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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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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.

KEYWORDS: hepatic encephalopathy; ammonia; glutamate receptors; NMDA receptors; MK-801.

Abbreviations used in the text: NMDA_N-methyl-D-aspartate; NH_4Ac__ammoniumacetate;NaKAc__sodium/potassium-acetate; AMPA__amino-3-hydroxy-5-methylisoxazole-4-propionic acid

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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 (Stahl, 1963), administration of ammonium-salts to patients with chronic liver disease provokes encephalopathy (Phillips *et al.*, 1952) and lowering of plasma ammonia by lactulose therapy often improves encephalopathy (Bircher *et al.*, 1971). Furthermore proliferation of astrocytes, the morphological substrate of chronic hepatic encephalopathy, is also found in patients with the congenital hyperammonemia syndromes (Norenberg *et al.*, 1985). 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 (Schenker, 1989). 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 (Fonnum, 1984), and the metabolism of glutamate is related closely to ammonia metabolism (Cooper and Plum, 1987). 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 (Hindfelt *et al.*, 1977; Watanabe *et al.*, 1985; Lavoie *et al.*, 1987; Bosman *et al.*, 1990; Swain *et al.*, 1992). 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 (De Knegt *et al.*, 1993*a*). 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 (Van Sande *et al.*, 1970; Vergara *et al.*, 1974; Watanabe *et al.*, 1984). 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 (Mukherjee *et al.*, 1985), which in turn impairs glutamate neurotransmission.

To determine whether the hepatic 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 (Fagg *et al.*, 1991), 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 (Fick *et al.*, 1987; Fick *et al.*, 1989). 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 (Fick *et al.*, 1989). After insertion of a Venflon cannula (diameter 0.8 mm, Viggo, Helsingborg, Sweden) into an ear vein, 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 (Da Fonseca-Wolheim, 1973). 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 (Van der Rijt *et al.*, 1990). 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 (Wong *et al.*, 1988, Kornhuber *et al.*, 1989*a*, Kornhuber *et al.*, 1989*b*). 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 (Bradford, 1976). 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 mM L-glutamate, 5 mM glycine, 10 mM $MgCl_2$. 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)(Kloog *et al.*, 1988). 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 mM of unlabelled MK-801. Final estimates of binding parameters were determined with a computerized, non-linear, least squares regression analysis (Munson and Rodbard, 1980).

Because experimental acute liver failure and acute hyperammonemia are known to increase brain water content by about ca. 2% (Swain *et al.*, 1991; De Knegt *et al.*, 1993*b*), 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 ± 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±0.2 and 15.5±1.9 hours respectively (mean±SEM, n=6). Two rabbits with acute liver failure died just before decapitation. Control studies 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 mmol/l), due to their portacaval shunt, when compared to rabbits with acute hyperammonemia (9±3 mmol/l) or controls (33±10 mmol/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 levels between rabbits with acute liver failure (533±62 mmol/l) and rabbits with acute hyperammonemia (955±120 mmol/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 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

Table I

Mean arterial ammonia levels during acute ischemic liver 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

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.

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.

Table IV

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

Reference	Animal	Model	Glutamate total	NMDA	Kainate	AMPA
Ferenci 1984b	Rabbit	Galactosamine	K_D nc B_{max} ↓		K_D nc B_{max} ↓	
Ferenci 1984a	Rabbit	Hyperammonemia	K_D nc B_{max} nc			
Watanabe 1988	Rat	Hepatectomy/CCl_4	K_D nc B_{max} nc			
Zimmerman 1989	Rat	Thioacetamide	K_D nc B_{max} nc			
Peterson 1990	Rat	Portacaval shunt		binding ↓	binding nc	binding nc
Maddison 1991	Dog	Congenital pc shunt		K_D nc B_{max} nc	K_D nc B_{max} ↓	abolition of low affinity receptor
Rao 1991	Rat	Acute hyperammonemia Chronic ..	K_D nc B_{max} ↓ K_D nc B_{max} ↓	binding ↓	binding nc	binding nc
Present study	Rabbit Rabbit	Acute liver failure Acute hyperammonemia		K_D nc B_{max} nc K_D nc B_{max} nc		

↓ decrease, nc no change

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 several other groups (Quarum *et al.*, 1990, Steele *et al.*, 1991). As discussed before (Kornhuber *et al.*, 1989*b*), the high affinity binding sites as found with the present technique may be identical to those found previously in rat brain (Wong *et al.*, 1988). 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 (Callachan *et al.*, 1988, Kavanaugh *et al.*, 1989, Ramoa *et al.*, 1990, and Amador *et al.*, 1991). With increasing ligand concentrations in saturation experiments, the amount of specific binding is decreasing from about 75% to 40% (Kornhuber *et al.*, 1989*a* and *b*). 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)(Rao *et al.*, 1992). In rabbits with galactosamine induced acute liver failure [^{3}H]glutamate binding was diminished (Ferenci *et al.*, 1984*a*), which was subsequently shown to be the result of a decreased number of kainate receptors (Ferenci *et al.*, 1984*b*). However, in acute liver failure from hepatectomy and tetrachloride in the rat (Watanabe *et al.*, 1988), thioacetamide in the rat (Zimmermann *et al.*, 1989) 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 receptors. In rats with chronic hepatic encephalopathy glutamate binding to the NMDA receptor was diminished (Peterson *et al.*, 1990); in dogs with chronic hepatic encephalopathy NMDA receptor binding appeared normal whereas changes occurred in the kainate and AMPA receptors (Maddison *et al.*, 1991). No changes in glutamate receptors were found in models of acute hyperammonemia in rabbits (Ferenci *et al.*, 1984*a*, present study). However, the number of all three subgroups of glutamate receptors were found to be diminished in hyperammonemic rats (Rao *et al.*, 1991). Thus, during experimental hepatic encephalopathy from acute liver failure there may be a selective loss of kainate receptors (Ferenci *et al.*, 1984*a*), with a preservation of NMDA receptors (present study). Chronic hepatic encephalopathy appears to be associated with variable changes of the glutamate receptor

populations (Peterson *et al.*, 1990; Maddison *et al.*, 1991). Conflicting results have also been found during hyperammonemia (Ferenci *et al.*, 1984*a*; Rao *et al.*, 1991; and present study).

Experimental acute hepatic encephalopathy and acute hyperammonemia appear to be associated with increased extracellular glutamate levels (Moroni *et al.*, 1983; Hamberger and Nystrom, 1984; De Knegt *et al.*, 1993a). Increased glutamate concentrations in the cerebrospinal fluid of patients with hepatic encephalopathy suggest that increased extracellular brain glutamate also occurs clinically (Van Sande *et al.*, 1970; Vergara *et al.*, 1974; Watanabe *et al.*, 1984). 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 (Engelsen, 1986). In these conditions most toxic effects of glutamate are thought to result from a prolonged depolarizing action at the postsynaptic receptor sites (Rothman and Olney, 1986; Rothman and Olney, 1987), in particular the NMDA receptor sites (Fagg and Massieu, 1991). This action is assumed to give rise to membrane permeability changes which lead to impaired ion homeostasis (Rothman and Olney, 1986; Rothman and Olney, 1987). Especially increased neuronal calcium influx may be responsible for cell swelling and degeneration (Collins *et al.*, 1989). 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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