanism of ammonia-lowering of OA following portacaval shunting involves increased glutamine synthesis by skeletal muscle. Additional ammonia removal would also be expected via glutamine synthesis in brain. Consistent with these mechanisms are the findings in the present study of significant elevations of glutamine in the blood and CSF of OA-treated, portacaval-shunted rats.

Other potential consequences of increased availability of glutamate would (as expected) include increased GABA synthesis (observed in plasma but not in CSF) resulting from increased flux via glutamic acid decarboxylase as well as increased synthesis of alanine via pyruvate aminotransferase. Consistent with the latter, alanine concentrations were found to be significantly increased both in plasma and CSF of OA-treated animals. In a study of the effects of OA on plasma amino acids in patients with cirrhosis, increases in plasma glutamate and alanine were also reported (Staedt et al., 1993) suggesting that amino acid changes similar to those observed in the present study occur in humans with chronic liver disease following OA treatment. Finally, increased glutamate availability would be expected to stimulate branched-chain amino acid (BCAA) aminotransferases resulting in increased synthesis (or decreased catabolism) of BCAAs. In favour of this mechanism were the findings in the present study of increased plasma concentrations of BCAAs and of leucine in CSF of OA-treated animals. Whether or not increased plasma concentrations of BCAAs contributed to the ammonia-lowering effect of OA is unclear. Data from controlled clinical trials have yielded equivocal results on the ammonia-lowering capacity of BCAAs (Conn and Eriksson, 1989).

In summary, results of the present study clearly demonstrate that OA is effective in the prevention of ammonia-precipitated encephalopathy in portacaval-shunted rats. Findings are consistent with two mechanisms namely: (i) increased urea synthesis (albeit at a reduced rate compared to normal animals) and (ii)

displacement of multiple transaminase reactions giving rise to increased glutamate followed by the facilitation of ammonia removal via glutamine synthetase both in peripheral tissues and in brain. Further studies of urea and glutamine synthesis are required in order to confirm these possibilities. OA has the potential to lower blood ammonia in ammoniagenic conditions associated with chronic liver disease and in this way, is useful in the prevention of PSE. OA prophylaxis could be particularly useful in the prevention of PSE in cirrhotic patients with a history of recurrent gastrointestinal bleeding.

ACKNOWLEDGMENT

These studies were funded by the Medical Research Council of Canada (grant #PG11118) and Merz + Co., Frankfurt, Germany. A. Michalak and G. Therrien were recipients of a Postdoctoral Fellowship and Research Studentship respectively from The Canadian Liver Foundation. The authors thank Dominique D. Roy for her assistance with the preparation of the manuscript.

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2.3 Article 3

L-Ornithine-L-aspartate lowers plasma and CSF ammonia and prevents brain edema, in rats with acute liver failure.

Hepatology, 30 (3), 1999

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ABSTRACT

Brain edema sufficient to cause intracranial hypertension and brain herniation remains a major cause of mortality in acute liver failure (ALF). Studies in experimental animal models of ALF suggest a role for ammonia in the pathogenesis of both encephalopathy and brain edema in this condition. As part of a series of studies to evaluate the therapeutic efficacy of ammonia-lowering agents, groups of rats with ALF caused by hepatic devascularization were treated with L-ornithine-L-aspartate (OA), an agent shown previously to be effective in reducing blood ammonia concentrations in both experimental and human chronic liver failure. Treatment of rats in ALF with infusions of OA (0.33 g/kg/h, intravenously) resulted in normalization of plasma ammonia concentrations and in a significant delay in onset of severe encephalopathy. More importantly, brain water content was significantly reduced in OA-treated rats with ALF. These protective effects of OA were accompanied by increased plasma concentrations of several amino acids including glutamate, γ-aminobutyric acid (GABA), taurine, and alanine, as well as the branched-chain amino acids, leucine, isoleucine, and valine. Increased availability of glutamate following OA treatment provides the substrate for the major ammonia-removal mechanism (glutamine synthetase). Plasma (but not cerebrospinal fluid) glutamine concentrations were increased 2-fold ($p<0.02$) in OA-treated rats, consistent with increased muscle glutamine synthesis. Direct measurement of glutamine synthetase activities revealed a 2-fold increase following OA treatment. These findings demonstrate a significant ammonia-lowering effect of OA together with a protective effect on the development of encephalopathy and brain edema in this model of ALF.

INTRODUCTION

Brain swelling culminating in increased intracranial pressure and subsequent brain herniation remains the major cause of death in acute liver failure (ALF). Although the pathogenesis of brain edema in ALF has not been fully elucidated, there is a growing body of evidence to suggest that ammonia (either directly or indirectly) plays a predominant role. In experimental animal models of ALF resulting from hepatectomy (Holmin et al., 1983), hepatic devascularization (Mans et al., 1979, Swain et al., 1992a) or toxic liver injury (Swain et al., 1992b), brain edema is a consistent finding, and brain ammonia frequently reaches millimolar concentrations. Exposure of various brain preparations to millimolar concentrations of ammonia *in vitro* results in significant cell swelling (Ganz et al., 1989, Norenberg et al., 1991). Furthermore, precipitous increases in blood ammonia concentrations are associated with brain edema in conditions such as Reye's Syndrome (Jenkins et al., 1987) and urea cycle enzymopathies (Brusilow, 1985).

L-ornithine-L-aspartate (OA) has been proven to be effective in lowering blood ammonia concentrations in both experimental and human chronic liver failure (Staedt et al., 1993, Kircheis et al., 1997, Rose et al., 1998). A recent randomized, clinical trial of OA treatment revealed significant reductions in blood ammonia in cirrhotic patients concomitant with a significant improvement of neuropsychiatric symptoms (Kircheis et al., 1997). In view of these findings and of the proposed role of ammonia in the pathogenesis of brain edema in ALF, the present study had 2 aims, namely 1) to study the efficacy of OA in the prevention of encephalopathy and brain edema in experimental ALF and 2) to relate this protective effect of OA to altered plasma and cerebrospinal fluid (CSF) concentrations of ammonia and ammonia-related metabolites as well as activities of the ammonia-related, glutamine synthetase. The rat with ALF caused by hepatic devascularization was chosen as an animal model for these studies, because this animal model recapitulates many of the features of ALF in humans including a rapid, progressive deterioration of neurological status, the appearance of cytotoxic brain edema at severe stages of encephalopathy,

sustained hyperammonemia, and a characteristic pattern of alterations of plasma and CSF amino acids similar to that observed in ALF in humans (Swain et al., 1992a, Swain et al., 1992b, Blei et al., 1992)

MATERIALS and METHODS

O-Phthalaldehyde reagent solution, 2-mercaptoethanol, and amino acid standards were purchased from Sigma Chemical Co., St-Louis, MO; Methanol (high performance liquid chromatography [HPLC] grade), sodium phosphate (monobasic) and tetrahydrofuran (HPLC grade) were from Anachemia, Montreal, Quebec, Canada. All reagents and solvents were reagent-grade purity unless otherwise stated. Double-distilled deionized water was used for preparation of amino acid solutions and buffers. The mobile phase was filtered through 0.45 μm filters (Type GS, Millipore Corporation, Bedford, MA., U.S.A) and degassed under vacuum prior to use.

Experimental design

Twenty-four hours after portacaval anastomosis (PCA), animals were reanesthetized with halothane, and cisterna magna catheters inserted. The following day (48 hours after PCA), animals were reanesthetized with halothane and underwent a second laparotomy at which time arterial, venous and peritoneal catheters were implanted, and hepatic artery ligation (HAL) was performed.

Five hours following HAL, rats were infused with OA (0.33g/kg body weight/h, intravenously) or isotonic saline over a 5-hour period. Arterial glucose levels were monitored throughout the experimental period. During development of ALF body temperature was maintained at 37±1 °C, using a rectal thermometer coupled to a thermostated heating pad. Hypoglycemia was prevented by intraperitoneal administration of glucose (through the peritoneal catheter) as needed. Neurological status was assessed every 30 minutes, and severe encephalopathy was

defined as loss of righting ability (precoma stage) progressing through loss of corneal reflex (coma stage).

Portocaval anastomosis (PCA)

Adult male Sprague-Dawley rats weighing 175 to 200 g were anesthetized with halothane and an end-to-side PCA was performed according to the guidelines of Lee and Fisher (1961). Rats underwent a laparotomy, the inferior vena cava and portal vein were isolated, allowing for the inferior vena cava to be clamped (anastomosis clamp, Roboz Instruments Inc., Washington, DC), and the removal of an elliptical piece of vein 1.5 times the portal vein diameter. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was <15 minutes. Following surgery, all animals were housed individually with free access to standard laboratory chow and water under constant conditions of temperature, humidity, and light cycles.

Venous and arterial catheters

Rats were maintained under halothane anesthesia for the duration of the operation. During the second laparotomy, two PE-50 catheters were inserted; a venous catheter in the vena cava and an arterial catheter in the aorta. A constant infusion of OA or saline was circulated through the venous catheter, and blood samples were collected through the arterial catheter.

Cisterna Magna Catheters

Rats were maintained under halothane anesthesia for the duration of the operation. Cisterna magna catheters were installed as previously described (Swain et al., 1992a). Briefly, the animal's head was mounted with the skull in a horizontal position in a stereotaxic apparatus. A 3-cm incision was made in the skin at the back of the head, and the overlying connective tissue was removed to expose the skull. A small hole was drilled in the skull using a dental burr (009) on the sagittal midline

immediately rostral to the interparietal-occipital bone suture. The hole was drilled in such a way that the occipital bone could be used as a guide while inserting the cannula (PE-10 tubing, Clay Adams, Parsippany, NJ). The catheter was then slowly inserted into the cisterna magna. Correctness of placement of the CSF-inflow window was checked by clearing the CSF outway, at which time a spontaneous flow of clear CSF occurred. When successful implantation had been confirmed, the skull was cleaned and dried and a small amount of dental acrylic cement (Yates and Bird, Chicago, IL) applied to anchor the inserted cannula. The entire operation took approximately 20 min. Rats were housed individually and allowed to recover overnight. Twenty-four hours after implantation, Silastic tubing (Dow Corning, Midland, MI) was connected to the cannula for serial CSF sampling.

Measurement of plasma and CSF amino acids

Amino acids were derivatized with o-phthalaldehyde, separated and analyzed using HPLC with fluorescence detection according to the method of Spink et al. (1986). Plasma or CSF samples were treated with acidified methanol (1:10), the mixture was left for 20 minutes at 4°C and centrifuged for 10 minutes at 15,400 g. Twelve µl of the supernatant was then withdrawn and mixed with 3 µl of homoserine (0.01 mM) as internal standard and reacted at room temperature with 20 µl o-phthalaldehyde reagent for 45 seconds, in a total volume of 35 µl. Twelve µl of this solution was then injected onto the HPLC column (C18 Pecosphere, Perkin Elmer) with a CMA/200 autosampler. The HPLC system consisted of a solvent delivery system (Perkin Elmer, Norwalk, CT, Series 400) coupled to a fluorometer (Perkin Elmer, LC-10 Fluorescence Detector, excitation 370 nm, emission 418-700 nm). Samples were injected in a 50 µl loop of a Rheodyne 7125 injector system (Rheodyne, Cotati, CA, U.S.A). Peak area measurements were computed using a programmable recording integrator (Perkin-Elmer LC-100). The chromatograph was run with gradients of increasing organic solvent concentration generated by differential pumping rates of solvents A and B. Solvent A contained 30 mM

disodium phosphate, 30 mmol/L sodium acetate, 2% (vol/vol) methanol, and 0.05% (wt/vol) sodium azide adjusted to pH 6.2 with glacial acetic acid. Tetrahydrofuran 1% (vol/vol) was added just before running the system. Solvent B consisted of a mixture of water: methanol:acetonitrile:tetrahydrofuran in 19:15:15:1 ratio by volume. Each gradient program ended with a 10 min. hold at 100% solvent B, followed by a 5-min re-equilibration with 100% solvent A. Amino acid content of plasma was calculated by peak area analysis using standard curves and the internal standard.

Ammonia levels were estimated in plasma using a commercial blood ammonia test kit, which uses an ion-exchange method, followed by colorimetric measurement of isolated ammonia nitrogen with the Berthelot phenate-hypochlorate reaction (Dienst, 1961). Ammonia was measured within 24 h of collection.

Measurement of brain water

One cerebral hemisphere was kept at 4 °C and brain water measured within 15 minutes of death. The brain was cut into 2-mm slices, and 1-mm punch biopsy specimens were obtained from the gray matter of the cerebral cortex. Water content of each specimen was measured gravimetrically using a density gradient of bromobenzene-kerosene precalibrated with K_2SO_4, as previously described (Marmarou et al., 1978). The cortical samples were placed onto the fluid column, and the equilibration point was measured within 2 min. The specific gravity of the tissue was calculated, and results were expressed as percentage of water content. Eight measurements were made per animal, and values were arithmetically averaged.

Measurement of muscle glutamine synthetase

Glutamine synthetase in skeletal muscle was assayed following the method of Girard and Butterworth (Girard and Butterworth, 1992). At coma stages of encephalopathy in saline-treated ALF rats, or equivalent time points in OA-treated animals, following killing, 100-mg samples of skeletal muscle were homogenized

(20% wt/vol) in 50 mmol/L Tris-HCl-ethylenediaminetetraacetic acid buffer (pH 7.4) using a polytron homogenizer. The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C, and 0.1 mL of the supernatant was preincubated for 10 minutes at 37°C in an assay mixture containing 100 mmol/L imidazole (pH 7.2), 12.5 mmol/L $MgCl_2$, 20 mmol/L mercaptoethanol; 10 mmol/L adenosine triphosphate, 4 mmol/L ammonium chloride, 13 mmol/L phosphoenolpyruvate, 50 units of of pyruvate kinase, and 0.16% Triton X-100. The reaction was started by adding [1-^{14}C] glutamate (2 μCi; specific activity; 45 mCi/mmol; NEN Dupont, Boston, MA) diluted with cold glutamate to a final concentration of 50 mmol/L and incubated for 30 minutes at 37°C. The reaction was terminated by addition 1 mL of ice-cold water, and the mixture as immediately passed through an anion exchange (Dowez AG-1 X 8, acetate form; Biorad Laboratories, Richmond, CA) column to separate radioactive glutamate (retained on the column) from glutamine (eluted through the column) formed by glutamine synthetase. Columns were further washed with 2 mL of distilled water to ensure complete recovery of glutamine, and the radioactivity in the eluate was determined by liquid scintillation spectrometry. In all assays, boiled homogenates served as blanks. Protein content of homogenates was determined by the method of Lowry et al. (1951). Enzyme-specific activities were expressed as micromoles of glutamine formed per hour per milligram of protein.

Statistical analysis

Blood and CSF data at each time point in OA versus saline-treated rats were analysed by Student's *t* test or ANOVA with Tukey Test for between group comparisons. $p<0.05$ was considered to represent a statistically significant main effect; p values for comparisons using post-hoc tests are indicated in the figure legends.

RESULTS

Following PCA and HAL, all rats exhibited reproducible neurological changes progressing through loss of activity, loss of righting ability (precoma stage), to loss of corneal reflex (coma stage). OA treatment significantly lengthened the time to precoma and coma stages of encephalopathy (Table 1).

Table 1: Neurological status in rats with ALF: Effect of OA

	Saline-treated (n) (min)	OA-treated (n) (min)
Time to loss of righting ability (precoma)	485.1±47.5 (6)	628.4±36.5* (6)
Time to loss of corneal reflex (coma)	694.8±51.2 (6)	915.7±47.8* (6)

NOTE: Values represent the mean ± S.D. of determinations from n=6 rats per treatment group. *Values significantly different from saline-treated controls, $p<0.02$ by Student *t* test.

Plasma ammonia concentrations were significantly reduced in concentration following OA infusions (Figure 1) compared with saline-treated controls. The significant ammonia lowering effect of OA on plasma ammonia was evident at all time points following the start of infusion. Saline-treated rats with ALF manifested plasma ammonia concentrations in the 500 μg/dL range. OA treatment of these animals, on the other hand, resulted in reduction of plasma ammonia concentrations to within normal ranges (ie: 40-60 μg/dL). OA treatment resulted in a significant reduction of ammonia concentrations in CSF of rats with ALF (saline-treated ALF rats, CSF [ammonia]: 970.94±90.6; OA-treated ALF rats, CSF [ammonia]: 640.27±77.8; $p<0.01$).

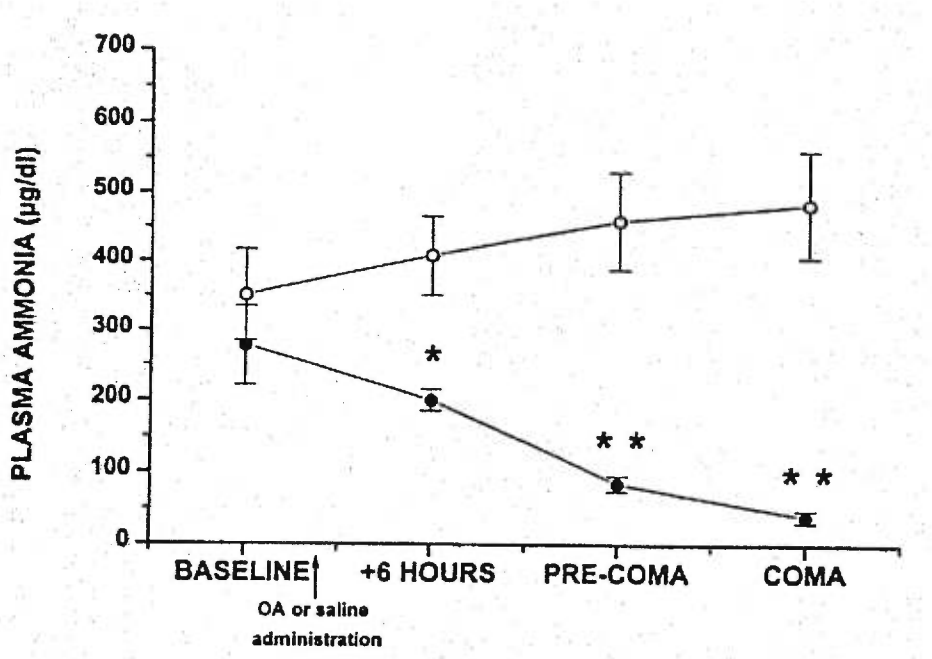

Figure 1: Effect of OA infusions (330 mg/kg/h) (●) versus saline (○) on plasma ammonia concentrations at various time points during the development of hepatic encephalopathy in rats with ALF caused by hepatic devascularization. Baseline indicates values indicate values before the start of infusions, +6 h time point taken at 6 h. following HAL. Precoma and coma time points are defined by loss of righting ability and loss of corneal reflex respectively. Data points represent mean values ± S.E. of duplicate determinations from 6 rats per treatment group. Values significantly different from saline-infused ALF rats at each time point indicated by *$p<0.05$ and **$p<0.01$ by ANOVA.

Furthermore, OA treatment significantly reduced brain water content in rats with ALF compared with saline treated controls ($p<0.001$) as shown in Figure 2.

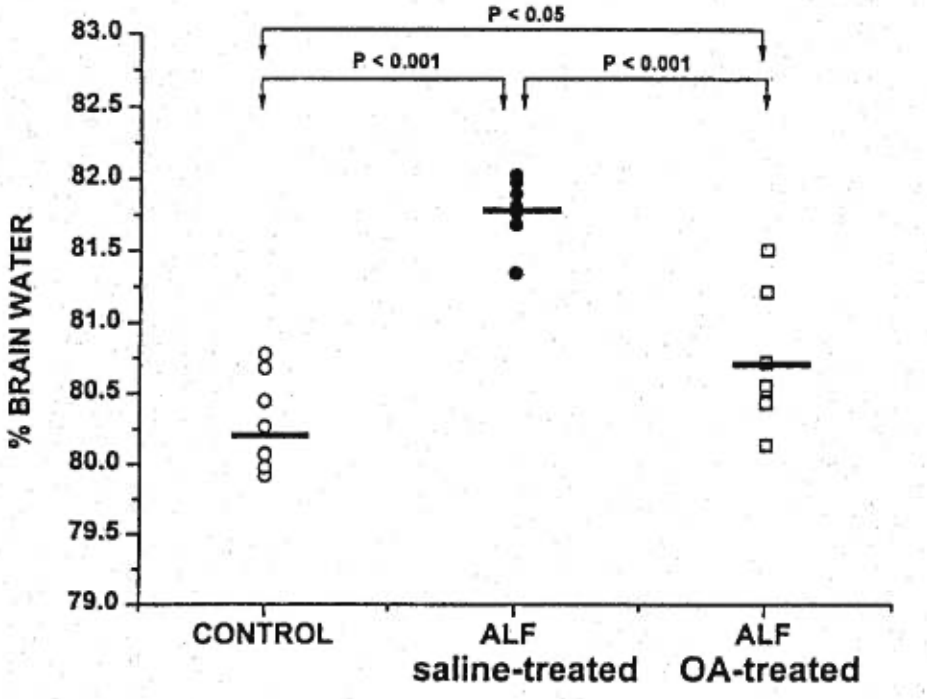

Figure 2: Effects of OA infusions (330 mg/kg/h) (□) versus saline (●) on brain water content in rats with ALF caused by hepatic devascularization, compared to normal controls (O). Data points indicate individual values and horizontal bars indicate means of n=7 animals per experimental group. Brain water content of ALF (saline-treated) rats was significantly increased compared with controls ($p<0.001$). OA treatment resulted in a significant ($p<0.001$) lowering of brain water content compared with saline-treated ALF controls by ANOVA.

OA infusions in rats with ALF resulted in a generalized increase of plasma amino acid concentrations (Table 2). Such changes included a 12-fold increase in plasma glutamate ($p<0.01$), increased glutamine (2-fold. $P<0.01$), increased alanine and GABA (3-fold, $p<0.01$), and significant 1.5- to 2-fold increases of branched-chain amino acids.

Table 2: Plasma metabolite concentrations in rats with ALF: Effect of OA

	Amino acids concentration (μmol/L) (mean ±S.D.)	
Amino acid	Saline-treated (coma stage)	OA-treated
Glutamate	499.2±38.9	6760.4±589.4*
Aspartate	107.4±13.5	4485±495.8*
Glutamine	7433.7±648.2	14973.7±596.2*
Arginine	999.7±108.4	1462.6±246.4*
Tyrosine	456.8±47.1	525.8±91.2
Tryptophan	86.3±8.3	144.8±13.5*
Phenylalanine	570.5±63.5	596.1±91.4
Leucine	193.4±19.4	419.1±50.5*
Isoleucine	128.8±16.2	476.9±87.3*
Valine	324.7±12.9	437.5±15.5*
GABA	14.9±1.7	51.9±8.5*
Glycine	2046.3±154.1	3461.7±409.4*
Taurine	983.1±83.9	3796.5±416.7
Alanine	2300.9±189.5	7201.3±424.3*

NOTE: Amino acids were measured in plasma samples at coma stages of encephalopathy in 6 ALF rats treated with saline infusions versus 6 ALF rats treated with OA (330 mg/kg/h) at equivalent time points. *Values significantly different from saline-treated controls, $p<0.01$ by Student's *t* test.

Alterations of CSF amino acids following OA treatment of rats with ALF are shown in Table 3. OA treatment resulted in a significant decrease in CSF glutamate, but no significant increases of glutamine in contrast to the findings in blood. Concentrations of the aromatic amino acids, tyrosine and phenylalanine, were significantly reduced in CSF following OA treatment. Concentrations of GABA and

branched-chain amino acids, on the other hand, were significantly increased in CSF of OA-treated animals compared with saline-treated controls.

Table 3: Cerebrospinal fluid metabolite concentrations in rats with acute liver failure: Effect of OA

	Amino acids concentration (μmol/L) (mean± S.D.)	
Amino acid	Saline-treated (coma stage)	OA-treated
Glutamate	9.4±1.2	4.3±1.2*
Aspartate	43.6±6.6	46.6±13.1
Glutamine	2453.6±131.6	2327.7±258.1
Arginine	20.9±2.7	19.2±1.8
Tyrosine	131.5±4.1	108.1±13.7*
Tryptophan	10.9±1.1	12.1±2.3
Phenylalanine	133.2±14.5	98.2±9.4*
Leucine	17.7±1.1	45.8±4.9*
Isoleucine	10.7±1.4	25.1±2.7*
Valine	60.1±9.1	169.8±4.5
GABA	1.5±0.2	7.7±1.1*
Glycine	30.7±2.3	44.9±4.2*
Taurine	43.9±5.1	41.6±4.6
Alanine	198.2±5.6	317.2±16.7*

NOTE: Amino acids were measured in CSF samples obtained via indwelling cisterna magna catheters at coma stages of encephalopathy in 6 ALF rats treated with saline infusions versus 6 ALF rats treated with OA (330 mg/kg/h) at equivalent time points.*Values significantly different from saline-treated controls, *$p<0.01$ by Student's *t* test.

OA treatment resulted in a significant 2-fold increase in glutamine synthetase activities as shown in Table 4.

Table 4. Muscle glutamine synthetase activity in saline versus OA-treated rats.

Treatment group	Muscle glutamine synthetase Activity (μmol/mg prot/h)
Saline	0.21±0.03 (6)
OA	0.39 ±0.04 (7)*

NOTE: Values represent the mean ± S.D. of duplicate determinations. Number of animals per treatment group in parentheses. *Values significantly different from saline-treated group, $p<0.01$ by Student's t test.

DISCUSSION

Results of the present study revealed that 1) OA administration leads to a significant lowering of plasma and CSF ammonia in this experimental model of ALF; and 2) this ammonia-lowering effect of OA is accompanied by a reduction in brain water content and a delay in the time of onset of severe encephalopathy. These findings add to the body of evidence that ammonia is implicated in the pathogenesis of brain edema in ALF. It has long been established that hyperammonemia in experimental animals with or without liver failure results in cerebral edema (Levin et al., 1989, Takahashi et al., 1991, Blei et al., 1994). Swelling of cerebral cortical astrocytes, the characteristic feature of experimental ALF (Ganz et al., 1989) is observed following ammonia infusions to young primates (Voorhoies et al., 1989) and exposure of rat cortical astrocytes in culture to ammonia results in significant cell swelling (Norenberg et al, 1991). Furthermore, precipitous increases in blood ammonia, similar to those reported in ALF are associated with brain edema in other conditions such as Reye's Syndrome (Jenkins et al, 1987) and urea cycle enzymopathies (Brusilow, 1985).

The hepatic devascularized rat has been widely used for the study of pathophysiologic mechanisms implicated in the phenomenon of brain edema in ALF (Blei et al., 1992). Extensive studies of peripheral and central metabolism have been performed in these animals and findings include increased blood and brain ammonia concentrations (Mans et al., 1979, Swain et al., 1992b). In one study using this model of ALF in which correlations between various metabolite concentrations and degree of neurological dysfunction were assessed, the best correlation observed was with plasma ammonia (correlation coefficient 0.76, $p<0.01$) followed by brain glutamine (correlation coefficient 0.69, $p<0.01$) (Mans et al., 1994). Other characteristics of this model of ALF include a characteristic plasma and CSF amino acid profile consisting of increased glutamine, glutamate, alanine and aromatic amino acids (Mans et al., 1979, Swain et al., 1992, Mans et al., 1994). Similar amino acid profiles have been reported in patients with ALF (Record et al., 1976). In the present

study, OA administration to rats with ALF caused by hepatic devascularization resulted in a significant attenuation of plasma and CSF ammonia concentrations, together with a significant, 2-fold further increase of plasma (but not CSF) glutamine. These findings suggest that OA stimulates glutamine synthesis in the periphery, an action which, in the hepatic devascularized rat, must come primarily from skeletal muscle. In support of this possibility, it has been reported that, in severe liver failure, muscle ammonia uptake is enhanced and that the subsequent glutamine synthesis is the major pathway for ammonia detoxification (Lockwood et al., 1979).

Once in solution, OA splits into its component amino acids, L-ornithine and L-aspartate. Ornithine is a substrate for both the urea cycle and for ornithine transaminase, which gives rise to glutamate semialdehyde via the reaction catalyzed by ornithine:α-ketoglutarate amino transferase (OAT). Glutamate semialdehyde dehydrogenase then transforms glutamate semialdehyde into a second molecule of glutamate. The glutamate formed from ornithine is thus available as substrate for several reactions including the following synthetase, and, in all probability, the major beneficial effect of OA, namely, its blood ammonia-lowering action, results from the stimulation of glutamine synthesis in skeletal muscle via glutamine synthetase. Consistent with this are the findings in the present study of significantly increased plasma glutamine in OA-treated animals with ALF. Furthermore, direct measurement of muscle glutamine synthetase activities revealed a significant 2-fold increase following OA treatment of rats with ALF. On the other hand, glutamine concentrations are not increased in CSF of OA-treated rats with ALF, an observation that undoubtedly results from the fact that both substrates for the glutamine synthetase reaction (glutamate and ammonia) are reduced in CSF following OA treatment. Previous studies had proposed that cellular glutamine accumulation could be the cause of ammonia-induced swelling. In favor of this, inhibition of the synthesis of glutamine was shown to prevent cell swelling induced by ammonia both *in vitro* (Norenberg et al., 1994) and *in vivo* (Blei et al., 1994). However, a beneficial effect of OA on cell swelling as a result of lowering brain glutamine synthesis is not

indicated by the results of the present study in which CSF glutamine concentrations in OA and saline-treated groups were of a similar magnitude. Further studies will be required to study the role of glutamate/glutamine-related mechanisms in the pathogenesis of cerebral edema in ALF.

As expected, blood aspartate concentrations increased precipitously following OA administration, an effect that could stimulate transaminase reactions, resulting in increased formation of oxaloacetate and glutamate. In this way, aspartate could contribute to ammonia removal, like ornithine, by providing the substrate (glutamate) for the only major peripheral ammonia detoxifying mechanism available in ALF, namely, muscle glutamine synthesis. Increased glutamate availability following OA treatment is also the most likely explanation for the increases of plasma GABA and branched-chain amino acids.

A consistent finding in experimental animal models of ALF is increased concentrations of glutamate in brain extracellular fluid (Michalak et al., 1996), a phenomenon which appears to result from decreased uptake of glutamate into the astrocytes as a consequence of reduced astrocytic glutamate transport (Knecht et al., 1997). In view of previous reports that exposure of cultured astrocytes to glutamate causes cell swelling, it is possible that increased extracellular glutamate contributes to brain edema in ALF. Such a possibility is supported by results of the present study which demonstrate a 60% lowering of CSF glutamate and a concomitant reduction in brain water content following OA treatment of rats with ALF.

ACKNOWLEDGMENT

The authors are grateful to Dominique Roy for her assistance with the preparation of the manuscript.

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2.4 Article 4

Manganese deposition in basal ganglia structures results from both portal-systemic shunting and liver dysfunction.

Gastroenterology, 117 (3), 1999.

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ABSTRACT

Background and aims. There is increasing evidence to suggest that manganese (Mn) deposition is responsible for the T_1-weighted magnetic resonance signal hyperintensities and neuropsychiatric symptoms observed in patients with chronic liver disease. However, the regional specificity of the Mn increase as well as their relationship to the presence of portal-systemic shunting or hepatobiliary dysfunction in these patients remains unclear. The present series of experiments were designed to assess these issues. ***Methods.*** Mn concentrations were measured by a neutron activation technique in (1) brain tissue samples from basal ganglia structures (pallidum, putamen, caudate nucleus) and cerebral cortical structures (frontal, occipital cortex) obtained at autopsy from 12 cirrhotic patients who died in hepatic coma and from 12 appropriately matched controls and (2) brain samples (caudate/putamen, globus pallidus (pallidum), frontal cortex) from groups (n = 8) of rats either (i) four weeks following end-to-side portacaval anastomosis, (ii) with liver cirrhosis due to common bile duct ligation or (iii) with fulminant hepatic failure following hepatic devascularization as well as 2 control groups (iv) appropriate sham-operated group of rats and (v) a group of normal Sprague Dawley rats. ***Results.*** Mn content of control human brain was highest in pallidum > putamen > caudate > occipital >frontal cortex and was significantly increased ($p<0.05$) in frontal cortex (by 38%), occipital cortex (by 55%), pallidum (by 186%), putamen (by 66%) and caudate (by 54%) of cirrhotic patients. Increased brain Mn content did not correlate in any brain region with patient age, etiology of cirrhosis or history of chronic hepatic encephalopathy (n=6). In cirrhotic and portacaval shunted rats, Mn content was increased in pallidum (by 27% and 57% respectively) and in caudate/putamen (by 57% and 67% respectively) when compared to control groups. Mn concentration in pallidum was significantly higher in portacaval-shunted rats compared to cirrhotic rats. No significant changes in brain Mn concentrations were observed in rats with acute liver failure. ***Conclusions.*** These findings suggest that brain Mn deposition results both from portal-systemic shunting and from liver dysfunction.

INTRODUCTION

Pallidal signal hyperintensity on magnetic resonance imaging (MRI) has been observed in a majority of cirrhotic patients (Pujol et al., 1993). Increasing evidence suggests that accumulation of manganese could best explain this finding. Pallidal samples obtained at autopsy from cirrhotic patients who died in hepatic encephalopathy (HE) contain up to 7 fold increased manganese content (Pomier Layrargues et al., 1995). Histological examination of these samples excluded other potential causes of MRI signal hyperintensities. Furthermore, similar pallidal images have been observed in patients receiving manganese as part of total parenteral nutrition and hyperintensities disappeared after cessation of manganese administration (Mirowitz et al., 1991, 1992).

We previously reported significant positive correlations between blood manganese, pallidal index (a measure of the degree of MR signal hyperintensity) and the presence of portal-systemic shunting in cirrhotic patients (Spahr et al., 1996). However studies attempting to correlate neurological symptoms with MR signal hyperintensities have so far yielded conflicting results (Spahr et al., 1996, Krieger et al., 1996, Kulisevsky et al., 1992, Taylor-Robinson et al., 1995, Weissenborn et al., 1995). These inconsistencies could result from the large variability in patient population as well as the different methods of evaluation of neuropsychological status. The aims of the present series of experiments were:

1) To measure the regional selectivity of manganese accumulation in the brains of cirrhotic patients versus appropriate material from matched-controls.

2) To determine the relative influence of portal-systemic shunting and cholestasis (both factors being suggested as major determinants for brain manganese deposition) on manganese accumulation in the brains of rats with chronic liver failure.

MATERIAL AND METHODS

Human studies

Brain samples were obtained from 12 cirrhotic patients who died in hepatic coma and 12 control subjects free from hepatic, neurological or psychiatric disorders at the time of death and were matched for age, sex and autopsy delay times. In no cases had patients or control subjects received total parenteral nutrition prior to death. Brain dissection was performed using a ceramic knife according to a standardized dissection protocol (De Armond et al., 1976). Brain samples were frozen at -80°C until time of assay. Manganese was measured in the following brain regions: frontal and occipital cortex, globus pallidus, putamen and caudate nucleus.

Rat studies

Animals

5 groups of male Sprague Dawley rats (250-300 g) were evaluated. Group 1 (n = 8) comprised normal rats. Group 2 (n = 8) were rats with end-to-side portacaval shunt performed according to the guidelines of Lee and Fisher (1961); rats underwent a laparotomy and the inferior vena cava and portal vein were isolated. The inferior vena cava was partially clamped and an elliptical piece of vein 1.5 times the portal vein diameter was cut and removed. The portal vein was ligated and cut and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was 15 minutes. Group 3 (n = 8) included sham-operated control rats matched for weight; they underwent a laparotomy and the inferior vena cava and portal vein were isolated and occluded for 15 minutes. Studies in portacaval shunted and sham-operated rats were performed 4 weeks after surgery. Group 4 (n = 8) were rats with secondary biliary cirrhosis induced by intracholedochal injection of formalin followed by bile duct ligation as previously described (Colombato et al., 1994). These animals were studied 6 weeks after surgery. Group 5 (n = 8) were rats with acute liver failure induced by a combination of portacaval anastomosis (as above) followed 24 hours later by hepatic artery ligation (Websters et al., 1991). Blood

glucose levels were monitored and maintained at normal levels in all animals. Body temperature was maintained at 37°C. These rats were studied 15-18 hours after devascularization at coma stage of encephalopathy. All groups of rats were anesthesized with halothane throughout the surgery and post operatively, animals were allowed to recover in individual cages under constant conditions of temperature, humidity and light cycles, with free access to standard rat chow and water.

Rats were sacrificed by decapitation and brains were quickly removed and dissected with non-metallic surgical instruments. Manganese was measured in the following brain regions: frontal cortex, globus pallidus and caudate/putamen.

Biochemistry

Blood samples were obtained after sacrifice by decapitation. The following biochemical tests were performed using routine techniques: serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin and alkaline phosphatase. Blood ammonia was measured using an ion-exchange method followed by a colorimetric measurement of isolated ammonia nitrogen with the Berthelot phenate-hydrochlorate reaction (Dienst, 1961).

Portosystemic shunting (PSS)

This parameter was evaluated in the group of rats with biliary cirrhosis. After injection of ^{141}cerium radiolabelled microspheres in the mesenteric vein, PSS was calculated from the ratio between lung and liver radioactivities as previously described (Groszmann et al., 1982).

Manganese

Manganese was measured in brain tissue using neutron activation analysis (Kennedy, 1990). Briefly, neutron activation analysis is a two step technique. The first step involves the tissue being irradiated with the neutron flux of a nuclear

reactor. Neutrons are then captured by the manganese nuclei and subsequently become radioactive. Once removed from the nuclear reactor, radiation is emitted as gamma rays. The second step involves detection and measurement of the energy emitted from the gamma rays. This measurement gives the total amount of the manganese in the sample and divided by the sample weight results in the average concentration of manganese per gram of tissue.

Statistical calculations

Results are expressed as mean ± SEM. Comparison between groups were made using analysis of variance with the Bonferroni correction and correlations were evaluated using the Pearson's r test. The level of statistical significance was set at $p < 0.05$.

RESULTS

Human studies

Patient characteristics (cirrhotics and controls) are summarized in table 1. Groups were rigorously matched for age, sex and time between death and sample freezing.

Table 1: Patient characteristics

	Controls (n = 12)		Cirrhotic (n = 12)	
Age (years)	64.3 ± 3.7		60.6 ± 2.5	
Sex (m/f)	10/2		9/3	
Diagnosis	Respiratory failure:	3	Alcoholic cirrhosis:	7
	Myocardial infarction:	3	B viral cirrhosis:	3
	Septic shock:	2	C viral cirrhosis:	1
	Lung cancer:	2	Cryptogenic cirrhosis:	1
	Aortic aneurysm:	1		
	Multiorgan failure:	1		
Delay between death and autopsy (hours)	13 ± 2		13 ± 4	

As shown in figure 1, in both controls and cirrhotics, brain manganese were found to be higher in basal ganglia structures (pallidum, putamen and caudate nucleus) than in cortical areas. In cirrhotics, a general increase in brain regional manganese concentration was observed compared to controls; manganese

accumulation was much more pronounced in pallidum (+ 186%; $p < 0.01$) compared to putamen (+66%; $p < 0.01$) and caudate nucleus (+54%; $p < 0.05$). Increased brain manganese content did not correlate with patient age, etiology of cirrhosis or history of recurrent hepatic encephalopathy (6 patients).

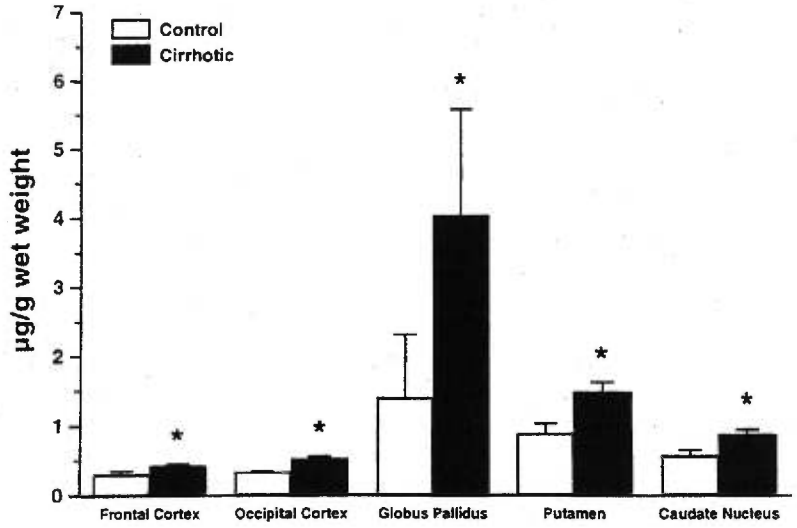

Figure 1: Manganese concentrations in post-mortem human brain tissue. Data are expressed as mean ± SEM; * statistically different from controls ($p<0.05$).

Rat studies

Characteristics of the 5 study groups are summarized in table 2. Serum bilirubin levels were higher in cirrhotic rats (38 fold) and in hepatic devascularized rats (13 fold) compared to control groups of animals. Serum transaminase, (aspartate and alanine), were also elevated in these same rats, particularly in hepatic devascularized rats. Serum albumin was found to be significantly lower only in cirrhotic rats, with 40 % decrease compared to controls. Alkaline phosphatase was found to be elevated only in rats with acute liver failure with a 45% increase relative to controls. Blood ammonia was elevated in all three experimental liver failure groups of rats, with acute liver failure rats demonstrating the highest degree of hyperammonemia.

Table 2: Characteristics of rat study groups

	Weight (g)	Bilirubin (mg/dL)	AST (UI/L)	ALT (UI/L)	ALP (UI/L)	ALB (g/L)	Ammonia (µg/dL)	PSS (%)	PP (mmHg)
controls (n=8)	355±25	3.1±0.7	155±16	55±3	178±21	25.3±0.6	181±5	---	---
sham (n = 8)	356±9	1.8±0.2	202±28	62±4	191±11	24.9±0.6	120±4	---	---
PCS (n = 8)	281±10	2.4±0.3	149±13	58±4	241±18	23.6±0.4	232±16[a]	---	---
Cirrhotics (n = 8)	420±11	117±11[b]	493±51[b]	90±8[a]	256±41	14.5±0.7[b]	268±21[b]	57±10	18.8±0.5
ALF (n = 8)	218±4	39±4[b]	15071±3340[b]	7137±2471[b]	400±25[b]	25.5±0.6	452±20[b]	---	---

Note: Results given as mean ± SEM
PCS: Portacaval shunt; ALF: Acute liver failure (devascularization); AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; ALB: albumin; PSS: portal-systemic shunting; PP: portal pressure significantly different from controls and sham groups; a: $p < 0.05$; b: $p < 0.01$. Portal-systemic shunting was found to be 57% ± 10% in cirrhotic rats with a range of 29% to 100%. Portal hypertension was found in cirrhotic rats with an average portal pressure of 18.8 mmHg.

In control and sham-operated rats, manganese content was higher in globus pallidus compared to other brain regions (Figure 2). In cirrhotic and portacaval-shunted rats, manganese content was increased in pallidum (by 27% and 57% respectively) and in caudate/putamen (by 57% and 67% respectively) compared to control rats; furthermore, manganese deposition was much more pronounced in the pallidum compared to other brain regions in these groups of animals. Pallidal manganese concentrations were higher in portacaval-shunted rats than cirrhotic rats (1.05 ± 0.07 vs 0.72 ± 0.06 µg/g/wet weight; $p < 0.05$). In contrast, no significant changes could be demonstrated in any brain regions of rats with acute liver failure when compared to control animals.

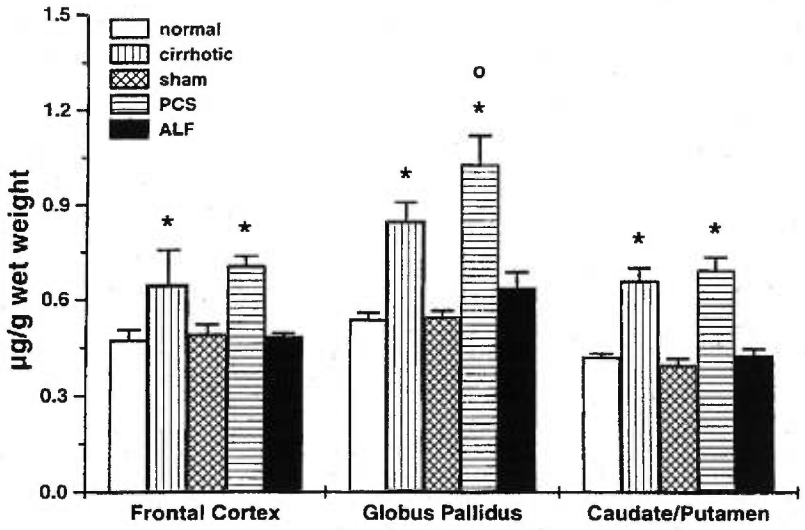

Figure 2: Manganese concentrations in rat brain tissue. Data are expressed as mean ± SEM.; * statistically different from controls and shams ($p<0.05$); ° statistically different between PCS and cirrhotic.

In cirrhotic rats, no significant correlation was observed between either serum albumin, serum bilirubin, serum transaminase, alkaline phosphatase levels, blood ammonia, portal pressure or the degree of portal-systemic shunting and brain manganese concentrations.

DISCUSSION

The present study confirms and extends previous reports of increased pallidal manganese concentrations in cirrhotic patients (Krieger et al., 1995, Maeda et al., 1997). Furthermore, results of the present study reveal that increased manganese in brain is a region-selective phenomenon with concentrations being highest in globus pallidus > putamen > caudate nucleus >> cerebral cortex in these patients.

It has been suggested that selective manganese deposition in the pallidum could be the cause of the high signal intensity in T_1-weighted images that has been consistently reported in the majority of cirrhotic patients (Pujol et al., 1993, Spahr et

al., 1996, Krieger et al., 1996, Kulisevsky et al., 1992, Taylor-Robinson et al., 1995, Weissenborn et al., 1995,Inoue et al., 1991, Zeneroli et al., 1991). Further evidence suggesting that MR pallidal hyperintensities are best explained by manganese deposition are provided by observations in patients receiving long term parenteral nutrition where typical pallidal images observed when patients received manganese supplementation disappeared after cessation of manganese administration (Mirowitz et al., 1991, 1992). Furthermore exposure of non-human primates to manganese administered either by inhalation or intravenously resulted in selective pallidal hyperintensities on MR imaging (Shinotoh et al., 1995, Yamada et al., 1986).

A cause-effect relationship between chronic liver disease and region-selective brain manganese accumulation is supported by several clinical observations. For example, a patient with decompensated cirrhosis caused by Alagille syndrome who manifested extrapyramidal symptoms and pallidal hyperintensity on MRI was found to have increased blood manganese and two months following liver transplantation, the extrapyramidal symptoms resolved, blood manganese returned to normal and pallidal MR signal hyperintensity disappeared in this patient (Devenyi et al., 1994). Other investigators have reported a similar disappearance of pallidal MR hyperintensities several months after liver transplantation in cirrhotic patients (Kulisevsky et al., 1992).

Possible mechanisms responsible for manganese accumulation in the pallidum of patients with cirrhosis include a decrease in elimination via biliary excretion and an increased systemic availability due to portal-systemic shunting. Average daily oral manganese intake is 2.5-3mg. Only 1-3.5% of this normally reaches the systemic circulation because ingested manganese is rapidly cleared by the liver and excreted into the bile (Papavasiliou et al., 1966). Cirrhosis is associated with a variable degree of cholestasis that can affect biliary manganese excretion. In addition, abnormalities of the microcirculation (capillarization of the sinusoids and/or intrahepatic shunting) are present in the cirrhotic liver. Alternatively (or additionally) increased blood manganese in cirrhotic patients could result from the presence of

portal-systemic collaterals produced either spontaneously or following surgical portacaval anastomosis or transjugular intrahepatic portalsystemic shunt.

To assess the relative importance of portal-systemic shunting and hepatobiliary dysfunction in the phenomenon of increased brain manganese in liver failure, we made use of animal models of portal-systemic shunting, of cholestasis associated with chronic liver failure and of acute liver failure, a condition associated with neither cholestasis nor portal-systemic shunting. Both shunting and chronic cholestasis resulted in significant region-selective brain manganese accumulation.

In rats with biliary cirrhosis, there was a combination of chronic cholestasis, as evidenced by increased serum bilirubin and alkaline phosphatase levels, of liver function impairment as shown by decreased serum albumin levels and of spontaneous shunting. All of these factors contribute to manganese accumulation in the brain. Therefore it is not surprising that brain manganese concentration did not correlate with any single parameter.

Pallidal manganese concentrations were higher in shunted rats compared to cirrhotic rats which confirms the notion that shunting is a major determinant of manganese accumulation in the brain. We observed in a previous study that blood manganese concentrations were particularly elevated in cirrhotic patients with previous portacaval shunts (Spahr et al., 1996). Furthermore, T_1-weighted MR signal intensity in pallidum significantly increased after transjugular portosystemic shunt placement but not after elective sclerotherapy in similar patients (Krieger et al., 1997). Moreover, pallidal signal hyperintensity (Yanai et al., 1995) on MRI was previously reported in a child with patent ductus venosus in the absence of significant liver disease.

Although evidence for a link between brain manganese deposition and MRI signal hyperintensities is convincing, a pathophysiological role for brain manganese deposition in the neurological symptoms in patients with liver disease and/or portalsytemic shunts remains to be demonstrated. Manganese toxicity had previously been reported in miners after a prolonged exposure to manganese dust, resulting in

extrapyramidal symptoms resembling Parkinson's disease (Nelson et al., 1993). In cirrhotic patients, a high incidence of extrapyramidal symptoms, in particular rigidity, similar to that observed in Parkinson's disease, is observed when a detailed and careful neurological evaluation is performed (Spahr et al., 1996, Krieger et al., 1997). However, recent reports evaluating the relationship between blood manganese, pallidal hyperintensity and neurological symptoms have yielded conflicting results (Spahr et al., 1996, Krieger et al., 1996, Kulisevsky et al., 1992, Taylor-Robinson et al., 1995, Weissenborn et al., 1995). These discrepancies may result from the fact that blood manganese levels do not accurately reflect concentrations of the metal in the brain.

In summary, region-selective increases in brain manganese concentration were observed in cirrhotic patients and in rats with either biliary cirrhosis or portacaval shunts. Brain manganese concentrations were particularly high in the pallidum of portacaval-shunted rats. These findings suggest that pallidal manganese accumulation observed in cirrhotic patients results from both portal-systemic shunting and impaired hepatobiliary elimination. Future studies in portacaval shunted and cirrhotic rats could be useful in the evaluation of the potential of chelating agents to reverse manganese brain overload in chronic liver failure.

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2.5 Article 5

Mild hypothermia delays the onset of coma and prevents brain edema and extracellular brain glutamate accumulation in rats with acute liver failure

Hepatology, 31 (4), 2000

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ABSTRACT

Mild hypothermia is effective in the prevention of brain edema associated with cerebral ischemia and traumatic brain injury. Brain edema is also a serious complication of acute liver failure (ALF). In order to assess the effectiveness of hypothermia in ALF, groups of rats were subjected to hepatic devascularization (portacaval anastomosis followed 48 h later by hepatic artery ligation) and body temperatures were maintained at either 35 °C (hypothermic) or 37 °C (normothermic). Mild hypothermia resulted in a significant delay in the onset of severe encephalopathy and in reduction of brain water content compared to normothermic ALF rats (control (n=8) 80.22%; ALF-37 °C (n=8) 81.74%; ALF-35°C (n=8) 80.48% ($p< 0.01$ compared to ALF-37°C)). This protective effect was accompanied by a significant reduction of cerebrospinal fluid (CSF) (but not plasma) ammonia concentrations (CSF ammonia: control: 0.05 mg/dl; ALF-37°C: 1.01mg/dl; ALF-35 °C: 0.07 mg/dl, $p<0.01$ compared to ALF-37°C). *In vivo* cerebral microdialysis studies revealed that mild hypothermia resulted in a significant reduction of extracellular glutamate concentrations in the brains of rats with ALF (control: 1.06 uM; ALF-37°C: 2.74 uM; ALF-35°C: 1.49 uM ($p<0.01$ compared to ALF-37°C)). These findings suggest that (1) mild hypothermia is an effective approach to the prevention of the CNS consequences of experimental ALF and that (2) the beneficial effect of hypothermia is mediated via mechanisms involving reduced blood-brain transfer of ammonia and/or reduction of extracellular brain glutamate concentrations. Mild hypothermia may be an effective approach to prevent brain edema in patients with ALF awaiting liver transplantation.

INTRODUCTION

The major cause of death in patients with acute liver failure is brain herniation resulting from raised intracranial pressure caused by a progressive increase in brain water content (Blei., 1991). It has been estimated that up to 40% of patients with ALF die whilst on the waiting list for liver transplantation (Makin et al., 1995) where, in almost all cases, death results from brain herniation. Despite several decades of investigation, the pathophysiologic mechanisms responsible for the central nervous system consequences of acute liver failure (ALF) have not been fully elucidated. However, ammonia remains a prime candidate. Hyperammonemia is a consistent finding in experimental animal models of ALF resulting from hepatectomy (Holmin et al., 1983), hepatic devascularization (Mans, et al., 1979, Swain et al., 1992a) or toxic liver injury (Swain et al., 1992b), and, in these cases, brain edema is invariably observed. In acute liver failure, brain ammonia may reach millimolar concentrations (Butterworth, 1992) and exposure of various *in vitro* brain preparations to millimolar concentrations of ammonia results in significant cell swelling (Ganz et al., 1989, Norenberg et al., 1991). Hyperammonemia is also a feature of Reye Syndrome (Jenkins et al., 1987) and congenital urea cycle enzymopathies (Brusilow et al., 1985), conditions in which cerebral edema is also consistently observed.

Previous studies showed that hypothermia extended the survival time and prevented the development of brain edema in rats with ALF (Traber et al., 1989). Furthermore, mild hypothermia (33°C - 35°C) has been shown to be neuroprotective in experimental models of traumatic (Dietrich et al., 1994) and ischemic brain injury (Busto et al., 1987). In the case of the latter, it was suggested that the beneficial effects of hypothermia were due to inhibition of the release of neurotransmitters rather than to an effect on cerebral energy metabolism (Ginsberg et al., 1992).

The goals of the present investigation, therefore, were to study the effect of hypothermia on the development of hepatic encephalopathy and brain edema in relation to blood and cerebrospinal fluid ammonia levels and to extracellular brain

glutamate and glutamine concentrations in rats with ALF due to hepatic devascularization.

MATERIALS AND METHODS

O-phthaldialaldehyde (OPA) reagent solution, 2-mercaptoethanol, Tris-acetate, Ringer`s solution constituents and amino acid standards were purchased from Sigma Chemical Co. (St-Louis., MO, USA). Sodium phosphate (monobasic), methanol (high-performance liquid chromatography [HPLC]-grade), and tetrahydrofuran (HPLC grade) were obtained from Anachemia (Montreal, Quebec, Canada). Double-distilled deionized water was used for preparation of standard amino acid solutions and buffers. The mobile phase used for HPLC was filtered through 0.45mm-pore-size membrane filters (Millipore Corp., Bedford, MA, USA) and degassed under vacuum before use.

Portacaval anastomosis (PCA)

Adult male Sprague-Dawley rats weighing 175-200g were anesthesized with halothane and an end-to-side PCA was performed according to the guidelines of Lee and Fisher (Lee and Fisher, 1961). Briefly, rats underwent a laparotomy and the inferior vena cava and portal vein were isolated allowing the inferior vena cava to be clamped (anastomosis clamp, Roboz Instruments Inc., Washington, D.C.), and the removal of an elliptical piece of vein 1.5 times the portal vein diameter. The portal vein was then ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was under 15 min. Overall mortality for shunted rats was less than 10%. Sham-operated control rats were matched for weight, anesthesized with halothane and a laparotomy was performed. The inferior vena cava and portal vein were clamped for 15 minutes, and then released. Following surgery, all animals were housed individually with free access to standard laboratory chow and water under constant conditions of temperature, humidity, and light cycles.

Hepatic artery ligation

Twenty four hours after PCA surgery, animals were reanesthetized with halothane and subjected to hepatic artery ligation (HAL) or laparotomy (controls) along with the insertion of PE-50 arterial catheters in the aorta to allow for blood sampling throughout the experiment.

In vivo cerebral microdialysis

Twenty-four hours after HAL, an intracerebral guide cannula was stereotactically implanted into the frontal cortex as previously described (Michalak et al., 1996). Briefly, PCA animals and their sham-operated controls were anesthetized with halothane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A medial incision was made over the skull, a burr hole was drilled to allow vertical access to the frontal cortex. A disposible plastic/siliconized intracerebral guide cannula was inserted through the burr hole to the required depth (coordinates: +3.2 mm [sagittal], -1.5 mm [lateral], and -2.5 mm [depth] relative to the bregma, based on the atlas of Paxinos and Watson, 1982). The guide cannula was secured in place using dental acrylic and closed with a dummy probe. Total surgery time was approximately 15 min. Throughout all surgical procedures, a rectal temperature probe was inserted, and the temperature was maintained at 37°C using a CMA/150 temperature controller. The microdialysis system (CMA/Microdialysis AB, Stockholm, Sweden; and Bioanalytical Systems, West Lafayette, IN) consisted of a microinjection pump (CMA/100), a syringe selector (CMA/111), a temperature controller (CMA/150), a microfraction controller (CMA/140), and a housing system for freely-moving animals (CMA/120).

Following HAL or sham surgery (i.e 48 h after portacaval shunting), the dummy probe was removed from the intracerebral guide cannula implanted on the previous day (described above), and microdialysis probes (CMA/12; 2 mm long, 500 mm optical diameter) were carefully inserted into frontal cortex of animals, all of which were freely moving. The probes were perfused by means of a microinjection

pump (CMA/100) at a constant flow rate of 2 ul/min. The perfusion medium consisting of Ringer`s solution (147 mmol/L NaCl, 4 mmol/L KCl, 2.4 mmol/L $CaCl_2$) was prepared daily using deionized water, filtered through 0.45-μm-pore-size Millipore filters and degassed before use. Preliminary studies demonstrated that extracellular amino acid concentrations had stabilized within 5h of the start of microdialysis (Raghavendra Rao, 1995). After this initial stabilization period, 20-min fractions were collected using a CMA/140 microfraction collector. Microdialysis fractions were capped, frozen immediately after collection, and stored at -70°C until time of analysis.

CSF removal

In a separate study, cisterna magna catheters were installed as previously described (Swain et al., 1992b) in groups of animal's, twenty-four hours after HAL or sham operation. Briefly, the animals head was mounted with the skull in a horizontal position in a stereotaxic apparatus. A 3 cm incision was made in the skin from the back of the head and the overlying connective tissue was removed to expose the skull. A small hole was drilled in the skull using a dental burr (009) on the midline immediately rostral to the interparietal-occipital bone suture. The hole was drilled in such a way that the occipital bone could be used as a guideline while inserting the cannula (PE-10 tubing, Clay Adams, Parsippany, NJ). The catheter was slowly inserted into the cisterna magna. Correctness of placement was accompanied by a spontaneous flow of clear CSF. When successful implantation had been confirmed, the skull was cleaned and dried and a small amount of dental acrylic cement (Yates and Bird, Chicago, IL) was applied to anchor the inserted cannula.

Body temperature and blood glucose monitoring

Following the insertion of the microdialysis probe, body temperature was monitored and maintained at 37°C by means of heating lamps (the ALF-37 group). In the ALF-35 group hypothermia occurred spontaneously in the absence of external

heating as previously described (Traber et al., 1989). Body temperatures in this group of animals were maintained at 35°C by warming with heating lamps from time to time when necessary. Arterial blood glucose levels were monitored and supplemental glucose was administered intraperitoneally as needed to maintain normoglycemia.

Neurological evaluation

Animals were assessed neurologically every 30 min during the progression of ALF. Animals that could no longer right themselves after being placed on their back were considered to have lost their righting ability (defined as the precoma stage); animals in which both righting ability and a corneal reflex could not be elicited were considered to be in coma (defined as the coma stage). Samples for amino acid analysis in extracellular fluid were taken hourly and amino acids measured hourly and at coma and precoma stages of encephalopathy. At the end of the experiment (coma stage), animals were sacrificed and the brains were removed. Half the brain was frozen and stained with cresyl violet for neuropathological verification of probe placement. The frontal cortex of the other half of the brain was used for brain water measurement. In the rats with cisterna magna catheters, CSF and blood ammonia samples were taken at baseline (following HAL) and at precoma and coma stages. Animals were sacrificed at the end of the experiment. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Brain water

One cerebral hemisphere was kept at 4°C and brain water measured at coma stages of encephalopathy. The brain was cut into 2-mm slices, and 1-mm punch biopsy specimens were obtained from the gray matter of the cerebral cortex. Water

content of each specimen was measured gravimetrically using a density gradient of bromobenzene-kerosene precalibrated with K_2SO_4 as previously described (Marmarou, 1978). The cortical samples were placed into the fluid column, and the equilibration point was measured within 2 min. The specific gravity of the tissue was calculated and results were expressed as percentage of water content. Eight measurements were made per animal, and values were arithmetically averaged.

Extracellular brain amino acid measurements

Dialysate samples obtained at coma stage, precoma stage, and at hourly intervals prior to precoma stage, were analyzed using Perkin-Elmer reverse-phase HPLC system with fluorescence detection and precolumn o-phthalaldehyde derivatization. The HPLC system consisted of a solvent delivery system (series 400; Perkin-Elmer; Norwalk, CT) coupled to a filter fluorometer (LC-10 fluorescence detector; Perkin-Elmer; excitation at 370 nm, emission at 418-700nm). Sample injections were performed using a 50 µl loop of a CMA/200 autosampler with derivatization accessories. The column used was a reverse-phase Perkin-Elmer C18.5m fitted with a Vydec reverse-phase C18 guard column. The chromatogram was performed with a gradient between methanol and sodium phosphate buffer (50 mmol/L [pH 5.25] containing 2% tetrahydrofuran) at a constant flow rate of 1ml/min for a further 10 min. The gradient was then run from 25% to 50% methanol over 45 min, held at 50% methanol for a further 10 min, and returned to 25% methanol/75% buffer over 15 min, before re-equilibration for reuse. Chromatograms were computed using a programmable recording integrator (LC-100; Perkin-Elmer). From standard chromatograms of amino acids, detector response factors for the individual amino acids were calculated relative to the internal standard. Concentrations of glutamate and glutamine in the extracellular liquid samples were calculated by reference to the internal standards (homoserine and norvaline).

Ammonia

Ammonia concentrations were estimated in plasma and CSF using a commercial ammonia test kit, which uses an ion-exchange method followed by colorimetric measurement of ammonia nitrogen with the Bertholet phenate-hypochlorate reaction (Dienst et al., 1961).

Statistical analysis

Data are expressed as mean ± S.E.M. from 8 animals per treatment group. Differences between groups (ALF-35, ALF-37, Sham) at the same time point were compared by one-way ANOVA (Bonferroni's correction), and differences within groups at different time points were compared by means of one-way ANOVA with repeated measures (Bonferroni's correction). P values of <0.05 were considered to be significant.

RESULTS

Following PCA and HAL, rats whose body temperatures were maintained at 37°C (ALF-37) developed progressive encephalopathy consisting of loss of activity, loss of righting ability (precoma stage) progressing to loss of corneal reflex (coma stage). ALF-37 rats developed pre-coma with an average delay time of 8.1 ± 0.8 h and coma at 13.4 = 1.1 h. Mild hypothermia (35°C) afforded complete protection against hepatic encephalopathy in rats with ALF (ALF-35). Thus, at times at which the ALF-37 rats were comatose, none of the ALF-35 rats had developed significant neurological deterioration. This protective effect was observed in all of eight ALF-35 rats. No rats in the sham-operated groups developed neurological symptoms.

Both ALF-37 and ALF-35 groups of rats were hyperammonemic. However plasma ammonia concentrations were not significantly different between ALF-37 and ALF-35 groups at any time point studied (Fig. 1a).

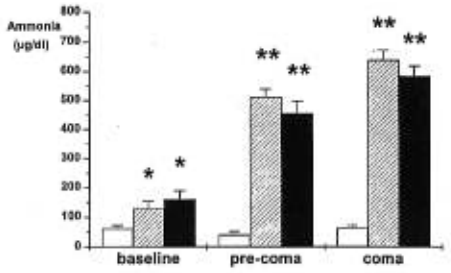

Fig 1a

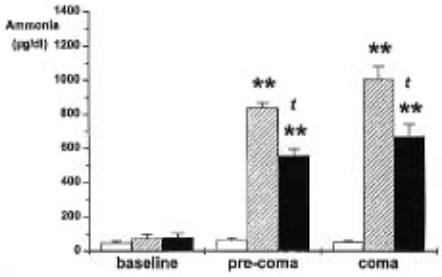

Fig 1b

Figure 1: Effect of mild hypothermia (35°C) ■ versus normothermia (37°C) □ on plasma (upper panel) and cerebrospinal fluid (lower panel) ammonia concentrations at various time points during the development of hepatic encephalopathy in rats with ALF resulting from hepatic devascularization compared to sham-operated controls □ . Baseline indicates data point 5h following ALF surgery or equivalent. Pre-coma and coma time points are defined as loss of righting and corneal reflexes, respectively. Data points represent mean values ± S.E.M of duplicate determinations from 8 rats per group. Values significantly different from sham-operated controls at each time point indicated by * $p < 0.05$ and ** $p < 0.001$ by ANOVA. Values significantly different from ALF-37 at a given time point indicated by † $p < 0.05$.

CSF ammonia concentrations, on the other hand, were significantly reduced by 34% ($p<0.01$) in ALF-35 rats (671.7 = 72.8 ug/dl) compared to ALF-37 rats at coma stages of encephalopathy (1007.3 = 77.8 ug/dl) (Fig. 1b). There was also a significant difference ($p<0.01$) in CSF ammonia between the two groups at pre-coma stages; ALF-35, 556.9 ± 39.6 ug/dl vs ALF-37, 837.6 ± 31.5 ug/dl. There were no significant differences in CSF ammonia concentrations between the normothermic and hypothermic ALF groups at baseline.

Rats with ALF whose body temperatures were maintained at 37°C manifested an increase in brain water whereas mild hypothermia led to a significant attenuation of this increase (sham, 80.22%; ALF-37, 81.74%; ALF-35, 80.48%, $p<0.01$ compared to ALF-37 group) (Fig.2).

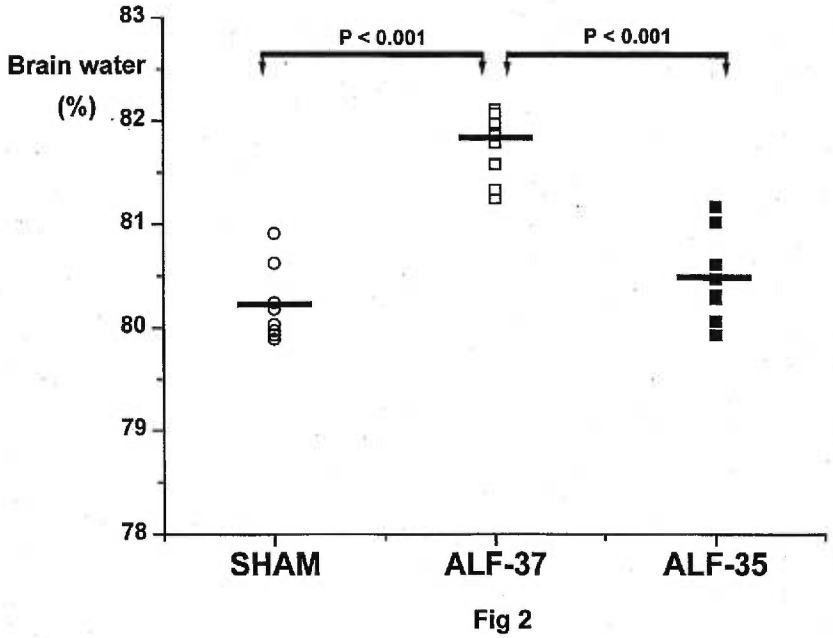

Figure 2: Effect of mild hypothermia (35°C) ■ versus normothermia (37°C) □ on brain water content in rats with ALF resulting from hepatic devascularization, compared to sham-operated controls ○. For definition of time points, please refer to legend to figure 1. Data points indicate individual values, and horizontal bars indicate means of n = 8 rats per experimental group. Brain water content of ALF-37 rats was significantly increased compared with controls ($p< 0.001$). Mild hypothermia (ALF-35) resulted in a significant lowering of brain water content ($p< 0.001$) compared to ALF-37 by ANOVA.

Extracellular (microdialysate) glutamate concentrations increased progressively with time in ALF-37 rats compared to controls resulting in a 2.6 fold ($p<0.01$) increase at coma stages of encephalopathy (Fig. 3).

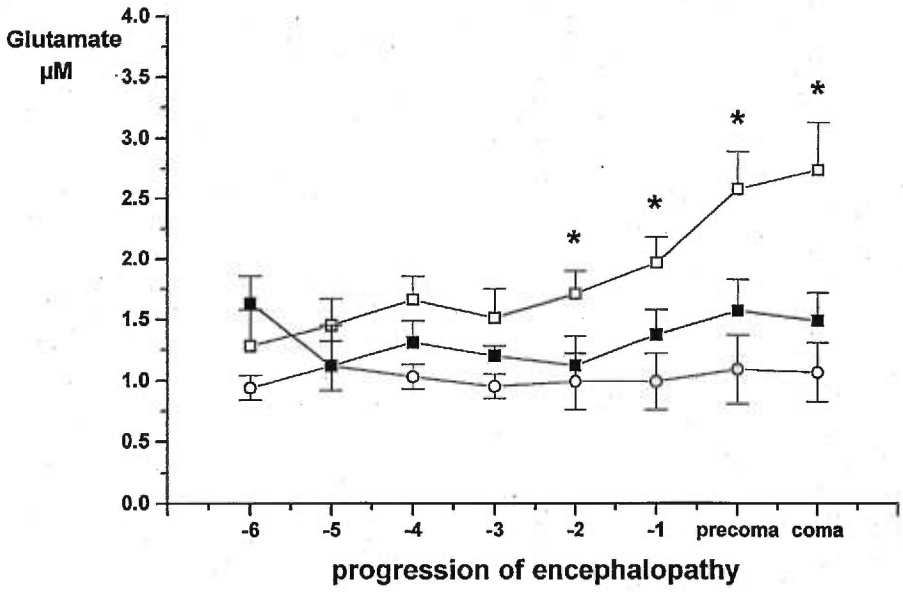

Figure 3: Extracellular brain concentrations of glutamate as a function of the progression of encephalopathy in rats with ALF. ○ sham-operated controls; □ ALF-37; ■ ALF-35. Data points represent means of duplicate determinations in 8 rats per treatment group. Vertical bars indicate S.E.M. Values significantly different from baseline and from sham-operated controls at a given time point are indicated by * $p < 0.05$.

In hypothermic rats, extracellular glutamate concentrations were not significantly increased compared to sham-operated control animals at any time point studied. On the other hand, increased extracellular glutamine concentrations were not significantly altered by hypothermia in rats with ALF at any time point. (Fig.4).

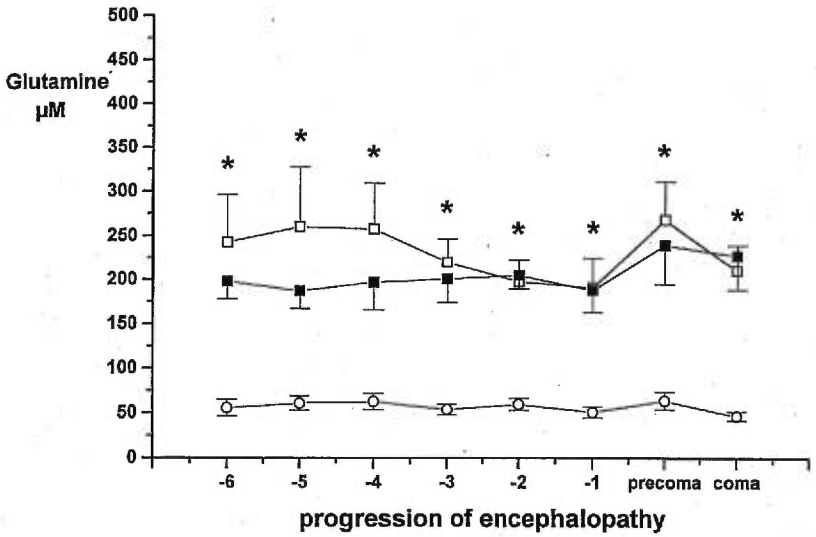

Figure 4: Extracellular brain concentrations of glutamine as a function of the progression of encephalopathy in rats with ALF. ○ sham-operated controls; □ ALF-37; ■ ALF-35. Data points represent means of duplicate determinations in 8 rats per treatment group. Vertical bars indicate SE.M. Values significantly different from sham-operated controls at a given time point are indicated by * $p < 0.05$.

DISCUSSION

Results of the present study clearly demonstrate that mild hypothermia delays the time of onset of hepatic encephalopathy and prevents brain edema in rats with ALF due to hepatic devascularization. These findings confirm and extend those from previous studies in which more severe decreases in body temperature were found to double survival times and prevent brain edema in ALF rats (Traber et al., 1989). Hypothermia was also previously shown to prevent the central nervous system consequences of pure hyperammonemia (Schenker et al., 1962) and of hepatectomy (Peignoux et al., 1982) and to delay ammonia-induced brain edema in rats following portacaval shunting (Cordoba et al., 1999).

Within hours of hepatic devascularization in the rat, brain ammonia concentrations rise rapidly to attain millimolar levels at coma stages of encephalopathy at which time brain edema is apparent (Swain et al., 1992a). On the basis of these observations, it has been proposed that ammonia toxicity is (directly or indirectly) the cause of brain edema in this model of ALF (Cordoba et al., 1996). Findings from the present study reveal that the beneficial effects of hypothermia are not mediated by an effect on blood ammonia. However, CSF ammonia concentrations were significantly reduced in hypothermic rats with ALF at times (following hepatic devascularization) when blood ammonia levels were unchanged. These findings suggest that one of the beneficial mechanisms of action of mild hypothermia in ALF may be to limit blood-brain barrier transfer of ammonia.

Ammonia is removed by glutamine formation via the enzyme, glutamine synthetase (GS). One explanation which has been proposed to explain ammonia-induced brain edema in ALF involves alterations in intracellular (astrocytic) osmolarity resulting from glutamine accumulation. In favour of this hypothesis, administration of the GS inhibitor methionine sulfoximine, reduces ammonia-induced brain edema both *in vitro* (Norenberg et al., 1994) and *in vivo* (Takahashi et al., 1991, Chodobski et al., 1986). However, in the present study, hypothermia-induced reductions in brain water content in ALF rats were not accompanied by significant reductions of extracellular brain glutamine at time points associated with brain edema. These findings suggest that mild hypothermia's major protective effect on brain edema in this model of ALF is not mediated via an effect on brain glutamine synthesis.

A consistent finding in experimental animal models of ALF is that of increased extracellular brain glutamate (Michalak et al., 1996, Bosman et al., 1992, de Knegt et al., 1994). Based upon these findings, it has been proposed that increased extracellular brain glutamate could be implicated with the pathogenesis of hepatic encephalopathy and brain edema in ALF (Michalak et al., 1997). In this same model of ALF, it has been proposed that increased extracellular glutamate

concentrations in brain are the consequence of a loss in expression of the astrocytic glutamate transporter GLT-1 (Knecht et al., 1997). Furthermore, studies in cultured astrocytes suggest that the loss of glutamate transporters on astrocytes is the result of ammonia (Chan et al., in press). Results of the present study reveal a significant lowering effect of mild hypothermia on extracellular (dialysate) brain glutamate concentrations in rats with ALF, concomitant with the prevention of encephalopathy and brain edema. These findings add further support to the notion that increased availability of glutamate within the extracellular space and therefore, increased glutamatergic neurotransmission is implicated in the pathogenesis of these central nervous system complications of ALF (Butterworth, 1997). Further support for this hypothesis is provided by a previous report that memantine, a non-competitive antagonist of glutamate (NMDA) receptors in brain, reduces the severity of neurological signs of hepatic encephalopathy in rats with ALF (Vogels et al., 1997).

Mild hypothermia is increasingly being used in clinical medicine for the prevention and treatment of brain edema of various origins. For example, a controlled clinical trial of mild hypothermia showed increased survival and improved clinical outcome in patients with neurotrauma who had been maintained at 34°C for 24h (Marion et al., 1997). Mild hypothermia is also beneficial in cerebral ischemia where the beneficial effect may (as appears to be the case in the present study in ALF), be mediated by a reduction in extracellular brain glutamate concentrations (Busto et al., 1989).

Other possible mechanisms whereby mild hypothermia may be beneficial in the prevention of brain edema in ALF include effects on cerebral blood flow and on cerebral oxygen uptake (Cordoba et al., 1999, Lanier, 1995).

In conclusion, results of the present study demonstrate a significant protective effect of mild hypothermia on brain edema and hepatic encephalopathy due to ALF in rats following hepatic devascularization. Possible mechanisms implicated in the beneficial action of mild hypothermia include decreased blood-brain transfer of ammonia and decreased extracellular brain concentrations of the excitatory amino

acid, glutamate. These findings reinforce the suggestion (Cordoba et al., 1999) that mild hypothermia may be an effective means of prevention of the central nervous system complications of fulminant hepatic failure (FHF) in humans. Indeed, hypothermia has already been used as part of a multiple therapeutic strategy in a patient with FHF (Rozga et al., 1993) and a recent preliminary study revealed a beneficial effect of mild hypothermia in the control of intracranial hypertension in several FHF patients awaiting liver transplantation (Jalan et al., 1999). Further evaluation of the value of mild hypothermia in the prevention of brain edema and hepatic encephalopathy in these patients is clearly warranted.

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CHAPTER 3

GENERAL DISCUSSION

An accumulating body of evidence suggests that HE results from alterations of multiple neurotransmitter systems in the brain (see review of the literature) as opposed to brain energy failure. Studies in autopsied brain tissue from HE patients as well as complementary studies in experimental animal models of FHF and PSE reveal significant modifications in different neurotransmitter systems, particularly in the glutamatergic system. In most cases these alterations appear to result from the accumulation in brain of neuroactive or neurotoxic substances that are normally removed by liver such as ammonia and manganese.

1. PATHOPHYSIOLOGY OF PORTAL-SYSTEMIC ENCEPHALOPATHY

1.1 Role of ammonia

Ammonia toxicity best explains the clinical, pathological and neurochemical features of PSE (Butterworth, 1991). Blood ammonia concentrations are frequently increased in patients with PSE and prevention and treatment of PSE is still generally aimed at lowering blood ammonia. Also, precipitation of encephalopathy occurs in patients with cirrhosis following administration of ammoniagenic substances such as high protein diet, urea and ammonia-releasing resins. In experimental animals with PSE, hepatic coma is accompanied by brain ammonia concentrations in the 2-5 mM range (Butterworth, 1991). Recent studies of patients with PSE using PET and $^{13}NH_3$ reveal increased brain uptake and metabolism of ammonia as well as increased permeability of the BBB to ammonia (Lockwood et al., 1991). This apparent ease with which ammonia moves from blood to brain in cirrhotic patients may account for the sometimes imperfect correlation between PSE grade and venous blood ammonia concentrations in these patients.

Neuropathologically, studies reveal that chronic hyperammonemia resulting from liver cirrhosis in adults and children leads to a characteristic pattern of astrocytic changes known as Alzheimer Type II astrocytosis (Dolman et al., 1988;

Harding et al., 1984; Von Hosselin and Alzheimer, 1912).

Ammonia exerts a deleterious effect on cerebral function by both direct and indirect mechanisms. Concentrations of ammonia in the millimolar range (equivalent to those encountered in brain in experimental liver failure) impair postsynaptic inhibition by blocking chloride extrusion from the postsynaptic neuron (Raabe, 1989) and inhibit excitatory neurotransmission as well (Fan et al., 1990). Chronic exposure of the brain to millimolar concentrations of ammonia results in impaired neuron-astrocytic trafficking of key substrates including neurotransmitters such as glutamate (Butterworth, 1993). Ammonia inhibits both neuronal and astrocytic uptake of glutamate and leads to changes in postsynaptic glutamate receptors.

Ammonia, if present in sufficiently high concentrations, has the potential to cause cerebral energy failure. Addition of millimolar concentrations of ammonia to brain preparations results in inhibition of α-ketoglutarate dehydrogenase, a key rate-limiting tricarboxylic acid cycle enzyme (Lai and Cooper, 1986). Chronic liver failure results in increased brain concentrations of lactate (Hindfelt et al., 1977) and increased CSF lactate concentrations correlate well with deterioration of neurological function both in experimental subacute PSE (Therrien et al., 1991) and in cirrhotic patients (Yao et al., 1987). Increased brain lactate production could result from decreased entry of pyruvate into the tricarboxylic acid cycle following ammonia-induced inhibition of α-ketoglutarate dehydrogenase. Administration of ammonium salts to PCS rats results in coma and in decreased brain ATP content (Hindfelt et al., 1977). However, this energy deficit is only apparent after prolonged coma suggesting that, in this experimental animal model of PSE, a cerebral energy deficit is only apparent at late, preterminal stages of the disorder. Supporting this, cerebral energy metabolism in relation to PSE is nowadays facilitated by the availability of NMR techniques. Several studies using ^{31}P-NMR have been undertaken in cirrhotic patients but, unfortunately, there is little consensus in the findings as they relate to brain energy metabolism. A significant decrease in Pi/ATP ratios has been found in the brains of eight cirrhotic patients with mild PSE (Ross et al., 1987). It was

interpreted as an indicator of a defect in brain energy metabolism. However, two subsequent studies could find no significant alterations of cerebral energy metabolism in similar groups of patients (Chamuleau et al., 1994; Taylor-Robinson et al., 1994).

1.2 Role of manganese

Blood manganese concentrations are increased during the active phase of acute hepatitis as well as in post-hepatitic cirrhosis and a significant correlation exists between blood manganese and activities of liver enzymes in patients with hepatitis (Versieck et al., 1974) or cirrhosis (Spahr et al., 1996). Blood manganese concentrations are consistently increased in cirrhotic patients and are correlated with pallidal T_1-weighted MR signal hyperintensities (Spahr et al., 1996; Hauser et al., 1994).

Similar pallidal MR signal hyperintensities have been described in a patient with Alagille's Syndrome, an autosomal dominant disorder characterized by cholestasis, intrahepatic bile duct paucity, end-stage liver disease, extrapyramidal symptoms and increased blood manganese (Devenyi et al., 1994). After liver transplantation, in cirrhotic patients and Alagille's Syndrome, neurological symptoms are resolved, blood manganese levels return to normal and pallidal MR signal hyperintensity disapppears. MR signal hyperintensities in pallidum have also been reported in patients during total parenteral nutrition and in cases of industrial manganese poisoning (Nelson et al., 1993), where it has been suggested that the MR signal hyperintensities were the result of manganese deposition in the brain (Mirowitz et al., 1991). This MR signal hypertensity disappeared after cessation of manganese exposure (Mirowitz and Westrich, 1992). Furthermore, exposure of nonhuman primates to manganese administered either by inhalation or intravenously resulted in selective pallidal hyperintensities on MRI (Shinotoh et al., 1995; Yamada et al., 1986).

Many studies have shown and confirmed that manganese is increased in brain in a region-selective phenomenon in cirrhotic patients (Krieger et al., 1995; Maeda et al., 1997). Our study supported this finding and further displayed manganese concentrations being highest in globus pallidus > putamen > caudate nucleus > cerebral cortex in cirrhotic patients (Figure 3.1; for details see article 4).

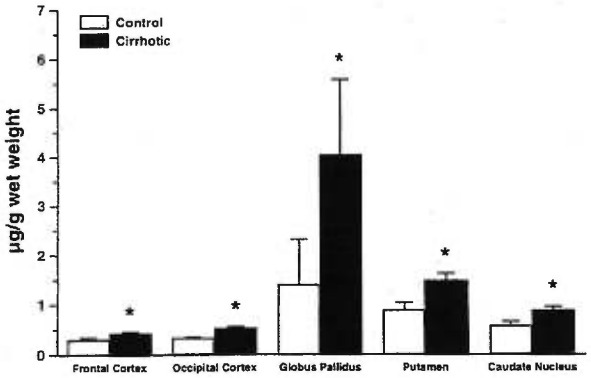


Figure 3.1: Manganese levels in cirrhotic and control human brain tissue.For details see chapter 2.4; article 4.

Manganese accumulation in brains of cirrhotic patients could be caused by a decrease in elimination via biliary excretion and an increase in systemic availability due to portal-systemic shunting. Most of ingested manganese is normally eliminated by the liver and excreted into the bile. Cirrhosis is associated with a variable degree of cholestasis that can affect biliary manganese excretion. Increased blood manganese in cirrhotic patients could result from the presence of portal-systemic collaterals produced either spontaneously or after PCA or TIPS.

In order to further evaluate the relative importance of portal-systemic shunting and hepatobiliary dysfunction in the phenomenon of increased brain manganese in liver failure, article 4 of the present thesis investigated manganese accumulation in different animal models of portal-systemic shunting, and cholestasis both associated with chronic liver failure. Furthermore, manganese deposition was studied in an animal model of FHF, a condition associated with neither cholestasis

nor portal-systemic shunting. Both shunting and chronic cholestasis resulted in significant region-selective brain manganese accumulation (Figure 3.2; for details see article 4). The degree of portal-systemic shunting was correlated with pallidal manganese concentrations (r = 0.72).

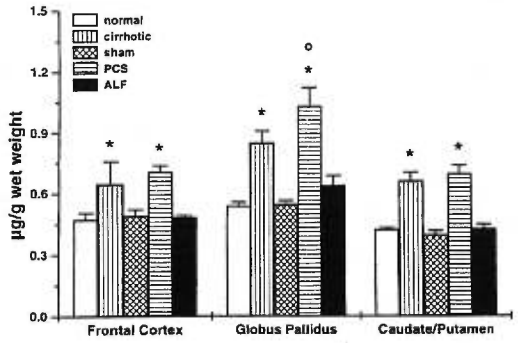


Figure 3.2: Manganese levels in brains from different experimental rat models of hepatic encephalopathy. For details see chapter 2.4; article 4.

Rats with biliary cirrhosis, manifest chronic cholestasis, (as was evident by increased serum bilirubin and alkaline phosphatase levels) and liver function impairment (as shown by decreased serum albumin levels and of spontaneous shunting). Both of these factors may contribute to manganese accumulation in the brain. Therefore it is not surprising that brain manganese concentrations did not correlate with any single parameter.

Portal-systemic shunting plays a major role in the region-selective accumulation of manganese in the brain. Correlations have been found between blood manganese, pallidal hyperintensity and the presence of portal-systemic shunting in cirrhotic patients (Spahr et al., 1996). Also, a significant increase of pallidal hyperintensity has been reported to occur after TIPS (Krieger et al., 1997). Typical pallidal MR images were also described in a patient with patent ductus

venosus (spontaneous portacaval shunt) in the absence of liver disease (Yanai et al., 1995) as well in 6 patients with portal vein thrombosis, portal-systemic collaterals and a normal liver (Nolte et al., 1998). Furthermore, an angiographic study revealed large portal-systemic collateral vessels originating from the superior mesenteric vein in all of nine patients who manifested pallidal MR signal hyperintensities whereas only 2 of 17 patients with normal MRI showed this angiographic pattern suggesting that pallidal MR signal hyperintensity in cirrhotic patients is mainly the consequence of portal-systemic shunting (Inoue et al., 1991). We found pallidal manganese concentrations were higher in PCS rats compared to cirrhotic rats which confirms the notion that shunting is a major determinant of manganese accumulation in the brain.

Although evidence for a link between brain manganese deposition and MRI signal hyperintensities is convincing, the evidence remains circumstantial. A pathophysiological role for brain manganese deposition in the neurological symptoms in patients with liver disease and/or portal-systemic shunts remains to be demonstrated. Manganese toxicity has previously been reported in miners after a prolonged exposure to manganese dust, resulting in extrapyramidal symptoms resembling Parkinson's disease (Nelson et al., 1993). In cirrhotic patients, a high incidence of extrapyramidal symptoms, in particular rigidity, similar to that observed in Parkinson's disease, is observed when a detailed and careful neurological evaluation is performed (Spahr et al., 1996; Krieger et al., 1997). However, recent reports evaluating the relationship between blood manganese, pallidal hyperintensity and neurological symptoms have yielded conflicting results (Spahr et al., 1996; Krieger et al., 1996; Kulisevsky et al., 1992; Taylor-Robinson et al., 1995; Weissenborn et al., 1995). These discrepancies may result from the fact that blood manganese levels do not accurately reflect concentrations of the metal in the brain. Liver transplantation normalizes blood manganese concentrations and results in the disappearance of the MRI signal in cirrhotic patients suggesting that the pallidal "lesion" is of a functional rather than a pathological nature (Kulisevsky et al., 1992). Manganese is toxic to brain and dopaminergic neurons in basal ganglia structures are

particularly susceptible to the toxic effects of manganese (Bird et al., 1984). The same pallidal samples that displayed high manganese obtained at autopsy from cirrhotic patients were found to contain significantly reduced densities of postsynaptic dopamine D_2 receptors (Mousseau et al., 1993). These findings suggest that increased pallidal manganese concentrations could, via a toxic action on dopaminergic neurons, be the cause of the extrapyramidal symptoms (tremor, rigidity, athetosis) encountered as part of the spectrum of PSE in humans.

The high capacity of astrocytes to accumulate manganese (Aschner et al., 1992) suggests that its uptake into these cells may play a role in the development of Alzheimer Type II astrocytosis, the neuropathologic hallmark of PSE. In support of this concept, histopathologic evaluation of brain tissue from cirrhotic patients who had manifested T_1-weighted MRI signal hyperintensities revealed Alzheimer Type II astrocytosis (Kulisevsky et al., 1992; Weissenborn et al., 1995). Furthermore, manganese intoxication in non-human primates has been shown to result in Alzheimer Type II astrocytosis (Pentschew et al., 1963), indicating that exposure to manganese could (in addition to ammonia) contribute to the change in astrocyte function and morphology that is characteristic of PSE.

In recent studies, manganese exposure has been found to decrease glutamate uptake in cultured astrocytes (Hazell and Norenberg, 1997) and to increase the expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hazell et al., 1999), suggesting that this metal may influence both the glutamatergic system and cerebral glucose metabolism in PSE.

Manganese accumulation has not been studied in patients with FHF. In our FHF animal model, rats with liver devascularization, no increase in manganese deposition is found in brain which strongly suggests that portal-systemic shunting rather than parenchymal liver cell loss is the major cause of pallidal manganese deposition in brain in PSE.

2. TREATMENT OF PORTAL-SYSTEMIC ENCEPHALOPATHY

2.1 Ammonia-lowering strategies: L-ornithine-L-aspartate

Since ammonia plays a significant role in the pathogenesis of PSE, therapeutic strategies involving lowering of blood and brain ammonia are the mainstays in the prevention and treatment of PSE.

Restriction of dietary protein is effective but should be used only in the short term to avoid harmful nutritional consequences (Cordoba and Blei, 1997). A positive nitrogen balance is necessary to promote liver regeneration and as well as to increase the capacity for skeletal muscle to remove ammonia in the form of glutamine (Lockwood et al., 1979). Non-absorbable disaccharides such as lactulose and lactitol, act by ensuring bowel movement and by affecting bacterial metabolism, including production and absorption of ammonia (Cordoba and Blei, 1997). However, uncomfortable side effects make them unpopular treatments. Antibiotics such as neomycin, are effective for modifying the bacterial flora and reducing blood ammonia levels however, they are toxic. L-carnitine is a potentially useful agent to protect against ammonia neurotoxicity by increasing the ammonia-induced depleted metabolic reserves (Therrien et al., 1997). However, the results of clinical studies with L-carnitine are conflicting, and controlled studies are needed to establish a role for L-carnitine in the treatment of PSE. Benzoate is also effective in reducing blood ammonia both in patients with inherited urea cycle disorders and in cirrhotic patients (Ferenci et al., 1996). The efficacy of this drug may be of limited value to patients with poor liver function due to the inability to carry out the conjugation step.

Ornithine and aspartate, two substrates for the urea cycle, have demonstrated ammonia lowering effects and improvement of clinical symptoms in hyperammonemic patients with PSE (Kircheis et al., 1997). In our studies, OA administration demonstrated a significant protective effect in ammonia-precipitated coma in rats following PCA compared to saline-treated PCS rats. The protective

effect was accompanied by significant reductions in plasma ammonia and a small but significant increase in plasma urea suggesting that the reduction in plasma ammonia levels by OA is therefore partly the result of increased urea synthesis in liver (Figure 3.3; for details see article 2).

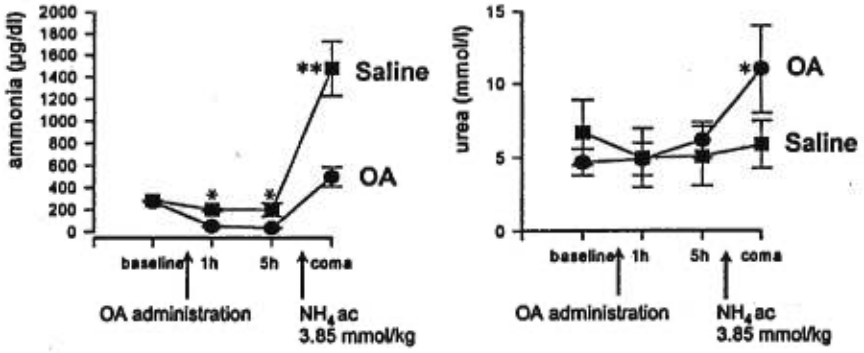


Figure 3.3: Effect of OA on plasma ammonia and urea. For details see chapter 2.2; article 2.

Increasing the ornithine concentration may stimulate flux via ornithine transcarbamylase (OTC) whereas aspartate affords a second nitrogen donor for the synthesis of urea (Figure 3.4; for details see article 2). Consistent with increased urea cycle flux are the findings of 2-3-fold increases of plasma arginine following OA administration in the present and a previous (Vogels et al., 1995) study.

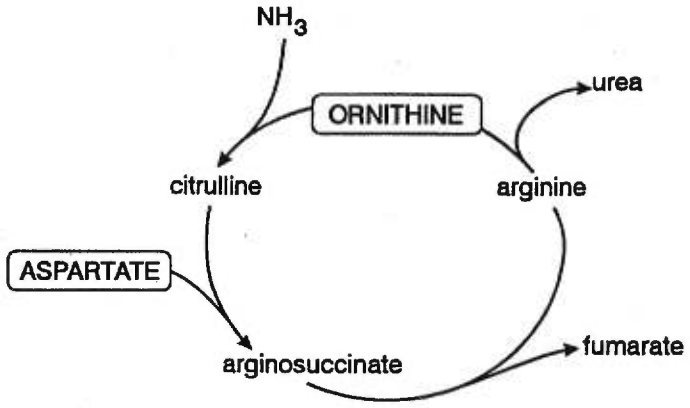


Figure 3.4: Schematic diagram of urea cycle.

Increased urea synthesis by the liver following OA treatment despite the presence of an end-to-side PCA is not surprising since the liver still receives some blood from hepatic veins and arteries. A previous study revealed enhanced urea synthesis and a lowering of blood ammonia following OA administration to cirrhotic rats (Gebhardt and et al. 1997).

Other potentially beneficial actions of L-ornithine and L-aspartate probably involve their ability to cause increased glutamate concentrations following stimulation of transaminases (Figure 3.5; for details see article 2).

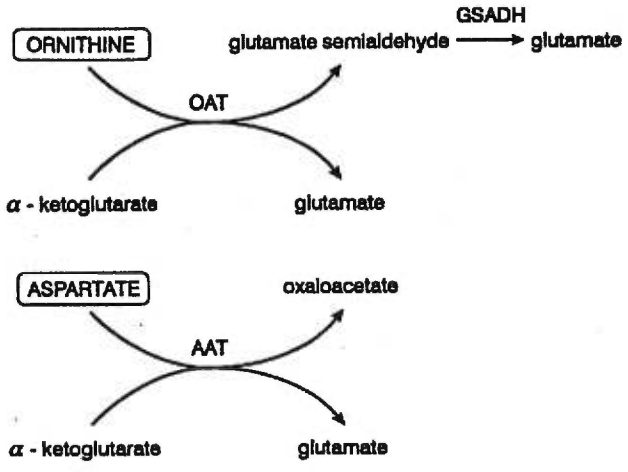


Figure 3.5: Possible transamination pathways for ornithine and aspartate.

Displacement by OA of the ornithine transaminase equilibrium towards glutamate and glutamate semialdehyde (and subsequently glutamate by the action of glutamate semialdehyde dehydrogenase, GSADH) and the aspartate aminotransferase equilibrium towards glutamate formation potentially affords 3 molecules of glutamate. That such mechanisms are stimulated following OA treatment of PCS rats is supported in our study where comparable increases of glutamate in both plasma and CSF following OA treatment suggest that the above mechanisms are operative both in brain and in peripheral tissues. L-ornithine is a substrate for the γ^+ transporter at the BBB, which is almost completely impermeable to glutamate or aspartate. Therefore, increased BBB uptake of L-ornithine would be expected to result in increased synthesis of glutamate in brain via the transamination reactions. It could be suggested that OA is involved in lowering ammonia peripherally and hence decreasing ammonia uptake into the brain. Centrally, only ornithine is involved in lowering ammonia by forming the precursor glutamate.

The increased CSF glutamate caused some concern and a neuronal count was done to establish whether the OA-induced CSF glutamate increase produced any neuronal cell death. It was confirmed that no neuronal cell death occurred (Rose et al., unpublished results) suggesting that the increased glutamate was not in an extracellular compartment.

Increased astrocytic concentrations of glutamate would then have the potential to stimulate the synthesis of glutamine via glutamine synthesis (GS). GS is present with relatively high specific activities in liver, brain and skeletal muscle. Furthermore, portacaval shunting results in induction of GS in muscle (Girard and Butterworth, 1992). Given the increased availability of the enzyme substrate (glutamate) and the possibility of enzyme induction in muscle, it is therefore likely that an important mechanism of OA following portacaval shunting also involves increased glutamine synthesis by skeletal muscle. Additional ammonia removal could also be expected via glutamine synthesis in the brain. Consistent with these mechanisms are the findings in the present study of significant elevations of

glutamine in the blood and CSF of OA-treated PCS rats.

Other potential consequences of increased availability of glutamate would (as expected) include increased GABA synthesis (observed in plasma but not in CSF) resulting from increased flux via glutamic acid decarboxylase as well as increased synthesis of alanine via pyruvate aminotransferase. Consistent with the latter, alanine concentrations were found to be significantly increased both in plasma and CSF of OA-treated animals. In a study of the effects of OA on plasma amino acids in patients with cirrhosis, increases in plasma glutamate and alanine were also reported (Staedt et al., 1993) suggesting that amino acid changes similar to those observed in the present study occur in humans with chronic liver disease following OA treatment. Finally, increased glutamate availability would be expected to stimulate branched-chain amino acid (BCAA) aminotransferases resulting in increased synthesis (or decreased catabolism) of BCAAs. In favour of this mechanism were the findings in the present study of increased plasma concentrations of BCAAs and of leucine in CSF of OA-treated animals. Whether or not increased plasma concentrations of BCAAs contributed to the ammonia-lowering effect of OA is unclear. Data from controlled clinical trials have yielded equivocal results on the ammonia-lowering capacity of BCAAs (Conn and Eriksson, 1989).

It is interesting to study the mechanism whereby OA lowers plasma and brain ammonia. OA lowers ammonia and displays a protective effect against ammonia-induced coma in PCS rats. Therefore at times when saline-treated PCS rats are in coma, OA-treated PCS rats manifest no neurological deterioration. In CSF, glutamate, glutamine, leucine, alanine and serine are increased after OA treatment. Whether the increased levels of these amino acids comes from the respective peripheral increases, remains to be investigated. In the case of glutamate this is impossible considering it cannot cross the BBB. This suggests that ammonia may suppress the production of these amino acids directly or indirectly and that these amino acids, specifically glutamate, may be implicated in the pathogenesis of PSE.

3. PATHOPHYSIOLOGY OF BRAIN EDEMA AND ENCEPHALOPATHY IN FULMINANT HEPATIC FAILURE

3.1 Role of ammonia

FHF results in brain edema and consequently increased ICP and death. Ammonia-related mechanisms are considered to be implicated in the pathogenesis of brain edema in FHF. For example, experimental animal models of FHF have demonstrated an increase in brain ammonia levels in the range of 0.5-5 mM (Swain et al., 1992b). Brain water is increased in dogs with urease-induced hyperammonemia (Levin et al., 1989). Normal rats as well as primates, manifest increased brain water following i.v. ammonia infusions. Cerebral edema is also commonly encountered in Reye's syndrome where hyperammonemia is attained without liver failure (Jenkins et al., 1987). Treatment of cerebral cortical slices (Ganz, M. et al., 1989) and cultured astrocytes (Norenberg et al., 1991) with ammonia results in significant cell swelling (Ganz, M. et al., 1989). Recently, a correlation was found in patients with FHF between cerebral herniation and arterial ammonia concentrations (Clemmesen et al., 1999). Whether brain edema results from a direct or an indirect action of ammonia remains to be established.

It has been proposed that ammonia contributes to brain edema in FHF following conversion of glutamate to the organic osmolyte glutamine. It has been suggested that glutamine accumulates in astrocytes and impairs cellular osmoregulation (Cordoba and Blei, 1996; Blei, 1991; Brusilow, 1986). This is supported *in vivo* and *in vitro* where brain edema (astrocytic swelling) is prevented by MSO, an irreversible inhibitor of glutamine synthetase (Blei et al., 1994; Norenberg and Bender, 1994).

3.2 Role of brain amino acids

To further investigate the pathophysiology of FHF, using *in vivo* cerebral microdialysis we measured extracellular levels of amino acids in relation to the degree of neurological impairment in frontal cortex in rats following liver devascularization. Extracellular concentrations of glutamate were significantly elevated 3 hours before the onset of precoma and continued to increase until the onset of coma (Figure 3.6; for details see article 1). Aspartate, another excitatory amino acid, was also elevated at several time points during ischemic liver failure but in contrast to glutamate showed no significant correlation with the progression of encephalopathy. The finding of two to three fold increases in glutamate in extracellular fluid during progression of encephalopathy in FHF confirms the results of previous studies showing increases of glutamate in CSF at coma stages of encephalopathy in the same experimental animal model of FHF (Swain et al., 1992a). Increased extracellular concentrations of glutamate have also been previously reported in other experimental animal models of FHF in the rat (Bosman et al., 1992) and in the rabbit (de Knegt et al., 1994). These results suggest that experimental acute liver failure results in increased glutamate release and/or diminished glutamate reuptake from the extracellular space.

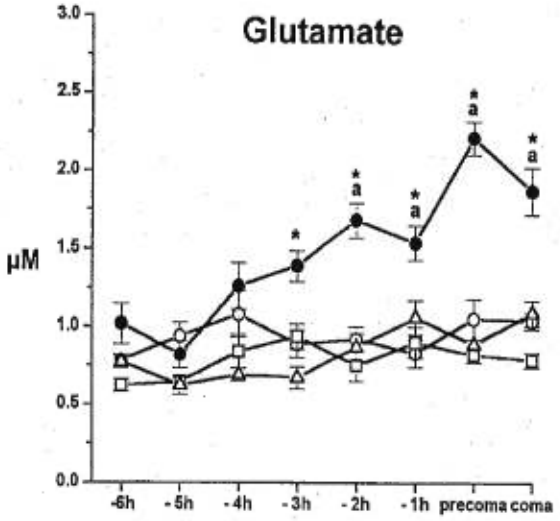


Figure 3.6: Extracellular brain glutamate levels in rats with FHF. For details see chapter2.1; article 1.

Increased extracellular glutamate is also found in other neurological impairments, like epilepsy and brain trauma both of which may be associated with neuronal loss. In contrast, FHF does not result in neuronal loss despite increased extracellular glutamate levels. This may be due to the very high (millimolar) concentrations of extracellular glutamate found in epilepsy and brain trauma compared to the modest (micromolar) increases in FHF. It has been recently suggested that increased extracellular glutamate levels arise due to a decrease in glutamate uptake resulting from defective astrocytic glutamate transporters. Studies of brain tissue from encephalopathic rats with FHF reveal decreased protein and gene expression of GLT-1, an astrocytic glutamate transporter (Knecht et al., 1997; Michalak et al., 1997; Huo et al., 1996). Decreased uptake of glutamate into nerve terminal preparations from rats with thioacetamide-induced FHF has also been described (Oppong et al., 1995), suggesting that reuptake of glutamate into the pre-synaptic terminal (in addition to the astrocyte) may also be decreased in FHF.

A likely explanation for the decreased uptake in FHF may relate to the exposure of brain to high concentrations of ammonia. Previous studies in the

ischemic model of FHF reveal brain ammonia concentrations in the 2 to 5 mM range (Swain et al., 1992b) and a significant inhibitory effect of 5 mM ammonia on glutamate uptake by synaptosomes has been described (Mena and Cotman, 1985). High affinity uptake of glutamate into astrocytes is also inhibited by exposure to millimolar concentrations of ammonia (Norenberg et al., 1985). In the experimental model of ischemic liver failure used in our studies, brain ammonia concentrations are elevated to levels shown to inhibit glutamate uptake mechanisms *in vitro*. Furthermore, the magnitude of extracellular glutamate concentrations in ischemic liver failure observed in the present study are positively correlated with arterial blood ammonia concentrations (Figure 3.7; for details see article 1).

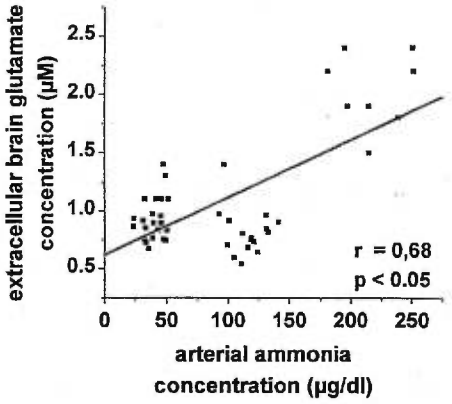


Figure 3.7: Correlation between extracellular brain glutamate and arterial ammonia in rats with hepatic encephalopathy. For details see chapter 2.1; article 1.

Assuming such mechanisms pertain also to the *in vivo* situation, the present findings of increased extracellular concentrations of glutamate *in vivo* are likely the consequence of ammonia-induced diminished removal of glutamate from the synaptic cleft.

Brain edema in FHF could result from exposure of the astrocyte to increased concentrations of glutamate. Deterioration of cerebral function parallels increased extracellular glutamate concentrations in several animal models of FHF (Bosman et

al., 1992; de Knegt et al., 1994). Furthermore, the time course of this increase reflects the time course of the establishment of brain edema in these animals (Swain et al., 1992b). Glutamate, when injected directly into brain in submillimolar quantities, causes marked astrocytic swelling (Van Harreveld and Fifkova, 1971). Moreover, a novel anion exchange inhibitor was shown to inhibit astrocytic swelling in traumatic-hypoxic brain injury caused by glutamate and it was suggested that the mechanism responsible for astroglial swelling involved the exchange transport inhibitor-sensitive glutamate uptake system (Kimelberg et al., 1989).

Several studies have addressed the issue of glutamate receptor changes in the brain in acute liver failure. Total glutamate binding site densities, assessed using [^{3}H]-glutamate as radioligand, were unchanged in the brains of rabbits with acute liver failure caused by galactosamine (Ferenci et al., 1984). Subsequent studies in the hepatectomized rat (Watanabe et al., 1988) and in the rat with acute liver failure due to thioacetamide administration (Zimmermann et al., 1989) likewise could find no evidence of alterations of total [^{3}H]-glutamate binding. On the other hand, results of a recent study reveal a selective loss of [^{3}H]-kainate binding sites in rats with ischemic liver failure (Michalak and Butterworth, 1997b). Furthermore, it was shown by us that in rats with ischemic liver failure NMDA receptor antagonist (MK-801) binding did not change compared to control groups. Also, densities of NMDA binding sites were unchanged in ischemic liver failure in rabbits (de Knegt et al., 1993). The selective loss of AMPA sites could be the consequence of exposure of the brains of these animals to increased extracellular glutamate concentrations generated in FHF. It has been suggested that acute ammonia neurotoxicity is mediated by the activation of NMDA receptors. In favour of such a mechanism, MK-801 has been shown to prevent the death of mice and rats administered lethal doses of ammonia (Marcaida et al., 1992).

Interestingly, extracellular brain concentrations of the inhibitory amino acids GABA and taurine remained within the range of those of control groups at all time points during the development of encephalopathy in rats with FHF. The findings of

unchanged GABA confirm the results of a previous study in the same animal model of FHF in which CSF and brain GABA concentrations were likewise found to be unaltered (Swain et al., 1992a). Furthermore, neither brain (Record et al., 1976) nor CSF (Moroni et al., 1987) GABA concentrations are increased in human HE associated with FHF. Thus, it may be concluded that increased extracellular GABA concentrations and, GABA-mediated transmission do not play a role in the pathogenesis of HE nor of brain edema in FHF. The finding in the present study of unchanged taurine concentrations in cerebral cortical dialysates of rats with ischemic liver failure contrasts those of a previous study (Bosman et al., 1992) in which increased taurine concentrations were reported. Possible explanations for this discrepancy include different experimental paradigms, different rat strains and much slower flow rates used for dialysis in the present study.

In article 1, we found that concentrations of glutamine in the extracellular compartment of the brain were elevated 2 to 5-fold early during the progression of encephalopathy in ischemic liver failure and that this increase was maintained throughout the progression of the disorder. Increased glutamine is undoubtedly the consequence of increased ammonia removal by brain in ischemic liver failure. Brain, being devoid of a urea cycle, removes excess ammonia by glutamine synthesis and a previous report described 3 to 5-fold increases in brain glutamine in this model of FHF (Swain et al., 1992b). Interestingly, in line with the results of the present study, extracellular glutamine concentrations although consistently elevated, were not well correlated with the severity of encephalopathy. This rather than playing a role in the pathogenesis of encephalopathy in FHF, increased brain glutamine content may be implicated in the phenomenon of brain edema (Blei, 1991; Blei et al., 1994). In favour of such a possibility, treatment of animals with MSO, an inhibitor of glutamine synthesis in the brain, inhibits water accumulation and the subsequent increase in intracranial pressure in experimental liver failure (Blei et al., 1994).

4. TREATMENT OF BRAIN EDEMA AND ENCEPHALOPATHY IN FULMINANT HEPATIC FAILURE

4.1 Ammonia-lowering strategies: L-ornithine-L-aspartate

As FHF develops, brain edema and ICP increase and result in brain herniation and death. Grade IV FHF is often non-reversible and liver transplantation is necessary. New treaments are needed until a liver donor is available.

Hypertonic solutions such as mannitol appear to be the mainstay of therapy leading to decreased ICP in patients with FHF. Mannitol doses require close monitoring since mannitol infusions may cause hyperosmolarity and fluid overload, which could exacerbate cerebral edema (Ede and Williams, 1996). Mannitol treatment is ineffective in FHF patients with renal failure.

A recent study shows that cerebral herniation in patients with FHF is strongly correlated with arterial ammonia concentrations (Clemmesen et al., 1999). Consequently, ammonia-lowering strategies have been suggested. The goal in such cases is to produce a drug which will decrease plasma ammonia rapidly. OA (given i.v) was shown by us to fit this need and was found to decrease plasma and CSF ammonia levels in rats with FHF due to liver devascularization (Figure 3.8; for details see article 3).

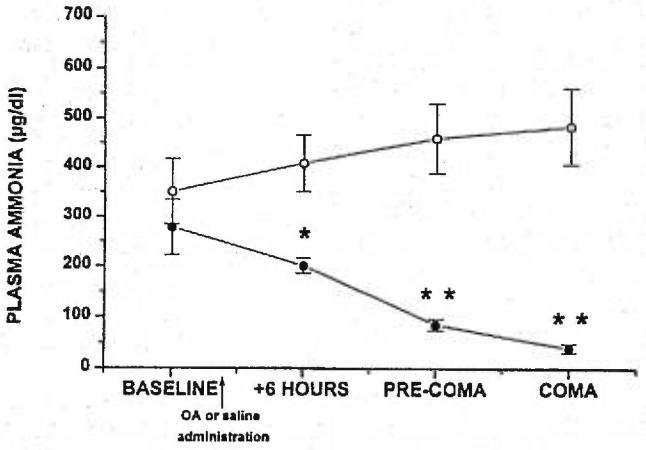


Figure 3.8: The effect of OA on plasma ammonia in rats with FHF. For details see chapter 2.3; article 3.

This ammonia-lowering effect is accompanied by a reduction in brain water content (Figure 3.9; for details see article 3) and a delay in the time to onset of severe encephalopathy.

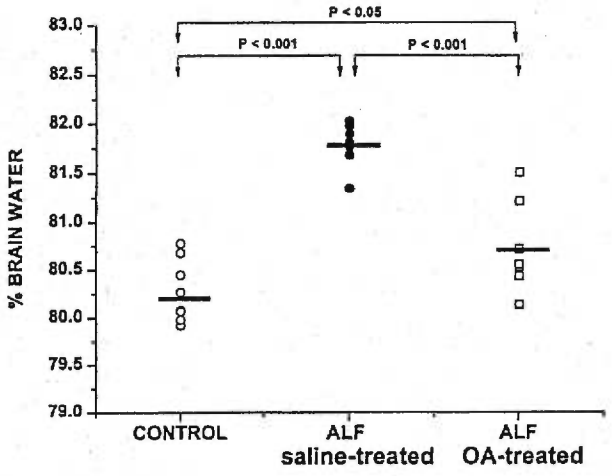


Figure 3.9: The protective effect of OA against increased brain water in rats with ALF. For details see chapter 2.3; article 3.

These findings add to the body of evidence that ammonia is implicated in the pathogenesis of brain edema in ALF. It has long been established that hyperammonemia in experimental animals with or without liver failure results in cerebral edema (Levin et al., 1989; Takahashi et al., 1991; Blei et al., 1994). Swelling of cerebral cortical astrocytes, the characteristic feature of experimental ALF (Ganz et al., 1989) is observed following ammonia infusions to young primates (Voorhoies et al., 1983) and exposure of rat cortical astrocytes in culture to ammonia results in significant cell swelling (Norenberg et al., 1991). Furthermore, precipitous increases in blood ammonia, similar to those reported in ALF are associated with brain edema in other conditions such as Reye's Syndrome (Jenkins et al., 1987) and urea cycle enzymopathies (Brusilow, 1985).

The hepatic devascularized rat has been widely used for the study of pathophysiologic mechanisms implicated in the phenomenon of brain edema in ALF (Blei et al., 1992). Extensive studies of peripheral and central metabolism have been performed in these animals and findings include increased blood and brain ammonia concentrations (Mans et al., 1979; Swain et al., 1992a). In one study using this model of ALF in which correlations between various metabolite concentrations and degree of neurological dysfunction were assessed, the best correlation observed was with plasma ammonia (correlation coefficient 0.76, $p<0.01$) followed by brain glutamine (correlation coefficient 0.69, $p<0.01$) (Mans et al., 1994). Other characteristics of this model of ALF include a typical plasma and CSF amino acid profile consisting of increased glutamine, glutamate, alanine and aromatic amino acids (Mans et al., 1979; Swain et al., 1992a; Mans et al., 1994). Similar amino acid profiles have been reported in patients with ALF (Record et al., 1976). In the present study (article 3), OA administration to rats with ALF caused by hepatic devascularization resulted in a significant attenuation of plasma and CSF ammonia concentrations, together with a significant 2-fold increase of plasma (but not CSF) glutamine. These findings suggest that OA stimulates glutamine synthesis in the periphery, an action which must come primarily from skeletal muscle. In support

of this possibility, it has been reported that in severe liver failure, muscle ammonia uptake is enhanced and that the subsequent glutamine synthesis is the major pathway for ammonia detoxification (Lockwood et al., 1979).

As mentioned previously, once in solution, OA splits into its component amino acids, L-ornithine and L-aspartate. Ornithine is a substrate for both the urea cycle and for ornithine transaminase, which gives rise to glutamate semialdehyde via the reaction catalyzed by ornithine:α-ketoglutarate aminotransferase (OAT). Glutamate semialdehyde dehydrogenase then transforms glutamate semialdehyde into a second molecule of glutamate. The glutamate formed from ornithine is thus available as a substrate for several reactions including GS, and the major beneficial effect of OA, namely its blood ammonia-lowering action, results from the stimulation of glutamine synthesis in skeletal muscle via GS. Consistent with this are the findings in the present study (article 3) of significantly increased plasma glutamine in OA-treated animals with ALF. Furthermore, direct measurement of muscle GS activities revealed a significant 2-fold increase following L-OA treatment of rats with ALF. On the other hand, glutamine concentrations are not increased in CSF of OA-treated rats with ALF, an observation that undoubtedly results from the fact that both substrates for the GS reaction (glutamate and ammonia) are reduced in CSF following OA treatment. Previous studies have proposed that cellular glutamine accumulation could be the cause of ammonia-induced swelling. In favor of this, inhibition of the synthesis of glutamine was shown to prevent cell swelling induced by ammonia both *in vitro* (Norenberg and Bender, 1994) and *in vivo* (Blei et al., 1994). However, a beneficial effect of OA on cell swelling as a result of lowering brain glutamine synthesis is not indicated by the results of the present study in which CSF glutamine concentrations in OA and saline-treated groups were of a similar magnitude. Further studies will be required to study the role of glutamate/glutamine-related mechanisms in the pathogenesis of cerebral edema in ALF.

As expected, blood aspartate concentrations increased precipitously following OA administration, an effect that could stimulate transaminase reactions, resulting

in increased formation of oxaloacetate and glutamate. In this way, aspartate could contribute to ammonia removal, like ornithine, by providing the substrate (glutamate) for the only major peripheral ammonia detoxifying mechanism available in ALF, namely, muscle glutamine synthesis. Increased glutamate availability following OA treatment is also the most likely explanation for the increases of plasma GABA and branched-chain amino acids.

A consistent finding in experimental animal models of ALF is increased concentrations of glutamate in brain extracellular fluid (article 1), a phenomenon which appears to result from the decreased uptake of glutamate into astrocytes as a consequence of reduced astrocytic glutamate transport (Knecht et al., 1997). In view of previous reports that exposure of cultured astrocytes to glutamate causes cell swelling, it is possible that increased extracellular glutamate contributes to brain edema in ALF. Such a possibility is supported by results of the present study (article 3) which demonstrate a 60% lowering of CSF glutamate and a concomitant reduction in brain water content following OA treatment of rats with ALF.

4.2 Hypothermia

A striking change in experimental acute hepatic failure, is the development of hypothermia (Traber et al., 1989). Deep hypothermia can be achieved with temperature dropping to an average of 27°C. Survival of the animal is influenced by this factor; devascularized rats kept at 37°C lose their righting reflex 8-12 hrs after the ligation of the hepatic artery, compared to a mean of 18 hrs when the temperature is allowed to decrease spontaneously (Traber et al., 1989). Survival up to 30 hrs have been recorded (Herlin and Holmin, 1984). These body core temperature changes may independently affect the neurobehavioral picture and thus require strict experimental control. Body temperature is generally best controlled using a heat lamp or heating pad.

There are many mechanisms involved in the neuroprotective action of

hypothermia. It is well known that an overall reduction in metabolic activities is associated with progressive hypothermia. This reduced metabolic activity results in a decreased cerebral metabolic rate for glucose and oxygen consumption (Michenfelder and Milde, 1991). This was supported with decreased rates of ATP depletion and lactate accumulation (Welsh et al., 1990). Hypothermia can prevent the biosynthesis, release and uptake of some neurotransmitters. Specifically, hypothermia attenuates the release of the excitatory amino acids glutamate and aspartate and subsequently their associated neurotoxicity.

Conventional hypothermia (defined as a body temperature <30°C) can exert protective effects on the brain in conditions of elevated ICP (Jalan et al., 1999). Hypothermia has also been shown to block ischemia-induced increases in BBB permeability and subsequently reduce brain edema formation. It decreases CBF and the cerebral metabolic rate for oxygen (Jalan et al., 1998b; Jalan et al., 1998a), thus reducing the metabolic requirements of the brain. Additionally, lowering brain temperature has also been reported to decrease ischemia-induced production of toxic levels of nitric oxide. However, the systemic effects of conventional hypothermia have limited its clinical applicability.

Results of the present study (article 5) clearly demonstrate that mild hypothermia delays the time of onset of HE and prevents brain edema in rats with ALF due to hepatic devascularization. These findings confirm those from previous studies in which more severe decreases in body temperature were found to double survival times and prevent brain edema in ALF rats (Traber et al., 1989). Hypothermia was also previously shown to prevent the central nervous system consequences of pure hyperammonemia (Schenker and Warren, 1962) and of hepatectomy (Peignoux et al., 1982) and to delay ammonia-induced brain edema in rats following portacaval shunting (Cordoba et al., 1999).

Within hours of hepatic devascularization in the rat, brain ammonia concentrations rise rapidly to attain millimolar levels at coma stages of encephalopathy when brain edema is apparent (Swain et al., 1992b). On the basis of

these observations, it has been proposed that ammonia toxicity is (directly or indirectly) the cause of brain edema in this model of ALF (Cordoba and Blei, 1996). Findings from the present study reveal that the beneficial effects of hypothermia are not mediated by an effect on blood ammonia. However, CSF ammonia concentrations were significantly reduced in hypothermic rats with ALF at times (following hepatic devascularization) when blood ammonia levels were unchanged (Figure 3.10; for details see article 5). These findings suggest that one of the beneficial mechanisms of action of mild hypothermia in ALF may be to limit BBB transfer of ammonia.

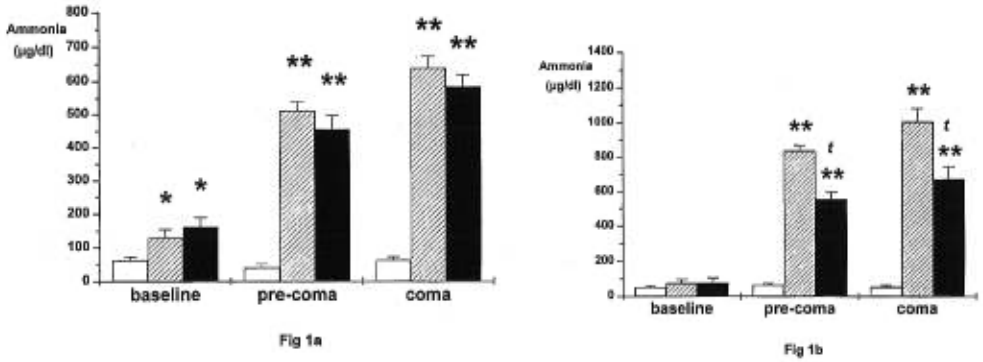


Figure 3.10: The effect of hypothermia on plasma (left) and CSF (right) ammonia. For details see chapter 2.5; article 5.

Ammonia is removed by glutamine formation via the enzyme GS. One explanation which has been proposed to explain ammonia-induced brain edema in ALF involves alterations in intracellular (astrocytic) osmolarity resulting from glutamine accumulation. In favour of this hypothesis, administration of the GS inhibitor MSO reduces ammonia-induced brain edema both *in vitro* (Norenberg and

Bender, 1994) and *in vivo* (Takahashi et al., 1991; Chodobski et al., 1986). However, in the present study (article 5), hypothermia-induced reductions in brain water content in ALF rats were not accompanied by significant reductions of extracellular brain glutamine at time points associated with brain edema (Figure 3.11; for details see article 5). These findings suggest that mild hypothermia's major protective effect on brain edema in this model of ALF is not mediated via an effect on brain glutamine synthesis.

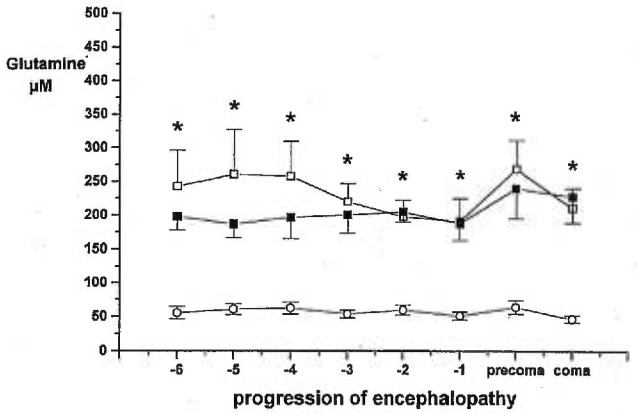


Figure 3.11: The effect of hypothermia on extracellular brain glutamine levels in rats with FHF.. For details see chapter 2.5; article 5.

A consistent finding in experimental animal models of ALF is that of increased extracellular brain glutamate (article 5) (Bosman et al., 1992; de Knegt et al., 1994). Based upon these findings, it has been proposed that increased extracellular brain glutamate could be implicated with the pathogenesis of HE and brain edema in ALF (Michalak and Butterworth, 1997b). In this same model of ALF, it has been proposed that increased extracellular glutamate concentrations in brain are the consequence of a loss in expression of the astrocytic glutamate transporter GLT-1 (Knecht et al., 1997). Furthermore, studies in cultured astrocytes suggest that the loss of astrocytic glutamate transporters is the result of ammonia exposure (Chan et al., in press). Results of the present study reveal a significant lowering effect of mild hypothermia on extracellular (dialysate) brain glutamate concentrations in rats

with ALF, concomitant with the prevention of encephalopathy and brain edema (Figure 3.12; for details see article 5). These findings add further support to the notion that glutamate levels are increased within the extracellular space and therefore, increased glutamatergic neurotransmission may be a culprit in the pathogenesis of these CNS complications of ALF (Butterworth, 1997). Further support for this hypothesis is provided by a previous report that memantine, a non-competitive antagonist NMDA receptors in the brain, reduces the severity of neurological signs of HE in rats with ALF (Vogels et al., 1997).

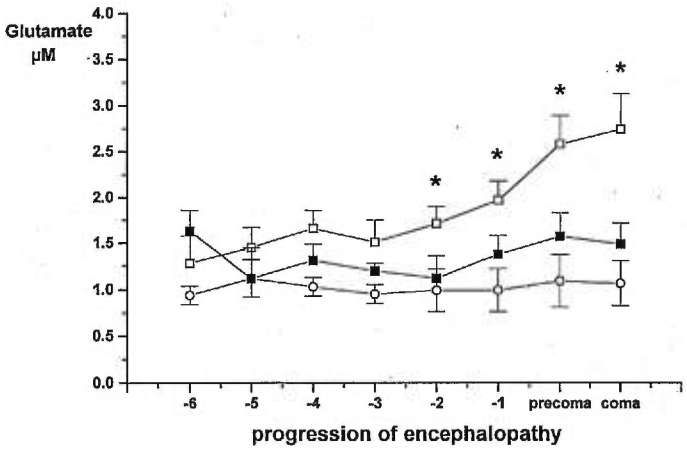


Figure 3.12: The effect of hypothermia on extracellular brain glutamate levels in rats with FHF.. For details see chapter 2.5; article 5.

Mild hypothermia is increasingly being used in clinical medicine for the prevention and treatment of brain edema of various origins. For example, a controlled clinical trial of mild hypothermia showed increased survival and inproved clinical outcome in patients with neurotrauma who had been maintained at 34°C for 24h (Marion et al., 1997). Mild hypothermia is also beneficial in cerebral ischemia where the beneficial effect may (as appears to be the case in the present study in ALF) be mediated by a reduction in extracellular brain glutamate concentrations (Busto et al., 1989).

Other possible mechanisms where mild hypothermia may be beneficial in the prevention of brain edema in ALF include effects on CBF and on cerebral oxygen uptake (Cordoba et al., 1999; Lanier, 1995).

CHAPTER 4

CONCLUSIONS

The results of the present study demonstrate:

1. An increase in extracellular glutamate correlating with arterial ammonia and neurological deterioration in rats with FHF due to liver devascularization. No change in NMDA receptor densities were observed (chapter 2.1; article 1).

2. A protective effect of OA was observed against ammonia-precipitated coma in PCS rats. OA lowers plasma ammonia and increases plasma urea (chapter 2.2; article 2). Ornithine and aspartate are transaminated to glutamate, the precursor for GS, which is thought to be stimulated in skeletal muscle in chronic liver failure.

3. OA protects against brain edema and coma in rats with FHF. OA lowers plasma ammonia levels and increases plasma glutamate levels. GS activity in muscle is increased in OA-treated rats with FHF (chapter 2.3; article 3).

4. Increased manganese deposition in the globus pallidus was observed in autopsied brain from cirrhotic patients who died in hepatic coma. In experimental animal models of chronic liver failure, brain manganese levels were also increased. A correlation was found between portal-systemic shunting and increased brain manganese in globus pallidus (chapter 2.4; article 4).

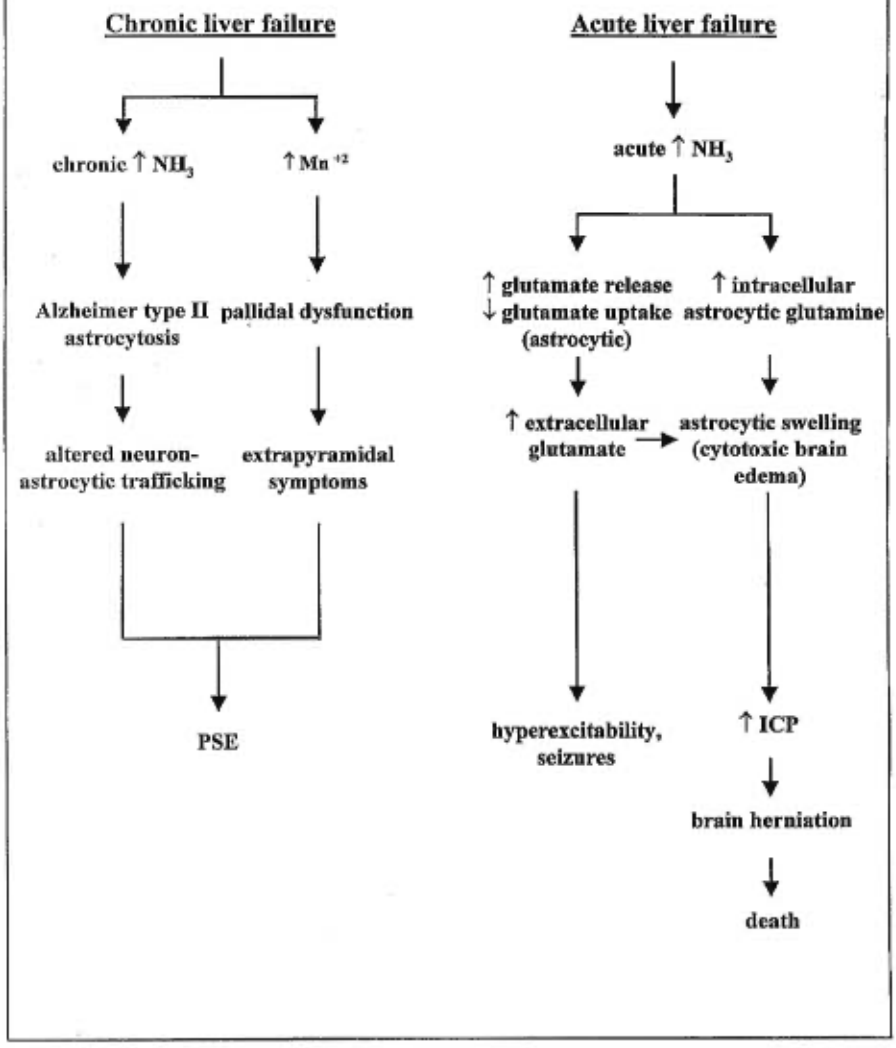
5. Mild hypothermia protects against brain edema and neurological deterioration in rats with FHF. This protective effect was accompanied by a decrease in CSF ammonia and in extracellular brain glutamate (chapter 2.5; article 5).

CHAPTER 5

SUMMARY

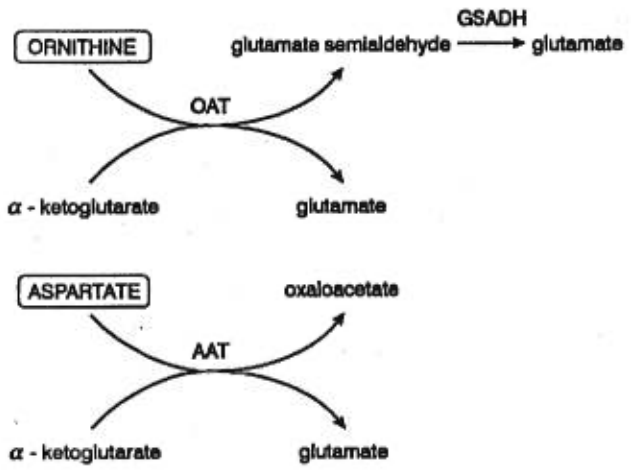
HE occurs in both acute and chronic liver failure, depending on the duration and degree of hepatic dysfunction. Hyperammonemia is a common finding in both types of HE and remains the main etiologic factor in this neuropsychiatric disease (see figure 5.1). However, the consequences of acutely increased brain ammonia, as is seen in acute liver failure are distinct from that encountered in chronic liver failure where adaptive mechanisms come into play.

Figure 5.1: Ammonia and manganese in HE: mechanisms of toxic action



From the results described in the manuscripts which constitute this thesis, treatment with OA demonstrated a lowering of plasma and CSF ammonia which subsequently delayed the onset of coma in PCS rats. These findings add new support for the hypothesis that ammonia plays a key role in the development of PSE. New information was derived on the ammonia-lowering mechanism of OA in chronic liver failure which includes increased residual hepatic urea synthesis and synthesis of glutamine both centrally and peripherally (liver and muscle). OA's mechanism of action involve stimulation of urea synthesis since ornithine and aspartate are precursors of the urea cycle. Furthermore, another mode of action involves the transamination of ornithine and aspartate towards glutamate. One molecule of OA could possibly transaminate to three molecules of glutamate and stimulate glutamine synthetase activity in muscle (see figure 5.2).

Figure 5.2: Possible transamination pathways for ornithine and aspartate.



OAT: ornithine aminotransferase;

AAT: aspartate aminotransferase;

GSADH: glutamate semialdehyde dehydrogenase

Manganese has also been found to be implicated in PSE. From the results described in the thesis, high manganese levels were found in the globus pallidus of cirrhotic patients who died in hepatic coma as well as in animal models of PSE. On the other hand, manganese levels were not increased in the brains of rats with acute liver failure. It was concluded that manganese is implicated only in PSE associated with chronic liver failure. A new finding was revealed, namely that manganese deposition in globus pallidus of cirrhotic patients resulted from both impaired hepatobiliary removal and portal-systemic shunting. Manganese deposition in globus pallidus may be responsible for the extrapyramidal symptoms found in patients with PSE.

The neuropathological picture in acute liver failure consists of astrocytic swelling leading to brain edema and intracranial hypertension. In acute liver failure, an acute increase of brain ammonia leads to many deleterious effects on the brain. One effect of increased brain ammonia is on glutamate release. Hyperammonemia stimulates pre-synaptic release of glutamate. A manuscript included in the thesis describes increases in extracellular concentrations of glutamate in a rat model of acute liver failure. Moreover, decreases in the expression of the astrocytic glutamate transporter GLT-1 were found in the brains of these rats. Inhibiting glutamate uptake by astrocytes is a probable cause of the increased extracellular brain glutamate in acute liver failure. Increased extracellular glutamate can cause hyperexcitability leading to seizures sometimes observed in patients with acute liver failure. Hyperammonemia also increases intracellular astrocytic glutamine through the activation of the only ammonia detoxifying pathway available to brain, namely glutamine synthetase. Brain edema subsequently occurs in acute liver failure resulting in cytotoxic brain edema, increased ICP and consequently brain stem herniation and

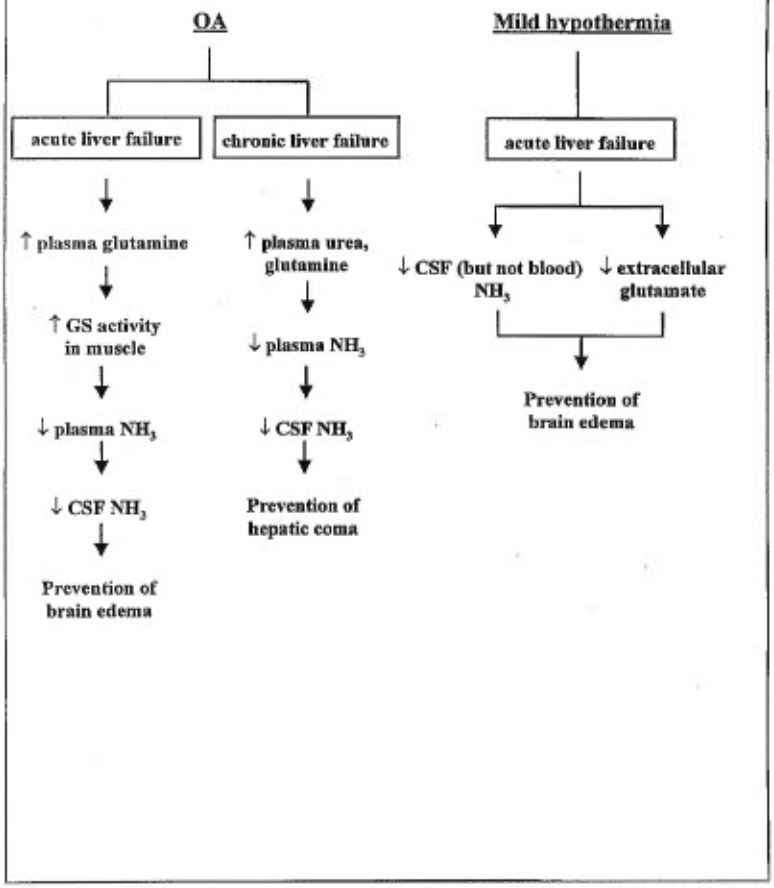
death.

From studies in the present thesis, OA treatment in rats with acute liver failure resulted in lowering of plasma ammonia and protection against brain edema further supporting the important role of ammonia in the pathogenesis of brain edema in acute liver failure. Since these findings were reported, Clemmesen et al., (1999) demonstrated a correlation between arterial ammonia and the phenomenon of brain herniation in patients with acute liver failure. Lowering of plasma ammonia in acute liver failure as a result of increased glutamine synthetase activity in muscle due to the formation of the precursor glutamate by transamination of ornithine and aspartate.

A novel finding in the present thesis was the protective effect of mild hypothermia which protected against brain edema in rats with acute liver failure. With this treatment, CSF ammonia was lowered whereas plasma ammonia was unchanged. This again supports an important role of brain ammonia in acute liver failure. The central ammonia-lowering effect of mild hypothermia is believed due to the normalization of ammonia permeability of brain in acute liver failure. Furthermore, mild hypothermia decreased extracellular brain glutamate in rats a finding which supports the involvement of extracellular glutamate in the pathophysiology of brain edema in acute liver failure.

OA and mild hypothermia proved to be efficient ammonia-lowering strategies in experimental animal models of acute and chronic liver failure. The different strategies involved separate mechanisms of action with lowering CSF ammonia as a common entity (see figure 5.3).

Figure 5.3: Therapeutic mechanisms of action of OA and mild hypothermia in acute and chronic liver failure



Future studies will be required in order to examine the pertinence of these findings to the development of new therapeutic strategies in the treatment of HE and brain edema in liver failure. In the case of OA, controlled clinical trials have demonstrated a clear ammonia-lowering effect in patients with end-stage chronic liver disease. However, studies in patients with acute liver failure have not been performed. In the case of mild hypothermia, there are results from uncontrolled trials in patients with acute liver failure and beneficial effects have been reported. Adequately controlled clinical trials are now required.

There can be little doubt that manganese deposition in globus pallidus of cirrhotic patients is the cause of the magnetic resonance signal hyperintensities observed in these patients. Such depositions could be causally related to the extrapyramidal symptoms described in PSE. Further studies are required to assess the impact of manganese chelation on motor symptoms of PSE in experimental models following appropriate clinical trials.

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