Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain

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Cauli O, Rodrigo R, Boix J, Piedrafita B, Agusti A, Felipo V. Acute liver failure-induced death of rats is delayed or prevented by blocking NMDA receptors in brain. Am J Physiol Gastrointest Liver Physiol 295: G503–G511, 2008. First published July 3, 2008; doi:10.1152/ajpgi.00076.2008.—Developing procedures to delay the mechanisms of acute liver failure-induced death would increase patients’ survival by allowing time for liver regeneration or to receive a liver for transplantation. Hyperammonemia is a main contributor to brain herniation and mortality in acute liver failure (ALF). Acute ammonia intoxication in rats leads to N-methyl-D-aspartate (NMDA) receptor activation in brain. Blocking these receptors prevents ammonia-induced death. Ammonia-induced activation of NMDA receptors could contribute to ALF-induced death. If this were the case, blocking NMDA receptors could prevent or delay ALF-induced death. The aim of this work was to assess 1) whether ALF leads to NMDA receptors activation in brain in vivo and 2) whether blocking NMDA receptors prevents or delays ALF-induced death of rats. It is shown, by in vivo brain microdialysis, that galactosamine-induced death of NMDA receptors activation in brain. Blocking NMDA receptors by continuous administration of MK-801 or memantine through miniosmotic pumps affords significant protection against ALF-induced death, increasing the survival time approximately twofold. Also, when liver injury is not 100% lethal (1.5 g/kg galactosamine), blocking NMDA receptors increases the survival rate from 23 to 62%. This supports that blocking NMDA receptors could have therapeutic utility to improve survival of patients with ALF.

hyperammonemia; hepatic encephalopathy; galactosamine; MK-801

ACUTE LIVER FAILURE (ALF) may lead to massive liver cell death and severe liver dysfunction. As a consequence, hepatic encephalopathy and multiorgan failure develop rapidly and may lead to rapid death of patients unless they receive a liver transplantation. However, the number of livers available for transplantation is low, leading to long waiting times for transplantation and to death of a relevant percentage of patients before a liver suitable for transplantation becomes available.

The liver has a high capacity for regeneration that may allow complete recovery even in patients who have severe liver injury and liver mass loss. It would be therefore of great clinical interest to have procedures to delay ALF-induced death. This would increase survival of patients with ALF either by allowing enough time for regeneration of the liver by itself or to receive a suitable liver for transplantation.

The mechanisms by which ALF leads to death are not well understood. The primary event is the death of liver cells by necrosis and apoptosis. This leads to inflammatory responses and decreased functionality of the liver, resulting in decreased elimination of toxic substances, including ammonia, and decreased synthesis and release of substances such as albumin, etc. Progression of ALF leads to multiorgan failure, systemic inflammatory response, hepatic encephalopathy, cerebral edema, and increased intracranial pressure, which seem to be the most important immediate causes of mortality in patients with ALF. Preventing or delaying these processes may help to delay ALF-induced death. One of the contributors to hepatic encephalopathy and cerebral edema is the increase in ammonia levels due to severe impairment of ammonia detoxification in the liver. Circulating ammonia may reach 250–600 μM in both experimental and human ALF (5, 6). Arterial ammonia concentrations greater than 150 μM have been proposed as a predictor of brain herniation and mortality in patients with ALF (6). Brain ammonia may reach millimolar concentrations in ALF and such concentrations are well established to have deleterious effects on cerebral function and may lead to death (16, 31). Jalan et al. (15) have shown that arterial ammonia concentration, ammonia delivery to the brain and its metabolic rate are higher in patients with high intracranial pressure. Moreover, increased arterial ammonia correlates with increased cerebral blood flow. These data and those reported by Bernal et al. (1) and Bhatia et al. (2) support an important role of hyperammonemia in the pathogenesis of intracranial hypertension and death in ALF.

Acute intoxication with large doses of ammonia also leads to death of animals with normal liver function. It is therefore likely that the high ammonia levels contribute to the process by which ALF leads to death. Acute ammonia intoxication affects different cerebral processes (11, 24) and leads to activation of the N-methyl-D-aspartate (NMDA) type of glutamate receptors in brain (13) and, subsequently, to oxidative stress (17), altered mitochondrial calcium homeostasis (19), depletion of ATP (16), proteolysis of microtubule-associated protein 2 and microtubule disaggregation (10), and neuronal damage (4).

These NMDA receptor-mediated processes in brain lead finally to death. Ammonia-induced death of mice and rats is prevented by blocking NMDA receptors with selective antagonists acting on three different sites of the receptors (12, 21), indicating that excessive activation of these receptors plays a main role in ammonia-induced death. Blocking NMDA receptors also improves hyperammonemia-induced encephalopathy and acute hepatic encephalopathy in rats (32).

It is therefore very likely that ammonia-induced excessive activation of NMDA receptors could also contribute to the process by which ALF leads to death. If this were the case, blocking NMDA receptors could be useful to prevent in some cases, or at least to delay, death induced by ALF. The aim of the present
work was to assess 1) whether ALF leads to activation of NMDA receptors in brain in vivo and 2) whether blocking NMDA receptors with MK-801 or memantine prevents or delays ALF-induced death of rats.

Activation of NMDA receptors in brain in vivo can be followed by microdialysis in freely moving rats (13). Activation of these receptors leads to increased calcium in the postsynaptic neuron. Calcium binds to calmodulin and activates nitric oxide synthase, increasing nitric oxide, which in turn activates soluble guanylate cyclase, increasing cGMP levels. Part of this cGMP is released to the extracellular fluid and this allows to measure activation of NMDA receptors in brain in vivo by determining the extracellular concentration of cGMP by microdialysis (13). One of the more common models of ALF is injection of the hepatotoxin galactosamine (3, 7, 25). We have assessed whether injection of galactosamine to rats leads to activation of NMDA receptors in brain in vivo and whether continuous administration of MK-801 or memantine, antagonists of NMDA receptors, through miniosmotic pumps prevents or delays galactosamine-induced death of rats.

**MATERIALS AND METHODS**

**Drugs.** MK-801 was from Sigma-Aldrich (St. Louis, MO), dissolved in sterile saline (2 mg/ml) and used to fill Alzet osmotic minipumps (200 μl each pump). For control rats osmotic pumps were filled with sterile saline. Memantine hydrochloride was from Tocris (Cokson, UK), dissolved in 10% dimethylsulfoxide (DMSO) in sterile saline (40 mg/ml) and used to fill Alzet osmotic minipumps (200 μl in each pump). For control rats of the memantine study osmotic pumps were filled with 10% DMSO in sterile saline. α-Galactosamine hydrochloride was from Sigma Chemical (St. Louis, MO), dissolved in saline, and injected intraperitoneally at 2.5 or 15 g/kg.

**Continuous administration of MK-801, memantine, or vehicle to rats and in vivo brain microdialysis.** In vivo brain microdialysis was carried out as previously described (8). Male Wistar rats (220 – 270 g) were anesthetized with halothane and the animal’s head was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A microdialysis guide (CMA/12 guide, CMA, Stockholm, Sweden) was implanted in the cerebellum at the following stereotaxic coordinates (from bregma): anteroposterior −10.2 mm, mediolateral +1.6 mm, dorsoventral −1 mm according to Pellegrino et al. (26).

Immediately after implantation of the microdialysis guide an osmotic pump (ALZET, model 2001) was implanted subcutaneously in the back of the rats. These pumps released 1 μl/h during 7 days. Two groups of nine rats were used: one group was implanted with osmotic pumps filled with vehicle (0.9% NaCl in sterile water) and the other group with osmotic pumps filled with MK-801 (2 mg/ml in sterile 0.9% NaCl) to keep the NMDA receptor continuously blocked.

In this experiment each rat was its own control for the effects of galactosamine.

Two days after implantation of the pumps, microdialysis probes (CMA/12) were implanted carefully in the freely moving rats. The probes were continuously perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 3 μl/min. The composition of aCSF was (in mM) 145 NaCl, 3.0 KCl, 2.26 CaCl₂, buffered at pH 7.4 with 2 mM phosphate buffer. Following a stabilization period of at least 2 h, microdialysis samples were collected every 30 min just after neurological examination. After six consecutive basal fractions, when we had enough samples to determine basal levels, both groups of rats (treated with saline or MK-801) were injected with galactosamine (2.5 g/kg ip), and collection of microdialysis samples was continued until death of the rats. Samples were stored at −80°C until analysis of cGMP and glutamate concentrations.

In a second type of experiment other groups of rats (13 rats per group) were implanted with the osmotic pumps as above but without the microdialysis experiments. These rats were injected, 48 h after osmotic pump implantation, with galactosamine at a lower dose (1.5 g/kg) to evaluate the survival rate during 7 days after injection.

In a third type of experiment other groups of rats (10 rats per group) were implanted with the osmotic pumps as above, without the microdialysis experiments, but the miniosmotic pumps were filled with memantine (40 mg/ml) instead of MK-801. These rats were injected, 48 h after osmotic pumps implantation, with galactosamine (2.5 g/kg) to evaluate the survival rate during 7 days after injection.

**Neurological evaluation.** Animals were assessed neurologically every 30 min after injection of galactosamine and during the progression of ALF. The grades of hepatic encephalopathy were assigned as follows: grade I: decreased motor activity, poor gesture control, and mild ataxia; grade II: severe ataxia, sedation, righting reflex present; grade III: no righting reflex to pain stimuli (precoma stage); grade IV: no reaction to pain stimuli and no corneal reflex (coma stage).

**Determination of cGMP.** cGMP was measured by using the BIOTRAK cGMP enzyme immunoassay kit from Amersham (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Determination of ammonia in blood and cerebellum.** Ammonia was measured in plasma and cerebellum as previously described (29). Blood (150 μl) was taken from the tail vein, deproteinized with one volume of ice-cold 6% trichloroacetic acid, and kept on ice for 15 min. After centrifugation at 12,000 g, for 10 min at 4°C, the supernatants were collected, neutralized with 2 M KHCO₃, and centrifuged at 12,000 g for 10 min at 4°C. The neutralized supernatants were used to measure ammonia.

Cerebella were homogenized and deproteinized in perchloric acid ethanol as previously reported (20). The neutralized supernatants were used to measure ammonia and glutamine. Ammonia was measured by using glutamate dehydrogenase as previously described (13).

**Determination of amino acids.** The glutamate content in the microdialysis samples and the content of glutamate, glutamine, aspartate, alanine, phenylalanine, tyrosine, tryptophan, isoleucine, leucine, and valine in serum were analyzed by HPLC using a Waters reverse-phase HPLC system with fluorescence detection and precolumn o-phthalaldehyde derivatization (Waters, Milford, MA) as previously described (28). It is well known that in liver failure aromatic amino acids increase and branched chain amino acids decrease. This is the reason by which we measured these amino acids. Glutamine, glutamate, aspartate, and alanine were measured because all of them are involved in ammonia metabolism.

Glutamine in cerebellum was determined by HPLC using the same samples prepared for ammonia determination (see above).

**Brain edema.** Water content was determined in the frontal cortex and in cerebellum by measuring tissue specific gravity as described by Marmarou et al. (22). After decapitation, brain hemispheres of four animals from each group were cut into coronal slices, and samples (~2 mm²) were taken from the frontal cortex and cerebellum. The samples were placed into a kerosene/bromobenzene gradient column, and the equilibrium point was recorded within 2 min. Brain water content was calculated as reported by Marmarou et al.

The animal experiments were approved by the Center (Centro de Investigacion Principe Felipe) and met the guidelines of the European Community for care and management of experimental animals.

**Determination of transaminases in serum.** GOT (glutamate-oxaloacetate transaminase) was determined as described by Rej and Horder (27) and GPT (glutamate-pyruvate transaminase) as described by Horder and Rej (14).

**Statistical analysis.** All data (except data from microdialysis experiments) were analyzed by unpaired Student’s t-tests. Data from microdialysis experiments were analyzed by two-way ANOVA followed by Bonferroni post hoc test. All values are given as means ± SE. P values below 0.05 were considered statistically significant.
RESULTS

Blockade of NMDA receptors does not prevent galactosamine-induced liver damage. To maintain NMDA receptors blocked during the progression of ALF MK-801 was continuously administered through osmotic pumps implanted in the back of the rats 48 h before injection of galactosamine. As shown in Table 1, the treatment with MK-801 did not prevent the increase in serum alanine and aspartate transaminases or increase in serum ammonia and decrease in alpha-amino acids in treated rats with MK-801.

Table 1. Chronic administration of MK-801 through osmotic pumps does not prevent galactosamine-induced liver failure nor amino acids changes in serum

<table>
<thead>
<tr>
<th></th>
<th>Galactosamine 2.5 g/kg</th>
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<th>Galactosamine 1.5 g/kg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MK-801</td>
<td>MK + Gal</td>
<td>Vehicle</td>
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<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ammonia, nmol/g</td>
<td>281 ± 29 (10)</td>
<td>291 ± 21 (10)</td>
<td>522 ± 41* (10)</td>
<td>515 ± 20* (10)</td>
</tr>
<tr>
<td>Glutamine, mmol/g</td>
<td>4.5 ± 0.7 (4)</td>
<td>4.6 ± 0.7 (4)</td>
<td>10 ± 2* (4)</td>
<td>11 ± 3* (4)</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
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<tr>
<td>GOT, IU/l</td>
<td>173 ± 19 (14)</td>
<td>142 ± 15 (12)</td>
<td>520 ± 1,394* (10)</td>
<td>1,020 ± 1,043* (10)</td>
</tr>
<tr>
<td>GPT, IU/l</td>
<td>68 ± 9 (14)</td>
<td>56 ± 7 (12)</td>
<td>1,826 ± 975* (10)</td>
<td>6,812 ± 764* (10)</td>
</tr>
<tr>
<td>Ammonia, µM</td>
<td>77 ± 5 (13)</td>
<td>84 ± 5 (10)</td>
<td>165 ± 11* (13)</td>
<td>122 ± 20* (10)</td>
</tr>
<tr>
<td>Glutamate, µM</td>
<td>385 ± 51 (14)</td>
<td>598 ± 65* (12)</td>
<td>663 ± 69* (10)</td>
<td>715 ± 56* (10)</td>
</tr>
<tr>
<td>Glutamine, µM</td>
<td>58 ± 5</td>
<td>48 ± 4</td>
<td>108 ± 12</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>Aspartate, µM</td>
<td>18 ± 2</td>
<td>16 ± 4</td>
<td>36 ± 4</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>Alanine, µM</td>
<td>202 ± 11</td>
<td>182 ± 13</td>
<td>352 ± 36</td>
<td>391 ± 22*</td>
</tr>
<tr>
<td>Aromatic amino acids, µM</td>
<td>27 ± 2</td>
<td>26 ± 6</td>
<td>130 ± 6</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>Branched-chain amino acids, µM</td>
<td>98 ± 9</td>
<td>84 ± 8</td>
<td>55 ± 8</td>
<td>85 ± 6*</td>
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</table>

Rats were implanted in the back with osmotic pumps containing sterile saline (control and Gal groups) and the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (MK-801 and MK+Gal groups). Rats were injected 48 h later with galactosamine (Gal) (2.5 or 1.5 g/kg ip) or saline. At 24 h after galactosamine administration rats were killed by decapitation and serum from blood neck was taken to measure cGMP. The results indicate that MK-801 was significantly different from control rats injected with saline: * P < 0.05, † P < 0.01, †† P < 0.001.
implanted in the back of the rats as described in METHODS. Two days later all rats were injected with 2.5 g/kg of galactosamine. The neurological status was examined every 30 min as described in METHODS. Onset time is the time from the injection of galactosamine (basal) and at the onset of grades I, II, III, and IV of encephalopathy. Values are given as percentage of the basal level and are significantly different from rats treated with saline: *P < 0.05, **P < 0.01, ***P < 0.001.

In rats treated with MK-801, galactosamine increased extracellular cGMP only at grade I. However, the increase in cGMP at grade grades II and III was completely prevented in these rats. This indicates that the initial increase in cGMP induced by galactosamine at grade I is not dependent on activation of NMDA receptors, whereas the large increases induced at grades II and III are a consequence of activation of NMDA receptors. This confirms that galactosamine-induced ALF leads to excessive activation of NMDA receptors in brain at grades II and III of encephalopathy.

To assess whether this activation of NMDA receptors is due to increased extracellular glutamate concentration, we measured it in the microdialysis samples. As shown in Fig. 3, extracellular glutamate is not affected in grades I, II, or III of encephalopathy in control rats nor in rats treated with MK-801. Extracellular glutamate increased only at grade IV of encephalopathy, reaching 484 ± 32% of basal in control rats. The

Table 2. MK-801 delays the onset and increases the duration of grades III and IV of encephalopathy and delays death induced by 2.5 g/kg of galactosamine

<table>
<thead>
<tr>
<th>Grade</th>
<th>Control</th>
<th>MK-801</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I → II</td>
<td>6±3</td>
<td>12±3</td>
<td>NS</td>
</tr>
<tr>
<td>II → III</td>
<td>19±3</td>
<td>27±4</td>
<td>NS</td>
</tr>
<tr>
<td>III → IV</td>
<td>23±3</td>
<td>32±3</td>
<td>0.04</td>
</tr>
<tr>
<td>Grade IV</td>
<td>24±3</td>
<td>35±3</td>
<td>0.02</td>
</tr>
<tr>
<td>Death</td>
<td>24.5±3</td>
<td>37.5±3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

GRADE OF ENCEPHALOPATHY

Control MK-801

GRADE OF ENCEPHALOPATHY

Fig. 2. Blocking NMDA receptors with MK-801 prevents the increase in extracellular cGMP in cerebellum induced by acute liver failure. Rats were implanted with microdialysis guides in the cerebellum and miniosmotic pumps containing sterile saline or MK-801 in the back as described in METHODS. Two days later microdialysis probes were inserted in the guides and samples of the extracellular fluid of the cerebellum were taken every 30 min. After 6 samples were taken to determine basal levels of extracellular cGMP, all rats were injected with 2.5 g/kg of galactosamine. The neurological status was examined every 30 min. The figure shows the extracellular concentration of cGMP before injection of galactosamine (basal) and at the onset of grades I, II, III, and IV of encephalopathy. Values are given as percentage of the basal level and are significantly different from basal in each group: *P < 0.05, **P < 0.01, ***P < 0.001. Significantly different from rats treated with saline: *P < 0.05, **P < 0.001.

Fig. 3. Galactosamine-induced increase in extracellular cGMP is not due to increased extracellular glutamate. Experiments were the same shown in Fig. 2. An aliquot of the same samples used in Fig. 2 to measure cGMP was used to determine glutamate by HPLC. Values are given as percentage of the basal concentration before injection of galactosamine and are means ± SE of 9 rats per group. Significantly different from basal in each group: **P < 0.01, ***P < 0.001. Significantly different from control rats treated with saline: *P < 0.05.
increase was significantly lower (294 ± 39%) in rats treated with MK-801.

Injection of galactosamine increases ammonia levels in brain; blocking NMDA receptors does not prevent the increase. We believe that activation of NMDA receptors in ALF induced by galactosamine is due to the high ammonia levels reached in brain. Ammonia in brain is rapidly metabolized to glutamine. We therefore assessed the effects of galactosamine injection on ammonia and glutamine levels in blood and in cerebellum.

Ammonia levels in blood were not different in rats treated with MK-801 alone (84 ± 5 μM) than in control rats treated with vehicle (77 ± 5 μM). Injection of galactosamine (2.5 g/kg) increased (P < 0.001) blood ammonia levels, which reached 165 ± 11 μM 24 h after galactosamine injection. In rats treated with MK-801 and galactosamine, ammonia reached 122 ± 20 μM, which was not different (P = 0.12) from rats injected with galactosamine alone.

Ammonia levels in cerebellum were not significantly different in rats treated with MK-801 alone (291 ± 21 nmol/g tissue) than in control rats treated with vehicle (281 ± 29 nmol/g tissue). Injection of galactosamine (2.5 g/kg) increased (P < 0.001) ammonia levels in cerebellum, reaching 522 ± 41 nmol/g tissue 24 h after galactosamine injection. In rats treated with MK-801 and galactosamine ammonia reached 515 ± 20 nmol/g tissue, which was not different from rats injected with galactosamine alone (Table 1).

Glutamine levels in cerebellum were not significantly different in rats treated with MK-801 alone (4.6 ± 0.7 μmol/g tissue) than in control rats treated with vehicle (4.5 ± 0.7 μmol/g tissue). Injection of galactosamine (2.5 g/kg) increased (P < 0.001) glutamine levels in cerebellum, reaching 10 ± 2 μmol/g tissue 24 h after galactosamine injection. In rats treated with MK-801 and galactosamine ammonia reached 11 ± 3 μmol/g tissue, which was not different from rats injected with galactosamine alone (Table 1).

Similar results were obtained in the experiments with rats injected with 1.5 g/kg of galactosamine, although the increases in blood ammonia and in brain glutamine were lower than for 2.5 g/kg (Table 1).

Ammonia levels in cerebellum were not significantly different in rats treated with MK-801 alone (343 ± 36 nmol/g tissue) than in control rats treated with vehicle (303 ± 27 nmol/g tissue). Injection of galactosamine (1.5 g/kg) increased (P < 0.001) ammonia levels in cerebellum, reaching 554 ± 44 nmol/g tissue 24 h after galactosamine injection. In rats treated with MK-801 and galactosamine ammonia reached 543 ± 37 nmol/g tissue, which was not different from rats injected with galactosamine alone (Table 1).

Glutamine levels in cerebellum were not significantly different in rats treated with MK-801 alone (4.2 ± 0.5 μmol/g tissue) than in control rats treated with vehicle (4.0 ± 0.6 μmol/g tissue). Injection of galactosamine (1.5 g/kg) increased (P < 0.001) glutamine levels in cerebellum, reaching 7.7 ± 0.9 μmol/g tissue 24 h after galactosamine injection. In rats treated with MK-801 and galactosamine, glutamine reached 6.6 ± 0.9 μmol/g tissue, which was not different from rats injected with galactosamine alone (Table 1).

Blocking NMDA receptors with MK-801 increases survival of rats after galactosamine-induced liver failure. The above results show that ALF leads to increased ammonia levels and activation of NMDA receptors in brain in vivo. Moreover, as shown in Table 2, blocking NMDA receptors delays the death of rats induced by a very large dose (2.5 g/kg) of galactosamine. This dose is 100% lethal. We also tested whether blocking NMDA receptors could afford protection against ALF-induced death when the injury is milder. We tested whether MK-801 is able to reduce or delay mortality of rats induced by injection of 1.5 g/kg of galactosamine. With this dose of galactosamine the progression of encephalopathy is slower than for 2.5 g/kg (Table 3). The effects induced by both doses of galactosamine on different parameters related with liver failure or ammonia metabolism at 24 h after galactosamine injection are given on Table 1. The lower dose of galactosamine induced milder changes than the large dose in blood ammonia, serum GOT, aspartate, aromatic amino acids, and, especially, brain glutamine (Table 1), reflecting a milder, or at least slower, liver failure.

Injection of 1.5 g/kg of galactosamine led to death of 77% (10 of 13) of control rats. MK-801 afforded a substantial protection against death; only 38% (5 of 13) of the rats died. Moreover, for the rats that died, the time to death in control rats injected with 1.5 g/kg of galactosamine was 48 ± 2 h and MK-801 significantly (P < 0.05) increased survival time to 77 ± 17 h (Table 3).

The progression of the encephalopathy in rats injected with 1.5 g/kg of galactosamine with or without MK-801 is shown in Table 3. The onset of grades I and II was not significantly different in control rats and in rats treated with MK-801. However, the time from injection of galactosamine to the onset of grades III and IV was significantly longer in rats treated with MK-801 than in control rats. Table 3 also shows the duration of each grade of encephalopathy. The times that control rats

Table 3. MK-801 delays the onset and increases the duration of grades III and IV of encephalopathy and delays death induced by 1.5 g/kg of galactosamine

<table>
<thead>
<tr>
<th>Grade</th>
<th>Control</th>
<th>MK-801</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>onset time, h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I → II</td>
<td>11 ± 4</td>
<td>12 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>II → III</td>
<td>10 ± 3</td>
<td>22 ± 5</td>
<td>0.04</td>
</tr>
<tr>
<td>III → IV</td>
<td>2 ± 0.5</td>
<td>9 ± 3</td>
<td>0.01</td>
</tr>
<tr>
<td>IV → death</td>
<td>1 ± 0.5</td>
<td>7 ± 3</td>
<td>0.03</td>
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<table>
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<th>time spent in each grade of encephalopathy, h</th>
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<tr>
<td>11</td>
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<td>NS</td>
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<tr>
<td>10</td>
<td>22</td>
<td>0.04</td>
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<tr>
<td>2</td>
<td>9</td>
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<tr>
<td>1</td>
<td>7</td>
<td>0.03</td>
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</table>

Miniosmotic pumps containing sterile saline or the NMDA receptor antagonist MK-801 were implanted in the back of the rats as described in METHODS. Two days later all rats were injected with 1.5 g/kg of galactosamine. The neurological status was examined every 30 min as described in METHODS. Onset time is the time from injection of galactosamine until the onset of the indicated grade of encephalopathy. The time spent in each grade of encephalopathy is also shown. Values are the mean ± SE of 13 rats per group for grades I and II and for 10 control rats and 5 rats injected with MK-801 for grades III and IV and for death. The other 3 control rats and 8 rats injected with MK-801 never reached grade III and survived. The P values of the statistical comparison of the values in rats treated with saline or MK-801 are also given.

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and rats injected with MK-801 stayed in grade I were not significantly different. However, in rats treated with MK-801 the duration of grades II, III, and IV of encephalopathy were significantly ($P < 0.01$) longer than in control rats. Also the time from injection of galactosamine to death was significantly ($P < 0.01$) longer in rats treated with MK-801 (77 ± 17 h) than in control rats (48 ± 2 h).

It is noteworthy that all rats entered grades I and II of encephalopathy but none of the rats that survived entered grade III. Values in Table 3 are means of 13 rats per group for grades I and II and for 10 control rats and 5 rats injected with MK-801 for grades II and IV and for death. The other 3 control rats and 8 rats injected with MK-801 never reached grade III and survived.

Blocking NMDA receptors does not prevent galactosamine-induced brain edema. We also assessed whether MK-801 reduces the induction by ALF of brain edema. We determined by gravimetry the water content in cerebral cortex and cerebellum of rats. As shown in Fig. 4, water content was not increased 16 h after injection of galactosamine but was significantly increased in both brain areas at 24 h. MK-801 did not prevent induction of edema in cerebral cortex nor in cerebellum (Fig. 4).

Blocking NMDA receptors with memantine also increases survival of rats after galactosamine-induced liver failure. MK-801 is an excellent tool for research on mechanisms but it is not adequate in therapeutic treatment because it has secondary effects. Memantine is another NMDA receptor antagonist that can be used in clinical practice. We therefore tested whether memantine also increases survival of rats with ALF. As shown in Table 4, memantine significantly ($P < 0.01$) increased the survival time of rats after injection of 2.5 g/kg of galactosamine. Control rats died at 38 ± 4 h and rats injected with memantine at 56 ± 5 h.

The onset of the different grades of encephalopathy in control rats and in rats treated with memantine occurred at the times given in Table 4. The onset of grades I and II was not significantly different in control rats and in rats treated with memantine. However, the time from injection of galactosamine to the onset of grade IV was significantly longer in rats treated with memantine than in control rats. The onset of grade III was also delayed from 32 to 40 h, but the difference was not statistically significant. Table 4 also shows the duration of each grade of encephalopathy. The times that control rats and rats injected with memantine stayed in grade I was not significantly different. However, in rats treated with memantine the durations of grades II and III of encephalopathy were significantly ($P < 0.01$) longer than in control rats. Also the time from injection of galactosamine to death was significantly ($P < 0.01$) longer in rats treated with memantine (56 ± 6 h) than in control rats (38 ± 4 h). These control rats survived longer than rats in the study with MK-801 and 2.5 g/kg galactosamine. The study with memantine was performed several months later and used a different batch of galactosamine. This would explain this difference. Variability in the effects of different batches of galactosamine has been already reported. However, the variability of the results within the same experiment, using the same batch of galactosamine was not high and allowed to obtain consistent results.

**DISCUSSION**

Different factors contribute to the pathogenesis of intracranial hypertension and death in ALF. A main contributor is hyperammonemia. Arterial ammonia levels greater than 150 µM has been proposed to predict cerebral herniation and death in ALF (6). Jalan et al. (15) showed that arterial concentrations of ammonia, its delivery to the brain, and its metabolic rate are significantly higher in the patients with higher intracranial pressure, confirming the important role of ammonia in the pathogenesis of intracranial hypertension and death in ALF.

**Fig. 4.** Blocking NMDA receptors with MK-801 does not prevent induction of cerebral edema by acute liver failure. Miniosmotic pumps containing sterile saline or MK-801 were implanted in the back of the rats as described in METHODS. Two days later rats were injected with 2.5 g/kg of galactosamine. Rats were killed 16 or 24 h after galactosamine injection (GalN). For reference, 1 group (veh) of rats implanted with pumps containing saline and 1 group (MK-801) implanted with pumps containing MK-801 were injected with vehicle instead of galactosamine and killed 24 h later. The presence of cerebral edema was assessed by gravimetry in the prefrontal cortex (A) and in the cerebellum (B) as described in METHODS. Two different samples were analyzed from each rat in each brain area. Values are means ± SE of 4 rats per group and are expressed as percentage of water in the cerebral tissue. Significantly different from control rats injected with vehicle: *$P < 0.05$, **$P < 0.01$.**
blocking NMDA receptors with MK-801 or memantine does
encephalopathy. This is further supported by the fact that
the increase in cGMP in
in cGMP is a consequence of activation of NMDA receptors
This galactosamine-induced increase in cGMP is completely
prevented by ten different antagonists (12, 21), indicating that
Memantine also increases the duration of grades II and
III (Table 4). Also none of the rats that survived to the injection
of 1.5 g/kg of galactosamine (3 controls and 8 injected with
MK-801) reached grade III of encephalopathy, further supporting
that blocking NMDA receptors prevents or delays the entry
in grade III.

As shown in Fig. 2, cGMP levels returned to basal in grade
IV of encephalopathy, suggesting that NMDA receptors are not
activated at this stage. The same occurs following acute
ammonia intoxication, which induces a large, NMDA receptor-
mediated increase in extracellular cGMP, followed by a return
to basal levels in rats that die subsequently (13). It is well
known that Ca\(^{2+}\) influx through NMDA receptors leads to
channel inactivation as a feedback regulatory mechanism to
prevent excessive activation of NMDA receptors and excito-
toxicity under physiological conditions (30, 33). This inactiva-
tion of NMDA receptors would explain the decrease in extrac-
cellular cGMP in grade IV of encephalopathy. However, under
the pathological situation (ALF) studied here, the previous
strong activation of NMDA receptors in grades II and III has
already triggered the processes leading to death, and the
reduction in activation of NMDA receptors in grade IV is not
efficient to prevent death in rats injected only with galac-
tosamine.

We also assessed whether the activation of NMDA receptors
contributes to ALF-induced death and whether blocking
NMDA receptors could have beneficial effects by increasing
the survival rate or the survival time in rats injected with
galactosamine.

Injection of a large dose of galactosamine (2.5 g/kg) was
100% lethal both in rats treated with saline and in those treated
with MK-801. However, survival time was increased nearly
twice (from 24.5 to 37 h) by MK-801, supporting a beneficial
role of blocking NMDA receptors either in cases of severe liver
failure. In the case of humans with severe liver injury, this
increase in the survival time could be enough to allow arrival
of a suitable liver for transplantation and could increase the net
survival rate of patients.

In cases of milder liver failure, the additional survival time
afforded by blocking NMDA receptors could also increase
survival rate by allowing regeneration of the liver in individ-
uals who would have died without treatment with NMDA
receptor antagonists. This is clearly illustrated by the results
obtained in rats injected with 1.5 g/kg of galactosamine. Only
23% of rats treated with saline survived; however, blocking
NMDA receptors increased survival to 62% of the rats.

We have previously shown that the activation of NMDA
receptors induced by acute ammonia intoxication in naive rats
is not a consequence of increased extracellular concentration of
 glutamate (13). We show here that also in ALF activation of
NMDA receptors is not a consequence of increased extracel-
lular glutamate. Activation of NMDA receptors occurs at
grades II and III of encephalopathy (Fig. 2) and extracellular

We have shown previously that, in normal rats and mice, an
acute increase in ammonia levels leads to activation of NMDA
receptors in brain in vivo (13). Moreover, ammonia-induced
death of mice or rats is prevented by blocking NMDA recep-
tors with ten different antagonists (12, 21), indicating that
excessive activation of NMDA receptors is responsible for
ammonia-induced death in animals with normal liver function.
It seems therefore likely that excessive activation of NMDA
receptors in brain should contribute to the deleterious effects
induced by hyperammonemia in ALF.

We show here that ALF induced in rats by injection of
galactosamine also leads to increased ammonia levels in brain
and to activation of NMDA receptors in brain in vivo, resulting
in activation of the glutamate-nitric oxide-cGMP pathway and
in increased concentration of extracellular cGMP when the
progression of the encephalopathy reached grades II and III.
This galactosamine-induced increase in cGMP is completely
prevented in rats in which the NMDA receptor has been
blocked with MK-801. This clearly indicates that the increase
in cGMP is a consequence of activation of NMDA receptors
and confirms that ALF leads to activation of NMDA receptors
in brain in vivo. Moreover, blocking NMDA receptors prevents
the increase in cGMP in grades II and III of encephalopathy
but does not prevent the appearance of edema. This indicates
that edema is not involved in the increase in cGMP.

Figure 2 shows that extracellular cGMP also increases
slightly in grade I of encephalopathy in rats injected with
galactosamine and that this increase is not prevented by block-
ing NMDA receptors with MK-801. This increase in cGMP is
due therefore to an NMDA receptor-independent mechanism.
This slight increase in cGMP could be due to altered activation
of AMPA or GABA receptors (9). These data support the idea
that NMDA receptors are not activated in grade I of hepatic
encephalopathy. This is further supported by the fact that
blocking NMDA receptors with MK-801 or memantine does

Table 4. Memantine increases the duration of grade III
of encephalopathy and delays death induced by 2.5 g/kg
of galactosamine

<table>
<thead>
<tr>
<th>Grade</th>
<th>Control</th>
<th>Memantine</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>onset time, h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>9 ± 3</td>
<td>14 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Grade II</td>
<td>23 ± 5</td>
<td>26 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Grade III</td>
<td>32 ± 3</td>
<td>40 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Grade IV</td>
<td>37 ± 4</td>
<td>55 ± 5</td>
<td>0.03</td>
</tr>
<tr>
<td>Death</td>
<td>38 ± 4</td>
<td>56 ± 6</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Time spent in each grade of encephalopathy, h

<table>
<thead>
<tr>
<th>Grade</th>
<th>1 ± 0.5</th>
<th>1 ± 0.8</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I → II</td>
<td>14 ± 2</td>
<td>15 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>II → III</td>
<td>9 ± 1</td>
<td>14 ± 1</td>
<td>0.04</td>
</tr>
<tr>
<td>III → IV</td>
<td>5 ± 1</td>
<td>15 ± 4</td>
<td>0.01</td>
</tr>
<tr>
<td>IV → death</td>
<td>1 ± 0.5</td>
<td>1 ± 0.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Miniosmotic pumps containing sterile saline or the NMDA receptor antag-
onist memantine were implanted in the back of the rats as described in
METHODS. Two days later all rats were injected with 2.5 g/kg of galactosamine.
The neurological status was examined every 30 min as described in METHODS.
Onset time is the time from injection of galactosamine until the onset of the
indicated grade of encephalopathy. The time spent in each grade of encepha-
lopathy is also shown. Values are the mean ± SE of 10 rats per group. The
P values of the statistical comparison of the values in rats treated with saline
or memantine are also given.
glutamate is not increased at these grades of encephalopathy (Fig. 3). As discussed previously for acute ammonia toxicity, activation of NMDA receptors in ALF could be a consequence of neuronal depolarization, which would release the voltage-dependent block by Mg$^{2+}$ of the ion channel of the NMDA receptor (23), allowing increased activation of the receptor without increasing extracellular glutamate.

We also show that the protective effect of blocking NMDA receptors in ALF is not associated with decreased cerebral edema, which remains unaltered in cerebral cortex and cerebellum after treatment with MK-801. This agrees with a recent report (4) showing by magnetic resonance in vivo that acute ammonia intoxication in rats induces cerebral edema (changes in apparent diffusion coefficient and myoinositol levels), neuronal damage (changes in N-acetylaspartate), and changes in T1 and T2 in some brain areas. Changes in N-acetylaspartate, T1, and T2 are prevented by MK-801 whereas ammonia-induced edema (changes in apparent diffusion coefficient or myoinositol) is not prevented by blocking NMDA receptors with MK-801. We show here that galactosamine increases ammonia levels in brain and induces cerebral edema. Neither the increase in brain ammonia nor the induction of edema is prevented by blocking NMDA receptors, supporting the idea that hyperammonemia leads to cerebral edema by mechanisms independent of NMDA receptors.

These data and those presented here show that acute hyperammonemia, including that present in ALF, induces different types of effects in brain. Some of these effects are mediated by activation of NMDA receptors whereas others (e.g., induction of edema) are independent of NMDA receptors.

The fact that blocking NMDA receptors increases survival time and survival rate in rats with ALF without affecting the presence of edema indicates that edema is not the only main contributor to death in ALF and that activation of NMDA receptors also plays a main role, independent of edema, in the mechanisms leading to death in ALF.

Moreover, 24 h after injection of 2.5 g/kg of galactosamine both rats treated with vehicle or MK-801 show similar grades of edema (Fig. 4). However, at this time, rats injected with galactosamine are in grade IV of encephalopathy whereas rats injected with MK-801 and galactosamine are still entering grade II of encephalopathy (Fig. 1, Table 2). This indicates that the progression of grades II and III of encephalopathy depends on NMDA receptors activation but is independent of edema. Also, Fig. 4 shows that 16 h after galactosamine injection, when the rats are in grade I (Fig. 1), there is no edema. However, at 24 h rats injected with galactosamine are in grade II whereas those injected with MK-801 and galactosamine remain in grade I and both groups show similar grades of edema. This further supports that the progression of the grades of encephalopathy is independent of edema.

Both NMDA receptor-dependent and independent effects of ammonia would contribute to death induced by ALF. We show here that blocking NMDA receptors affords a significant protection against this death, increasing about twice the survival time both in severe liver failure (2.5 g/kg of galactosamine) and in milder liver injury (1.5 g/kg of galactosamine). In the case of human patients, a twofold increase in survival time would be very beneficial, allowing additional time for arrival of an appropriate liver for transplantation and in this way increasing the survival rate.

Moreover, when liver injury is not so severe (1.5 g/kg of galactosamine), blocking NMDA receptors increases directly the survival rate 2.7-fold, from 23 to 62%. A similar increase in survival rate could be expected in patients with not too massive ALF. These data strongly support that blocking NMDA receptors would improve survival of patients with ALF.

We have used MK-801, a strong NMDA receptor antagonist, as an experimental tool to evaluate the role of these receptors in the mediation of ALF. Strong antagonists of NMDA receptors like MK-801 have some secondary effects making them not very adequate for routine or chronic clinical use. However, we also show that milder NMDA receptor antagonists like memantine, which is being used routinely in clinical practice without relevant secondary effects, also delays ALF-induced death. Memantine and other mild NMDA receptor antagonists would be therefore useful to increase survival of patients with ALF either by allowing more time for liver regeneration or to get a liver suitable for transplantation.

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REFERENCES


