

## Prenatal Exposure of Rats to Ammonia Impairs NMDA Receptor Function and Affords Delayed Protection Against Ammonia Toxicity and Glutamate Neurotoxicity

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**Abstract.** The aim of this work was to assess whether perinatal hyperammonemia impairs the function of NMDA receptors and whether this impairment affords protection against acute ammonia toxicity and glutamate and NMDA neurotoxicity. Rats were exposed to ammonia during the prenatal and lactation periods by feeding the female rats an ammonium-containing diet since day 1 of pregnancy. After weaning (at postnatal day 21), the pups were fed a normal diet with no ammonia added. This treatment resulted in a marked decrease of the growth rate of the animals, which was maintained even 1 month after normalization of ammonia levels. Rats exposed to ammonia were more resistant than controls to acute ammonia toxicity 13 days after feeding a normal diet but not at 3 months. Primary cultures of cerebellar neurons from hyperammonemic rats showed decreased binding of [<sup>3</sup>H]MK-801 and were remarkably more resistant than controls to glutamate and NMDA toxicities. Also, the increase in aspartate aminotransferase activity induced by low concentrations of NMDA was not produced in such cultures. These results indicate that exposure to ammonia during the prenatal and lactation periods results in long-lasting impairment of NMDA receptor function. This would be the reason for the delayed protection afforded by exposure to low ammonia levels against acute ammonia toxicity in animals and against glutamate and NMDA toxicity in neuronal cultures.

**Key Words:** Ammonia toxicity; Cerebellar neurons; Glutamate toxicity; Hyperammonemia; NMDA receptor.

### INTRODUCTION

Hepatic encephalopathy is one of the main causes of death in Western countries. In spite of much work, its pathogenesis is not well understood and several hypotheses have been proposed as explanations for its effects. Hyperammonemia is considered one of the main factors responsible for hepatic encephalopathy. Elevated levels of ammonia accompany a number of human diseases, including cirrhosis and acute liver failure. A five- to tenfold increase in normal ammonia concentrations in the blood induces toxic effects in most animal species, with functional disturbances of the central nervous system (CNS).

These effects are especially marked in newborn infants. Neonatal hyperammonemia can be due to congenital deficiencies of any of the urea cycle enzymes: ornithine transcarbamylase (1, 2), carbamyl phosphate synthetase I (3), argininosuccinate synthetase (4), argininosuccinase (5) and arginase (6), or in acetylglutamate synthetase (7). Severe, transient hyperammonemia can also be encountered in critically ill newborns, without deficiencies of the above enzymes, particularly in those who are preterm or have asphyxia (8, 9). Acute neonatal hyperammonemia due to deficiencies of the urea cycle enzymes can be lethal. Affected neonates appear clinically normal at birth; however, within 24–48 hours they

develop vomiting and lethargy that quickly progresses to coma and death caused by rapidly rising hyperammonemia. Immediate and aggressive therapy is essential to prevent brain damage and death. Hyperammonemia in the newborn period is commonly associated with significant CNS damage, both in treated urea cycle deficiencies and in neonatal transient hyperammonemia (9). An inverse correlation of the IQ with the duration of hyperammonemic coma has been reported (10, 11).

The mechanism by which ammonia causes these deleterious effects is unknown. A possible role for excitatory neurotoxic amino acids in the pathogenesis of hepatic encephalopathy has been suggested by Moroni et al (12–15). These authors have shown that acute ammonia intoxication leads to increased release of glutamate from the brain surface of the rat (13) and that the content of quinolinic acid, an excitotoxic tryptophan metabolite, is increased in brain regions of rats used as experimental models of hepatic encephalopathy (14) as well as in cerebrospinal fluid and frontal cortex from patients with hepatic failure (15). In agreement with this, we have recently found that antagonists of the NMDA type of glutamate receptors prevent death of animals injected with large (lethal) doses of ammonium acetate, suggesting that acute ammonia toxicity is mediated by activation of the NMDA receptor (16).

It has been shown that chronic, moderate hyperammonemia prevents acute ammonia toxicity in rats (17). As this toxicity is mediated by activation of NMDA receptors, we considered of interest to assess: 1) whether chronic moderate hyperammonemia affects the amount or function of NMDA receptors and 2) whether this alteration could explain the protective effect of chronic moderate hyperammonemia against acute toxicity of large amounts of ammonium salts.

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As mentioned above, the effects of hyperammonemia in humans are especially marked in the neonatal period and result in death or in serious CNS damage with subsequent deterioration of the IQ. Moreover, the NMDA receptor has been implicated in the processes of memory and learning. It has been shown that NMDA receptor antagonists block long-term potentiation and impair learning (18, 19). It is therefore possible that mental retardation induced by neonatal hyperammonemia could be due to impairment of NMDA receptor function. We therefore considered that the neonatal period would be more suitable to test the two questions mentioned above. Also, primary cultures of neurons were considered the most suitable model to test the function of NMDA receptors because they allow assessment of glutamate and NMDA toxicity as well as induction of aspartate amino transferase (AAT) by low concentrations of NMDA. We have therefore studied whether exposure to ammonia during the prenatal and lactation periods could affect the amount or function of NMDA receptors and whether this alteration results in reduced sensitivity to ammonia toxicity in animals or to glutamate or NMDA toxicity in cultured neurons. We also tested the effect on another parameter induced by activation of NMDA receptor, i.e. the induction in primary cultures of neurons of AAT by exposure to low concentrations of NMDA (20).

It has been reported that persistent blockade of NMDA receptors during the neonatal period results in impaired growth hormone secretion and a remarkable decrease in body weight (21, 22). We also determined the effect of pre- and neonatal exposure to ammonia on the body weight of rats as an additional parameter related to NMDA receptor function.

We found that such exposure to low ammonia levels results in reduced growth of the animals, decreased ability of the NMDA receptor to be activated by glutamate or NMDA, and delayed protection against ammonia toxicity in rats and against glutamate toxicity in cultured neurons.

## MATERIALS AND METHODS

### Animals and Diets

Wistar rats of 220–260 g were used. For prenatal exposure to ammonia, female rats were made hyperammonemic by feeding them a diet containing ammonium acetate (20% by mass) as previously described (23, 24). Rats were fed the ammonium-containing diet starting on day 1 of pregnancy and were maintained on this diet until weaning (at postnatal day 21). After weaning, pups were either fed a normal diet, with no ammonium acetate added, or continued on ammonium until sacrifice.

### Primary Neuronal Cultures

Primary cultured neurons were prepared from the cerebellum of 8-day-old Wistar rats as previously reported (25). Exactly the same conditions were used for cultures from control pups

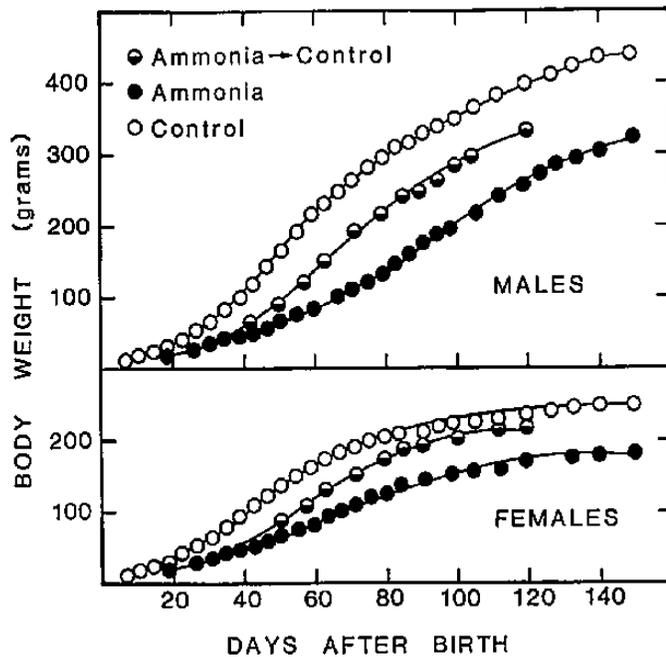
and from pups exposed to ammonia during pregnancy and lactation. In brief, tissue was mechanically dissociated with a pipette. The cell suspension was filtered through a mesh with a pore size of 90  $\mu\text{m}$  and resuspended in 3.3 ml/brain of basal Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100  $\mu\text{g/ml}$  gentamycin and 25 mM KCl. Cells were seeded onto polylysine-coated plates. After 15 minutes (min) at 37°C, the medium containing unattached cells was removed and fresh medium was added. To prevent proliferation of non-neuronal cells, cytosine arabinoside (10  $\mu\text{M}$ ) was added to the culture medium 20 hours after seeding. Cells were incubated at 37°C in 5%  $\text{CO}_2$  atmosphere.

### Binding of [ $^3\text{H}$ ]MK-801 to Primary Cultures of Neurons

It has been shown that [ $^3\text{H}$ ]MK-801 is able to gain access to its binding site located within the NMDA channel only when it is gated, and thereby [ $^3\text{H}$ ]MK-801 is useful for radiolabeling the open NMDA channels (26). We therefore used the binding of [ $^3\text{H}$ ]MK-801 to neurons as a tool to assess the effect of perinatal exposure to ammonia on NMDA receptor function. Primary cultures of cerebellar neurons were prepared as above using 24-well plates. After 9–10 days *in vitro*, cells were washed with prewarmed phosphate buffered saline and 1 ml of incubation mixture containing 5 mM Tris-citrate buffer, pH 7.4, 100  $\mu\text{M}$  glycine, 100  $\mu\text{M}$  glutamate and 50–1,000 nM [ $^3\text{H}$ ]MK-801 (1.8 Ci/mmol, New England Nuclear) was added. After incubation for 15 min at 37°C, medium was removed and the cells were washed four times with 5 mM Tris-citrate, containing 100  $\mu\text{M}$  glycine and 100  $\mu\text{M}$  glutamate (to maintain association of MK-801). The cells were resuspended in 0.5 N NaOH plus 0.4% sodium deoxycholate. Aliquots of this suspension were used for determination of protein and for counting of radioactivity. Nonspecific binding was determined in the presence of 0.25 mM unlabeled MK-801.

### Assay of Glutamate and NMDA Toxicity and Intravital Staining of the Culture

Glutamate (and NMDA) toxicity in primary cultures of cerebellar neurons is mainly mediated by activation of NMDA receptors and is prevented by antagonists of these receptors. We therefore used the assays of glutamate or NMDA toxicity as an index of the functional state of NMDA receptors. Following exposure to high concentrations of glutamate or NMDA, neurons would die if NMDA receptors are active but would survive if the receptors are not functional. Glutamate or NMDA toxicities were assayed as described previously (27). Monolayers were washed with Locke's solution without magnesium (154 mM NaCl, 5.6 mM KCl, 3.6 mM  $\text{NaHCO}_3$ , 2.3 mM  $\text{CaCl}_2$ , 5 mM HEPES, pH 7.4), containing 5.6 mM glucose. Cells were incubated with glutamate or NMDA in the same solution for 4 hours at 37°C. The incubation was terminated by washing the monolayer three times with the above solution. Cell viability was determined immediately by staining for 5 min at 23°C with a mixture of fluorescein diacetate (15  $\mu\text{g/ml}$ ) and propidium iodide (4.6  $\mu\text{g/ml}$ ). The stained cells were immediately examined with a fluorescence microscope. The percentage of surviving neurons was calculated by assessing the ratio of fluorescein



**Fig. 1.** Effect of exposure to ammonia on the growth of rats. Female Wistar rats were fed control or ammonium-containing diet from day 1 of pregnancy until weaning at postnatal day 21. After weaning, a group of the pups exposed to ammonia were fed a normal diet without ammonia added (Ammonia → control group) while others were maintained on the ammonium diet (Ammonia group). Values are the mean of the following number of animals: for controls, 7 males and 8 females; for ammonia group 12 males and 9 females; for the ammonia → control group 8 males and 13 females. Standard deviations were less than 13% for all values.

diacetate/propidium iodide staining directly under the microscope. At least 400 cells were counted for each point.

#### Assay of Aspartate Aminotransferase and of Lactate Dehydrogenase in Primary Cultures of Cerebellar Neurons

It has been shown that long-term exposure to low concentrations of NMDA induces an increase in the activity of AAT in cultured neurons while the activity of lactate dehydrogenase is not affected. This effect is mediated by activation of the NMDA receptor and is prevented by antagonists of this receptor (20). We therefore considered that the induction of AAT in cultured neurons could serve as an index of the function of NMDA receptors; if the receptor is not active, AAT would not be induced. We therefore tested the effect of perinatal exposure to ammonia on the induction of AAT by low concentrations of NMDA. Cultures were prepared as above except that cells were grown in the presence of 10 mM KCl instead of 25 mM. After 48 hours of culture, NMDA (50  $\mu$ M final concentration) was added. After 6–7 days *in vitro*, culture medium was removed and cells were washed with phosphate buffered saline at 37°C, resuspended in 500  $\mu$ l of 10 mM imidazole buffer, pH 7.0 and sonicated. Aspartate aminotransferase activity was determined essentially as described by Morán and Rivera-Gaxiola (20). Briefly, the assay mixture contained 90 mM HEPES-Tris buffer,

**TABLE 1**  
Effect of Prenatal Exposure to Ammonia on Acute Ammonia Toxicity after Different Times on Normal Diet

Time after weaning	Experiment	Treatment	Animals died/injected
13 days	1	Control	7/10
		Ammonia	3/14
	2	Control	15/20
		Ammonia	7/16
1 month	1	Control	10/12
		Ammonia	7/11
3 months	1	Control	8/12
		Ammonia	6/8
	2	Control	8/12
		Ammonia	8/11

For prenatal exposure to ammonia, female Wistar rats were fed the ammonium-containing diet starting on day 1 of pregnancy; this diet was maintained until weaning (at postnatal day 21). Experiments with control pregnant rats (fed a normal diet without addition of ammonia) were carried out simultaneously. At the day of weaning all pups from controls or hyperammonemic rats were fed the normal diet for the time indicated. At this time rats were injected intraperitoneally with 7 mmol/Kg of ammonium acetate.

pH 7.4, 0.17% Triton X-100, 17 U/ml malate dehydrogenase, 17 mM L-aspartate and cell homogenate (5–15  $\mu$ g of protein). After 5 min at 25°C, the reaction was initiated by addition of  $\alpha$ -ketoglutarate (8.5 mM final concentration). Lactate dehydrogenase was determined as described by Vassault (28).

## RESULTS

The effect of the ammonia treatment on the growth of animals is shown in Figure 1. The body weight of the rats maintained on ammonium was significantly less than that of controls throughout the time period examined. The difference increased until day 80 and was thereafter relatively constant. For rats exposed to ammonia during pregnancy and lactation and fed a normal diet after weaning (day 21), the growth rate was significantly lower than in controls until approximately day 60, indicating that the consequences of ammonia exposure were not completely overcome even after one month of ammonia withdrawal.

As shown in Table 1, rats exposed to ammonia during pregnancy and lactation and fed a normal diet (without ammonia) after weaning are significantly more resistant to acute ammonia toxicity 13 days after weaning, but not 3 months later.

As shown in Figure 2, prenatal exposure to ammonia markedly reduced (by approximately 60%) the binding of [<sup>3</sup>H]MK-801 to NMDA receptors. We then tested the effect on two parameters which are well known to be affected by activation of the NMDA receptor in primary cultures of cerebellar neurons: the induction of AAT by low concentrations of NMDA and the neurotoxicity of high concentrations of glutamate or NMDA. As shown

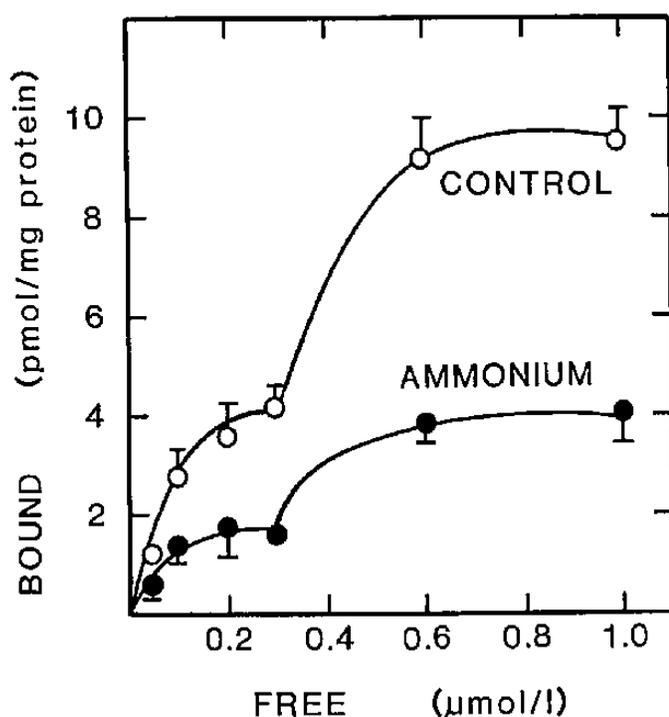


Fig. 2. Effect of prenatal exposure to ammonia on the binding of [<sup>3</sup>H]MK-801 in primary cultures of cerebellar neurons. Cultures of cerebellar neurons were prepared from 7–8 day old rats exposed (ammonium) or not (control) to ammonia during pregnancy and lactation. All culture conditions were the same for control and experimental samples. Experiments were carried out 9–10 days after seeding as indicated in Materials and Methods. Values are the mean  $\pm$  standard deviation of triplicate samples from five different experiments using three different cultures. For points lacking standard deviation, this was less than the width of the point.

in Table 2, AAT activity was increased in control neurons by treatment with low doses of NMDA. The increase in AAT activity was prevented by blocking the NMDA receptor with the selective antagonist MK-801 (data not shown). When the neurons were prepared from rats exposed to ammonia, NMDA was not able to induce AAT activity. Lactate dehydrogenase activity was not affected by any of the treatments.

As shown in Figure 3, prenatal exposure to ammonia affords a significant delayed protection against the neurotoxicity of glutamate and NMDA in cultures of cerebellar neurons. For neurons from control rats, the concentrations of glutamate and NMDA at which 50% of the neurons died were approximately 0.1 mM and 0.2 mM, respectively, while for neurons from ammonia-exposed rats it was approximately 1 mM for glutamate and more than 1 mM for NMDA (Fig. 3).

#### DISCUSSION

It has been reported that persistent blockade of NMDA receptors in immature rats results in impaired growth hor-

TABLE 2  
Prenatal Exposure to Ammonia Prevents the Induction of Aspartate Aminotransferase by NMDA in Primary Cultures of Cerebellar Neurons

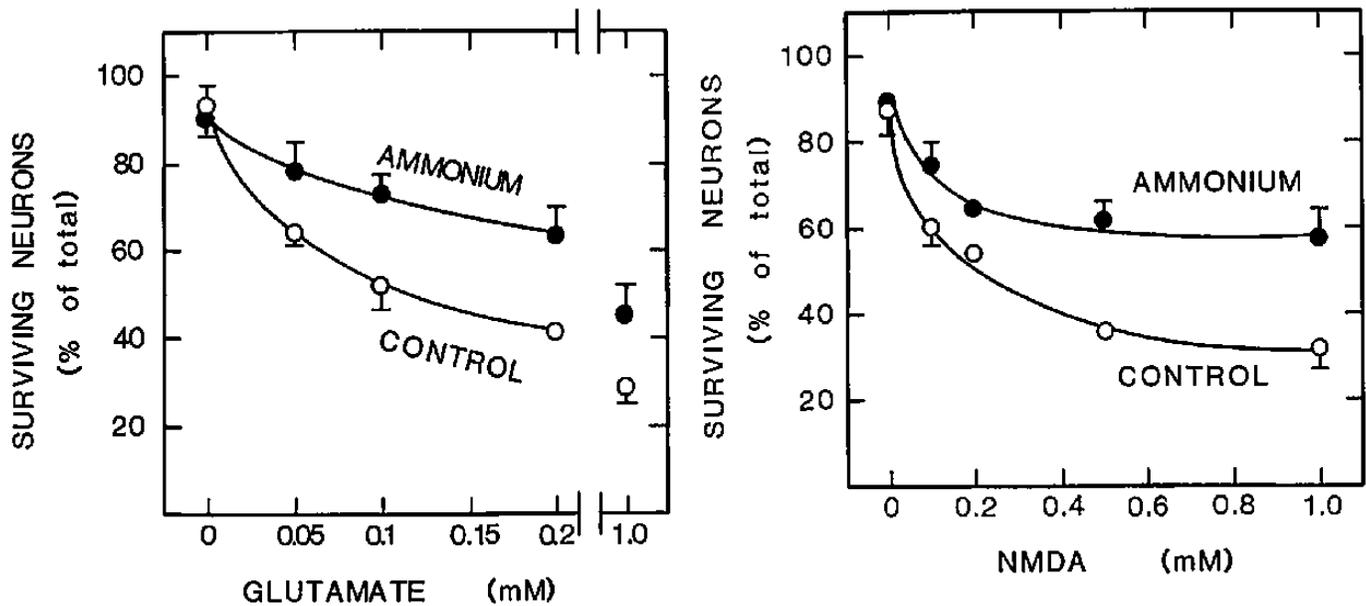
Treatment	NMDA	Aspartate amino-transferase	Lactate dehydrogenase
Control	No	17.5 $\pm$ 1.1	31 $\pm$ 3
	Yes	23.9 $\pm$ 0.6*	29 $\pm$ 3
Ammonia	No	16.7 $\pm$ 1.7	34 $\pm$ 4
	Yes	16.9 $\pm$ 1.6	33 $\pm$ 4

Primary cultures of cerebellar neurons were prepared from 7–8 day old rats exposed (ammonia) or not (controls) to ammonia during pregnancy and lactation. The cultures were prepared and the enzyme activities analyzed as indicated in Materials and Methods. Values are the mean  $\pm$  standard deviations of four experiments using four different cultures. For each experiment samples were assayed in quintuplicate. The value significantly different from the same culture not treated with NMDA ( $p \leq 0.001$ ) is indicated by an asterisk.

more secretion (21). Also, neonatal blockade of these receptors by daily injections of selective antagonists from postnatal day 1 to 22 results in a remarkable decrease of body weight by 50–65% at postnatal day 24 and by 25–32% at 70 days of age (22). This indicates that functional NMDA receptors are necessary for normal growth of animals. As shown in Figure 1, exposure to ammonia during pregnancy and lactation results in a remarkable decrease in the body weight of the rats; the body weight decrease induced by ammonium exposure is similar to that induced by injecting selective antagonists of the NMDA receptor. It is therefore possible that the reduced growth of ammonia-exposed rats could be a consequence of impaired function of NMDA receptors.

It is remarkable that the normal growth rate is not resumed until about 1 month after ammonia withdrawal, while normal ammonia levels in blood and tissues are reached within 48 hours. This suggests that at least some of the deleterious effects of ammonia exposure during pregnancy and lactation (probably including impairment of NMDA receptor function) are maintained for some time after normalization of ammonia levels in tissues. These results are in agreement with a long-lasting impairment of NMDA receptor function by hyperammonemia.

It has been shown that blocking the NMDA receptor with the selective antagonist MK-801 prevents acute ammonia toxicity (16). As mentioned above we suspected that prenatal exposure to ammonia results in a long-lasting impairment of NMDA receptor function. We therefore tested whether this treatment prevents ammonia toxicity. As shown in Table 1, rats exposed to ammonia during pregnancy and lactation but fed a normal diet without ammonia after weaning were significantly more resistant to acute ammonia toxicity than controls 13 days after



**Fig. 3.** Prenatal exposure to ammonia prevents glutamate and NMDA toxicity in primary cultures of cerebellar neurons. Primary cultures of cerebellar neurons were prepared as in Figure 2 from rats exposed (ammonium) or not (control) to ammonia during pregnancy and lactation. Glutamate (A) and NMDA (B) toxicities were determined 8–10 days later as described in Materials and Methods. For glutamate toxicity, values are the mean  $\pm$  standard deviation of six different experiments using four different culture preparations. For NMDA toxicity, values are the mean  $\pm$  standard deviation of four experiments using three different cultures. At least 400 cells were counted for each point. For points lacking standard deviation this was less than the width of the point.

weaning; the protective effect is very slight or missing 1 month after weaning and has disappeared at 3 months. These results support the ideas that hyperammonemia leads to long-lasting impairment of NMDA receptor activation and that impaired NMDA receptor function affords protection against acute ammonia toxicity.

It has been reported that acute ammonia toxicity is mediated by activation of the NMDA type of glutamate receptors (16). On this basis, we hypothesized that the protective effect against ammonia toxicity shown in Table 1 could be due to a reduced number of NMDA receptors or to an impairment of activation of this receptor. To test this possibility we used primary cultures of neurons prepared from cerebellum from controls or from rats exposed to ammonia. We assessed whether exposure of rats to ammonia results in a decreased content or function of NMDA receptors. The cultures were carried out and maintained under exactly the same conditions so that, at the time of the assays, the neurons from hyperammonemic rats had been in a medium without ammonia added for 8–10 days.

Binding of [ $^3$ H]MK-801 seems to reveal two types of NMDA receptors in these neurons (Fig. 2). This is in agreement with a recent report in which ifenprodil discriminates two NMDA receptor populations in cerebellar granule cells (29). Our results also show that the number of NMDA receptors accessible to [ $^3$ H]MK-801 is markedly reduced in neurons from hyperammonemic rats, in-

dicating that NMDA receptor function is impaired even after 9–10 days of culture under normal conditions (in the absence of ammonia). Both types of NMDA receptor were affected in the same way. These results also support the idea that hyperammonemia induces a long-lasting impairment of NMDA receptor function.

Another parameter that we used to assess the functional state of NMDA receptors was the induction of AAT by low doses of NMDA (20). As shown in Table 2, NMDA induced AAT in control neurons, in agreement with the previous reports of Morán and Rivera-Gaxiola (20). The induction of AAT was prevented by MK-801, indicating that it is mediated by activation of NMDA receptors. However, NMDA was not able to increase the activity of AAT in primary cultures of neurons from hyperammonemic rats (Table 2). This supports the idea that hyperammonemia results in long-lasting impairment of NMDA receptor function.

To further confirm that activation of NMDA receptor is impaired in neurons from hyperammonemic rats, we assessed the toxicity of glutamate and of NMDA in these neuronal cultures. It is well known that glutamate or NMDA at high concentrations lead to the death of these neurons and that their neurotoxicity is mediated by activation of the NMDA receptor (e.g. is prevented by selective antagonists of this receptor). The results shown in Figure 3 clearly indicate that neurons from hyperammonemic rats are much more resistant to glutamate or

NMDA toxicity than those from control rats. This indicates that activation of the NMDA receptor is impaired in these neurons and that this impairment affords protection against glutamate and NMDA toxicity.

Chronic blockade of NMDA receptors through daily systemic administration of competitive antagonists from postnatal days 1–22 results in long-lasting alterations in neurotransmission, as well as in decreased excitotoxic sensitivity (30–36). The long-lasting effects of prenatal and neonatal exposure to ammonia on NMDA receptor function shown here are in good agreement with those reports.

In summary, the results shown here indicate that hyperammonemia results in long-lasting impairment of NMDA receptor function, which is maintained even after normalization of ammonia levels for at least 2 weeks. It is possible that such impairment of the NMDA receptor function could be involved in the origin of the mental retardation observed in many cases of neonatal hyperammonemia in humans. Moreover, the impaired function of NMDA receptors in chronic hyperammonemia could explain its protective effect against acute ammonia toxicity in animals and against glutamate and NMDA toxicity in cultured neurons.

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