HEPATIC ENCEPHALOPATHY: EXPERIMENTAL STUDIES ON THE PATHOGENESIS.

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Experimentele studies naar de pathogenese

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voor mijn ouders

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CHAPTER 1

Introduction
What is the evidence that hyperammonemia causes hepatic encephalopathy?

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INTRODUCTION

Despite our expanding insight into brain function (and dysfunction) and the yearly production of several hundreds indexed papers on hepatic encephalopathy, our understanding of the syndrome has improved only marginally. The recognition of the syndrome of portosystemic encephalopathy in the fifties stimulated a search for gut-derived substances that could reach the brain because of defective hepatic clearance (1). At first ammonia was considered the prime neurotoxin. However, the ammonia hypothesis was rejected by many because the correlation between plasma ammonia level and degree of encephalopathy was found to be poor and therapy aiming at lowering increased plasma ammonia concentrations in patients was often ineffective. New theories on the pathogenesis with implications for treatment were developed, but -unfortunately- promising new approaches to treatment (branched chain amino acids, hemoperfusion, hepatocyte transplantation, benzodiazepine antagonists) have not yet yielded convincing results. Currently there is renewed interest for ammonia and its role in the pathogenesis of hepatic encephalopathy.

For a substrate to be considered a toxin relevant for the development of hepatic encephalopathy three criteria should be fullfilled (2): 1. the toxin should be present in abnormally high amounts in the presence of hepatic encephalopathy, 2. administration of the toxin should induce hepatic encephalopathy, and 3. correction of the abnormality should reverse hepatic encephalopathy. In the following discussion these criteria will be applied to ammonia.

Ammonia should be present in abnormal amounts in the presence of hepatic encephalopathy.

Blood ammonia concentrations are usually elevated in patients with liver disease with or without hepatic encephalopathy (3-7). Plasma ammonia levels tend to increase with the degree of hepatic encephalopathy (8,9) and frequently correlate well with the changing neuropsychiatric status in the individual patient (9,10). Cerebrospinal fluid glutamine levels, the cerebral metabolic end product of ammonia, have been shown to correlate closely with the degree of encephalopathy (11-14). Besides portosystemic encephalopathy there are other hyperammonemia syndromes (inherited urea cycle enzyme deficiency syndromes (15), inherited organic acidemias (15), transient hyperammonemia

in premature infants (16,17), Reye's syndrome (18,19) and valproic acid induced hyperammonemia (20)), all of which are associated with high plasma ammonia levels and encephalopathy.

In experimental hepatic encephalopathy in the rat from acute ischemic liver failure, a clear correlation between the brain ammonia concentration and the neurological status exists (21). In addition, most other currently accepted animal models used for the study of hepatic encephalopathy universally demonstrate the coexistance of elevated plasma and brain ammonia concentrations (22,23). Canine congenital portosystemic encephalopathy is a "spontaneous animal model" characterized by many of the pathological features of chronic hepatic encephalopathy in man, including elevated plasma ammonia levels (24,25). See table I.

Thus, ammonia appears to be present in abnormally high amounts in both clinical and experimental hepatic encephalopathy. However, in the clinical condition the correlation between plasma ammonia level and degree of encephalopathy is often found to be poor (8,9). Because of this poor correlation, and the description of comatose patients with normal plasma ammonia levels (4,8), many tend to reject the ammonia hypothesis on the development of hepatic encephalopathy. There are, however, several reasons for this discrepancy. First, arterial plasma ammonia levels indicate the ammonia delivery to the brain, but do not give information about the brain ammonia concentration. In addition, in liver disease there is increased entrance of ammonia into the brain, which may cause ammonia induced encephalopathy even though arterial ammonia levels are only slightly elevated (26). The strong correlation between cerebrospinal fluid levels of glutamine and degree of encephalopathy supports this view. Second, a strong correlation between plasma ammonia and degree of encephalopathy would implicate simple, direct toxic effects of ammonia on brain function, whereas it is more likely that ammonia interferes with normal brain function through the induction of a variety of disturbances which need time to become expressed and to resolve. The observation that the blood ammonia level frequently correlates well with the changing neuropsychiatric status in the individual patient favours this concept (9,10). Third, patients with severe liver disease can develop liver failure associated complications like renal failure (27,28), acid-base and electrolyte disturbances (29) and infections (30), which by themselves may induce or aggravate metabolic/hepatic encephalopathy thus masking a probable correlation between plasma ammonia and degree of encephalopathy (31). In addition, the number of patients with liver disease prescribed hypnotics, sedatives, analgesics or tranquilizers should not be overlooked (pseudo-encephalopathy)(32).

TABLE I

Experimental hepatic encephalopathy from acute liver failure

Species	Model	First author (reference)	Plasma ammonia μmol/l	Brain ammonia μmol/l
Rabbit	Ischemia	De Knegt (42)	450-700	-
	Galactosamine	Blitzer (80)	150-200	-
	Hepatectomy	Berlinger (81)	600-800	-
Rat	Ischemia	Bosman (22)	700-1000	1000-1500
	CCl_4	Yamamoto (82)	250-400	-
	Hepatectomy	Schalm (83)	600-1100	-
	Galactosamine	Zeneroli (23)	-	1500-4000
Dog	Paracetamol	Francavilla (84)	400-650	-
Pig	Ischemia	De Groot (85)	300-700	-

- not measured

None of these different causes can be deduced from the clinical symptoms of encephalopathy in the individual patient, because no presentation of encephalopathy is specific, or in other words coma is coma (32). In conclusion, hepatic encephalopathy is usually accompanied by elevated plasma ammonia levels and the blood ammonia level frequently correlates well with the changing neuropsychiatric status in the individual patient. A poor correlation between plasma ammonia and degree of encephalopathy can probably be explained by the diverse toxic actions of ammonia which need time to

become expressed and to resolve, and by the coexistence of other liver failure associated complications which by themselves may induce or aggravate encephalopathy. In case of normal or near normal plasma ammonia levels in patients with encephalopathy, there may be increased ammonia entry into the brain or there may be pseudo-encephalopathy.

Induction of the abnormality, i.e. the administration of ammonia, should induce hepatic encephalopathy.

In the fifties it was observed that the administration of ion exchange resins which liberate ammonia in the gut posed patients with liver cirrhosis at increased risk for the development of hepatic encephalopathy (33). Van Caulaert et al. (34) were the first to observe that ammonium chloride ingestion precipitated hepatic encephalopathy in patients with liver cirrhosis. These experiments have been repeated, yielding seemingly conflicting results (table II). From these studies it becomes apparent that one single dose of 30-90 mmol ammonia (i.e. ca. 0.5-1.5 mmol/kg body weight) does not have important immediate effects on clinical symptoms (9,35,36), pyschometric performance (37) or the electroencephalogram (38). However, such a dose may give rise to clinical symptoms of encephalopathy after several hours (8). Larger doses given for a longer period induce severe signs of encephalopathy in all patients with liver cirrhosis (1,39). It can thus be concluded that ammonia induces encephalopathy in patients with liver cirrhosis, the occurrence depending on dose and duration.

Infusion of ammonia into rabbits, simulating the plasma ammonia levels of acute ischemic liver failure, induces an encephalopathy with many of the characteristics of encephalopathy from acute liver failure (40). At present there are no other extensive studies on experimental hyperammonemia. However, from the limited description of experimental hyperammonemia found in the literature, discrepancies between experimental hepatic encephalopathy and hyperammonemic encephalopathy appear to be predominantly determined by the amount and rate of ammonia administration. Bolus injections, which give acutely increased plasma ammonia levels, are frequently followed by convulsions (32), whereas more gradual increases to levels found in acute liver failure induce symptoms like disappearance of spontaneous activity and lethargy which can also be observed in hepatic encephalopathy (40,42). See table III.

TABLE II

Ammonia tolerance tests in patients with liver cirrhosis

First author (reference)	Ammonia dose in mmol	Time of observation	Number of patients with hepatic encephalopathy/ number of patients studied
Marchesini (36)	60/one bolus	45 minutes	2/17
Loguercio (37)	90/one bolus	45 minutes	0/30
Eichler (35)	30/one bolus	60 minutes	0/3
Cohn (38)	60/one bolus	60 minutes	0/19
Stahl (9)	60/one bolus	90 minutes	2/170
Jonung (86)	75-150/in 2 doses	6 hours	0/7
Summerskill (8)	40-50/one bolus	7 hours	5/5
Sherlock (1)	50-150/day	4-6 days	8/8
Phillips (39)	150-225/day	4-22 days	9/9
Summerskill (87)	150-1250/one bolus	?	6/6

Correction of hyperammonemia should reverse hepatic encephalopathy

In theory, high blood ammonia should be decreased by the extraction of ammonia and/or its substrates, and the inhibition of the involved enzymes. Current therapy is directed towards lowering gut-derived toxins, in particular ammonia. Ammonia is formed in the bowel by degradation of intestine-derived glutamine, degradation of dietary proteins and degradation of urea by urease-producing enteric bacteriae (43). Therefore the therapeutic aim is to reduce the degredation of dietary protein, urea and glutamine. The theoretical options consist of a low protein diet, the acceleration of the bowel transit time, the removal of urease-producing bacteriae from the bowel and the reduction of the body

TABLE III

Experimental encephalopathy from acute hyperammonemia

Species	Model	First author (reference)	Plasma ammonia μmol/l	Brain ammonia μmol/kg
Rabbit	Infusion	De Knegt (42)	600-1000	-
	Infusion	Ferenci (88)	250-350	-
Rat	Bolusinjections	Zieve (89)	-	2000-3750
	Bolusinjections	Zeneroli (23)	-	3200-5000
	PCS+injections	Zieve (89)	-	2500-3500
	PCS+infusion	Bosman (90)	650-750	2000-2300
	Urease	Bosman (22)	800-1500	1000-2000

- not measured

urea pool.

The association between a high protein diet and the occurrence of hepatic encephalopathy is well recognized, with the blood ammonia concentration varying with the dietary protein content (39,44). The amount of ammonia released from food is variable (45); the ammonia content of meat is high, whereas the ammonia content of vegetarian proteins is low. Indeed in patients with liver cirrhosis vegetarian proteins are better tolerated (46). Furthermore a vegetarian diet contains more fibre which stimulates bowel movements and thereby facilitates the elimination of toxic metabolites (47,48). In addition bowel cleansing with an enema is also effective in lowering plasma ammonia (49,50). Antimicrobial drugs like neomycin are known to suppress intestinal bacteria responsible for ammonia production, and were subsequently shown to improve the mentalstate of patients with hepatic encephalopathy (51).

Lactulose has many therapeutic effects and therefore forms the cornerstone of current

treatment of hepatic encephalopathy: it accelerates the bowel transit time, it modifies the colonic flora with displacement of urease containing bacteria, it stimulates the incorporation of ammonia into the bacterial wall and it inhibits ammonia generation in the gut (52). The beneficial effects of lactulose in hepatic encephalopathy were early recognized (53); and controlled trials have clearly shown the therapeutic capacity of lactulose in hepatic encephalopathy with lowering plasma ammonia levels (54).

Other promising treatment options are the ammonia fixation and enhancement of renal excretion by sodium benzoate (55), and the inhibition of bacterial urease by nicotinohydroxamic acid (56).

Although the benefits of the treatment modalities discussed are greater in patients with low-grade hepatic encephalopathy and the application is often less successful in severe liver disease (due to increased occurrence of other liver failure associated complications), it can be concluded that the current treatment of hepatic encephalopathy is effective mainly through lowering plasma ammonia.

In conclusion, it appears that ammonia fullfills largely the criteria originally named by Zieve (2): 1. a patient with hepatic encephalopathy usually has an elevated plasma ammonia level, which correlates more or less with the changing mental state; 2. ammonia induces encephalopathy in patients as well as in experimental animals, the occurrence and presentation of encephalopathy depends on dose and duration; and 3. lowering the ammonia production and plasma ammonia ameliorates most cases of hepatic encephalopathy. *"If the role of ammonia in the genesis of hepatic coma is not yet satisfactorily defined, there is no other theory that comes so near to conforming to the observed facts"* (57).

AIMS OF THIS THESIS

The pathogenesis of hepatic encephalopathy is largely unknown. Ammonia was the first toxin seriously considered to be of importance in the pathogenesis, has been subsequently rejected, but now appears to return to the forefront of "hepatic neurochemistry" (58). In theory, hyperammonemia can disturb normal brain function through interference with basic cell metabolism (e.g. pH-regulation and water and energy balance)(59-61), electrophysiologic membrane function (depending on ion pumps)(62) and biochemical communication between neurons, i.e. neurotransmission (depending on

neurotransmitters). With regard to neurotransmission, one of the current opinions on the pathogenesis of hepatic encephalopathy is a dysbalance between neuro-inhibitory and neuro-excitatory neurotransmitters (63). Several groups have proposed that neuro-inhibition via GABA or endogenous benzodiazepines is enhanced; we are investigating whether excitatory neurotransmission -especially glutamate neurotransmission- is altered during hepatic encephalopathy.

The metabolism of glutamate, the most important excitatory neurotransmitter in the mammalian brain, is linked to that of ammonia (64,65). In neurons the neurotransmitter glutamate is produced from glutamine, through the enzymatic action of glutaminase. In vitro the activity of glutaminase, which is essential for the formation of glutamate, is inhibited by ammonia (66,67). Thus it was assumed that ammonia decreases the amount of glutamate in the brain, which probably would have an effect on normal brain function. Subsequently, the amount of glutamate was shown to be decreased in autopsied brain tissue from patients as well as animals with hepatic encephalopathy (22,68-70). However, these were whole brain studies in which the total amount of glutamate was assessed in all anatomical compartments together (vascular, intracellular and extracellular). The concentration of a neurotransmitter in the synaptic cleft, a part of the extracellular space, is probably the best reflection of neurotransmitter function. Since less than 1% of the total amount of amino acid in the brain is found in the extracellular space, total brain measurements are not suitable for determining neurotransmitter function (71). The brain dialysis technique has made the extracellular space more accessible for research (72-75). Furthermore, with brain dialysis one can distinguish between glutamate derived from basal cell metabolism and that attributable to neurogenic events (76,77).

The aim of the present investigation was to determine in-vivo whether there is a hyperammonemia-induced deficit of the excitatory neurotransmitter glutamate in the extracellular space of the brain during encephalopathy from acute liver failure and from acute hyperammonemia in the rabbit.

In our laboratory we have rabbit models for both acute ischemic liver failure and acute hyperammonemia (78,79). In the latter model ammonia is infused such that the plasma ammonia levels simulate those measured during acute ischemic liver failure (78). Because good animals models form a crucial part in experiments to unravel the mechanism of ammonia toxicity and its contribution to hepatic encephalopathy, this thesis also describes the clinical and biochemical details of our rabbit models for acute ischemic liver failure and acute hyperammonemia.

Aims of this thesis:

1. To study, in rabbits, the suitability of experimental acute liver failure and acute hyperammonemia simulating acute liver failure for the study of hepatic encephalopathy and ammonia toxicity.
2. To study glutamate neurotransmission in rabbits with acute liver failure and acute hyperammonemia.
3. To determine the role of the changes in glutamate neurotransmission found in 2 in the development of hepatic encephalopathy.

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CHAPTER 2

Encephalopathy from acute liver failure and from acute hyperammonemia in the rabbit.
A clinical and biochemical study.

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SUMMARY

To study the molecular basis of ammonia toxicity highly reproducible models of acute liver failure and acute hyperammonemia in the rabbit were developed. Acute liver failure was induced by two-stage liver devascularization, and acute hyperammonemia by prolonged ammonia infusion such that the plasma ammonia pattern found in acute liver failure was simulated. Clinical symptoms, spectral analysis of the E.E.G., biochemistry (blood gasses, renal function, electrolytes and markers of hepatic injury) and the presence of cerebral edema were studied. During acute liver failure severe encephalopathy developed after 10.2 ± 1.9 hours (n=6, mean$\pm$SEM). Other liver failure associated abnormalities were cerebral edema, lactic acidosis, renal dysfunction, hypothermia and septicaemia. During acute hyperammonemia severe encephalopathy developed after 18.2 ± 0.4 hours (n=6, mean$\pm$SEM). Other abnormalities found were cerebral edema and lactic acidosis. In both animal models comparable E.E.G. changes were observed (a decrease of mean dominant frequency and theta-activity, and an increase of delta-activity). However, these changes were not statistically significant, and non-specific as they also occurred in control-rabbits despite their clinical well-being.

This study demonstrates in the rabbit the similarity between encephalopathy due to acute ischemic liver failure and that due to hyperammonemia. An observed difference in hyperammonemia-induced encephalopathy was pronounced ataxia which did not occur during acute liver failure, whereas hypothermia, sepsis and renal failure occurred exclusively in acute liver failure. Our models appear satisfactory for the study of hepatic encephalopathy and ammonia toxicity.

INTRODUCTION

For the study of the pathogenesis and the treatment of hepatic encephalopathy reliable animal models are needed. With the renewed interest in the role of hyperammonemia (1), there is also a need for reliable models of acute hyperammonemia. During development of acute liver failure ammonia increases gradually. Therefore in models of hyperammonemia, ammonia should also increase gradually. In most studies ammonia is administered in doses which lead rapidly to levels according to or higher than the peak levels encountered in acute liver failure (2-4). Since the effects of ammonia are related to degree and duration of hyperammonemia, the toxic effects of a bolus injection of ammonia may differ from the effects of ammonia in liver failure (5).

In our laboratory, rabbits with acute ischemic liver failure have been used in studies on hepatic encephalopathy (6,7). Taking both dose and exposure time into account, we developed an ammonia infusion model which simulates the plasma ammonia level found in rabbits with acute ischemic liver failure (8). We use this more pathophysiological model to study the mechanism of ammonia toxicity.

Galactosamine induced acute liver failure in the rabbit is a well recognized model to study hepatic encephalopathy; it has been characterized for one type of inbred rabbit strain (9). However the time of development of hepatic encephalopathy is rather wide, ranging from 21 to 44 hours, and the hepatic necrosis is variable (9). Furthermore the hepatotoxic mechanism of galactosamine-induced hepatitis has not been completely elucidated and important toxic effects on other organs cannot be excluded (10,11). Therefore we favour a surgical model of acute liver failure.

This paper describes in detail the clinical and biochemical features of acute ischemic liver failure and acute hyperammonemia in the rabbit.

METHODS

Animal models

New Zealand white rabbits weighing 2-3 kg were used. Acute liver failure (ALF) was induced by two-stage liver devascularization (6). Under anesthesia laparotomy was performed: a ligature was placed around the hepatoduodenal ligament and guided through a plastic tube through the abdominal wall to the subcutaneous layer of the left subcostal region, and a small-diameter (5 mm) side-to-side portacaval shunt was constructed. The

superior mesenteric artery was clamped to reduce splanchnic blood stasis. To correct acidosis after release of the vascular clamps 5 ml 8.4% sodium bicarbonate were given intravenously. Postoperatively the rabbits were given 50 ml 10% glucose subcutaneously, followed by standard laboratory chow and water ad libitum. The second day after the operation, acute ischemic liver failure was induced by tightening the ligature around the hepatoduodenal ligament, after giving 50 mg amoxicillin intravenously. The rabbits were subsequently placed in a restraining box. To prevent hypoglycemia 10% glucose was given intravenously, starting at a volume of 3 ml/hr and which was adjusted according to the plasma glucose level when necessary.

Acute hyperammonemia (AMM) was induced by prolonged intravenous ammonium acetate (NH_4Ac) infusion (8). After insertion of a Venflon cannula (diameter 0.8 mm, Viggo, Sweden) into an earvein, NH_4Ac was infused at a constant volume of 6 ml/hr, starting with an initial dose of 0.8 mmol/kg/hr, which was subsequently increased by 0.2 mmol/kg/hr every two hours. After 16 hours the dose remained constant.

Control rabbits (C) received a sodium/potassium acetate (NaKAc) solution, which was infused -as far as the acetate concentration was concerned- as in the NH_4Ac experiments.

Design of the study

19 rabbits were studied: 7 with acute liver failure (ALF), 6 with acute hyperammonemia (AMM) and 6 controls (C). Before the start of the experiments and after the development of severe encephalopathy, clinical, neurophysiological and biochemical data were studied. A rabbit was considered to have severe encephalopathy when it layed in the cage, could not achieve the sitting position -even after stimulation- and could not lift its head (encephalopathy stage B, as described earlier (7)). After completion of the measurements, the rabbits were sacrificed with pentobarbital 1 g/kg body weight (Euthesate, Gist Brocades, the Netherlands). Necropsy was performed to exclude gross morphological abnormalities and in case of acute liver failure to confirm tightening of the ligature. Brains were rapidly removed for determination of water contents. Rabbits were excluded from evaluation when survival did not exceed at least 6 hours; in such cases death was considered to be due to postoperative complications.

Clinical investigation

Clinical investigation of the rabbits consisted of systematic neurological examination, performed hourly. Neurological signs studied were spontaneous activity,

body posture, righting reflex, muscle tone, hyperextension (opisthotonus), reaction to a painful stimulus, ataxia, nystagmus, pupillary and corneal reflexes, and the occurrence of convulsions. During the study the body temperature was measured (Philips Digital Thermometer, the Netherlands).

Electroneurophysiological investigation

One day before the experiment and under anesthesia three silver electrodes were implanted directly onto the dura for E.E.G. monitoring. Two were placed onto the right hemisphere, one anterior (3 mm) and one posterior (11 mm) to the sutura coronaria. The third electrode, the ground electrode, was placed onto the left hemisphere. The electrodes were fixed onto the skull with dental cement. Automated E.E.G. analysis (spectral analysis) was performed as follows (12). During 100 seconds, variations in the electric potentials between 0.53 and 70 cycles per second in the frontal-occipital lead were registered by an E.E.G.-apparatus (Van Gogh, the Netherlands). The data were fed in a personal computer (Olivetti M24, Italy). To avoid interference of high frequency energy, signals were filtered at 25.6 Hz. The power spectrum was constructed using Fast Fourier Transformation. Parameters calculated for the range 1-25.6 Hz were the mean dominant frequency (MDF) and the relative delta- and theta-activity (0.5-3.5 and 3.5-8.0 Hz respectively).

Biochemical investigation

After insertion of a Venflon cannula into an ear artery, arterial blood samples were taken at 0,1 and 2 hours, and every two hours thereafter for ammonia determination, using an enzymatic method (13). Blood glucose levels were measured hourly (Haemoglucotest, Boehringer, Germany). Before the start of the experiment and during encephalopathy stage B the blood was studied more extensively: arterial blood gasses and hemoglobin were determined using an acid-base laboratory (ABL 2, Radiometer Holland, the Netherlands). Blood chemistry was measured using an automatic analyser (CHEM 1, Technicon/Bayer, Germany): sodium, potassium, chloride, phosphate, urea, creatinin, total protein, albumin, total bilirubin, alkaline phosphatase, ALAT, ASAT, osmolarity and lactic acid. Immediately after sacrifice, blood was drawn for bacterial cultures.

Cerebral edema

The presence of brain edema was investigated by means of the dry weight method

(14,15). After removal of the brain, left and right cortex, hippocampus, cerebellum and brainstem were dissected. Samples of these brain regions were heated in an oven at 105 °C until the weight remained constant. Thus the water contents could be calculated.

Statistics

The results are presented as mean±S.E.M. For statistical analysis the paired or unpaired Student's *t* test was used. Statistical significance denotes $p < 0.05$.

All experiments were approved by the ethical committee on animal experiments of the Erasmus University Rotterdam.

RESULTS

Each group of rabbits consisted of 6 animals. One rabbit with acute liver failure died after 4 hours and was excluded for further evaluation. The cause of death remained unknown; autopsy did not reveal gross abnormalities.

Clinical data

All rabbits studied developed encephalopathy. Encephalopathy stage B was reached after 10.2±1.9 hr in rabbits with acute liver failure and after 18.2±0.4 hr in rabbits with acute hyperammonemia (ALF vs. AMM $p < 0.05$). Encephalopathy stage B from acute liver failure was characterized by complete loss of spontaneous activity, impaired body posture, absence of the righting reflex, marked hypotonia and unresponsiveness to a painful stimulus. Opisthotonus was observed in two rabbits with acute liver failure. Encephalopathy stage B from acute hyperammonemia was also characterized by loss of spontaneous activity and impaired body posture, but was preceded by pronounced ataxia. Absence of the righting reflex, hypotonia, opisthotonus and unresponsiveness to a painful stimulus were frequently observed. In one rabbit with acute hyperammonemia nystagmus occurred. Convulsions did not occur, neither during acute liver failure nor during acute hyperammonemia. Control rabbits were studied for 15 hours; they all behaved normally until the end of the experiment. See table I.

Body temperature

During the control studies and during ammonia infusion body temperature did not

Table I

Neurological examination of rabbits with severe encephalopathy from acute hepatic failure and acute hyperammonemia.

Neurological signs	C (n=6)		ALF (n=6)		AMM (n=6)	
	t_0	t_E	t_0	t_E	t_0	t_{EB}
diminished activity	0	0	0	6	0	6
impaired body posture	0	0	0	6	0	6
disturbed righting reflex	0	0	0	6	0	4
decreased muscle tone	0	0	0	6	0	3
opisthotonus	0	0	0	2	0	3
impaired reaction to pain	0	0	0	6	0	3
ataxia	0	0	0	0	0	6
nystagmus	0	0	0	0	0	1
abnormal pupillary reflexes	0	0	0	0	0	0
abnormal corneal reflexes	0	0	0	0	0	0
convulsions	0	0	0	0	0	0

C controls, ALF acute liver failure, AMM acute hyperammonemia

t_0 start of the experiment, t_E encephalopathy stage B/end of experiment.

change significantly (C from 38.6±0.4 °C at the beginning to 39.5±0.3 °C at the end of the study, and AMM from 39.5±0.3 °C to 39.2±0.6 °C). During acute liver failure there was a significant decrease of the body temperature: from 38.2±0.5 °C to 35.3±0.7 °C ($p<0.05$).

Plasma ammonia levels

During all experiments increases of arterial ammonia levels in the rabbits with acute liver failure and acute hyperammonemia, showed a similar pattern (table II). At the

Table II

Mean arterial ammonia levels (in μmol/l) at the start of all experiments and during severe encephalopathy.

Model	t_0	n	t_4	n	t_8	n	t_{12}	n	t_E	T_E	n
C	22±7	6	30±7	6	21±2	6	13±5	6	37±13	15	6
ALF	123±12 *	6	299±36	6	596±203	4	349±58 #	2	679±89	10.2±1.9	6
AMM	39±8	6	216±43	6	332±37	6	543±99	6	627±79	18.2±0.4	6

C controls, ALF acute liver failure, AMM acute hyperammonemia, t_0 start of the experiment, t_4-t_8-t_{12} after 4,8 and 12 hours respectively of acute liver failure/acute hyperammonemia, t_E encephalopathy stage B/end of experiment, and T_E time of development of encephalopathy stage B/end of experiment, in hours.

Values are derived from six experiments and are expressed as mean±SEM.

* $p<0.05$ ALF vs. C, and # $p<0.05$ ALF vs. AMM.

Table III

Spectral analysis of the E.E.G. at the start of the experiment and during severe encephalopathy stage B.

Mean dominant frequency (Hz)

Model	t_0	t_E
C	4.5±0.2	3.6±0.4
ALF	4.7±0.6	3.2±0.5
AMM	5.3±0.3	3.6±0.4 * $p<0.05$

Delta-activity (%)

Model	t_0	t_E
C	51.6±4.5	64.7±8.4
ALF	57.2±7.4	72.9±7.8
AMM	47.8±1.7	70.0±3.1 * $p<0.05$

Theta-activity (%)

Model	t_0	t_E
C	36.6±4.5	28.2±7.4
ALF	27.8±4.0	19.9±6.4
AMM	30.3±1.9	24.4±2.8

C controls, ALF acute liver failure, AMM acute hyperammonemia,
t_0 prior to induction of acute liver failure/acute hyperammonemia,
t_E encephalopathy stage B/end of experiment.
Data are derived from 6 experiments and are expressed as mean±SEM.
* $p<0.05$: statistical significance t_0 vs. t_E.

Table IV

Arterial blood gasses, hemoglobin, lactic acid and glucose at the start of the experiments and during severe encephalopathy stage B.

	Units	C-t_0	C-t_E	ALF-t_0	ALF-t_E	AMM-t_0	AMM-t_E
pH		7.45±0.02	7.56±0.02*	7.45±0.01	7.34±0.09	7.45±0.02	7.24±0.05*
bicarbonate	mmol/l	24.3±1.2	29.7±1.2*	22.3±0.6	15.5±2.2	21.7±1.2	9.2±1.0*
base excess	mmol/l	0.9±1.2	7.2±1.2*	-0.8±0.5	-9.2±4.4	-1.1±1.4	-16.5±1.9*
pCO_2	mm Hg	34.9±0.8	33.4±1.0	32.4±1.3	31.2±5.6	31.4±1.2	22.0±1.4*
pO_2	mm Hg	90.5±1.3	87.6±5.0	91.4±3.0	98.3±21.8	93.4±2.9	109.3±5.1*
O_2 saturation	%	96.4±0.2	96.8±0.5	96.6±0.2	83.1±13.8	96.7±0.3	95.5±0.9
hemoglobin	mmol/l	14.5±0.4	12.3±0.3*	12.7±0.8	12.9±0.8	13.6±0.4	11.9±0.5*
lactic acid	mmol/l	2.2±0.6	4.3±0.8	2.2±0.7	14.2±2.4*	3.0±0.9	16.5±3.6*
glucose	mmol/l	8.7±0.2	9.6±1.7	8.0±0.3	7.9±1.0	9.0±0.2	11.9±0.9*

C controls, ALF acute liver failure, AMM acute hyperammonemia.

t_0 start of the experiment, t_E encephalopathy stage B/end of experiment.

Data are derived from 6 experiments and are expressed as mean±SEM.

* $p < 0.05$ t_0 vs. t_E.

start of the experiments ammonia levels were significantly higher in ALF-rabbits, when compared to AMM- or C-rabbits ($p<0.05$). After 12 hours, ammonia levels were significantly lower in ALF-rabbits when compared to AMM-rabbits ($p<0.05$). However, during encephalopathy stage B plasma ammonia levels were similar.

Electroneurophysiological data

The mean dominant frequency tended to decrease: ALF from 4.7 ± 0.6 to 3.2 ± 0.5 Hz ($p>0.05$), AMM from 5.3 ± 0.3 to 3.6 ± 0.4 Hz ($p<0.05$) and C from 4.5 ± 0.2 to 3.6 ± 0.4 Hz ($p>0.05$)(table III). There was a concomitant increase of the delta-activity and decrease of the theta-activity (table III). Changes were significant for acute hyperammonemia only ($p<0.05$). It is important to note, that the changes occuring among the control rabbits were comparable to the changes in the ALF-and AMM-rabbits.

Biochemical data

All rabbits with acute ischemic liver failure had laboratory evidence of hepatic injury, indicated by elevated bilirubin and liver enzymes. Arterial blood gasses demonstrated normal oxygenation and ventilation in all animals studied. At the start of theexperiments ALF-rabbits showed a tendency towards anemia, which can most likely be attributed to the laparotomy two days earlier. During infusion of NH4Ac and NaKAc, hemoglobin levels decreased significantly, probably due to dilution and blood sampling. Similar changes were observed for plasma albumin. All rabbits with severe encephalopathy had elevated plasma levels of lactic acid, resulting in acidosis. Hypoglycemia was not observed; hypoglycemia during acute liver failure was prevented by glucose infusion such that plasma glucose levels remained constant. During acute hyperammonemia mild hyperglycemia was observed. Renal function was diminished inALF-rabbits at the beginning of the experiment, which worsened after the induction of liver ischemia. In all rabbits studied, plasma osmolarity increased significantly, due to infusion of Na/K-acetate, ammonium acetate and increases of lactic acid. Urea levels increased during ammonia infusion resulting from increased urea formation from ammonia. Two rabbits with acute liver failure had positive blood cultures (Staphylococcus epidermidis and Pseudomonas). After infusion of NH_4Ac or NaKAc blood cultures did not become positive. See tables IV and V.

Table V

Renal function, electrolytes and albumin during acute liver failure and acute hyperammonemia.

	Units	C-t_0	C-t_E	ALF-t_0	ALF-t_E	AMM-t_0	AMM-t_E
creatinine	μmol/l	63±5	79±16	178±76	321±65	111±20	131±20
urea	mmol/l	4.5±0.4	3.8±0.4	15.3±5.7	13.6±4.2	4.6±0.3	13.1±0.9*
sodium	mmol/l	138±0	150±1*	136±2	139±2	139±1	149±2*
potassium	mmol/l	4.9±0.2	3.7±0.0*	4.1±0.3#	5.5±0.8	4.7±0.1	5.3±0.5
chloride	mmol/l	104±2	114±1*	109±4	105±3	106±2	125±2*
calcium	mmol/l	3.6±0.1	2.7±0.1*	3.0±0.2#	2.7±0.2	3.6±0.1	2.8±0.1*
phosphate	mmol/l	1.8±0.1	1.6±0.1	2.3±0.5	3.8±0.2*	1.9±0.0	2.0±0.1
osmolarity	mosmol/l	292±3	303±3*	295±3	327±8*	288±2	326±6*
albumin	g/l	40.7±1.1	36.2±0.7*	35.2±1.2#	32.0±1.0	42.0±1.0	38.8±1.3

C controls, ALF acute liver failure, AMM acute hyperammonemia.

t_0 start of the experiment, t_E encephalopathy stage B/end of experiment.

Data are derived from 6 experiments and are expressed as mean±SEM.

* $p<0.05$ t_0 vs. t_E, # $p<0.05$ vs. controls at t_0.

Brain edema

Water contents of left and right brain structures did not reveal any significant differences; therefore, the means of left and right are presented. Control animals had cortical water contents of 80.96±0.37%. During both acute liver failure and acute hyperammonemia, cortical water contents were increased but differences did not reach statistical significance (ALF 82.06±0.58% and AMM 81.38±0.53%, $p>0.05$). During acute liver failure the water contents of hippocampus (ALF 82.77±0.49% vs. C 81.19±0.22%) and cerebellum (ALF 80.46±0.26% vs. C 79.43±0.30%) were significantly elevated ($p<0.05$). During acute hyperammonemia brain water contents of hippocampus and cerebellum were also elevated, but differences did not reach statistical significance. In brain stem no significant changes were found. When we defined the presence of cerebral edema or increased brain water contents as water contents above the upper limit of the 95% confidence interval of the control values in at least 2 brain regions (two out of cortex, hippocampus, cerebellum and brainstem), then 4 out of 6 rabbits with acute liver failure and 4 out of 6 rabbits with acute hyperammonemia had increased brain water contents or cerebral edema. Brain water contents are summarized in table VI.

DISCUSSION

In this study we have evaluated our rabbit models of acute liver failure and acute hyperammonemia, for their similarities and dissimilarities, and to assess their suitability for the study of hepatic encephalopathy and ammonia toxicity. Acute liver failure was induced by two-stage liver devascularizatione; for technical reasons we favoured liver devascularization over hepatectomy. Acute hyperammonemia was induced by ammonia infusion such that the plasma ammonia levels found in acute liver failure were simulated.

After laparotomy, rabbits appeared in good clinical shape, had moderate hyperammonemia indicating portacaval shunting, and showed signs of renal dysfunction which may be attributed to portocaval shunting (i.e. hepatorenal syndrome) but may also have been caused by the devascularization procedure itself. All rabbits with acute liver failure and acute hyperammonemia developed severe encephalopathy within a narrowrange of time. Development was earlier in rabbits with acute liver failure, as we have also shown in previous studies (16,17). It is intriguing, that encephalopathy stage B was reached at similar plasma ammonia levels (table II). In both models, encephalopathy was characterized by diminished activity, impaired body posture and disturbed

Table VI

Brain water contents of rabbits with severe encephalopathy from acute liver failure and acute hyperammonemia.

	C	ALF	AMM
Cortex	80.96±0.37	82.06±0.58	81.38±0.53
Hippocampus	81.19±0.22	82.77±0.49 *	81.68±0.46
Cerebellum	79.43±0.30	80.46±0.26 *	80.28±0.25
Brainstem	73.81±0.57	74.51±0.81	75.18±0.54

C controls, ALF acute liver failure, AMM acute hyperammonemia.
Values are derived from 6 experiments and are expressed as mean±SEM.
* $p<0.05$ vs. controls.

righting reflex; however in hyperammonemia pronounced ataxia preceded. We were not able to quantitate the encephalopathy in our rabbits with spectral analysis of the E.E.G., although this has been shown to be a sensitive technique to detect encephalopathy in rats (18).

In addition to encephalopathy, there was development of other liver failure associated complications. Cerebral edema and lactic acidosis occurred in both models, whereas renal dysfunction, septicaemia and hypothermia occurred exclusively in acute liver failure.These complications, except for hypothermia, are also of importance in the human situation (19).

In this study, we have demonstrated important similarities between acute liverfailure and acute hyperammonemia. However, important dissimilarities also became apparent. Major differences between the two models are a recent laparotomy and the presence of a portacaval shunt in rabbits with acute liver failure. These may have had an effect on the time of development of encephalopathy and the occurrence of other extrahepatic complications. Furthermore, hyperammonemia may exert different effects in

the presence of a portacaval shunt (20).

Acute liver failure in rabbits has been studied by others. The clinical syndrome of galactosamine induced liver failure encompasses the development of encephalopathy, renal failure and cerebral edema (9,15). The ability of electroencephalography to measure the encephalopathy appeared poor with significant changes occurring only late in course (9). Studies on hepatectomized rabbits demonstrated the development of encephalopathy (diminished activity, ataxia, impaired body posture, decreased muscle tone), renal failure and hypothermia (21,22,23). Thus, acute liver failure in the rabbit appears to be rather uniform. Longterm ammonia infusion has not been performed earlier.

Can we conclude that our models are suitable for the study of acute liver failure and ammonia toxicity? If acute liver failure is considered a syndrome resulting from the accumulation of toxic substances no longer cleared by the liver, than our model of ischemic liver failure clearly appears suitable. However, when one considers toxic substances deriving from necrotic liver tissue to be of importance too, than this model has also a drawback in that there is minimal liver blood flow. In addition, a long-term ammonia infusion seems to be the method of choice to study the mechanism of ammonia toxicity in hepatic encephalopathy, which has also been noted by others (24). However, it is important to realize that our rabbits receiving an ammonia infusion were completely healthy, i.e. had not undergone recent laparotomy and did not have a portacaval shunt.

In our opinion the animal models described here fulfill important requirements for satisfactory animal models to study encephalopathy from acute liver failure and acute hyperammonemia: the models are reproducible, encephalopathy develops in all rabbits within a narrow range of time and with similar plasma ammonia levels, frequent blood sampling is feasible, the models are not extremely costful, do not pose health hazards to the investigators and are approved by our university ethical committee on animal experiments. In addition, our models demonstrate important neurological and biochemical similarities between acute liver failure and acute hyperammonemia. Some of the differences between these models may be due to the fact that hyperammonemia may exert different effects in the presence of a portacaval shunt (20). Therefore development of a model of acute hyperammonemia in portacaval shunted rabbits would be of interest.

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CHAPTER 3

Extracellular brain glutamate during acute liver failure and during acute hyperammonemia simulating acute liver failure.
An experimental study based on in-vivo brain dialysis.

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SUMMARY

Hyperammonemia is thought to be important in the pathogenesis of hepatic encephalopathy. However, the mechanism leading to ammonia toxicity is still not known. Since the metabolism of the most important excitatory neurotransmitter glutamate is closely linked to that of ammonia, it has been postulated that hyperammonemia lowers the availability of the neurotransmitter glutamate. To study this hypothesis we used brain dialysis to measure glutamate levels in extracellular cerebral fluid from rabbits with acute ischemic liver failure or acute hyperammonemia. The basal glutamate concentration was found to be increased during both acute liver failure (start of experiments 4.9 ± 1.7 μmol/l; end of experiments 9.5 ± 2.1 μmol/l, $n=6$, difference $p<0.05$) and acute hyperammonemia (start of experiments 4.4 ± 1.2 μmol/l; end of experiments 7.3 ± 1.8 μmol/l, $n=7$, difference $p>0.05$)(mean$\pm$SEM). Both the veratridine and the potassium-evoked glutamate release were increased during acute liver failure but appeared normal during hyperammonemia. It is concluded that during acute liver failure and acute hyperammonemia in the rabbit there is no decreased glutamate availability in the extracellular space of the cortical brain; contrary we found evidence for increased extracellular glutamate concentrations in cortical brain, which was more pronounced in acute liver failure. Experimental hepatic encephalopathy is not due to cerebral glutamate deficiency.

INTRODUCTION

The pathogenesis of hepatic encephalopathy has not been completely elucidated yet. Currently, there is renewed interest in the role of hyperammonemia (1). The mechanism leading to ammonia toxicity is still not known. Hyperammonemia affects normal brain functioning in many ways; it may disturb basic cell functions (e.g. pH regulation and water and energy balance (2-4)), electrophysiology (5) and biochemical neurotransmission. In the present study we focussed on the effect of hyperammonemia on biochemical neurotransmission, in particular glutamate neurotransmission.

The metabolism of glutamate, the most important excitatory neurotransmitter in the brain, is linked to that of ammonia (6). In neurons the neurotransmitter glutamate is produced from glutamine, through the enzymatic action of glutaminase. In vitro the activity of glutaminase, which is essential for the formation of glutamate, is inhibited by ammonia (7-8). Thus it was assumed that ammonia decreases the amount of glutamate in the brain, which probably would have an effect on normal brain functioning. Subsequently, the amount of glutamate was shown to be decreased in autopsied brain tissue from patients as well as animals with hepatic encephalopathy (9-12). However, these were whole brain studies in which the total amount in all anatomical compartments (vascular, intracellular and extracellular) was assessed. The concentration of a neurotransmitter in the synaptic cleft, a part of the extracellular space, is probably the best reflection of neurotransmitter function. Since less than 1% of the total amount of amino acid in the brain is found in the extracellular space, total brain measurements are not suitable for determining neurotransmitter function (13). The brain dialysis technique has made the extracellular space more accessible for research (14-17). For brain dialysis a thin dialysis tube is implanted in a region of the brain of an animal. The tube is perfused with an electrolyte solution, and small molecules which pass through the dialysis membrane can be measured in the dialysate. Before analyzing the glutamate concentrations in extracellular cerebral fluid, one has to distinguish between glutamate derived from basal cell metabolism and that attributable to neurogenic events. Under normal conditions, the glutamate responsible for the "basal" concentration is derived mainly from the metabolic pool (18-19). After the addition of depolarizing agents, such as veratridine or potassium, "evoked" glutamate release from the neurotransmitter pool is observed (19-20).

In our laboratory we have rabbit models for both acute ischemic liver failure and acute hyperammonemia (21,22). In the latter model ammonia is infused such that the

plasma ammonia levels simulate these measured during acute ischemic liver failure (21). The aim of the present investigation was to determine in-vivo whether there is a hyperammonemia-induced deficit of the excitatory neurotransmitter glutamate in the extracellular space of the brain during experimental hepatic encephalopathy. Therefore we studied the basal concentrations and evoked release of glutamate in our two rabbit models.

METHODS

Animal models

New Zealand white rabbits weighing 2-3 kg were used. Acute liver failure (ALF) was induced by a two-stage liver devascularization procedure (22). Under anesthesia a laparotomy was performed: a loose ligature was placed around the hepatoduodenal ligament and guided through a plastic tube through the abdominal wall, and a small-diameter (5 mm) side-to-side portacaval shunt was constructed. On the first postoperative day a small dialysis tube was implanted in the cortex for brain dialysis, together with 3 silver electrodes on the dura for E.E.G. registration. On the second postoperative day acute liver failure was induced by tightening the ligature around the hepatoduodenal ligament. The rabbits were subsequently placed in a restraining box. To prevent hypoglycemia 10% glucose was given intravenously.

Acute hyperammonemia (AMM) was induced by a prolonged intravenous ammonium acetate (NH_4Ac) infusion (21). NH_4Ac was infused at a constant rate of 6 ml/hr, starting with an initial dose of 0.8 mmol/kg/hr and increasing this dose by 0.2 mmol/kg/hr every two hours. Control rabbits (C) received a sodium/potassium acetate (NaKAc) solution infused -as far as the acetate concentration was concerned- as in the NH_4Ac experiments.

Brain dialysis

Under anesthesia and after fixation of the head of the animal in a stereotaxic frame, the skull was exposed for bilateral trepanations 1 mm below the bregma. Then a dialysis tube (diameter 0.3 mm, Cordis Dow, Roden, the Netherlands) supported by a tungsten wire (Clark Tungsten Tubes TW 5.3, Reading, United Kingdom) was inserted through the trepanation holes into the cerebral cortex with the aid of a stereotaxic micromanipulator. The dialysis tube was fixed with glue in polyethylene tubing

(0.28x0.61 mm, Rubber, Hilversum, the Netherlands). Brain dialysis was performed between 24-48 hours after implantation (23-24). One end of the dialysis tube was connected to a syringe pump (Harvard Syringe Infusion Pump 22, U.S.A.) and perfused with Krebs-Ringer's solution (composition in mmol/l: NaCl 122; KCl 3; $MgSO_4$ 1.2; KH_2PO_4 0.4; $NaHCO_3$ 25; $CaCl_2$ 1.2; pH 7.35-7.45; 2.5 μl/min) (figure 1). To check the position of the dialysis tube in the cortex the brains of all rabbits were dissected.

Design of the study

Three sets of experiments were performed. The basal concentrations of glutamate were studied in the first set of 19 experiments (6 ALF, 7 AMM, 6 C), the veratridine-evoked glutamate release in the second set of 22 experiments (7 ALF, 7 AMM, 8 C) and the potassium-evoked glutamate release in the third set of 18 experiments (6 ALF, 6 AMM, 6 C).

All experiments started with flushing for one hour to achieve equilibration. During the basal studies samples were collected in one-hour fractions. After the first one-hour period, acute liver failure was induced or the NH_4Ac or NaKAc infusion was started. Brain dialysis was performed continuously until death from acute liver failure or acute hyperammonemia. Control studies were performed for 12 hours.

The studies of the veratridine and potassium-evoked release of glutamate started with a 60-minute period to determine the basal concentration. Then, veratridine (100 μmol/l, Sigma, U.S.A.) or potassium (KCl 100 mmol/l, NaCl concentration was reduced to 25 mmol/l to maintain iso-osmolarity) was added to the perfusate for 10 minutes with the aid of the liquid switch (Carnegie Medical, Stockholm, Sweden), and the dialysate was collected during 6 consecutive periods of 10 minutes. These 60-minute and 6 10-minute samples reflect glutamate release prior to the induction of acute liver failure or acute hyperammonemia. Ten hours after the induction of acute liver failure or the start of the NH_4Ac or NaKAc infusion, brain dialysis was again performed: one hour to achieve equilibration, one hour for measurement of the basal concentration and one hour (6x10) for measurement of the veratridine or potassium-evoked release.

Concentrations of glutamine and glutamate are presented as concentrations in the dialysate (μmol/l) or as the release into the dialysate (pmol/10 minutes).

Laboratory measurements

The amounts of glutamate and glutamine in the dialysates were quantified by high performance liquid chromatography on a liquid chromatograph (Hewlett Packard 1084B,

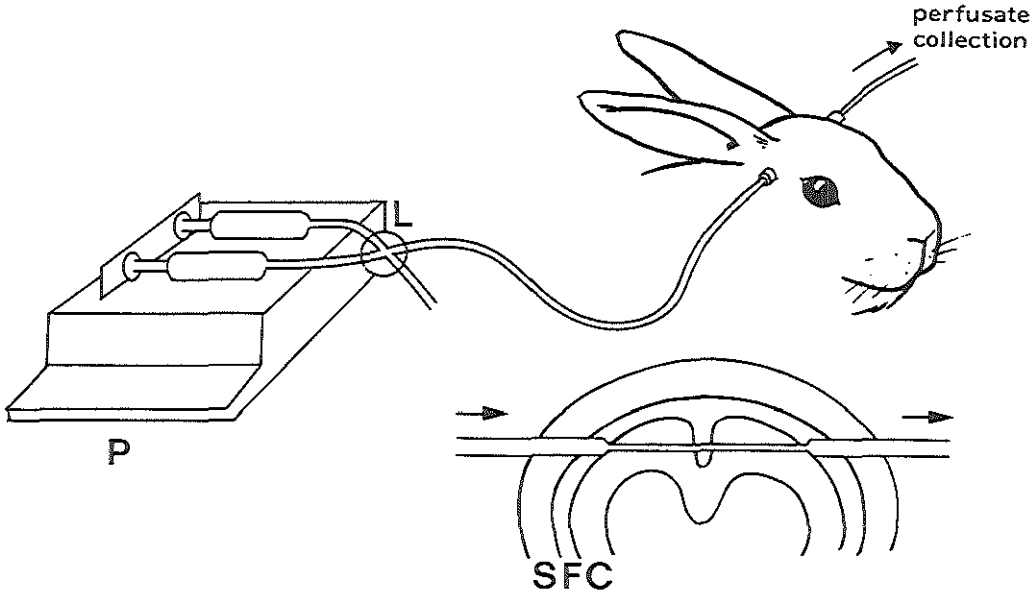


Figure 1

Schematic representation of brain dialysis. A thin dialysis tube is placed in the cerebral cortex (C) of the rabbit; the tube is connected to the syringe pump (P). The tube is perfused with Krebs-Ringer's solution, 2.5 μl/min. The "liquid switch" (L) ensures rapid changes to different perfusates. S skull, F cerebrospinal fluid.

Palo Alto, California, U.S.A.), using a modification of the procedure described by Kraak et al. (25). Samples of 25 μl were injected onto a 150x4.6 mm Hypersil ODS 5 μm column (Chrompack, Middelburg, the Netherlands). The mobile phase consisted of 4 mM NaH_2PO_4, 0.1% (w/v) sodium dodecylsulfate and 0.3% (w/v) tertiary amylalcohol, pH 2.80. A flow rate of 0.8 ml/min was used and the column was operated at 30°C.

Detection of the amino acids was accomplished after derivatization with orthophthaldialdehyde (26).

Arterial blood samples were taken from all rabbits at 0,1 and 2 hours and then every two hours for ammonia determinations (27). Blood glucose levels were measured hourly (Haemoglucotest, Boehringer, Germany). To prevent hypoglycemia in ALF rabbits the rate of the glucose infusion was adjusted to blood glucose levels.

Clinical signs of encephalopathy

During the study rabbits were restrained. Clinical signs could not therefore be evaluated during brain dialysis. However, after the studies of the veratridine and potassium-evoked release were completed, the rabbits were evaluated clinically. Clinical signs studied were spontaneous activity, righting reflex, ataxia, pupillary reflex and reaction to a painful stimulus.

Spectral analysis of the E.E.G.

During implantation of the dialysis tube 3 silver electrodes were also fixed directly on the dura for E.E.G. measurements. Two were placed on the right hemisphere, one anterior (3 mm) and one posterior (11 mm) to the sutura coronalis. The ground electrode was placed on the left hemisphere. The electrodes together with the polyethylene tube, containing the dialysis tube, were fixed onto the skull with dental cement. Automated E.E.G. analysis (spectral analysis)(28) was performed hourly during the determination of the basal glutamate concentrations. During the studies of the evoked glutamate release spectral analysis was performed just before each stimulus.

Statistics

The results are presented as the mean $\pm$ S.E.M. For statistical analysis the paired or unpaired Student's *t* test was used. Statistical significance was set at $p < 0.05$.

All experiments were approved by the ethical committee on animal experiments of the Erasmus University Rotterdam.

RESULTS

Survival time

During the first set of experiments all animals with acute liver failure or acute

hyperammonemia died after 11±2 and 14±1 hours, respectively. Control studies (NaKAc) were performed for 12 hours: one unexplainable death occurred after 11 hours. According to the protocol for the study of evoked release, the rabbits had to survive for at least 12 hours after the first stimulus. Thus, in the veratridine experiments the data on 7 out of 9 ALF rabbits, 7 out of 9 AMM rabbits and 8 out of 8 controls could be evaluated. In the potassium experiments these numbers were 6 out of 10, 6 out of 7 and 6 out of 6, respectively.

Plasma ammonia levels

The increases in arterial ammonia levels in rabbits with acute liver failure and acute hyperammonemia were similar. At the start ammonia levels tended to be higher in ALF rabbits (93±11 μmol/l), due to their portacaval shunt, than in AMM rabbits (31±10 μmol/l) or controls (25±10 μmol/l). During the experiments the mean arterial ammonia levels among rabbits receiving an ammonia infusion were not significantly different from those among rabbits with acute liver failure [$p > 0.05$]. One hour before the death of the rabbits used in the basal glutamate studies -a time when all rabbits were assumed to have similar degrees of encephalopathy- plasma ammonia levels also did not differ significantly [$p > 0.05$] between rabbits with acute liver failure (456±122 μmol/l) and rabbits with acute hyperammonemia (690±86 μmol/l). The plasma ammonia levels in the second and third sets were comparable to those of the first set. Plasma ammonia levels are summarized in figure 2 and table I.

Plasma glucose levels

None of the rabbits developed hypoglycemia. During acute liver failure plasma glucose levels did not change significantly (at the start: 8.4±0.4 mmol; one hour before death: 7.1±0.6 mmol/l [$p > 0.05$]), whereas the plasma glucose levels during ammonia infusion increased from 7.5±0.7 mmol/l to 19.0±2.3 mmol/l [$p < 0.05$]. During control studies plasma glucose levels did not change (at the start: 8.3±0.5 mmol/l; at the end: 8.9±0.9 mmol/l [$p > 0.05$]).

Spectral analysis of the E.E.G.

Spectral analysis of the E.E.G. showed a significant decrease in the mean dominant frequency (MDF) during development of encephalopathy as a result of acute liver failure (at the start: 4.3±0.3 Hz; one hour before death: 3.2±0.5 Hz, $p < 0.05$) as well as acute hyperammonemia (at the start: 5.8±0.4 Hz; one hour before death:

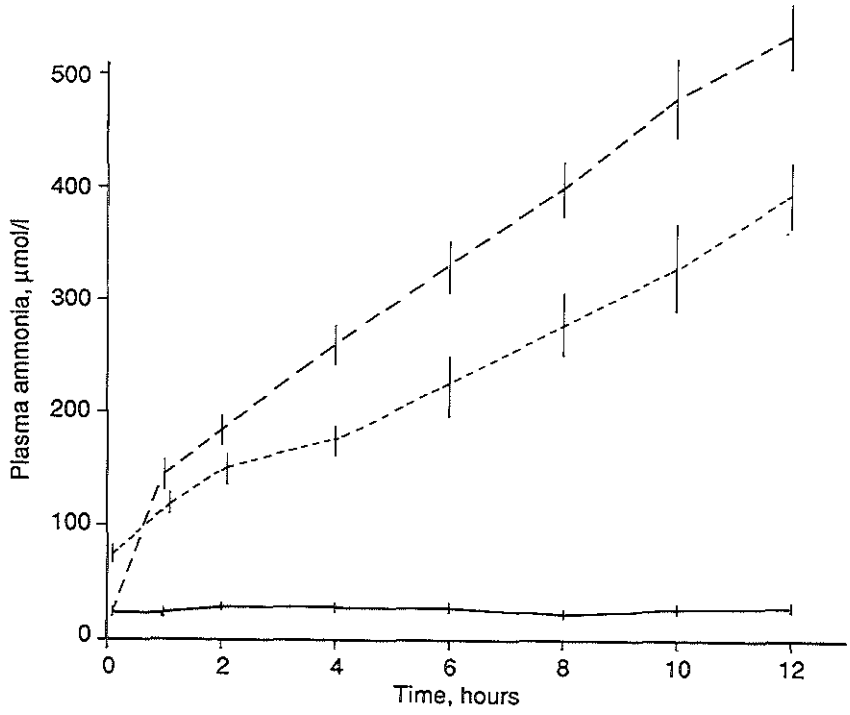


Figure 2

Time course of plasma ammonia levels in rabbits with acute ischemic liver failure or acute hyperammonemia and control rabbits. Values are the mean±SEM.

ALF: n=19, dotted line; AMM: n=20, interrupted line; C: n=20, continuous line.

3.1±0.3 Hz, $p<0.05$)). Probably the portacaval shunt explains why ALF rabbits tended to have a lower MDF at the beginning of the experiment than AMM rabbits. One hour before the death of the rabbits used to study basal concentrations, the severity of the encephalopathy (as indicated by the MDF) in ALF rabbits was similar to that in AMM rabbits. During the NaKAc infusion the MDF did not change (table I).

TABLE I

Plasma ammonia, mean dominant frequency of the E.E.G. and the basal extracellular concentrations of glutamine and glutamate.

		MDF of EEG (Hz)		Plasma ammonia (μmol/l)		Extracellular glutamine (μmol/l)		Extracellular glutamate (μmol/l)	
Study	Model (n)	t_0	t_{12}/t_{end}	t_0	t_{12}/t_{end}	t_0	t_{12}/t_{end}	t_0	t_{12}/t_{end}
B	ALF (6)	4.3±0.3[♦]	3.2±0.5[§]	93±11[♦]	456±122[§]	147±18	469±96[§]	4.9±1.7	9.5±2.1[§]
	AMM (7)	5.8±0.4	3.1±0.3[§]	31±10	690±86[§]	104±19	313±39[§]	4.4±1.2	7.3±1.8
	C (6)	5.4±0.4	5.3±0.3	25±10	39±10	99±6	78±9	3.9±1.2	3.4±1.1
V	ALF (7)	5.0±0.5	3.0±0.4[§]	74±21	373±47[§]	158±35	374±34[§]	3.3±0.8	8.4±3.3
	AMM (7)	5.2±0.6	4.6±0.4	19±4	465±52[§]	99±12	243±21[§]	4.1±0.8	5.5±0.9
	C (8)	5.3±0.4	5.3±0.3	28±5	29±14	86±5	77±3	3.4±0.7	3.4±0.5
K	ALF (6)	4.7±0.2	3.0±0.3[§]	64±18	396±24[§]	117±30	354±64[§]	4.0±0.4	7.2±0.9
	AMM (6)	5.4±0.2	4.0±0.4	20±8	611±79[§]	93±11	296±34[§]	2.8±0.6	3.6±0.7
	C (6)	5.2±0.4	4.7±0.2	24±3	29±8	89±24	59±7	1.9±0.3	1.8±0.2

Clinical signs of encephalopathy

After twelve hours of liver ischemia all 13 rabbits (7 veratridine and 6 potassium) showed signs of encephalopathy: there was no spontaneous activity, the rabbits were unable to sit or lift their heads, and the righting reflex was absent. Other abnormalities noted were: severe ataxia (3x), absence of pupillary reflexes (2x) and diminished reaction to a painful stimulus (4x). After twelve hours of ammonia infusion signs of encephalopathy could be detected in 7 out of 13 rabbits (7 veratridine and 6 potassium). These rabbits showed loss of spontaneous activity and absence of the righting reflex. Two were also unable to sit and lift their heads, and ataxia was observed in 3 rabbits. Convulsions were not observed. In the control group (8 veratridine and 6 potassium) two rabbits exhibited absence of spontaneous activity and loss of the righting reflex.

Glutamine level in cortical brain dialysate (Table I and figure 3)

During the basal studies the glutamine level in cortical brain dialysate rose significantly in parallel with plasma ammonia in rabbits with acute liver failure as well as those with acute hyperammonemia. During acute liver failure the concentration of glutamine in the dialysate increased from 147 ± 18 μmol/l to 469 ± 96 μmol/l [$p < 0.05$], during hyperammonemia from 104 ± 19 μmol/l to 313 ± 39 μmol/l [$p < 0.05$]. The differences between the two experimental groups did not reach statistical significance at any time [$p > 0.05$]. The controls exhibited no significant changes (99 ± 6 μmol/l at the beginning and 78 ± 9 μmol/l at the end of the experiments [$p > 0.05$]).

Table I

B: basal studies, V: studies of veratridine-evoked release and K: studies of potassium-evoked release.

ALF acute liver failure, AMM acute hyperammonemia, C controls, n number of experiments.

t_0 start of the experiment or prior to induction of acute liver failure/acute hyperammonemia, t_{12} 12 hours after induction of acute liver failure/acute hyperammonemia, and t_{end} one hour before death in basal studies.

Values are the mean$\pm$SEM. $^{\blacklozenge}$ $p < 0.05$ vs. controls, § $p < 0.05$ both vs. t_0 and controls.

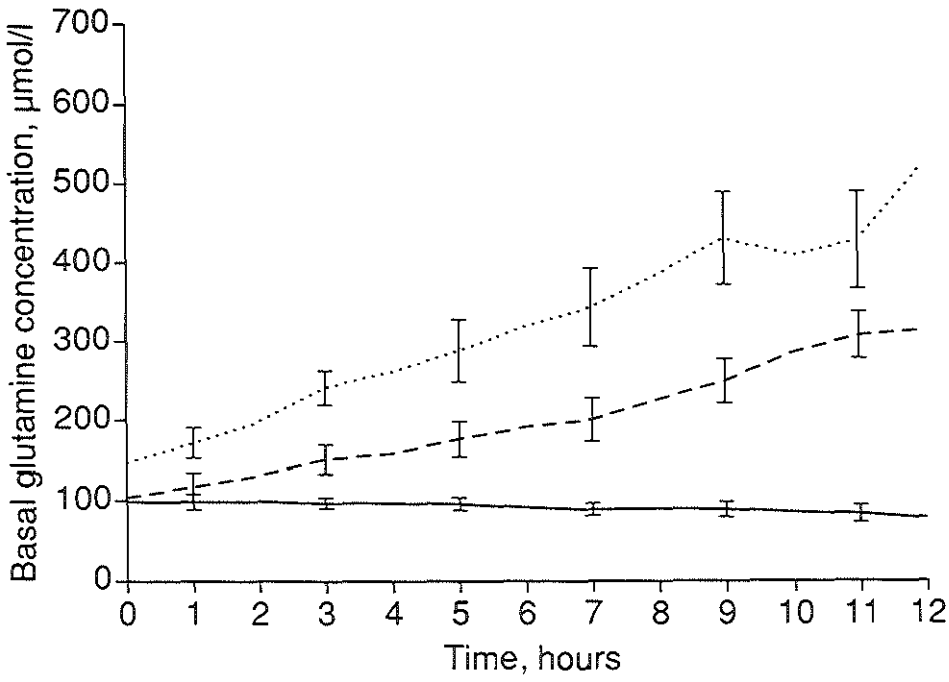


Figure 3

Changes in basal glutamine levels in cortical brain dialysate of rabbits with acute ischemic liver failure or acute hyperammonemia and control rabbits. Values are the mean±SEM.
ALF: n=6, dotted line; AMM: n=7, interrupted line; C: n=6, continuous line.

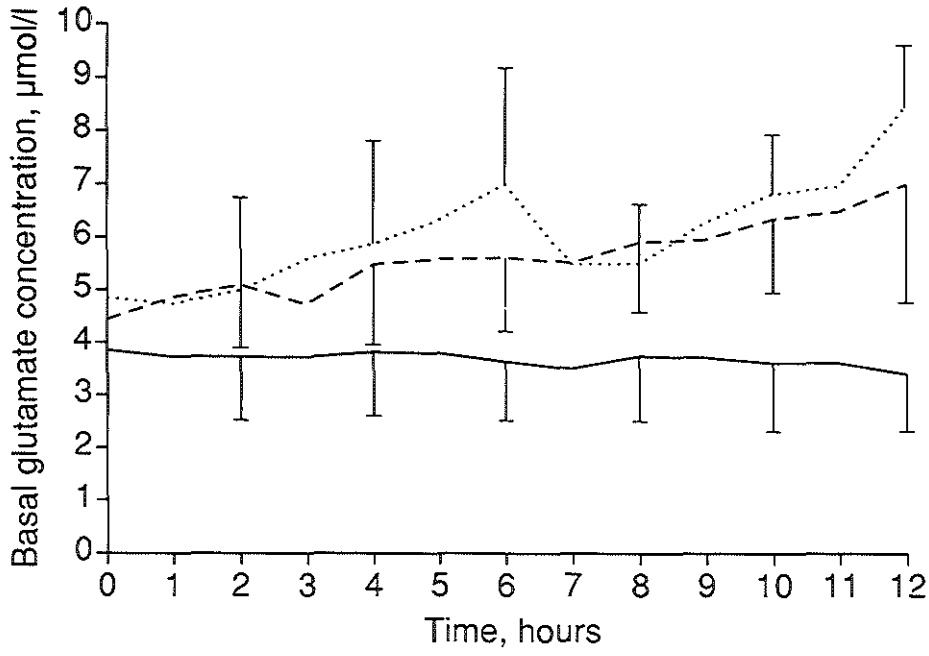
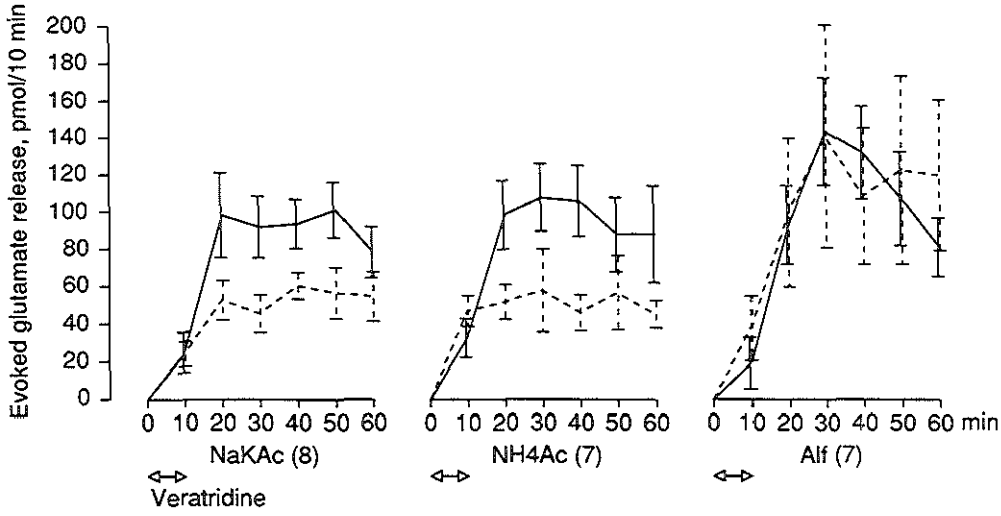


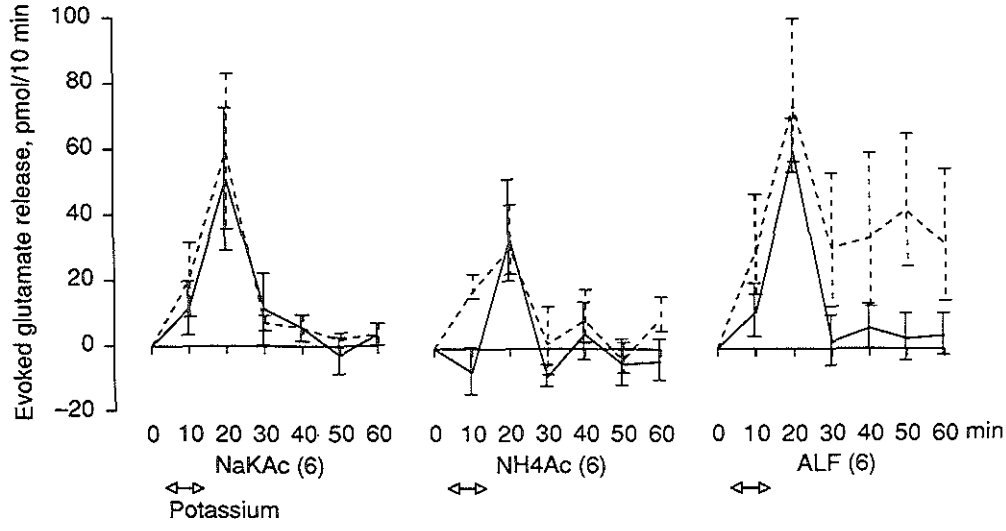
Figure 4

Changes in basal glutamate levels in cortical brain dialysate of rabbits with acute ischemic liver failure or acute hyperammonemia and control rabbits. Values are the mean±SEM.
ALF: n=6, dotted line; AMM: n=7, interrupted line; C: n=6, continuous line.

VERATRIDINE EVOKED GLUTAMATE RELEASE



POTASSIUM EVOKED GLUTAMATE RELEASE



Upon veratridine and potassium stimulation the glutamine concentration in the cortical brain dialysate decreased, indicating glutamine uptake by the nervous tissue surrounding the dialysis tube. Glutamine uptake was similar in the three experimental groups studied; twelve hours of ischemic liver failure or hyperammonemia did not induce significant changes.

Basal glutamate level in cortical brain dialysate (table I and figure 4)

In ALF rabbits the glutamate concentration in the dialysate increased from 4.9 ± 1.7 μmol to 9.5 ± 2.1 μmol/l [$p < 0.05$], in AMM rabbits from 4.4 ± 1.2 μmol/l to 7.3 ± 1.8 μmol/l [$p > 0.05$]. Control rabbits exhibited no changes (beginning with 3.9 ± 1.2 μmol/l and ending with 3.4 ± 1.1 μmol/l).

Veratridine-evoked glutamate release in cortical brain dialysate (figure 5, upper panel)

In the control group the first addition of veratridine to the perfusate for 10 minutes doubled the glutamate concentration in the dialysate, with a maximal increase in the glutamate concentration of 4.8 ± 0.7 μmol/l. After 12 hours of NaKAc infusion this effect had decreased significantly to 3.0 ± 0.4 μmol/l [$p < 0.05$]. Similar results were obtained for the AMM rabbits, although the differences were not statistically significant: maximal increase in the concentration prior to ammonia infusion was 5.2 ± 1.0 μmol/l versus 3.7 ± 0.8 μmol/l after 12 hours of ammonia infusion [$p > 0.05$]. In ALF rabbits the veratridine-evoked glutamate release did not change: before induction the maximal evoked increase in the concentration was 6.2 ± 1.3 μmol/l, and 12 hours after induction of acute liver failure it was 6.2 ± 2.0 μmol/l. The evoked glutamate release was higher in ALF rabbits; after 12 hours of acute liver ischemia the differences were significant, when

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Figure 5

Veratridine-evoked glutamate release (upper panel) and potassium-evoked glutamate release (lower panel) in control rabbits and in rabbits with acute hyperammonemia or acute ischemic liver failure. Values represent release in pmol/10 minutes. Values are the mean$\pm$SEM. Veratridine: C n=8, AMM n=7, ALF n=7. Potassium: C n=6, AMM n=6, ALF n=6. Continuous line represents stimulation prior to induction of acute liver failure/acute hyperammonemia; dotted line represents stimulation 12 hours after induction of acute liver failure/acute hyperammonemia.

compared with rabbits exposed to NH_4Ac or NaKAc [$p < 0.05$].

Potassium-evoked glutamate release in cortical brain dialysate (figure 5, lower panel)

In the controls the first addition of potassium to the perfusate for 10 minutes induced a maximal increase in the glutamate concentration of 2.4 ± 0.9 μmol/l. After 12 hours of NaKAc infusion the evoked glutamate release had not changed significantly (3.2 ± 0.9 μmol/l). The glutamate release in response to the first addition of potassium was somewhat less in AMM rabbits (1.8 ± 0.5 μmol/l), although these differences were not statistically significant. The potassium-evoked glutamate release did not change after 12 hours of NH_4Ac infusion (2.3 ± 0.3 μmol/l). Before the induction of acute liver failure, the evoked glutamate release was not significantly different from that in control rabbits (3.12 ± 0.24 μmol/l). After 12 hours of acute liver failure the maximal response had not changed significantly (4.1 ± 0.9 μmol/l, [$p > 0.05$]); however, the levels usually did not return to baseline values.

DISCUSSION

In this study, based on in-vivo brain dialysis of rabbits with acute ischemic liver failure or acute hyperammonemia, we found an increased basal concentration of the excitatory neurotransmitter glutamate in the cortical brain dialysate. The veratridine and potassium-evoked glutamate release appeared to be unchanged in rabbits with acute hyperammonemia, when compared with controls, but was increased in rabbits with acute liver failure. The veratridine-evoked release of glutamate prior to the induction of acute liver failure (i.e. after portosystemic shunting for 48 hours) tended to be higher than in control rabbits. After 12 hours of acute liver failure the release appeared to be unchanged; however, when compared to control rabbits in which the veratridine evoked release was decreased after 12 hours, we probably demonstrated an increase. After 12 hours of acute liver failure the maximal potassium-evoked glutamate release had not changed, but the levels usually did not return to baseline values. However, in interpreting microdialysis data it is important to note that the concentration of amino acids measured in the dialysate are a reflection of the total actual extracellular concentrations. It is unknown how changes of an amino acid in the dialysate correspond to fluctuations of the concentration in the synaptic cleft.

In the studies of the basal glutamate concentration rabbits with both acute liver

failure and acute hyperammonemia developed encephalopathy followed by death, which was accompanied by comparable elevations of the plasma ammonia levels. These findings demonstrate the potential of ammonia to induce encephalopathy and death. The findings of increased basal extracellular glutamate levels in rabbits with acute liver failure and with acute hyperammonemia suggest that hyperammonemia might have caused this increase. The more pronounced increase in basal glutamate levels and the increased evoked glutamate release in rabbits with acute liver failure, indicate that other factors may contribute to the increased extracellular glutamate level in acute liver failure. However, it should be noted that the reaction to hyperammonemia in rabbits with acute liver failure may have been different from that in normal rabbits, due to the existence of a portacaval shunt (29); furthermore, in the studies of the evoked release the degree of encephalopathy was less for rabbits with hyperammonemia than for those with acute liver failure.

Other studies based on brain dialysis have been reported: rabbits with galactosamine-induced liver failure as well as rats with a portacaval shunt were also found to have increased basal glutamate levels, whereas rats with a portacaval shunt exhibited a normal potassium-evoked release of glutamate (14-17). Most other studies on hepatic encephalopathy and hyperammonemia have demonstrated decreased glutamate levels. Studies of hippocampal slices and isolated nerves demonstrated decreased glutamate availability during hyperammonemia (30-32). Postmortem studies in rats and in patients, and a recent study using in vivo nuclear magnetic resonance spectroscopy in rats also indicated decreased glutamate levels (9-12). However, in these studies total brain concentrations were measured and since less than 1% of the total amount of amino acid is located in the extracellular space, total brain measurements are not suitable for determining amino acid neurotransmitter function (13). A study based on the cortical cup technique reported an ammonia-induced increase in extracellular basal glutamate concentrations with a concomitant decrease in the total brain glutamate content (33), which emphasizes the importance of measuring extracellular concentrations.

Thus during experimental encephalopathy due to acute liver failure or acute hyperammonemia there is likely to be an increase in the glutamate concentration in the extracellular space. It is still not clear whether this increase stems from the metabolic or neurotransmitter pool of glutamate. The results of our studies on the evoked release during acute liver failure suggest the neurotransmitter pool; changes in the potassium-evoked release point to impaired re-uptake, since the glutamate concentrations tended to remain elevated whereas the initial responses were similar. Ammonia-induced impaired re-uptake has been demonstrated in-vitro (32). However, although the evoked release of

glutamate comes mainly from the neurotransmitter pool under normal conditions, a contribution from the glial compartment cannot be excluded here since glial cells exhibit many abnormalities during hyperammonemia and hepatic failure (34).

How can increased extracellular brain glutamate levels give rise to hepatic encephalopathy, or is this just an epiphenomenon? After release into the synaptic cleft, glutamate binds to its receptors. An increased glutamate concentration will give rise to prolonged and/or increased exposure of these receptors to glutamate. This could have two effects: it could lead to compensatory down-regulation and/or there could be a direct toxic effect.

It appears logical to assume that increased exposure of receptors to glutamate induces down-regulation (35). Some studies on glutamate receptors in hepatic encephalopathy indicate a decreased number of receptors (36-40), whereas others could not find any changes (41).

The other effect of increased glutamate exposure may be a direct toxic effect. There is evidence indicating that prolonged glutamate exposure is deleterious to the brain (42). In-vitro experiments have demonstrated that increased glutamate levels are toxic to both neurons and glial cells (43-45). Most toxic effects of glutamate are thought to result from a prolonged depolarizing action of glutamate at its postsynaptic receptor sites (43-45). This action is assumed to cause changes in membrane permeability which lead to impaired ion homeostasis (43-44).

Increased glutamate concentrations in the cerebrospinal fluid of patients with hepatic encephalopathy suggest that increased glutamate exposure also occurs clinically (46-48). The increased extracellular glutamate levels in our animal models of hepatic encephalopathy and hyperammonemia, together with the knowledge that glutamate toxicity occurs in other diseases, indicate that glutamate metabolism and toxicity should receive further attention from investigators engaged in the field of hepatic encephalopathy.

In conclusion, during encephalopathy due to acute ischemic liver failure or acute hyperammonemia in the rabbit there is no deficit of the excitatory neurotransmitter glutamate. On the contrary, we found evidence for increased glutamate availability during hyperammonemia and hepatic encephalopathy.

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CHAPTER 4

Binding of the ligand [^{3}H]MK-801 to the MK-801 binding site of the N-methyl-D-aspartate receptor during experimental encephalopathy from acute liver failure and from acute hyperammonemia in the rabbit.

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SUMMARY

Binding of the ligand [^{3}H]MK-801 to the MK-801 binding site of the N-methyl-D-aspartate (NMDA) receptor population on brain homogenates in rabbits was studied during experimental encephalopathy from acute liver failure and from acute hyperammonemia in the rabbit. Homogenates were prepared from brain cortex, hippocampus and striatum. Hepatic encephalopathy was induced by a two-stage liver devascularization procedure and acute hyperammonemia by a prolonged ammonium acetate infusion; rabbits receiving a sodium/potassium acetate infusion served as controls. In these animal models extracellular brain glutamate levels are known to be elevated. However no significant alterations in the number nor the affinity of the MK-801 binding sites of the NMDA receptors were found during acute liver failure and acute hyperammonemia. These findings suggest that the NMDA receptor population remains unaltered in experimental encephalopathy from acute liver failure and acute hyperammonemia, despite alterations in extracellular brain glutamate levels.

INTRODUCTION

Hepatic encephalopathy is a neuropsychiatric syndrome caused by serious liver disease. Hyperammonemia is thought to be an important causative factor since plasma ammonia levels often correlate with the degree of encephalopathy (1), administration of ammonium-salts to patients with chronic liver disease provokes encephalopathy (2) and lowering of plasma ammonia by lactulose therapy often diminishes encephalopathy (3). Furthermore proliferation of astrocytes -the morphological substrate of chronic hepatic encephalopathy- is also found in patients with the congenital hyperammonemia syndromes (4). However, the exact mechanism how hyperammonemia contributes to the syndrome of hepatic encephalopathy remains to be elucidated.

One of the current opinions with regard to the pathogenesis of hepatic encephalopathy is a dysbalance between neuro-inhibition and neuro-excitation (5). Several groups have proposed that neuro-inhibition via GABA or endogenous benzodiazepines is enhanced; we are investigating whether excitatory neurotransmission -especially glutamate neurotransmission- is diminished during hepatic encephalopathy. A large portion of central mammalian neurons use the amino acid glutamate as an excitatory neurotransmitter (6), and the metabolism of glutamate is related closely to ammonia metabolism (7). In vitro and in vivo experiments have shown that both liver failure and hyperammonemia may alter brain glutamate homeostasis. Whole brain glutamate contents were found to be diminished during hepatic encephalopathy or hyperammonemia in patients as well as animals (8-12). Recently, using in vivo brain dialysis, we have demonstrated increased extracellular glutamate levels in the brains of rabbits developing encephalopathy from acute ischemic liver failure or acute hyperammonemia (13). The finding of increased extracellular glutamate concentrations indicates increased glutamate availability in the synaptic cleft, i.e. increased glutamate exposure at the receptor level, during hepatic encephalopathy and hyperammonemia. Increased glutamate concentrations in the cerebrospinal fluid of patients with hepatic encephalopathy suggest that increased glutamate exposure also occurs clinically (14-16). To explain the paradoxical observation of increased excitatory neurotransmitter molecules in a state of neuroinhibition, the following hypothesis was constructed. Initially, increased glutamate levels lead to increased excitation; thereafter prolonged activation of glutamate receptors leads to a compensatory down-regulation (17), which in turn impairs glutamate neurotransmission.

To determine whether the encephalopathy observed in our animal models is associated with changes in the glutamate receptors, we performed binding studies in

brains from rabbits developing encephalopathy from acute liver failure or acute hyperammonemia. As the N-methyl-D-aspartate (NMDA) receptor is the best characterized of the acidic amino acid receptor subtypes (18), we measured the binding of [^{3}H]MK-801 -a specific ligand of the NMDA receptor- to homogenates prepared from different regions from the brains of normal rabbits and rabbits with encephalopathy due to acute ischemic liver failure or acute hyperammonemia.

METHODS

Animals

18 (3 groups of 6) New Zealand white rabbits weighing 2-3 kg were used.

Animal models

Acute liver failure was induced by a two-stage liver devascularization procedure as described earlier (19,20). Under anesthesia a laparotomy was performed: a loose ligature was placed around the hepatoduodenal ligament and guided through a plastic tube through the abdominal wall to the subcutaneous layer of the left subcostal region, and a small-diameter (5 mm) side-to-side portacaval shunt was constructed. During this procedure the superior mesenteric artery was clamped to reduce splanchnic blood stasis. To correct acidosis after release of the vascular clamps 5 ml 8.4% sodium bicarbonate were given intravenously. Postoperatively the rabbits were given 50 ml 10% glucose subcutaneously, followed by standard laboratory chow and water ad libitum. The second day after the operation acute ischemic liver failure was induced by tightening the loose ligature around the hepatoduodenal ligament after giving 50 mg amoxicillin intravenously. The rabbits were subsequently placed in a restraining box. To prevent hypoglycemia 10% glucose was given intravenously, starting at a rate of 3 ml/hr and which was adjusted according to the plasma glucose level when necessary.

Acute hyperammonemia was induced by a prolonged intravenous ammonium-acetate (NH_4Ac) infusion as described earlier (20). After insertion of a Venflon cannula (diameter 0.8 mm, Viggo, Helsingborg, Sweden) into an earvein, NH_4Ac was infused at a constant rate of 6 ml/hr, starting with an initial dose of 0.8 mmol/kg/hr, which was subsequently increased by 0.2 mmol/kg/hr every two hours.

Control rabbits received a sodium/potassium acetate (NaKAc) solution, which was infused -as far as the acetate concentration was concerned- as in the NH_4Ac experiments.

Laboratory measurements

Arterial blood samples were taken from all rabbits studied at 0,1 and 2 hours, and every two hours thereafter for ammonia determination, using an enzymatic method (21). Blood glucose levels were measured hourly (Haemoglucotest, Boehringer, Germany).

Clinical signs of encephalopathy

During study all rabbits were kept in a restraining box. However, at regular time-intervals the rabbits were put into a large cage for clinical evaluation. Clinical signs studied were spontaneous activity, body posture, righting reflex, presence of ataxia and reaction to a painful stimulus. In most rabbits with acute hepatic encephalopathy due to ischemic liver cell necrosis two stages of hepatic encephalopathy are easily recognizable (22). Stage A is characterized by a disturbed righting reflex: the animal will not get up immediately when placed on its side. At stage B the rabbit lies in the cage and cannot achieve the sitting position, even after stimulation, and usually cannot lift its head. All rabbits with ischemic liver cell necrosis and all rabbits receiving NH_4Ac were sacrificed during encephalopathy stage B. The rabbits receiving NaKAc were sacrificed at the same time as the rabbits receiving NH_4Ac. All animals were sacrificed by decapitation.

[^{3}H]MK-801 binding to the MK-801 binding site of the NMDA receptor

Within 10 minutes after decapitation the brain was removed and hippocampus, frontal cortex and striatum were dissected. Tissue samples were sealed in vacuum plastic bags and stored at -70°C until further analysis. The binding of [^{3}H]MK-801 to the MK-801 binding site of the NMDA receptor was studied as described earlier (23-25). Rabbit brain tissue was homogenized in about 50 volumes of buffer with a glass-teflon homogenizer (at 2°C; 5 mM Tris-HCl buffer, pH7.4; Potter S. Braun, FRG). The homogenate was pelleted (15000 x g, 20 min), resuspended by vortexing and pelleted again. Then the homogenate was pelleted (40000 x g, 20 min) and resuspended 4 times in buffer. The protein concentration was measured in the final homogenate with bovine gamma-globulin as standard (26). Binding experiments were carried out at 21°C in plastic microtitre plates in a total volume of 0.22 ml. First, in hippocampus, cerebral cortex and striatum a one-point-binding assay was performed for which the incubation medium consisted of 0.22 ml 5 mM Tris-HCL (pH 7.4) containing 3 nM [^{3}H]MK-801, 5 μM L-glutamate, 5 μM L-glycine, 10 μM $MgCl_2$. Second, in the cerebral cortex saturation studies were performed for which 0.2-80 nM [^{3}H]MK-801 was used. After incubation for 22 hours bound ligand was separated from free ligand by rapid filtration through

Whatman GF/B filters with a Titertek cell harvester followed by a 10 second wash with cold assay buffer (4°C)(27). The filters were transferred into plastic vials, 5 ml of a toluene-based scintillation cocktail was added (Rotiszint 22) and they were monitored for tritium in a Beckman LS 1801 counter at about 54% efficiency. Non-specific binding was defined as that not displaced by 100 μM of unlabelled MK-801. Final estimates of binding parameters were determined with a computerized, non-linear, least squares regression analysis (28).

Because experimental acute liver failure and acute hyperammonemia are known to increase brain water content by about ca. 2% (29,30), calculation was based on pmol/mg protein unit. Such calculation is practically independent of the presence of cerebral edema, as opposed to a calculation based on tissue weight.

Necropsy

After sacrifice of the animal necropsy was performed to exclude gross pathological abnormalities. In the animals with acute ischemic liver cell necrosis the liver was examined carefully to confirm tightening of the ligature.

Statistics

All results are presented as mean$\pm$S.E.M. For statistical analysis the unpaired Student's *t* test when comparing two groups and the Student-Newman-Keuls test when comparing three groups were applied. Statistical significance denotes $p<0.05$.

All experiments were approved by the Ethical Committee on Animal Research of the Erasmus University Rotterdam.

RESULTS

Clinical signs of encephalopathy

Encephalopathy from acute liver failure was characterized by complete loss of spontaneous activity, impaired body posture, absence of the righting reflex, decreased muscle tone and a diminished reaction to a painful stimulus. Rabbits with encephalopathy from acute hyperammonemia exhibited similar symptoms, but in addition had severe ataxia. All animals with acute liver failure and acute hyperammonemia developed encephalopathy stage B, after 14.8$\pm$0.2 and 15.5$\pm$1.9 hours respectively (mean$\pm$SEM, n=6). Two rabbits with acute liver failure died just before decapitation. Control studies

Table I

Mean arterial ammonia levels during acute ischemic liver failure and acute hyperammonemia.

Model	t_0	t_4	t_8	t_{12}	t_{end}
ALF	44±7 μmol/l	176±16	277±35	400±43	533±62
AMM	9±3	299±81	430±85	693±184	955±120*
C	33±10	28±14	25±9	41±16	34±18

Values are derived from six experiments and are expressed as mean±SEM.
ALF acute liver failure, AMM acute hyperammonemia, C controls,
t_0 start of the experiment, t_4-t_8-t_{12} after 4,8 and 12 hours, t_{end} encephalopathy stage B.
* $p<0.05$ AMM vs. ALF

were performed for 14.7±1.8 hours (mean±SEM, n=6); among the control rabbits no spontaneous deaths occurred.

Plasma ammonia levels

During the experiments increases of arterial ammonia levels in the rabbits with acute liver failure and acute hyperammonemia showed a similar pattern. At the start of the experiments ammonia levels tended to be higher in rabbits with acute liver failure (44±7 μmol/l), due to their portacaval shunt, when compared to rabbits with acute hyperammonemia (9±3 μmol/l) or controls (33±10 μmol/l)(all three groups: mean±SEM, n=6). However, the difference did not reach statistical significance ($p>0.05$). During the experiments mean arterial ammonia levels tended to be higher among the rabbits receiving an ammonia infusion, but differences were not statistically different (table I). During encephalopathy stage B the difference in plasma ammonia

Table II

One-point binding assay of [^{3}H]MK-801 to synaptical membranes from normal rabbits and rabbits with severe encephalopathy stage B from acute liver failure or acute hyperammonemia.

Brain area	Model	[^{3}H]MK-801 binding (pmol/mg protein)
Hippocampus	ALF	0.829±0.044
	AMM	0.809±0.031
	C	0.811±0.032
Cerebral cortex	ALF	0.679±0.032
	AMM	0.656±0.038
	C	0.622±0.033
Striatum	ALF	0.453±0.020
	AMM	0.478±0.018
	C	0.480±0.029

Data are derived from six experiments and are expressed as mean±SEM.
ALF acute liver failure, AMM acute hyperammonemia, C controls.

levels between rabbits with acute liver failure (533±62 μmol/l) and rabbits with acute hyperammonemia (955±120 μmol/l) reached statistical significance ($p < 0.05$, mean±SEM, n=6)(table I).

[^{3}H]MK-801 binding to the MK-801 binding site of the NMDA receptor

Homogenates were prepared from the hippocampus, cerebral cortex and striatum

Table III

Saturation studies on binding of [^{3}H]MK-801 to synaptical membranes from cerebral cortex of normal rabbits and rabbits with severe encephalopathy stage B from acute liver failure or acute hyperammonemia.

Model	K_d1 (nM)	$B_{max}1$ (pmol/mg)	K_d2 (nM)	$B_{max}2$ (pmol/mg)
ALF	1.62±0.36	0.63±0.07	265.8±195.9	7.29±4.92
AMM	1.19±0.23	0.45±0.08	182.7±152.7	4.57±2.76
C	2.09±0.38	0.79±0.14	304.6±181.3	4.65±1.99

Data are derived from six experiments and are expressed as mean±SEM.
ALF acute liver failure, AMM acute hyperammonemia, C controls.
B_{max} expressed per mg protein.

as these are major anatomic regions in which glutamate is used for neurotransmission. In control rabbits [^{3}H]MK-801 binding to synaptical membranes in the one-point assay was high in the hippocampus, intermediate in the cortex and low in the striatum. No significant differences in [^{3}H]MK-801 binding were found in rabbits exhibiting severe encephalopathy stage B from acute liver failure or acute hyperammonemia (Table II). The single concentration of [^{3}H]MK-801 (3 nM) labels primarily the high affinity binding site (about 80%) compared to the low-affinity binding site (about 20%)(see below).

Saturation studies of the binding of [^{3}H]MK-801 to homogenates from cerebral cortex demonstrated two different binding sites, one with low and one with high affinity. Compared to corresponding data for controls, the values for K_D and B_{max} were not changed in rabbits with encephalopathy from acute liver failure or acute hyperammonemia (table III).

DISCUSSION

The results of the present study indicate there are no significant alterations in the NMDA receptor population in cerebral cortex -and probably also in hippocampus and striatum- of rabbits with severe encephalopathy from acute liver failure or acute hyperammonemia.

In this study heterogeneity of [^{3}H]MK-801 binding sites was found, as reported recently by other groups (31,32). As discussed before (25), the high affinity binding sites as found with the present technique may be identical to those found previously in rat brain (23). The exact nature of the low-affinity binding site is not clear at present. However, it has been proposed that [^{3}H]MK-801 labels an additional binding site, for instance the nicotinic receptor channel (33-36). With increasing ligand concentrations in saturation experiments, the amount of specific binding is decreasing from about 75% to 40% (24,25). With such low specific binding it is not possible to determine the K_d and B_{max} values of the low-affinity binding site (K_{d2} and B_{max2}) with high precision. This may explain the scatter of the K_{d2} and B_{max2} values.

Results of previous studies reported on glutamate receptors in experimental hepatic encephalopathy have been conflicting (table IV)(37). In rabbits with galactosamine induced acute liver failure, [^{3}H]glutamate binding was diminished (38), which was subsequently shown to be the result of a decreased number of kainate receptors (39). However, in acute liver failure from hepatectomy and tetrachloride in the rat (40), thioacetamide in the rat (41) and ischemic liver necrosis in the rabbit (present study) no changes were found, although in the latter we cannot exclude changes of the kainate and AMPA (amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors. In rats with chronic hepatic encephalopathy, glutamate binding to the NMDA receptor was diminished (42); in dogs with chronic hepatic encephalopathy, NMDA receptor binding appeared normal whereas changes occurred in the kainate and AMPA receptors (43). No changes in glutamate receptors were found in models of acute hyperammonemia in rabbits (38, present study). However, the number of all three subgroups of glutamate receptors were found to be diminished in hyperammonemic rats (44). Thus, during experimental hepatic encephalopathy from acute liver failure there may be a selective loss of kainate receptors (38), with a preservation of NMDA receptors (present study). Chronic hepatic encephalopathy appears to be associated with variable changes of the glutamate receptor populations (42,43). Conflicting results have also been found during hyperammonemia (38,44 and present study).

Table IV

Glutamate receptors in experimental hepatic encephalopathy. A review of the literature.

First author (ref.)	Animal	Model	Glutamate total	NMDA	Kainate	AMPA
Ferenci (39)	Rabbit	Galactosamine	K_D nc B_{max} ↓		K_D nc B_{max} ↓	
Ferenci (38)	Rabbit	Hyperammonemia	K_D nc B_{max} nc			
Watanabe (40)	Rat	Hepatectomy/CCl_4	K_D nc B_{max} nc			
Zimmerman (41)	Rat	Thioacetamide	K_D nc B_{max} nc			
Peterson (42)	Rat	Portacaval shunt		binding ↓	binding nc	binding nc
Maddison (43)	Dog	Congenital pc shunt		K_D nc B_{max} nc	K_D nc B_{max} ↓	abolition of low affinity receptor
Rao (44)	Rat	Acute hyperammonemia	K_D nc B_{max} ↓	binding ↓	binding nc	binding nc
		Chronic ..	K_D nc B_{max} ↓			
Present study	Rabbit	Acute liver failure		K_D nc B_{max} nc		
	Rabbit	Acute hyperammonemia		K_D nc B_{max} nc		

↓ decrease, nc no change

Experimental acute hepatic encephalopathy and acute hyperammonemia appear to be associated with increased extracellular glutamate levels (13,45,46). Increased glutamate concentrations in the cerebrospinal fluid of patients with hepatic encephalopathy suggest that increased extracellular brain glutamate also occurs clinically (14-16). Increased extracellular glutamate together with a normal NMDA receptor population, might lead to glutamate overexposure. In brain ischemia and epilepsy there is evidence indicating that prolonged glutamate exposure is deleterious to the brain (47). In these conditions most toxic effects of glutamate are thought to result from a prolonged depolarizing action at the postsynaptic receptor sites (48-49), in particular the NMDA receptor sites (18). This action is assumed to give rise to membrane permeability changes which lead to impaired ion homeostasis (48,49). Especially increased neuronal calcium influx may be responsible for cell swelling and degeneration (50). Maybe this mechanism is also of importance in encephalopathy from acute liver failure and acute hyperammonemia.

In conclusion, the NMDA receptor population is normal during encephalopathy from acute liver failure and acute hyperammonemia in the rabbit. In addition, as there is experimental evidence of increased extracellular brain glutamate concentrations in these conditions, the concept of glutamate overexposure should receive further attention in studies on the pathogenesis of hepatic encephalopathy.

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CHAPTER 5

$^{45}CaCl_2$ autoradiography in brain from rabbits with encephalopathy from acute liver failure and from acute hyperammonemia.

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SUMMARY

In experimental hepatic encephalopathy and hyperammonemia extracellular levels of glutamate are increased in hippocampus and cerebral cortex. It has been suggested that overstimulation of glutamate receptors causes a pathological calcium entry into neurons via receptor-operated (NMDA- and AMPA-type) or voltage-dependent calcium channels leading to calcium overload and cell death. Neurodegeneration as a result of exposure to excitotoxins, including glutamate, can be localized and quantified using $^{45}CaCl_2$ autoradiography. This approach was used to study cerebral calcium accumulation in rabbits with acute liver failure and acute hyperammonemia.

Acute liver failure was induced in 6 rabbits, acute hyperammonemia in 4 rabbits; 4 control rabbits received sodium-potassium-acetate. At the start of the experiment 500 μCi $^{45}CaCl_2$ was given intravenously. Decapitation followed after development of severe encephalopathy. Autoradiographs were made from brain slices. The ratio dorsal hippocampus:background labelling in whole brain slices was calculated in 12 consecutive brain slices, to obtain the relative optical density in the dorsal hippocampus.

All rabbits with acute liver failure and acute hyperammonemia developed severe encephalopathy, after 13.2 ± 1.7 and 19.3 ± 0.5 hours respectively (mean$\pm$SEM). Plasma ammonia levels were 425 ± 46 and 883 ± 21 μmol/l, then, respectively ($p < 0.05$). Control rabbits maintained normal plasma ammonia levels (13 ± 5 μmol/l), demonstrated normal behaviour throughout the study and were sacrificed after 16 hours.

There was no increased ^{45}Ca accumulation in the hippocampus of rabbits with severe encephalopathy from acute liver failure or acute hyperammonemia. This suggests that, despite increased extracellular brain glutamate levels in these conditions, glutamate neurotoxicity was not important for the development of encephalopathy in our rabbits.

INTRODUCTION

Hepatic encephalopathy is a neuropsychiatric syndrome caused by serious liver disease. Hyperammonemia appears to be an important causative factor (1). Ammonia may disturb normal brain function in many ways (1,2), but the precise mechanism of ammonia toxicity in liver disease is unknown.

The amino acid glutamate is the most common excitatory neurotransmitter in the mammalian brain (3) and the metabolism of glutamate is closely related to that of ammonia (4,5). Experimental acute hepatic encephalopathy and acute hyperammonemia appear to be associated with increased extracellular brain concentrations of glutamate (6-9). In addition, increased glutamate concentrations are also found in cerebrospinal fluid of patients with hepatic encephalopathy (10-12). Increased extracellular glutamate levels may cause overstimulation of glutamate receptors (ionotropic excitatory amino acid receptors: presynaptic kainic acid (KA), and postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartate (NMDA)). In brain ischemia and epilepsy there is evidence indicating that prolonged glutamate exposure is deleterious to the brain (13,14). In these conditions most toxic effects are thought to result from a prolonged depolarizing action at the postsynaptic receptor sites (15,16), in particular the AMPA receptor sites (17). This action is assumed to give rise to membrane permeability changes which lead to impaired ion homeostasis (15,16). Especially increased neuronal calcium influx via opening of the NMDA receptor channel may be responsible for cell swelling and degeneration (18). Maybe this mechanism is also of importance in encephalopathy from acute liver failure and acute hyperammonemia.

The regional distribution of ischemic (19) or excitotoxic (20) brain damage can be visualized and quantified (21) using $^{45}CaCl_2$ autoradiography of brain sections. To determine whether excitotoxic brain injury is of importance in hepatic encephalopathy and ammonia toxicity, we performed $^{45}CaCl_2$ autoradiography studies on brains from rabbits with encephalopathy from acute ischemic liver failure and acute hyperammonemia simulating acute liver failure.

METHODS

Animals

12 New Zealand white rabbits weighing 2-3 kg were used; 6 rabbits with acute

liver failure, 4 rabbits with acute hyperammonemia and 4 control rabbits.

Animal models

Acute liver failure was induced by a two-stage liver devascularisation procedure as described earlier (22,23). Under anesthesia a laparotomy was performed: a loose ligature was placed around the hepatoduodenal ligament and guided through a plastic tube through the abdominal wall to the subcutaneous layer of the left subcostal region, and a small-diameter (5 mm) side-to-side portacaval shunt was constructed. During this procedure the superior mesenteric artery was clamped to reduce splanchnic blood stasis. To correct acidosis after release of the vascular clamps, 5 ml 8.4% sodium bicarbonate were given intravenously. Postoperatively the rabbits were given 50 ml 10% glucose subcutaneously, followed by standard laboratory chow and water ad libitum. The second day after the operation acute ischemic liver failure was induced by tightening the loose ligature around the hepatoduodenal ligament after giving 50 mg amoxicillin intravenously. The rabbits were subsequently placed in a restraining box. To prevent hypoglycemia 10% glucose was given intravenously, starting at a volume of 3 ml/hr and which was adjusted according to the plasma glucose level when necessary.

Acute hyperammonemia was induced by a prolonged intravenous ammonium acetate (NH_4Ac) infusion as described earlier (23). After insertion of a Venflon cannula (diameter 0.8 mm, Viggo, Helsingborg, Sweden) into an earvein, NH_4Ac was infused at a constant volume of 6 ml/hr, starting with an initial dose of 0.8 mmol/kg/hr, which was subsequently increased by 0.2 mmol/kg/hr every two hours. After 16 hours the dose remained constant.

Control rabbits received a sodium/potassium acetate (NaKAc) solution, which was infused -as far as the acetate concentration was concerned- as in the NH_4Ac experiments.

Clinical signs of encephalopathy

During study all rabbits were kept in a restraining box. However, at regular time-intervals the rabbits were put into a large cage for clinical evaluation. Clinical signs studied were spontaneous activity, body posture, righting reflex, presence of ataxia and reaction to a painful stimulus. In most rabbits with acute hepatic encephalopathy due to ischemic liver cell necrosis two stages of hepatic encephalopathy are easily recognizable (24). Stage A is characterized by a disturbed righting reflex: the animal will not get up immediately when placed on its side. At stage B, the rabbit lays in the cage and cannot achieve the sitting position, even after stimulation, and usually cannot lift its head. All

rabbits with ischemic liver cell necrosis and all rabbits receiving NH_4Ac were sacrificed by decapitation during encephalopathy stage B. The rabbits receiving NaKAc were sacrificed after 16 hours.

Laboratory measurements

Arterial blood samples were taken from all rabbits studied, just before the start of the experiment and during severe encephalopathy (stage B). Ammonia was determined with an enzymatic method (25). Blood glucose levels were measured hourly (Haemoglucotest, Boehringer, Germany).

$^{45}CaCl_2$ autoradiography

Autoradiography was essentially performed as described previously (20). Just before the induction of acute liver failure or the start of ammonium acetate or sodium/potassium acetate infusion radioactive calcium chloride (500 μCi $^{45}CaCl_2$ (Amersham) in 1 ml of saline) was given intravenously into an ear vene. With development of encephalopathy stage B, rabbits with acute liver failure or acute hyperammonemia were sacrificed by decapitation; control rabbits were sacrificed after 16 hours. The brains were rapidly removed from the skull, immediately frozen in isobutanol, which was chilled with dry ice, and stored at -70°C prior to further use. Coronal sections (30 μm) through basal ganglia and hippocampus were cut in a cryostat at -20°C, and thaw-mounted onto gelatin-chromalum coated glass slides, dried on a hot plate (40°C) and stored at room temperature in the presence of silica gel for 1 day. Subsequently, the dried sections were exposed to ß-sensitive film (Hyperfilm ßmax, Amersham) in Kodak-X-O-matic cassettes for 7 days and the film was developed in D19 (Kodak).

Autoradiographs were analyzed in a semi-quantitative manner using relative optical density (ROD) and computerized image analysis (MCID software, Imaging Research Inc., Brock University, St. Catherines, Canada). Autoradiographs of ^{45}Ca standards indicated that, relative optical density and radioactivity increase linearly, up to 6 times background level (=ROD in control brain tissue), and that a relative ROD increase of δ% (versus background) represents a relative radioactivity increase (=^{45}Ca increase) of 2δ%. The ratio of ROD dorsal hippocampus versus ROD background (in the rest of the same brain section) was calculated for both hemispheres in 12 consecutive sections (distance between sections 60-90 μm), to obtain the relative increase in ROD in the dorsal hippocampus of each animal. Using this approach a ratio of <1 denotes decreased labelling, >1 increased labelling and 1 unchanged labelling as compared to the rest of the brain.

After exposure to autoradiographic film brain sections were stained with thionine according to standard procedures.

Necropsy

After sacrifice of the animals necropsy was performed to exclude gross pathological abnormalities. In the animals with acute ischemic liver cell necrosis the liver was examined carefully to confirm tightening of the ligature.

Statistics

The results are presented as mean $\pm$ S.E.M. For statistical analysis unpaired Student's *t* test was used. Statistical significance denotes $p<0.05$.

All experiments were approved by the Ethical Committee on Animal Research of the Erasmus University Rotterdam.

RESULTS

Clinical signs of encephalopathy

Encephalopathy from acute liver failure was characterized by complete loss of spontaneous activity, impaired body posture, absence of the righting reflex and decreased muscle tone. One rabbit with acute liver failure developed ataxia. Rabbits with encephalopathy from acute hyperammonemia exhibited similar symptoms, but in addition all four rabbits developed ataxia. One rabbit with hyperammonemia developed generalized seizures and died spontaneously immediately thereafter. All animals with acute liver failure and acute hyperammonemia developed encephalopathy stage B, after 13.2 ± 1.7 ($n=6$) and 19.3 ± 0.5 ($n=4$) hours respectively ($p>0.05$). Control studies were performed for 16 hours; all four control rabbits exhibited normal behaviour at the end of the experiments.

Plasma ammonia and glucose levels

At the start of the experiments ammonia levels tended to be higher in rabbits with acute liver failure (86 ± 22 μmol/l), due to their portacaval shunt, when compared to rabbits with acute hyperammonemia (19 ± 13 μmol/l) or controls (25 ± 9 μmol/l). However, the difference did not reach statistical significance ($p>0.05$). During encephalopathy stage B the difference in plasma ammonia levels between rabbits with

acute liver failure (425±46 μmol/l) and rabbits with acute hyperammonemia (883±21) reached statistical significance ($p<0.05$). At the end of the control experiments ammonia levels had not changed (13±5 μmol/l).

Hypoglycemia was not observed. In control rabbits plasma glucose levels were 8.4±0.5 mmol/l at the start, and 7.6±0.1 mmol/l ($p>0.05$) at the end of the experiments. In rabbits with acute liver failure these levels were 7.9±0.1 mmol/l and 8.2±0.6 respectively ($p>0.05$); and in rabbits with acute hyperammonemia 9.0±0.4 mmol/l and 16.4±2.8 mmol/l respectively ($p<0.05$).

^{45}Ca-autoradiography

Autoradiographs from control rabbits showed a diffuse greyish labelling of the brain slices, and no regional distribution of the label. Autoradiographs from rabbits with acute liver failure and acute hyperammonemia also showed a diffuse greyish labelling. In two rabbits with acute liver failure and in one rabbit with acute hyperammonemia a slight increased labelling of the hippocampal region could be seen (figure 1a). However, quantification of the ratio of ROD hippocampus versus ROD background (=ROD surrounding brain tissue in the same section), revealed no significant differences between rabbits with acute liver failure, acute hyperammonemia and controls (table 1). In addition, the histology of the hippocampal pyramidal cell layer in the sections used for autoradiography, appeared to be normal in both rabbits with acute liver failure and acute hyperammonemia (data not shown). Finally, also in striatum, or any other brain area in the forebrain of rabbits with acute liver failure or acute hyperammonemia, no ^{45}Ca accumulation was found (figure 1b).

DISCUSSION

The present results show that there is no ^{45}Ca accumulation in the brains of rabbits with severe encephalopathy from acute liver failure or acute hyperammonemia. Thus, despite findings of increased extracellular brain levels of glutamate (6-9), as well as increased brain tissue and cerebrospinal fluid levels of the excitotoxin quinolinic acid in both clinical and experimental hepatic encephalopathy (26,27), we could not demonstrate excitotoxic brain damage in experimental acute hepatic encephalopathy using $^{45}CaCl_2$ autoradiography. The latter technique has been shown to be a sensitive tool to demonstrate cerebral calcium accumulation, in pathological conditions associated with

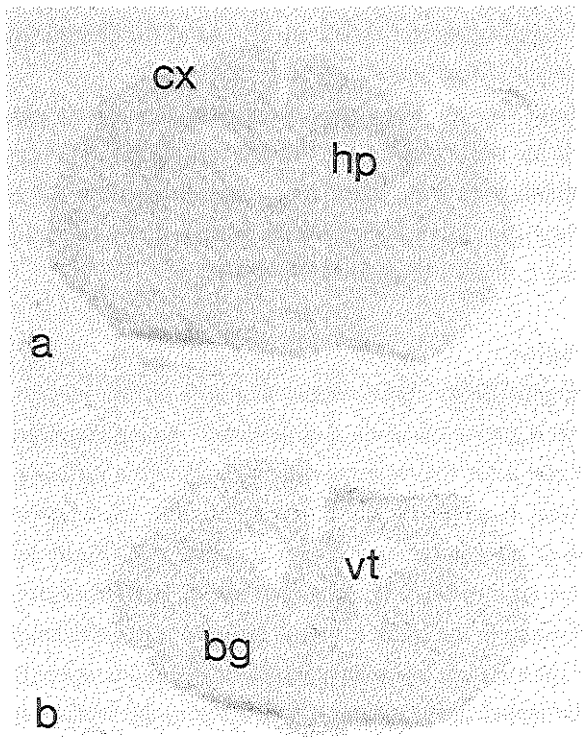

Figure 1a and 1b

^{45}Calcium-autoradiographs of coronal brain slices, from a rabbit with acute liver failure, through the hippocampus (a) and basal ganglia (b). Magnification 3x.
cx=cortex, hp=hippocampus, bg=basal ganglia, vt=ventricle.

excitotoxicity (19-21,28). Furthermore, increases of extracellular brain glutamate, during cerebral ischemia (3.5-8 fold)(29) and hypoglycemia (2.5 fold)(30), conditions considered to be associated with glutamate related neurototoxicity, are similar to those found during acute liver failure (6-8). However, glutamate levels obtained in brain dialysates are a resultant of release from the transmitter pool, leakage of metabolic glutamate from

TABLE

Ratio of relative optical density (ROD) of the hippocampal region and relative optical density of the background in brains from rabbits with acute liver failure and acute hyperammonemia.

Experimental animal		Hippocampus, left	Hippocampus, right
ALF	1	1.011±0.014	1.002±0.014
	2	0.997±0.017	0.999±0.016
	3	1.024±0.011	1.017±0.009
	4	1.021±0.015	0.965±0.009
	5	0.943±0.010	0.907±0.014
	6	1.003±0.007	0.975±0.010
	mean±SEM	1.000±0.012	0.978±0.016
AMM	1	0.961±0.014	0.909±0.007
	2	0.925±0.011	0.918±0.011
	3	0.979±0.012	0.971±0.016
	4	0.978±0.011	0.950±0.010
	mean±SEM	0.961±0.013	0.937±0.014
C	1	0.979±0.011	0.954±0.010
	2	0.937±0.006	0.930±0.010
	3	1.020±0.006	0.993±0.007
	4	1.021±0.011	1.009±0.015
	mean±SEM	0.989±0.020	0.972±0.018

ALF acute liver failure, AMM acute hyperammonemia and C control rabbits.
Ratios in individual rabbits represent the mean±SEM of 12 consecutive brain slices.

different cell types and of the uptake by high affinity glial and presynaptic glutamate uptake systems. Since several attempts to detect changes in extracellular glutamate during both normal and pathophysiological neuronal stimulation, which is supposed to release glutamate into the synaptic cleft, have failed (31), it is questionable whether glutamate in brain dialysates is a good indicator of the glutamate concentration in the excitatory synaps. Thus, increased extracellular brain concentrations of glutamate following experimental acute liver failure, may not be able to stimulate AMPA or NMDA receptors in order to precipitate excitotoxic injury.

Another explanation for our negative results, may be the relative short duration of the pathological condition. After ischemic or excitotoxic brain injury, calcium accumulation and cell death increase up to at least three days after the initial insult (21). We have studied our animals 12-20 hours after the induction of acute liver failure or acute hyperammonemia, i.e. the duration of significant increased extracellular glutamate was even less (6). Thus, in our experimental models there might have been not enough time to develop excitotoxic brain damage. This possible explanation, of course, should be tested in more chronic models of encephalopathy.

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CHAPTER 6

Neuronal and glial marker proteins in encephalopathy associated with acute liver failure, and acute hyperammonemia in the rabbit.

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SUMMARY

Neuronal and glial cell marker proteins were quantified in order to evaluate the possibility of increased proteolysis in the brain of rabbits with acute liver failure and acute hyperammonemia. Acute liver failure was induced by a two-stage liver devascularization procedure. Acute hyperammonemia was induced by a prolonged infusion of ammonium acetate, which simulates the plasma ammonia level in acute liver failure. Control animals received an infusion of sodium/potassium acetate. After development of severe encephalopathy, the animals were sacrificed (13.7 ± 1.3 hours for rabbits with acute liver failure and 20.2 ± 0.8 hours for rabbits with hyperammonemia [n=6, mean$\pm$SEM]) and their brains were dissected into cerebral cortex, hippocampus, cerebellum and brain stem. The total protein content and the concentrations of the neuronal cell marker proteins NSE (neuron specific enolase), NF-68 and NF-200 (68 kDA and 200 kDA neurofilament polypeptides) and the glial cell marker proteins GFAP (glial fibrillary acidic protein) and S-100 were determined. Total protein content was decreased in the brain stem in acute hyperammonemia only. The content of neuronal and glial cell markers was not affected in either of the two conditions. However, low molecular weight proteolytic fragments of the NF-68 polypeptide were observed in the hippocampus of three out of six animals in both experimental groups. No proteolytic degradation of GFAP was observed.

The results show that, in experimental encephalopathy due to acute liver failure and acute hyperammonemia, no major changes occur in the marker proteins. The finding of proteolytic fragments of the NF-68 polypeptide indicates that the neuronal population is affected prior to glial alterations. These findings are in agreement with the concept that acute hepatic encephalopathy is reversible and induces only slight structural changes.

INTRODUCTION

Hepatic encephalopathy is a neuropsychiatric syndrome caused by serious liver disease. Hyperammonemia appears to be an important causative factor (1). Ammonia disturbs normal brain function in many ways (1,2), but the precise mechanism of ammonia toxicity is unknown. We have concentrated on the interference of hyperammonemia with excitatory neurotransmission (3,4).

The amino acid glutamate is the most common excitatory neurotransmitter (5) and the metabolism of glutamate is closely related to that of ammonia (6,7). Increased extracellular concentrations of glutamate were found, in the brain of rabbits with acute ischemic liver necrosis, galactosamine-induced liver necrosis and acute hyperammonemia (3,4) and in the cerebral spinal fluid of rats with acute liver failure (8). Furthermore, increased glutamate concentrations have been demonstrated in the cerebrospinal fluid of patients with hepatic encephalopathy (9-11). This indicates that the receptors are exposed to an increased glutamate level in conditions of hyperammonemia. In vitro experiments have shown that hyperammonemia compromises glutamate re-uptake by astrocytes (12,13). Moreover, astrocytes are critical for ammonia metabolism (7,13) and the most characteristic neuropathological finding in hepatic encephalopathy is the transformation of astrocytes in grey matter into Alzheimer type II glia (13-15).

The extracellular brain glutamate concentration is found to increase 3.5-10 fold in brain ischemia (16) and this is considered crucial to the development of neuronal deficits and cell death in stroke, hypoglycemia and epilepsy (17). In brain ischemia, glutamate toxicity is mediated mainly through activation of postsynaptic receptors. The neurons swell within minutes (18) and die within a day due to excessive calcium influx (19,20). The extracellular brain glutamate concentration is increased 2-5 fold in experimental acute liver failure (3,4). Consequently, glutamate neurotoxicity may also be of importance in the pathogenesis of hepatic encephalopathy.

Glutamate receptor mediated neurotoxicity induces degenerative and/or proliferative changes in the brain. The concentrations of neuronal and glial cell marker proteins have been used to monitor these effects. These markers include: neuron specific enolase (NSE), a neuronal cytoplasmic enzyme (21,22), NF-68 and NF-200, two polypeptide fragments of neuronal intermediate filaments (23), glial fibrillary acidic protein (GFAP), a fibrillary component of the glial intermediate filament (24) and S-100 which is a glial cytoplasmic protein (21). The appearance of breakdown products of neuronal and glial intermediate filaments is a sensitive indicator of excitatory cell injury

(25). This report concerns degenerative and proliferative changes in neurons and glial cells in experimental acute liver failure and acute hyperammonemia.

METHODS

Animals

18 New Zealand white rabbits weighing 2-3 kg were used.

Animal models

Acute liver failure (ALF) was induced by a two-stage liver devascularization procedure as described earlier (26,27). Under anesthesia a laparotomy was performed: a loose ligature was placed around the hepatoduodenal ligament and guided through a plastic tube through the abdominal wall to the subcutaneous layer of the left subcostal region, and a small-diameter (5 mm) side-to-side portacaval shunt was constructed. During this procedure the superior mesenteric artery was clamped to reduce splanchnic blood stasis. To correct acidosis after release of the vascular clamps, 5 ml 8.4% sodium bicarbonate were given intravenously. Postoperatively the rabbits received 50 ml 10% glucose subcutaneously, followed by standard laboratory chow and water ad libitum. The second day after the operation acute liver failure was induced by tightening the loose ligature around the hepatoduodenal ligament after giving 50 mg amoxicillin intravenously. The rabbits were subsequently placed in a restraining box. To prevent hypoglycemia 10% glucose was given intravenously, starting at a volume of 3 ml/hr and adjusted according to the plasma glucose level when necessary. If survival after induction of liver ischemia was less than 6 hours, death was considered to be related to postoperative complications. Only rabbits surviving for at least 6 hours were included in the analysis.

Acute hyperammonemia (AMM) was induced by a prolonged intravenous ammonium acetate (NH_4Ac) infusion simulating the plasma ammonia pattern of the rabbits with acute liver failure (27). After insertion of a Venflon cannula (diameter 0.8 mm, Viggo, Helsingborg, Sweden) into an ear vein, NH_4Ac was infused at a constant volume of 6 ml/hr, starting with an initial dose of 0.8 mmol/kg/hr and increasing the dose by 0.2 mmol/kg/hr every two hours for 16 hours. Control rabbits (C) received a sodium/potassium acetate (NaKAc) solution, which was infused as in the NH_4Ac experiments.

Laboratory measurements

Arterial blood samples were taken at 0,1 and 2 hours, and then every two hours thereafter for ammonia determination, using an enzymatic method (28). Blood glucose levels were measured hourly (Haemoglucotest, Boehringer, Germany).

Clinical signs of encephalopathy

At regular intervals the rabbits were put into a large cage for clinical evaluation. Rabbits with acute liver failure exhibit two easily recognizable stages of encephalopathy (29). Stage A is characterized by a disturbed righting reflex: the animal will not get up immediately when placed on its side. In stage B the rabbit lies in the cage and cannot achieve the sitting position, even after stimulation, and cannot lift its head. All rabbits with acute liver failure and all rabbits receiving NH_4Ac were sacrificed during encephalopathy stage B. The rabbits receiving NaKAc were sacrificed at the same time as the rabbits receiving NH_4Ac. All animals were sacrificed by decapitation.

Brain sampling

Brains were rapidly removed and dissected. Dissection was performed on a plastic plate, placed on ice. Cerebral cortex (left and right fronto-temporal parts), hippocampus (left and right), cerebellum and brain stem were dissected. The samples were packed immediately in plastic bags, which were stored at -70°C until further analysis.

Quantitative analysis of neuronal and glial cell marker proteins

Brain samples were sonified at 90°C and a dot immunobinding procedure was performed (25,30): brain samples were diluted in buffer (120 mM KCl, 20 mM NaCl, 2 mM $NaHCO_3$ and 5 mM Hepes, pH 7.4/0.7% Triton X-100 (vol/vol) to a concentration of ca. 0.5 μg total protein per μl. Using the Minifold II slot-blot apparatus as a template, 20 μl samples were blotted onto nitrocellulose membrane filters. The filters were fixed in 10% acetic acid and 25% 2-propanol. The remaining reactive sites on the filters were blocked with a blocking solution containing 0.5% gelatin. The filters were incubated overnight at room temperature in an antibody solution (blocking solution, 0.1% Triton X-100 and antisera). The antibody preparations were diluted as follows: S-100, 1/500; GFA, 1/2500; NSE, 1/500; NF-68, 1/500 and the supernatant of the monoclonal antibody FE3 against NF-200, 1/100. The specificities of the antibodies have been described in detail (24). After antibody incubation, the filters were reacted with ^{125}I-protein A in the blocking solution containing 0.1% Triton X-100 at a radioactivity of 150,000-200,000

cpm/ml. Finally the filters were dried, cut and assayed for radioactivity in a gamma counter (Automatic Gamma Counter and Sample Change System 54, Nuclear Data Inc.). For determination of the NF-200 polypeptide with a monoclonal antibody, rabbit immunoglobulins to mouse immunoglobulins (diluted 1:1000 in the antibody solution) were used as bridging antibodies since protein A binds very weakly to mouse immunoglobulins. The amount of each of the specific proteins in the unknown samples was quantified. First, a reference sample was prepared from a mixture of all control samples from the frontotemporal cerebral cortex and standard curves were constructed by means of dilution. The amount of protein was found by interpolation of the standard curves. It was expressed in arbitrary units (AU), defined as the amount of protein in 20 μl of the reference sample. The total protein concentration in the samples was determined with a commercial assay (Pierce) on microtiter plates. Bovine serum albumin was used as a standard (30).

Qualitative analysis of neuronal and glial intermediate filaments

Equal amounts of proteins applied to SDS-PAGE gels were separated by electrophoresis with a linear 6-12 % acrylamide gradient (31). The proteins were then transferred to 0.45 μm nitrocellulose membranes (Schleicher & Schuell, Keen, NH, USA) for antibody incubation (32). The antibody preparations were diluted in the following manner: rabbit anti-human GFA, 1/10000 and rabbit anti-bovine neurofilament NF-68, 1/1000. Preparation of the rabbit antisera used in this study, as well as determination of their specificities, was described elsewhere (33). Goat-anti-rabbit IgG-alkaline phosophatase was used as the secondary antibody and BCIP/NBT as substrate (BIO-RAD, Richmond, CA, USA).

Necropsy

Necropsy was performed to exclude gross pathological abnormalities. In the animals with acute ischemic liver necrosis the liver was examined carefully to confirm tightening of the ligature.

Statistics

The results are presented as mean$\pm$S.E.M.. For statistical analysis the unpaired Student's *t* test and the Student-Newman-Keuls procedure were used. Statistical significance was indicated by $p<0.05$. All experiments were approved by the Ethical Committee on Animal Research of the Erasmus University Rotterdam.

Table I

Mean arterial ammonia levels (in μmol/l) during acute liver failure, acute hyperammonemia and control experiments.

	t_0	t_4	t_8	t_{12}	t_{end}
C	51±4	40±5	33±1	29±2	35±3
ALF	120±24*	247±26*	313±37*	481±109*	518±97*
AMM	49±3	216±23*	315±34*	370±39*	535±117*

C controls, ALF acute liver failure, AMM acute hyperammonemia.
t_0 start of the experiment, t_4 after 4 hours etc., t_{end} end of experiment during severe encephalopathy (stage B).
Values are expressed as mean±SEM; each group consisted of 6 rabbits.
* $p<0.05$ with respect to controls (Student-Newman-Keuls test).

RESULTS

Clinical signs of encephalopathy

Encephalopathy due to acute liver failure was characterized by complete loss of spontaneous activity, impaired body posture, absence of the righting reflex, decreased muscle tone and a diminished reaction to a painful stimulus. Rabbits with encephalopathy due to acute hyperammonemia exhibited similar symptoms but also had severe ataxia. All animals with acute liver failure and all animals with acute hyperammonemia developed encephalopathy stage B after 13.7±1.3 and 20.2±0.8 hours respectively (n=6, mean±S.E.M., $p<0.05$). Two rabbits with acute liver failure died just after stage B was recognized. Control studies were performed for 18-20 hours.

Table II

Brain total protein content (μg/mg wet weight) in acute liver failure, acute hyperammonemia and control experiments.

	C	ALF	AMM
Cortex[1]	73±1.5	73±1.4	73±1.2
Hippocampus[1]	83±1.1	86±1.3	81±1.4
Cerebellum	72±0.4	74±2.3	70±1.3
Brainstem	66±1.0	66±1.1*	60±1.9*

C controls, ALF acute liver failure, AMM acute hyperammonemia.

[1]Values are the mean of two (left and right) samples.

Each group consisted of 6 rabbits. Values are expressed as mean±SEM.

*$p < 0.05$ AMM with respect to C, and ALF with respect to AMM (Student-Newman-Keuls test).

Plasma ammonia levels

During the experiments the increases in the arterial ammonia levels in rabbits with acute liver failure and acute hyperammonemia were similar in pattern. At the start of the experiments ammonia levels were significantly higher in ALF-rabbits (120±24 μmol/l), due to their portacaval shunt, compared to AMM- (49±3 μmol/l) or C-rabbits (51±4 μmol/l)($p < 0.05$). During encephalopathy stage B, the plasma ammonia levels in ALF-rabbits (518±97 μmol/l) and AMM-rabbits (535±117 μmol/l) did not differ significantly ($p > 0.05$)(table I).

Table III

Neuronal and glial marker proteins (AU/mg wet weight) in acute liver failure, acute hyperammonemia and control experiments.

Cerebral cortex[1]	C	ALF	AMM
GFAP	0.91±0.11	0.80±0.04	0.93±0.16
S-100	0.95±0.04	0.98±0.04	0.96±0.06
NSE	0.79±0.03	0.84±0.03	0.78±0.04
NF-68	0.84±0.04	0.83±0.03	0.94±0.06
NF-200	0.86±0.06	0.85±0.07	0.85±0.08
Hippocampus[1]	**C**	**ALF**	**AMM**
GFAP	0.93±0.10	0.82±0.04	0.95±0.11
S-100	0.94±0.05	0.95±0.03	0.93±0.06
NSE	0.84±0.04	0.86±0.05	0.78±0.02
NF-68	0.83±0.02	0.88±0.06	0.81±0.05
NF-200	0.70±0.05	0.69±0.03	0.69±0.06

C controls, ALF acute liver failure, AMM acute hyperammonemia.

[1] Values are the mean of two (left and right) samples. Values are expressed in arbitrary units (AU), defined as the amount of protein in 20 μl of a reference sample (see methods). Each group consisted of 6 rabbits. Values are expressed as mean±SEM.

Total protein content and neuronal and glial cell marker proteins

Total protein content was measured in cerebral cortex, hippocampus, brain stem and cerebellum (table II). During acute liver failure no changes in the total protein content were found. During acute hyperammonemia the total protein content of the brain stem was found to be decreased, compared to control rabbits ($p < 0.05$); no changes were found in cortex, hippocampus and cerebellum. The amounts of the neuronal (NSE, NF-68 and NF-200) and glial (GFAP and S-100) cell marker proteins, in the cerebral cortex and hippocampus, during acute liver failure and acute hyperammonemia were not different from those found in control rabbits (table III). Similar results were obtained in the cerebellum and brain stem (not shown).

Qualitative alterations of glial and neuronal intermediate filaments

Sonified brain samples containing equal amounts of the NF-68 polypeptide and the GFAP from the hippocampus, were investigated by the immunoblot technique using polyclonal rabbit antisera against the polypeptides (25). In both experimental groups, three out of six experimental animals, showed a visual increase -compared to the controls- in low molecular breakdown products of the neurofilament 68 kD polypeptide with molecular weights of 46 and 48 kD (figure 1). The increase seemed to be more pronounced among the animals infused with ammonia. No alterations of the immunoblot staining pattern, of GFAP from the hippocampus of these animals were detectable (not shown).

DISCUSSION

The concentrations of neuronal (NSE, NF-68 and NF-200) and glial (GFAP and S100) cell markers were unaffected in experimental encephalopathy, due to acute liver failure and acute hyperammonemia; the total protein content was lowered only in the brain stem of animals infused with ammonia. However, an increased amount of low molecular breakdown products of NF 68kD was found in the hippocampus of both treated groups.

The degradation pattern of NF-68 is similar to that induced by calcium activated neutral proteases in vitro (34), since the major breakdown products have an approximate molecular weight of 50 kD. Similar breakdown products were observed after in vivo stimulation of hippocampal NMDA receptors in rats (25).

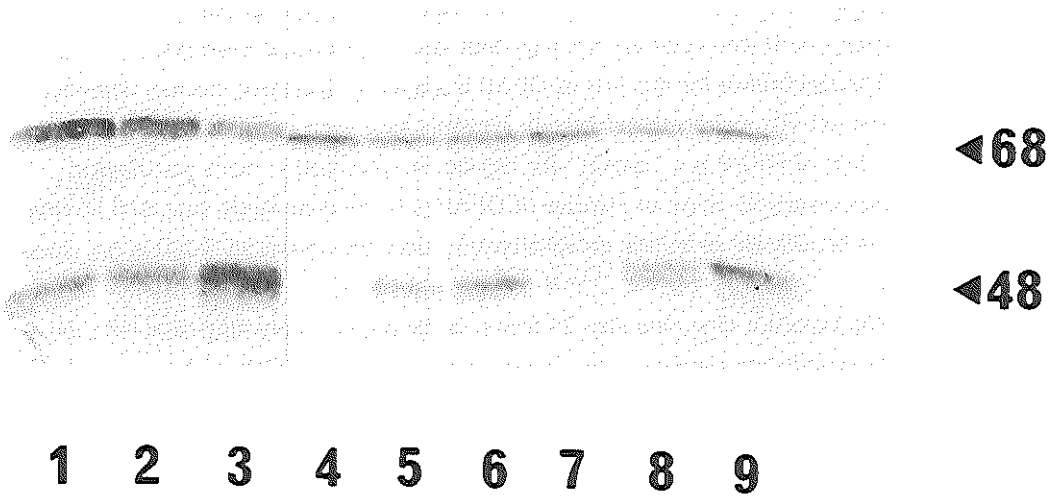

Figure 1

Immunoblots of NF68-like polypeptides in the rabbit hippocampus after acute liver failure (lane **2,5,8**) and after acute hyperammonemia (lane **3,6,9**), as compared to controls (lane **1,4,7**). 30 μg tissue protein were applied to each lane and rabbit anti-bovine NF-68 serum was used for detection. Molecular weights (kD) are indicated to the right of the immunoblots.

Changes in glial proteins are the principal finding in hepatic encephalopathy. Cultured astrocytes showed a selective decrease in GFAP after four days of exposure to 10 mM ammonium chloride (35), and there was a selective loss of GFAP in the grey matter of patients who died of liver cirrhosis and Alzheimer type II gliosis (36,37). Alzheimer type II astrocytes are not seen until after 5-10 days of treatment (15,38,39).

The mechanism for this loss of GFAP is unknown. Increased calcium-dependent proteinase activity and decreased protein synthesis have been proposed (35,40). This specific loss of GFAP is a "marker" for hepatic encephalopathy; other pathological conditions invariably show an increase of GFAP (13). Predominantly structural proteins appear to be affected in hepatic encephalopathy, since the content of S-100 is not altered (41,42).

The exposure time (less than 24 hours) in the present study may have been too short for quantitative changes in the proteins to occur. The qualitative change in NF-68 seen in the hippocampus of animals with acute liver failure and acute hyperammonemia, indicates the involvement of calcium-activated proteolytic enzymes. This could be the result of (early) NMDA-receptor mediated neurotoxicity (18); however, quantitative neurofilament degeneration is lacking. Whether these findings are of importance for the pathogenesis of hepatic encephalopathy remains uncertain. More experimental work on this issue is needed in animal models simulating chronic hepatic encephalopathy.

However, the results are in agreement with the concept that acute encephalopathy due to acute liver failure or acute hyperammonemia is a reversible condition (43), which is not characterized by extensive structural changes in the brain.

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CHAPTER 7

Discussion.
What is the mechanism of ammonia toxicity in hepatic encephalopathy?

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INTRODUCTION

Ammonia appears to fullfill largely the requirements named by Zieve (1), for a substrate to be considered a toxin relevant for the development of hepatic encephalopathy: 1. patients presenting with hepatic encephalopathy usually have elevated plasma ammonia levels (2), 2. the administration of ammonia to patients with liver disease or to animals induces encephalopathy (3,4), and 3. the treatment modalities aimed at lowering plasma ammonia levels are the most effective (5). The exact mechanism how hyperammonemia contributes to the development of encephalopathy in liver disease is, however, still unknown. Here we will attempt to summarize the pathophysiology of hyperammonemia, and to assess its significance in the pathogenesis of hepatic encephalopathy.

In theory, toxins may influence central nervous system functioning at three levels: 1. basic cell metabolism (e.g. pH regulation, water and energy balance), 2. electrophysiologic membrane function (e.g. ionic pumps) and 3. biochemical communication between neurons (i.e. synaptic neurotransmission). Ammonia has been shown to interfere with brain function at all three levels.

BASIC CELL METABOLISM

In hepatic encephalopathy there is a decrease of brain cell metabolism, reflected by decreased oxygen and glucose consumption (6). Recently it was shown that the changes in cerebral blood flow and glucose consumption, in patients with liver cirrhosis are comparable to those in experimental hyperammonemia in animals (7,8). These findings are highly suggestive for ammonia being the cause of decreased brain metabolism. Whether decreased brain metabolism is a direct effect of ammonia or is a reflection of lower energy demand because of lower neuronal activity, is not yet known.

Brain edema, an important complication of acute liver failure (9), reflects disturbed cerebral water balance. Brain edema also occurs in experimental acute liver failure and hyperammonemia (4,10-12). Probable mechanisms are inhibition of neuronal Na^+-K^+-ATPase and disturbances of the integrity of the blood-brain barrier (13,14). Recently, the accumulation of osmogenic amino acids such as glutamine, which increases during hyperammonemia, has been proposed to contribute to the development of cerebral edema in acute liver failure (15). These findings demonstrate the relevance of older

studies in which methionine sulfoximine, an inhibitor of glutamine synthesis, was shown to delay ammonia induced neurological deterioration in mice (16). The beneficial effects of methionine sulfoximine were confirmed recently (17). Others have proposed that cell swelling could be the result of activation of a calcium-dependent pathway (12). In this regard our findings of increased extracellular brain glutamate levels during experimental acute liver failure and acute hyperammonemia in relation to excitotoxicity are of particular interest (see below)(18).

ELECTROPHYSIOLOGIC MEMBRANE FUNCTION

Ammonia disturbs the generation of both inhibitory and excitatory postsynaptic potentials. Under normal conditions the inhibitory neurotransmitters GABA and glycine open chloride channels after binding to their receptors. This induces hyperpolarisation through an influx of chloride, and the neuron looses its ability to fire (19). Ammonia inactivates the chloride extrusion from neurons (20), thereby abolishing the concentration gradient for chloride across the neuronal membrane. As a consequence, the opening of chloride channels by GABA or glycine is no longer followed by an influx of chloride into the neurons. Thus ammonia impairs postsynaptic inhibition in cerebral cortex, thalamus, brainstem and spinal cord (21).

Ammonia decreases intracellular potassium and increases extracellular potassium, by a mechanism yet unknown (22-24). However, this has a depolarizing effect on neurons, which interferes with the generation of excitatory postsynaptic potentials (21). The changes in intra- and extracellular potassium may result in a presynaptic conduction block (25), which means that a decreased number of synaptic terminals excrete the excitatory neurotransmitter glutamate on postsynaptic neurons. Consequently, the excitatory synaptic potential decreases, and thereby the generation of action potentials in the postsynaptic neurons. This can reduce excitatory neurotransmission (21).

BIOCHEMICAL COMMUNICATION BETWEEN NEURONS

The effects of ammonia on synaptic biochemical neurotransmission are mainly mediated through glutamate. A large portion of central mammalian neurons use the amino acid glutamate as an excitatory neurotransmitter (26), and the metabolism of glutamate is

related closely to that of ammonia (27). In vitro and in vivo experiments, have shown that ammonia alters brain glutamate homeostasis. Whole brain glutamate contents were found to be diminished during hepatic encephalopathy or hyperammonemia in patients as well as animals (28-31). Thus, it was assumed that encephalopathy could originate from an ammonia induced deficit of glutamate. However, these were whole brain studies in which the total amount in all anatomical compartments (vascular, intracellular and extracellular) was assessed. The concentration of a neurotransmitter in the synaptic cleft, a part of the extracellular space, is probably the best reflection of neurotransmitter function. Since less than 1% of the total amount of amino acid in the brain is found in the extracellular space, total brain measurements are not suitable for determining neurotransmitter function (32). Extracellular brain glutamate concentrations appear to be increased (19,33-35). Increased glutamate concentrations in cerebrospinal fluid of patients with hepatic encephalopathy suggest that increased glutamate levels also occur clinically (36-38).

Increased extracellular glutamate levels in the brain may have two effects: it could lead to compensatory down-regulation of glutamate receptors and/or there could be a direct toxic effect. Results in experimental animals are conflicting with regard to receptor changes, some have found evidence for down-regulation (39-43), whereas others did not find any changes (44-46). Differences in outcome may have been caused by differences in animal models used, with more changes occurring in more chronic models. The only study on patients with chronic liver disease published until now, also failed to demonstrate changes in the glutamate receptor (NMDA-subtype)(47). Most investigators have studied only one subtype of glutamate receptors or have measured total glutamate binding. With the increasing knowledge on different subtypes with different functions it is important that a clear distinction between all the known glutamate receptor subtypes is made (NMDA, kainate and AMPA)(48).

Toxic effects may result from increased extracellular concentrations. It is logical to assume that, especially with a normal number of glutamate receptors, increased extracellular glutamate levels may lead to glutamate overexposure. In brain ischemia and epilepsy there is evidence indicating that prolonged glutamate exposure is deleterious to the brain (49). In these conditions most toxic effects of glutamate are thought to result from a prolonged depolarizing action at the postsynaptic receptor sites (50,51), in particular the NMDA receptor sites (48). This action is assumed to give rise to permeability changes, which lead to impaired ion homeostasis (50,51). Especially increased calcium influx may be responsible for cell swelling and degeneration (52). Until now only a few studies on glutamate toxicity and/or calcium metabolism in hepatic

encephalopathy and hyperammonemia have been reported. Ammonia decreases the calcium concentration in primary astrocyte cultures (53). No changes were found in brains from rabbits with acute liver failure or acute hyperammonemia using ^{45}Ca-autoradiography (54). The finding of proteolytic peptide fragments in the brains of rabbits with acute liver failure and acute hyperammonemia, suggests that hepatic encephalopathy and hyperammonemia may be associated with some excitotoxic brain damage (55). However, based on these studies important excitotoxic brain damage in hepatic encephalopathy appears rather unlikely, although this topic warrants further study.

In summary, ammonia has a variety of biochemical and neurological effects. Most important seem the effects on electrophysiologic membrane function (disturbance of the generation of inhibitory and excitatory postsynaptic potentials) and on glutamate neurotransmission and metabolism. The accumulation of glutamine may be basic to the development of brain edema. Based on current knowledge about ammonia toxicity, it is a challenge for future studies not to investigate whether ammonia has a role in the development of hepatic encephalopathy, but to improve our understanding of the major toxic actions of ammonia and to make them amenable to manipulation, i.e. treatment.

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CHAPTER 8

Summary and conclusions/Samenvatting en conclusies

SUMMARY AND CONCLUSIONS

Ammonia has been considered the prime toxin in the pathogenesis of hepatic encephalopathy. However, because the correlation between plasma ammonia and degree of encephalopathy is often found to be poor, the ammonia hypothesis has been rejected by many. Currently, there is renewed interest for ammonia and its role in the development of hepatic encephalopathy.

For a substrate to be considered a toxin, relevant for the development of hepatic encephalopathy, three criteria should be fullfilled: 1. the toxin should be present in abnormally high amounts in the presence of hepatic encephalopathy, 2. administration of the substrate should induce hepatic encephalopathy, and 3. correction of the abnormality should reverse hepatic encephalopathy. Indeed, ammonia fullfills these criteria largely (**chapter 1**). The aim of this thesis was to study the mechanism of ammonia toxicity in hepatic encephalopathy.

Hepatic encephalopathy has been thought of as a dysbalance between inhibitory and excitatory neurotransmitters. Glutaminase, an enzyme essential for the formation of glutamate, is inhibited by ammonia. Thus it was assumed that ammonia decreases the amount of glutamate in the brain, which would probably have an effect on normal brain function. Subsequently, the amount of glutamate was shown to be decreased in autopsied brain tissue. However, these were whole brain studies in which the total amount of glutamate was assessed in all anatomical compartments together. The concentration of a neurotransmitter in the synaptic cleft, a part of the extracellular space, is probably the best reflection of neurotransmitter function. Since less than 1% of the total amount of amino acid in the brain is found in the extracellular space, total brain measurements are not suitable for determining neurotransmitter function. The brain dialysis technique has made the extracellular space more accessible for research. Therefore, we decided to use brain dialysis in the study of brain glutamate in experimental hepatic encephalopathy.

Reproducible animal models are needed for the study of hepatic encephalopathy. In the rabbit we induce acute ischemic liver necrosis and acute hyperammonemia. In the latter model plasma ammonia levels found in acute ischemic liver necrosis are simulated. Our animal models appear reproducible and suitable to be used in the study on hepatic encephalopathy and ammonia toxicity (**chapter 2**): encephalopathy develops in all animals studied, and drop outs occur seldomly. There are important resemblances: in both models, encephalopathy is characterized by loss of spontaneous activity, loss of body posture, disappearance of the righting reflex and hypotonia; ataxia is observed more

frequently in acute hyperammonemia. Extrahepatic complications, which occur in both models are cerebral edema and lactic acidosis; renal failure, hypothermia and sepsis only occur in acute ischemic liver necrosis.

Using the brain dialysis technique in the cortex of rabbits with acute liver failure and acute hyperammonemia, we could not find a decrease of neurotransmitter glutamate. Contrary, our results showed evidence for increased basal levels of extracellular brain glutamate. During both conditions, the veratridine- and potassium-evoked release of glutamate appeared unchanged. We concluded that encephalopathy from acute liver failure or acute hyperammonemia, is not caused by a glutamate deficit. Contrary, we found evidence for increased exposure of glutamate receptors to their ligand.

How can increased extracellular brain glutamate levels induce encephalopathy? In theory, it could have two distinct effects: it could lead to down-regulation of glutamate receptors, or could have direct toxic effects. Binding of the ligand MK-801 to the MK-801 binding site of the N-methyl-D-aspartate receptor was unchanged in both acute liver failure and acute hyperammonemia (**chapter 4**).

In brain ischemia and epilepsy there is evidence indicating that prolonged glutamate exposure is deleterious to the brain. Prolonged glutamate exposure may lead to impaired ion homeostasis; especially increased calcium influx may be responsible for cell swelling and degeneration. We postulated that this mechanism could also be of importance in the pathogenesis of hepatic encephalopathy. However, using calcium-autoradiography we could not find increased calcium uptake in the brain of rabbits with encephalopathy from acute liver failure or acute hyperammonemia (**chapter 5**). In addition, there was no siginificant break-down of neuronal (neuron specific enolase, NF-68, NF-200) or glial (glial fibrillary protein, S-100) proteins, which are known to be increased after excitotoxic brain injury.

In conclusion, ammonia appears to be of importance in the pathogenesis of hepatic encephalopathy. Experimental acute hyperammonemia resembles acute liver failure in many important aspects. Hepatic encephalopathy is not due to an ammonia induced glutamate deficit; contrary, the availability of glutamate in the extracellular space appears to be increased. However, this increase does not give rise to important excitoxic brain damage in our animal models.

SAMENVATTING

In studies naar de pathogenese van hepatische encephalopathie, is ammoniak van begin af aan beschouwd als een belangrijk toxine. Vanwege echter het ontbreken van een duidelijke correlatie tussen de plasma ammoniak spiegel en de mate van encephalopathie, werd de ammoniak hypothese door velen verworpen. Sinds enige tijd staat de ammoniak hypothese weer in het middelpunt van de belangstelling.

Leslie Zieve heeft in het begin van de jaren tachtig criteria opgesteld, analoog aan de postulaten van Koch, waaraan een substraat moet voldoen voordat het als relevant toxine in de pathogenese van hepatische encephalopathie kan worden beschouwd: (1) het toxine moet in verhoogde concentratie voorkomen bij patienten met hepatische encephalopathie, (2) toediening van het toxine moet encephalopathie veroorzaken en (3) verwijdering van het toxine moet leiden tot een vermindering van de encephalopathie. Ammoniak blijkt grotendeels aan deze criteria te voldoen (**hoofdstuk 1**). Het doel van dit proefschrift was het bestuderen van het mechanisme van ammoniak-toxiciteit bij hepatische encephalopathie.

Bij aanvang van het hier beschreven onderzoek, was een verstoring van het evenwicht tussen inhibitoire en excitatoire neurotransmitters een belangrijke hypothese. Ammoniak zou door remming van het enzym glutaminase, de synthese van de belangrijkste excitatoire neurotransmitter glutamaat blokkeren en zo encephalopathie kunnen induceren. Vele studies leken dit te bevestigen, echter in de meeste van deze studies werd de glutamaatconcentratie in homogenaten bestudeerd, d.w.z. de som van de concentraties in de extra- en intracellulaire ruimten. Neurotransmissie vindt plaats in de synaps, een onderdeel van de extracellulaire ruimte. De hoeveelheid glutamaat in de extracellulaire ruimte bedraagt minder dan 1% van het totale glutamaat aanwezig in de hersenen; meting van de hoeveelheid glutamaat in hersenhomogenaten geeft dan ook geen informatie over de beschikbaarheid van glutamaat voor neurotransmissie. Met behulp van hersendialyse, een techniek waarmee extracellulaire concentraties van laagmoleculaire stoffen kunnen worden bestudeerd, wilden wij meer inzicht verkrijgen in de beschikbaarheid van glutamaat voor neurotransmissie, bij konijnen met acute leverinsufficiëntie en acute hyperammonemia.

Om het mechanisme van ammoniak-toxiciteit bij hepatische encephalopathie te bestuderen, dient men over reproduceerbare diermodellen te beschikken. Wij maakten gebruik van konijnen met een acute ischemische levernecrose, en konijnen met een acute hyperammonemie, waarbij het plasma ammoniak gehalte hetzelfde beloop heeft als bij

acute ischemische levernecrose. Deze diermodellen bleken in de praktijk zeer goed te functioneren (**hoofdstuk 2**). Bij alle konijnen ontstond encephalopathie, terwijl het aantal uitvallers buitengewoon laag was. Het klinische beeld van encephalopathie vertoonde belangrijke overeenkomsten. In beide modellen werd de encephalopathie gekarakteriseerd door verminderde activiteit, abnormale lichaamshouding, afwezigheid van de "righting reflex" en hypotonie. Encephalopathie ten gevolge van acute hyperammonemia werd bovendien gekarakteriseerd door ataxie, hetgeen slechts een enkele maal bij acute ischemische levernecrose werd gezien. Behoudens encephalopathie werden in beide modellen aanwijzingen gevonden voor cerebraal oedeem en lactaat acidose; nierinsufficiëntie, hypothermie en sepsis werden alleen bij acute ischemische levernecrose gevonden.

In de cortex van konijnen met acute leverinsufficiëntie en acute hyperammonemie werden geen aanwijzingen gevonden voor een tekort aan neurotransmitter glutamaat (**hoofdstuk 3**). Met behulp van continue dialyse, tijdens ontwikkeling van acute leverinsufficiëntie en acute hyperammonemie, vonden wij zelfs een toename van het glutamaatgehalte in het dialysaat (respectievelijk, een toename met een factor 2 en 1,7). De veratridine- en kalium-geïnduceerde afgifte van neurotransmitter glutamaat was ten opzichte van controle-konijnen niet afgenomen. Wij concludeerden, dat encephalopathie ten gevolge van acute leverinsufficiëntie en acute hyperammonemia niet gepaard gaat met een verminderde beschikbaarheid van glutamaat voor neurotransmissie. In tegendeel, wij menen dat de verhoogde glutamaat-concentraties duiden op een toegenomen bezetting van de glutamaatreceptoren.

Hoe kan een verhoogde glutamaat concentratie in de extracelullaire ruimte encephalopathie veroorzaken? De theoretische mogelijkheden zijn een down-regulation van de glutamaatreceptoren of direct toxische effecten. In dezelfde diermodellen werden bindingsstudies met MK-801 uitgevoerd, een stof die selectief aan een belangrijke klasse glutamaat-receptoren bindt (N-methyl-D-aspartaat)(**hoofdstuk 4**). Noch bij acute leverinsufficientie, noch bij acute hyperammonemie werden veranderingen in de binding van MK-801 gevonden.

De mogelijkheid van direct toxische effecten werd afgeleid uit de wetenschap dat bij hersenischemie een verhoogde extracellulaire cerebrale glutamaat concentratie voor een groot deel verantwoordelijk is voor de post-ischemische schade. Een verhoogde glutamaatconcentratie leidt tot een verhoogde intracellulaire calciumconcentratie, hetgeen toxisch en op den duur zelfs lethaal is voor een cel. Met behulp van calcium-autoradiografie konden wij geen verhoogde of afwijkende calciumopname aantonen in de

hersenen van konijnen met encephalopathie als gevolg van acute leverinsufficientie of acute hyperammonemia (**hoofdstuk 5**). Bovendien vonden wij geen belangrijk afwijkende hoeveelheden afbraakproducten van neuronale en gliale eiwitten aantonen (neuron specifiek enolase, NF-68, NF-200, "glial fibrillary protein" en S-100)(**hoofdstuk 6**), die vrijkomen bij excitatoir gemedieerde beschadiging.

Op grond van bovenstaande gegevens concluderen wij, dat ammoniak van belang is voor het ontstaan van hepatische encephalopathie; ammoniak infusie leidt tot encephalopathie welke zeer veel overeenkomsten vertoont met encephalopathie bij acute leverinsufficiëntie. Encephalopathie ten gevolge van acute leverinsufficiëntie en acute hyperammonemie gaat niet gepaard met een tekort aan neurotransmitter glutamaat; in tegendeel, de concentratie lijkt zelfs verhoogd. Echter, deze verhoging geeft geen aanleiding tot belangrijke glutamaattoxiciteit in de door ons bestudeerde modellen.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 19 juli 1961 te Schiedam. Hij groeide op in Schiedam, Maassluis en Spijkenisse, en behaalde zijn eindexamen V.W.O. aan de openbare scholengemeenschap "De Ring van Putten" te Spijkenisse in 1979. In verband met uitloting werd in dat jaar de studie Scheikunde aangevangen aan de Rijksuniversiteit Leiden. Een jaar later werd begonnen met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Als onderdeel van het Kandidaatsexamen deed hij, onder leiding van Prof. Dr. S.W. Schalm, een half jaar onderzoek op de afdeling Interne Geneeskunde II van de Erasmus Universiteit (hoofd: Prof. J.H.P. Wilson) naar de pathogenese van hepatische encephalopathie. Na het behalen van het kandidaatsexamen in 1983 continueerde hij als student-assistent dit onderzoek, waarmee de basis voor het huidige onderzoek werd gelegd. Na het behalen van het doctoraal examen was hij werkzaam op de Liver Unit (hoofd: dr. E.A. Jones) van de National Institutes of Health te Bethesda, U.S.A., in de periode oktober t/m december 1985, alwaar een begin gemaakt werd met dierexperimenteel onderzoek. Na het behalen van het artsexamen in 1987, werkte hij gedurende 3 jaar aan het onderzoek naar de pathogenese van hepatische encephalopathie. Tevens verzorgde hij toen de coördinatie van hepatitis B vaccinatie van medische studenten. Vanaf 1-1-1991 is hij in opleiding tot internist in het Westeinde Ziekenhuis te Den Haag (opleider: dr. E.J. Buurke).