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Maternal Hypoxia During Pregnancy Induces Fetal Neurodevelopmental Brain Damage: Partial Protection by Magnesium Sulfate

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Fetal low brain oxygenation may be an outcome of maternal complications during pregnancy and is associated with increased risk of cerebral palsy and periventricular leukomalacia in newborns. One treatment used for prevention of fetal brain damage is maternal treatment with $MgSO_4$. Although this treatment is indicated to reduce the risk of cerebral palsy in newborns, its use remains controversial. We have shown previously that pretreatment with $MgSO_4$ in a mouse model of maternal hypoxia prevented a delay in the development of motor reflexes induced by hypoxia. We demonstrate here that pretreatment with $MgSO_4$ reduces hypoxia-induced motor disabilities in adult offspring. This effect is associated with histologic protection of the Purkinje cells in the cerebellum and stabilization of brain-derived neurotrophic factor (BDNF) levels in the cerebellum. $MgSO_4$ did not prevent the reduction in cerebral cortex cell density and cell size induced by maternal hypoxia, however, nor did it interfere with the modulation of BDNF and nerve growth factor (NGF) expression in the cerebral cortex. $MgSO_4$ pretreatment also prevented the impairment of short-term memory (30 min, $P < 0.05$) but not long-term memory (7 days). Nevertheless, maternal pretreatment with $MgSO_4$ reduced CA1 cell layer width and induced alterations in both NGF and BDNF in the hippocampus. These results support the prophylactic effect of $MgSO_4$ against motor disabilities; however, they may also indicate possible harmful effects on the cerebral cortex and hippocampus.

Key words: hypoxia; neurotrophic factors; magnesium sulfate; neurogenesis

Several types of pathologies during pregnancy are known to be major causes of fetal brain damage, such as hypoxia, seizures, and infection. The connection between hypoxia and fetal brain damage is well documented and has been proven (Volpe, 1995). Low brain oxygenation is associated with an increased risk of cerebral palsy and periventricular leukomalacia (PVL) in newborns (Volpe, 2001).

Epidemiologic studies have described that $MgSO_4$ (Mg), administered to pregnant mothers with preeclampsia or in preterm labor, reduced the incidence of cerebral palsy in low birth-weight infants (Nelson and Grether, 1995; Schendel et al., 1996). An observational study showed that of 42 infants later diagnosed with cerebral palsy, 7.1% were exposed in utero to Mg versus 36% of the control survivors. They concluded that Mg might have a prophylactic effect against cerebral palsy in this population of infants with birth weights less than 1,500 g.

In the brain, hypoxia can produce temporary brain dysfunction or permanent brain injury, depending on the duration, degree of oxygen deprivation, and the age of the fetus. Evidence accumulated during the last decade suggests that glutamate-mediated excitotoxicity may play an important role in neuronal damage (McDonald et al., 1988). The hypoxia/ischemia cascade leads to neuronal cell death through overstimulation of the excitatory amino acid receptors (Sombati et al., 1991; Lipton and Rosenberg, 1994), cellular calcium influx, and the formation of

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free radicals and nitric oxide. The physiologic characteristics of the glutamate *N*-methyl-D-aspartate receptor (NMDAR) change markedly during development in a way that can also determine cell vulnerability to excitotoxicity. Indeed, several studies have indicated that neurotoxicity in the fetal brain resulted from overstimulation of the excitatory amino acid receptor, NMDAR, which was extremely active in the immature rat brain as compared to that in the adult rat brain (McDonald et al., 1988).

Studies have demonstrated that Mg can protect against brain damage and NMDA-induced neurodegeneration in neonatal rats. The prophylactic effect of Mg pretreatment on embryonic brain damage in rats was studied recently by Hallak et al. (2000). They demonstrated a reduction in brain size and elevated histologic brain damage in embryo brains after a 2-hr hypoxic episode; both effects were prevented by an Mg load. NMDAR is the first glutamate receptor expressed and is required for the establishment of neuronal circuits (Kleckner and Dingledine, 1991; Messesmith et al., 1997; Scheetz et al., 2000). The blocking of NMDAR by Mg may result in the disturbance of neurogenesis. In addition, studies have demonstrated that Mg can protect against brain damage and NMDA-induced neurodegeneration in neonatal rats, as well as convulsions in adult rats (McDonald et al., 1990; Wolf et al., 1990). Mg administration also had a definite prophylactic effect on central nervous system (CNS) oxygen toxicity (Katz et al., 1990). It reduced seizure duration and electroencephalogram (EEG) amplitude in convulsions due to oxygen toxicity. A neuroprotective effect of Mg was demonstrated even when Mg was administered 24 hr after an ischemic episode (Tsuda et al., 1991).

An animal model of maternal hypoxia and its effect on fetal brain has been established by Hallak et al. (2000). Using the same protocol, we have shown previously that developmental retardation of motor reflex development induced by maternal hypoxia at gestational Day 17 was prevented partially by pretreatment with Mg (Golan et al., 2004). In the present study, we have examined the behavioral consequences of maternal hypoxia and the prophylactic action of Mg treatment on adult offspring behavior. In addition, Mg efficacy in the prevention of histopathologic damage was demonstrated for some brain areas tested during development and in adult offspring. Finally, the possible involvement of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in neurodevelopmental damage was confirmed. In this context, the effect of Mg administration on mothers who breathed regular air was also examined.

MATERIAL AND METHODS

Animals

Pregnant Jackson Black C-57 mice at gestational Day 17 were assigned randomly to one of four groups: (1) intraperitoneal (i.p.) saline injections in room air exposure (S + A, $n = 18$); (2) Mg injections (i.p) in room air exposure (Mg + A, $n = 18$); (3) saline injections (i.p) with hypoxia chamber exposure (S + H, $n = 18$); and (4) Mg injections (i.p) with hypoxia chamber exposure (Mg + H, $n = 18$).

Mg Administration

The maternal Mg injection protocol involved an i.p. loading dose of 270 mg/kg followed by 27 mg/kg every 20 min for 4 hr, injections were given in a volume of 0.1 ml. A second loading dose of 270 mg/kg was given at the end of the 4-hr period. Control mice were injected with saline using the same volume and schedule. The protocol that was selected followed Hallak et al. (2000). Using this protocol, 30 min after the injection of Mg or saline, the magnesium values in the mothers' blood samples were 5.6 mg/dl compared to 3.6 mg/dl in the blood samples of the saline-injected mothers.

Hypoxia Induction

After Mg injections, pregnant mice were placed in a Plexiglas chamber (20 cm x 10 cm x 10 cm) that was perfused with a gas mixture of 9% oxygen, 3% CO_2, and balanced nitrogen for a 2-hr period immediately after the 4-hr injection protocol. Mice from the control groups (S + A; Mg + A) were placed in the same chambers, perfused with air for 2 hr periods.

Surgical Procedure

On gestation Day 17, 2 and 24 hr after treatment, five mice from each group were anesthetized with ketamine, concomitantly with Rompun (administered i.p.). Dissections were carried out after adequate anesthesia. Five fetal brains from each litter were isolated and deep-frozen at −80°C for later analysis. The heads of the remaining fetuses were removed and immersed in 4% paraformaldehyde (PFA) for histologic analysis. The rest of the mice were monitored for the day of delivery; from each litter, eight newborns were kept alive for developmental examination.

The mouse colony was maintained in a 12:12-hr light/dark schedule; food and water were provided ad lib. All procedures were carried out according to guidelines from the Israeli Council on Animal Care and approved by the Ben-Gurion University of the Negev Animal Care and Use Committee.

Tissue Preparation

At postnatal Day 1 (P1), P7, P14, P21, and 8 months, offspring were anesthetized and dissected. Their brains were removed rapidly into ice-cold, artificial cerebrospinal fluid (ACSF) and the brain regions were separated into cortex, hippocampus, cerebellum, and thalamus. Brain tissues were deep-frozen at −80°C for later analysis. One- to seven-day-old mice were anesthetized by hypothermia, whereas older mice were anesthetized by i.p. administration of ketamine and Rompun. For histologic analysis, offspring at P1, P7, P14, P21, and 8 months were anesthetized and perfused transcardially with 4% PFA.

Immunoassay

NGF and BDNF were examined in brain homogenates using a specific enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). ELISA sensitivity was 4 and 47 pg/ml for NGF and BDNF, respectively.

Histology

Sections from PFA-fixed (4%) paraffin-embedded tissue were used. Sagittal sections (4-μ thick), 0.48−0.6 mm from the

midline, were mounted on saline-coated slides, dried at 37°C for 48 hr and stored at room temperature. Afterward, hematoxylin staining images were sampled in an Olympus IX-70 microscope equipped with a SuperCam video camera (Applitec, Israel), and analysis was carried out using NIH Image software (Wayne Rasband, NINDS, NIH). The following parameters were measured: (1) cortex length; (2) cortex width (perpendicular to the CA1 region); (3) secondary motor cortex cell density and size; (4) corpus callosum width (above the posterior part of the lateral ventricle); (5) cerebellum area and cerebellum lobe length; (6) Purkinje cell density (measured in cerebral lobe 3); (7) granular layer thickness of cerebral lobe 3; (8) hippocampus area; (9) granular cell layer (measured in two areas of the inner blade); and (10) pyramidal cell layer width, measured in three areas of the CA1 region.

Behavioral Examination

Mouse behavior was examined at the age of 4–6 months. All experiments were videotaped and analyzed off-line.

Clinging to a grid. Mice were placed on a metal wire grid cover of a mouse cage. The grid was shaken gently so the mice would tighten their grip on the wires, and then the grid was turned upside-down for 60 sec, while the mice hung below the grid. The length of time the mice succeeded in holding on to the wires was measured (Crawley, 1999).

Balance beam. The balance beam measures motor coordination and balance. A beam, 8 mm in diameter and 70 cm long, was situated horizontally and elevated. Enclosed escape boxes were placed at the ends of the beam. The mice were placed in the center of the beam. The time required to reach these boxes, the time spent in an upright position, and the duration on the beam were measured (Crawley, 1999).

Vertical pole. The vertical pole measures motor coordination and balance. The mice were placed in the center of grooved wooden poles, 18 mm in diameter and 1 m long. Initially the poles were kept in a horizontal position, and then were lifted gradually to a vertical position and held there for 1 min. The time elapsed until the mice fell off the pole was measured (Crawley, 1999).

Ataxia measure. For a measure of ataxia, mice were placed at one end of a dark tunnel 60 cm long and the number of stops along the tunnel were counted.

Morris water maze. Mice were trained to swim to a hidden platform in the water maze (Morris, 1984). The maze consisted of a 150-cm diameter, 40-cm high circular pool filled with milk and maintained at 25–26°C. The 10-cm diameter platform was 0.5–1 cm below the surface of the milk. The pool was placed in the center of an enclosure with white walls and a colored geometric figure on each of the walls (triangle, square, circle, and strip) that served as spatial cues. Three- to four-month old mice were tested in a blind analysis and were assigned randomly and trained to find the platform in a given quadrant. One day before the training, the mice were placed on the platform for 10 sec; if they fell, they were replaced on the platform. For 3 consecutive days, each mouse was placed in the pool six times, starting in a random order of direction: north, south, east, or west with 30-min intervals between trials. After locating the platform, mice were dried and returned to their cages. If the mice could not locate the platform after 60 sec in the pool, the trial was stopped and the escape time recorded as 61 sec. As the probe test, after the last trial on the third day, the platform was removed from the pool. The mice were placed in the pool, starting at a location opposite the platform, and were allowed to swim for 60 sec. The time spent swimming in each quadrant was recorded. The amount of time spent swimming in the quadrant where the platform had been located was recorded as another index of spatial learning. A second probe test was carried out 7 days after the last day of training. All the data was recorded on videotape and a blind analysis was done. The mice were housed in individual cages with a 12-hr light/dark cycle and were tested between 9:00–16:00 daily. For visible platform training, spatial cues were removed and the location of the platform was made visible by the use of clear water, the marking of the platform by a blue flag, and its elevation to 1 cm above the surface of the water. The remaining details were similar to those described above for the hidden platform test.

Statistical Analysis

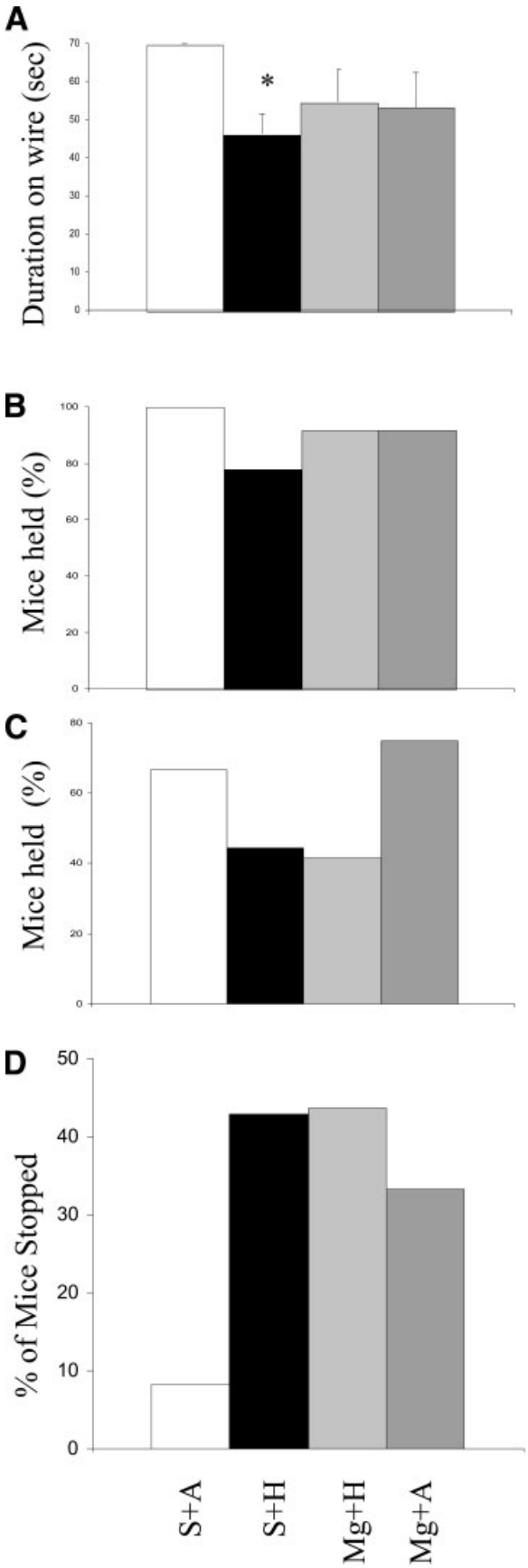
To evaluate maternal insult effects, a multivariate analysis of variance (MANOVA) was used. When the overall MANOVA was significant, Bonferroni post-hoc test for multiple comparisons was carried out. A χ^2 test was used to compare differences in the % of mice (of all mice tested) suceeded or failed to perform a task.

RESULTS

Behavioral Consequences in Adult Offspring of Maternal Hypoxia and Mg Treatment During Pregnancy

Motor function. Maternal hypoxia reduced the ability of offspring to grip a grid of metal wires (Fig. 1A). All mice from the control group, S + A, clung to the grid for the duration of the test (60 sec); however, offspring from the maternal hypoxia group (S + H), did so for a significantly shorter time, as illustrated in Figure 1A. Offspring pretreated with Mg, with or without the hypoxic episode (Mg + H and Mg + A), did not demonstrate a significant difference in their ability to cling to the grid, compared to that in the control group.

The adult offspring were tested on the balance beam, an additional test of motor function and coordination. None of the control group fell off the beam during the 60 sec of the experiment, whereas only 77.7% of the S + H group achieved similar success. In groups pretreated with Mg, 91.6% of the mice presented similar results (Fig. 1B). Motor function and strength was tested further on the vertical pole, as illustrated in Figure 1C. Of the offspring from the control group, 66.6% clung to the pole during the 60 sec of the experiment, whereas only 44.4% of the S + H offspring and 41.6% of the Mg + H offspring did so successfully. Pretreatment with Mg thus did not prevent failure in this test. In contrast, the Mg treatment (Mg + A) tended to increase the rate of success in this test, as 75% of the offspring were able to hold on to the pole for the duration of the experiment. Ataxia, as measured by the number of stops while walking in the 10-cm wide, 70-cm long tunnel (Fig. 1D), was observed in 8.3% of the offspring in the S + H group. A



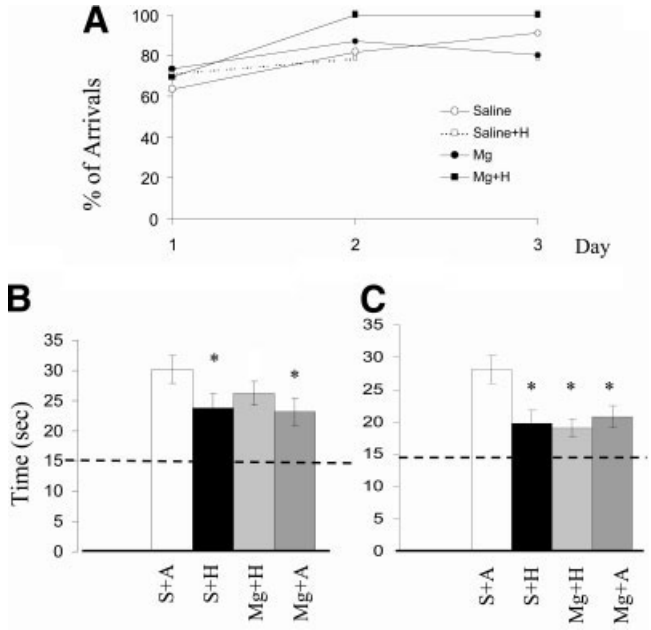


Fig. 2. The effect of maternal hypoxia and Mg administration on adult offspring learning and memory. Spatial learning in the Morris water maze is expressed as the percentage of mice that found the platform in less than 20 sec during the first test of each training day (**A**). The time mice searched in the quarter where the platform used to be, 30 min after the last training session (**B**) and 7 days after the last training session (**C**). $\star P < 0.05$, $n = 9$–12 in each group.

tendency of increase in the percent of ataxic offspring was observed in all the treated groups: 42.8% in the S + H group, 43.7% in the Mg + H group and 33.3% in the Mg + A group. Maternal hypoxia therefore tended to impair motor performance in all tasks examined, and pretreatment with Mg partially protected offspring against some aspects of this damage.

Learning and memory. Spatial learning, as examined in the Morris water maze, was slightly slower in offspring of the S + H and Mg + A groups during their training, as shown in Figure 2A. A trend for lower percent of mice found the platform in less than 20 sec during the first trial on the third day of training. Memory for the platform's location, tested 30 min after the last training in the probe test, was significantly poorer in offspring from the S + H and Mg + A groups ($P < 0.05$) compared to that in the control group. A good memory was indicated by a longer search time in the quarter of the pool where

Fig. 1. The effect of maternal hypoxia and Mg on adult offspring motor function. Adult offspring performance was examined in a "clinging to a grid test" (**A**), balanced beam test: the percent of mice that suceeded to hold on the beam is presented (**B**). Vertical pole test the percent of mice that suceeded to hold on the verticle pole for the duration of the test is presented (**C**). Ataxia measure is presented as percent of mice stopped (**D**). $\star P < 0.05$, $n = 9$–12 in each group.

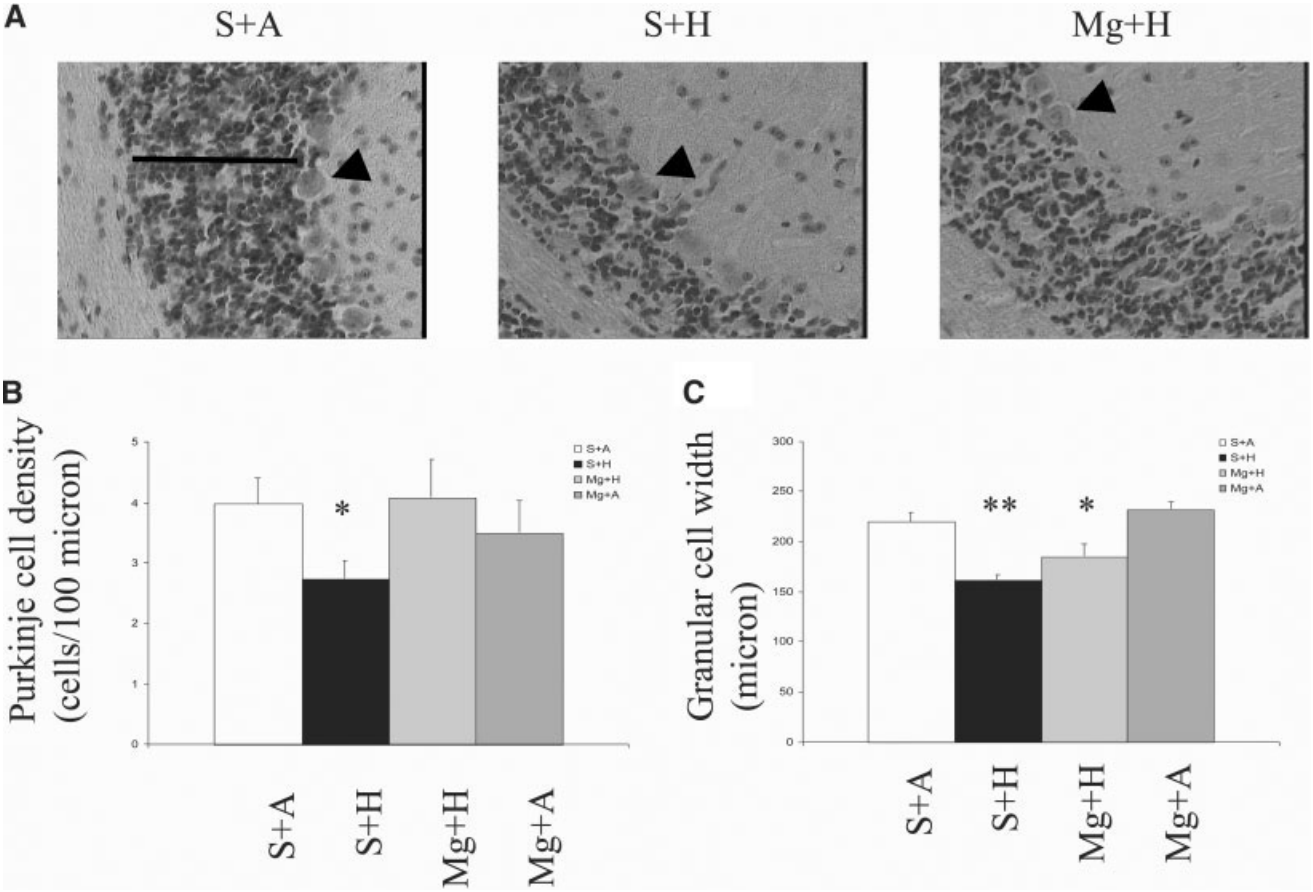


Fig. 3. The effect of maternal hypoxia and Mg on the histology of the adult offspring cerebellum. An example of Nissl staining in cerebellar lobe 3 of adult mice from the S + A, S + H and Mg + H groups (**A**). Purkinje cell density (**B**) and cerebellar granular layer width (**C**). $\star P < 0.05$, $\star\star P < 0.001$. Figure can be viewed in color online via www.interscience.wiley.com.

the platform used to be during training (correct quarter). Offspring from the control and the Mg + H groups searched in the correct quarter a similar amount of time (Fig. 2B). After 7 days, mice from the control group had adequately memorized platform location, as indicated by a 28.1-sec search in the correct quarter. All treated groups, however, searched a significantly shorter amount of time in this quarter of the pool ($P < 0.05$, Fig. 2C).

The possibility that impairment of motor function, as demonstrated above, could influence the offspring's performance of this task was examined by the visible version of the Morris water maze. Mice from all groups found the visible platform on the last day of training in a similar amount of time: 6.8 sec for S + A; 5.95 sec for S + H; 6.13 sec for Mg + H; and 8.06 sec for Mg + A. Similar performances were shown also in the probe test after this task. Only the Mg + A group showed a significantly shorter duration of searching in the correct quarter (less than 15 sec of the 60-sec test).

We conclude that maternal exposure to both hypoxia or Mg significantly impaired spatial learning and memory. The combination of both treatments rescued the offspring from short-term memory damage, but not from long-term memory loss (7 days).

Maternal Hypoxia During Pregnancy Modified Offspring Brain Morphology

Sagittal brain sections of newborns from all groups were examined for developmental consequences of the treatments. Gross measurements of different brain areas were analyzed at P1, P7, P14, P21, and in the adult offspring's brains (P270).

The cerebellum area and the cerebellar lobule length were not affected by the treatments at all ages examined. In adult offspring, however, Purkinje cell density was reduced to 68.8% in the control group (2.74 ± 0.31 cells in 100 μm in the S + H compared to 3.98 ± 0.42; $P < 0.05$). Mg + H and Mg + A group offspring had values similar to those of the controls (3.51 ± 0.63 and 4.08 ± 0.52 cells per 100 μm, respectively; Fig. 3A). The width of the granular layer was reduced from 220 ± 8.41 μm in the control group to 162.4 ± 5.2 μm ($P < 0.001$) in offspring

TABLE I. Effect of Hypoxia and Pretreatment with Mg on Cerebral Cortex Dimensions†

	S + A		S + H			Mg + A			Mg + H		
	Value	SD	Value	SD	% of control	Value	SD	% of control	Value	SD	% of control
Cortex length (μm)											
Adult	1,183.0	27.02	1,147.6	33.9	97.0	1,127.9	26.1	95.3	1,200.2	29.7	101.5
P21	1,283.4	107.2	1,218.1	48.8	94.9	1,203.6	35.8	93.8	1,321.8	91.9	103.0
P7	937.7	37.4	893.6	81.4	95.3	908.1	50.05	96.8	932.0	44.5	99.4
P1	574.3	46.2	511.1	114.1	89.0	540.1	34.2	94.0	556.7	43.7	96.9
Cortex width (μm)											
Adult	245.8	16.8	224.0	14.2	91.1	286.0	37.5	116.3	225.9	19.2	91.9
P21	278.6	26.8	268.0	31.7	96.2	253.7	37.4	91.1	261.6	17.1	93.9
P14	275.2	10.9	228.8★	28.5	83.1	264.8	39.4	96.2	268.9	45.5	97.7
P7	263.1	29.4	161.3★	14.2	61.3	247.5	56.9	94.1	211.1	4.5	80.2
P1	180.9	14.5	204.9	2.2	113.3	173.0	36.7	95.6	187.4	48.1	103.6
Corpus callosum width (μm)											
Adult	88.6	12.9	87.8	14.03	99.1	96.0	16.6	108.4	79.5	9.6	89.7
P21	72.5	10.1	118.4	18.8	163.2	71.6	11.7	98.8	70.1	8.3	96.6
P14	87.1	40.1	56.7	5.3	65.1	62.2	6.7	71.4	75.8	6.9	87.0
P7	84.3	16.1	59.9	9.8	71.0	70.6	8.1	83.7	113.0	27.7	133.9

†S + A, saline injections + air exposure; S + H, saline injections + hypoxia; Mg + A, Mg injections + air exposure; Mg + H, Mg injections + hypoxia; SD, standard deviation; P, postnatal day.
★$P < 0.05$ compared to controls of same age.

of the S + H group. Mg + H did not prevent the damage, as evaluated by the width of 185.7 ± 11.97 μm ($P < 0.05$). No significant difference in granular cell layer width was observed in offspring of the Mg + A group.

Analysis of cerebral cortex length and width, cerebral cortex layer 1 width, and corpus callosum width revealed no significant differences at all ages examined, except for a transient reduction in cerebral cortex width at ages P7 and P14 in offspring of the S + H group (Table I). A detailed analysis, however, of cell density and size in cerebral cortex of adult offspring revealed a significant reduction in cell density in all M2 cerebral cortex layers, as demonstrated in Figure 4.

The most affected layers were layers 2–3, in which cell density and size were both affected by all treatments (Fig. 4A). For example, in offspring from the S + H group, cell density was reduced by 24.8% compared to that in controls. The cell density in offspring of the Mg + H and Mg + A groups was reduced by 29.1% and 27.8% of the control group, respectively. Pretreatment with Mg did not prevent the reduction in cell density and size in cerebral cortex layers 2–3. In cerebral cortex layer 4, cell density, but not cell size, was affected by all the treatments. Cell density was reduced by hypoxia or Mg in layers 5–6. A combined treatment, however, (Mg + H) protected the cells from altered cell density and size (Fig. 4A,B). Overall, the superficial layers were the most susceptible to both interventions at gestational Day 17. Pretreatment with Mg partially protected the cerebral cortex from histopathologic damage only in layers 5-6.

Morphologic damage to the hippocampus region was observed only in offspring treated with Mg. No difference in the CA1 pyramidal layer width and dentate gyrus granular cell layer (DG) was detected in the offspring exposed to hypoxia. In adult offspring of the Mg + A group, there was a slight increase in DG width (105.2% of control) and significant expansion was observed in DG width in offspring of the Mg + H group ($P < 0.03$). In contrast, a reduction in the CA1 cell layer was observed in adult offspring of the Mg + A and Mg + H groups (86.2% and 82% of control CA1 width, respectively; $P < 0.001$; Fig. 5). The significant reduction in the CA1 width was consistent in these groups, also at younger ages (Fig. 5).

Taken together, the histologic data indicates major sensitivity to hypoxia in the cerebellum and in cerebral cortex cell density and size. Mg pretreatment showed a prophylactic action against part of these effects. In the hippocampus, however, Mg treatments had a deleterious effect, as reflected by modifications in cell layer width.

Maternal Hypoxia and Mg Treatment During Pregnancy Modulated Neurotrophic Factor Expression in Fetal Brains

Neurotrophic factor signaling has a major role in neurogenesis. It is involved in cell survival (Huang and Reichardt, 2001; Sofroniew et al., 2001) and in the control of synaptic function (McAllister et al., 1995, 1997). A reduction in BDNF expression was found after perinatal hypoxia (Schmidt-Kastner et al., 2002). We therefore examined the short- and long-term effects of maternal hypoxia during pregnancy and pretreatment with Mg on the expression of BDNF in the fetal brain.

BDNF expression levels in fetal brain homogenate 2 hr after treatments was high in groups pretreated with saline (0.28 and 0.25 pg/μg protein, S + A and S + H, respectively) and significantly lower ($P < 0.001$) in the groups pretreated with Mg (0.1 and 0.12 pg/μg protein,

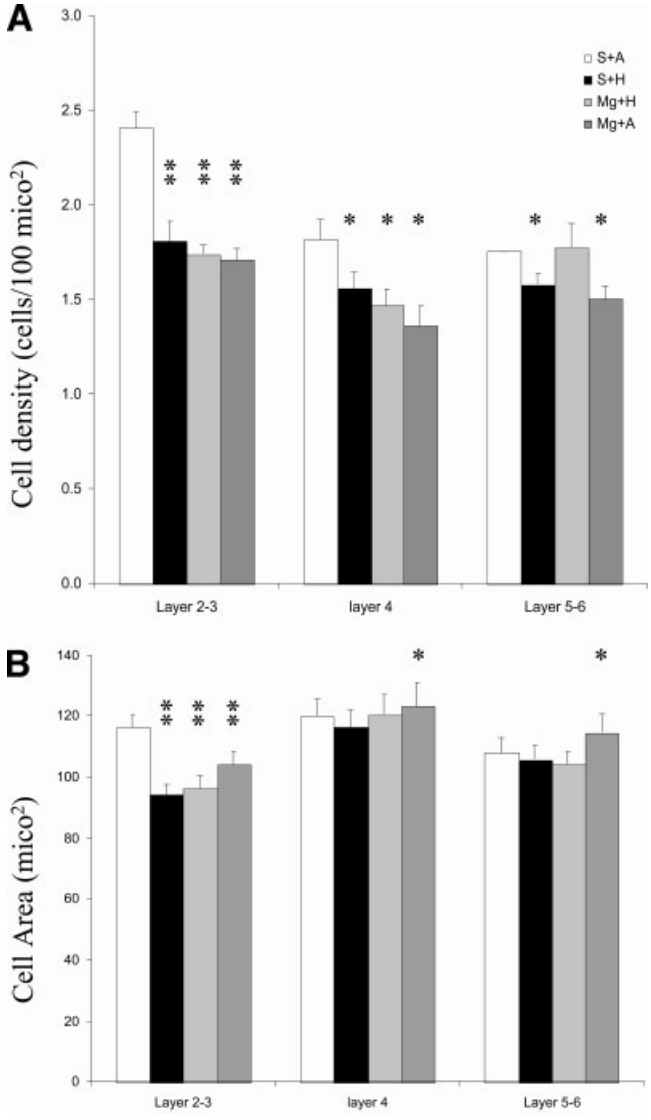


Fig. 4. The effect of maternal hypoxia and Mg on adult offspring cerebral cortex histology. Cell density (**A**) and cell size (**B**) in the different cerebral cortex layers are presented: layer 2–3, layer 4, and layer 5–6. $\star P < 0.05$, $\star\star P < 0.001$.

Mg + H and Mg + A, respectively; Fig. 6A). At 24 hr after treatments, a significant reduction in BDNF levels in fetal brains was observed in both groups pretreated with saline, as compared to their BDNF values 2 hr after the treatment ($P < 0.001$). At this time, however, BDNF levels in fetal brains were lower in the S + H group, compared to that in the control group ($P < 0.01$). There were no significant differences between the BDNF levels in the groups pretreated with Mg at the two time points examined (Fig. 6A). An examination of BDNF levels in brains of naive embryos demonstrated BDNF levels similar to those observed in the S + A group 24 hr after the treatment. This suggests that 4 hr of saline administration may stimulate an increase in BDNF levels. This enhancement in BDNF expression was prevented by pretreatment with Mg.

Long-term effects of maternal hypoxia and Mg treatment were observed in the cerebellums of adult offspring (Fig. 6B). BDNF levels in cerebellums of offspring of the S + H group tended toward higher values ($P < 0.08$), whereas pretreatment with Mg prevented this tendency. Elevated BDNF levels were seen also in the thalamus in offspring from all treated groups. All treatments significantly increased BDNF levels (from 1.01 pg/μg protein in the control group to 2.2 pg/μg protein in the S + H group, 1.99 pg/μg protein in the Mg + H group, and 2.32 pg/μg protein in the Mg + A group). A reduction in BDNF levels in cerebral cortex was observed in adult offspring from all groups (Fig. 6D). These levels went from 0.71 pg/μg protein in the control group to 0.49 pg/μg protein, 0.42 pg/μg protein ($P < 0.02$), and 0.42 pg/μg protein ($P < 0.03$) in the S + H, Mg + H, and Mg + A groups, respectively. In adult offspring hippocampus, BDNF levels were not modified by S + H treatment; however, adult offspring of both groups pretreated with Mg had significantly elevated levels ($P < 0.04$).

Maternal treatments, hypoxia, and Mg affected BDNF levels in offspring brains. Hypoxia modulated BDNF levels in fetal and adult offspring brains, whereas Mg had only a long-term effect. In the short-term, Mg prevented a reduction in BDNF expression. Long-term modifications were specific to brain regions; in some brain regions treatment elevated BDNF levels, whereas in others, it reduced them.

The NGF levels (Fig. 7) examined in homogenates of adult offspring cerebellum were similar for all groups. In the thalamus region of adult offspring, all treatments significantly increased NGF levels (0.48 pg/μg protein in the control group compared to 1.51, 2.33, and 1.2 pg/μg protein in the S + H, Mg + H, and Mg + A groups, respectively; $P < 0.03$). In contrast, all treatments caused a slight reduction in NGF expression in cerebral cortex of adult offspring. Whereas NGF levels were 0.82 pg/μg protein in the control group, a tendency to reduced NGF levels was observed in the cerebral cortex of offspring from the S + H, Mg + H, and Mg + A groups (0.37, 0.45, and 0.47 pg/μg protein, respectively; $P < 0.06$). In hippocampus, Mg treatments induced a significant increase in NGF levels, and hypoxia increased the NGF levels in P14 offspring ($P < 0.04$), but did not affect NGF expression in brains of adult offspring.

In conclusion, maternal hypoxia had a significant long-term effect on the expression of NGF in brains of adult offspring only in the thalamus region. Mg treatments, with or without exposure to hypoxia, significantly modified NGF levels in thalamus and hippocampus. As was depicted for BDNF, modifications in the protein level (increase or decrease) depended on the brain region being examined.

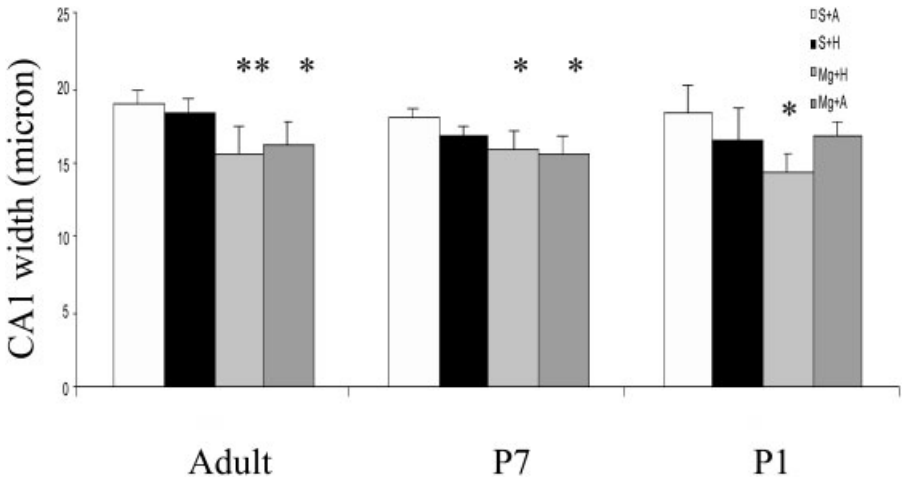


Fig. 5. The effect of maternal hypoxia and Mg on the morphogenesis of hippocampal CA1 cell layer. The width of CA1 was measured at P1, P7, and in adult offspring. $\star P < 0.05$, $\star\star P < 0.001$.

DISCUSSION

We examined the endpoint effect of maternal hypoxia during pregnancy and Mg pretreatment on offspring development. To evaluate the mechanism involved, we carried out a histologic study and examined the levels of neurotrophic factors in the brains of offspring. The hallmarks of maternal hypoxia effects were confirmed and the prophylactic effect of Mg was tested via adult offspring behavior and brain histology. The possibility that Mg effects were mediated by modulation of neurotrophic factor (BDNF and NGF) levels was examined.

Regulation of BDNF

The importance of BDNF for the development of different brain regions, particularly the hippocampus and cerebellum, has been shown previously (Segal et al., 1992; Mertz et al., 2000; Sherrard and Bower, 2002). A reduction in the expression of BDNF mRNA was observed after focal ischemia (Schmidt-Kastner et al., 2002). In addition, BDNF had a prophylactic effect against hypoxia-ischemia (IH) brain injury in neonatal rats (Cheng et al., 1997; Almli et al., 2000). This evidence suggests that part of the cell death observed in HI animal models is due to reduced BDNF levels. Our study demonstrates the prevention of hypoxia-induced reduction in fetal BDNF levels, 24 hr after the hypoxic episode, as a result of Mg pretreatment, as indicated by stable BDNF levels in the fetal brains (Mg + H and Mg + A) 2 and 24 hr after undergoing hypoxia. In our study, in addition to the initial regulation of BDNF levels by hypoxia and Mg treatments, long-lasting regulation of BDNF and NGF levels in brains of offspring was observed. This may be due to permanent changes in regulation of production of these proteins.

Motor Function

The prophylactic effect of Mg was found in the "clinging on a grid" test and a similar tendency was observed in the balance beam tests (Fig. 1), which require motor coordination for their performance (Crawley, 1999). One brain area participating in the coordination of motor function is the cerebellum. Indeed, the most consistent effects of Mg, as a prophylactic agent, were found in the cerebellum (Fig. 3 and 6). The cerebellum, specifically the Purkinje cell in the cerebellum, was noted previously for its sensitivity to HI; damage to the cell soma and the dendritic tree was reported previously (Lee et al., 2001). The present study is consistent with these findings. We have demonstrated a significant reduction in Purkinje cell density, which was not observed in offspring belonging to mothers pretreated with Mg. The changes in Purkinje cell density and the reduction of cerebellar granular cell layer width were associated with a trend of an elevation in BDNF levels in cerebellums of adult offspring. Although BDNF is known for its prophylactic effect, it is possible that an increase in its levels may have harmful effects, or may cause abnormal growth, such as sprouting. Mg pretreatment prevented an increase in BDNF level and thus supported normal growth.

Cell death in the sub- and cortical plate regions (McQuillen et al., 2003), due to apoptosis or necrosis, has been reported to be a result of HI, depending on the age of the exposed newborn (Grojean et al., 2003). The reduction in cell density observed in adult offspring cerebral cortexes in our study might be related to the long-lasting outcome of earlier hypoxic experience. Both BDNF and NGF are important factors for cell survival (Huang and Reichardt, 2001; Sofroniew et al., 2001) and neuron structure (McAllister et al., 1995; Bibel et al., 2000) in the cerebral cortex. The reduction in the levels of these factors in adult offspring cerebral cortex may therefore contribute to differences in cell size and density observed in this brain region. Unlike the cerebellum, most histopathologic damage observed in the cerebral cortex was not prevented by Mg pretreatment. Only the hypoxic damage to cell density was prevented. Interestingly, the superficial cortical layers, which are last occupied by neurons during embryonic development, were the most vulnerable to all treatments in our study. As we deepened our examination of layers 4 and 5–6, smaller effects of hypoxia were observed and the prophylactic effect of Mg was found to be greater. This

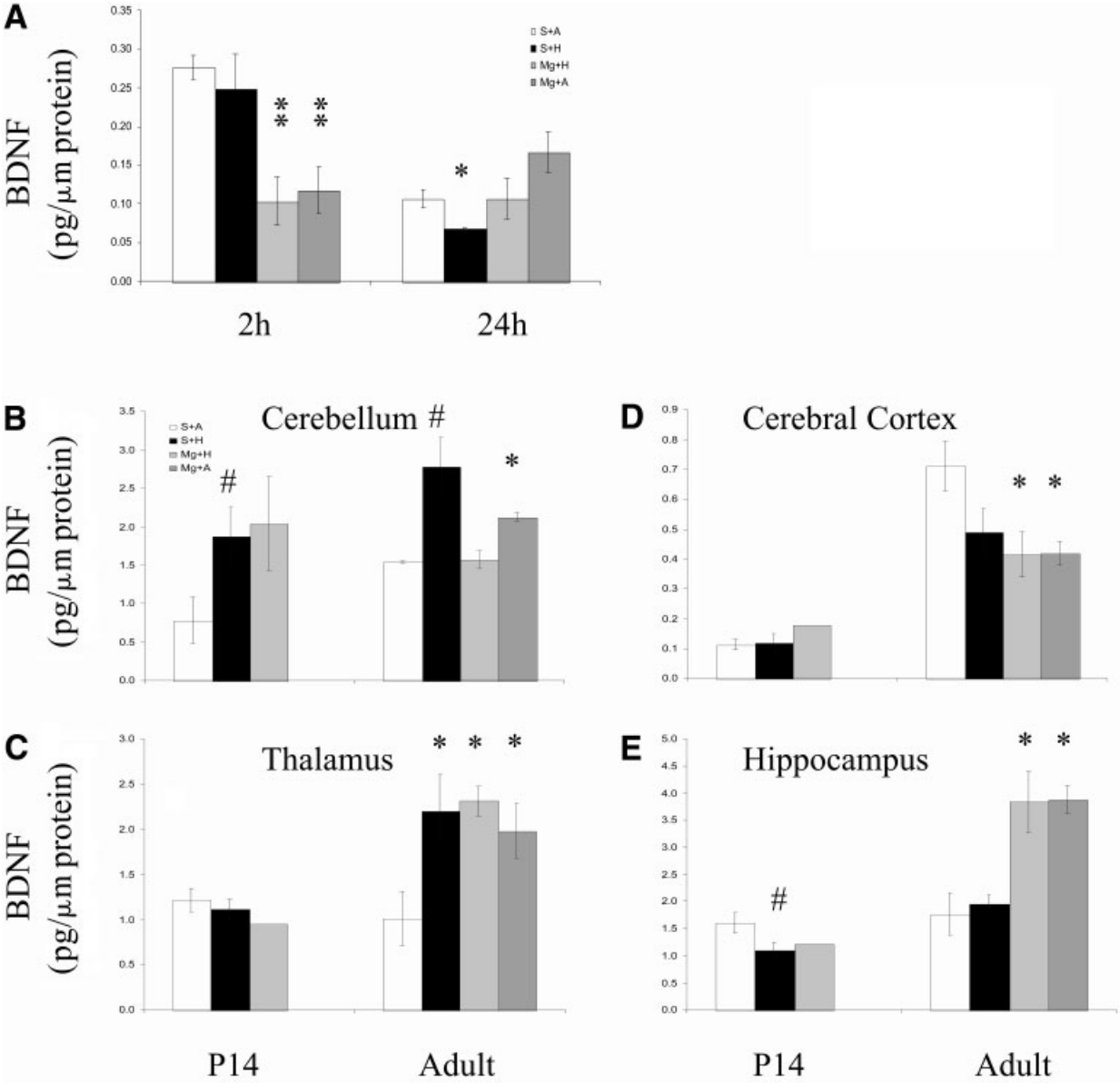


Fig. 6. The effect of maternal hypoxia and Mg on BDNF levels in fetal and newborn brains. BDNF ontogeny was examined in offspring at E17, E18, P14, and in the adult brain. **A:** BDNF levels in E17 2 hr after treatment and in E18 24 hr after treatment. BDNF levels in P14 and adult offspring in cerebellum (**B**), cerebral cortex (**C**), thalamus (**D**), and hippocampus (**E**). $n = 4-8$ samples at each point, apart from P14 cerebral cortex, thalamus, and hippocampus, in which $n = 1$. $^{\star}P < 0.05$, $^{\star\star}P < 0.001$, $\#P < 0.08$.

may also depend on the NMDAR subunit composition in the different cortical layers (Monyer et al., 1994). Paradoxically, in the offspring of air-breathing mice pretreated with Mg, the damage in the cerebral cortex was observed to be equal in all layers.

Learning and Memory

An additional skill that was examined and found affected by hypoxia is spatial learning. This finding is also in accordance with previous studies (Cai et al., 1999; Almli et al., 2000; Simonova et al., 2003). Spatial learning predominantly depends on the neuronal circuits of the hippocampal and cortical areas (Morris, 1984; D'Hooge and De Deyn, 2001). In cases of impaired short-term memory of the platform location, it was found only 30 min after being learned in the hypoxia and Mg groups, whereas the Mg + H group presented an unchanged performance, compared to the control group. Long-term

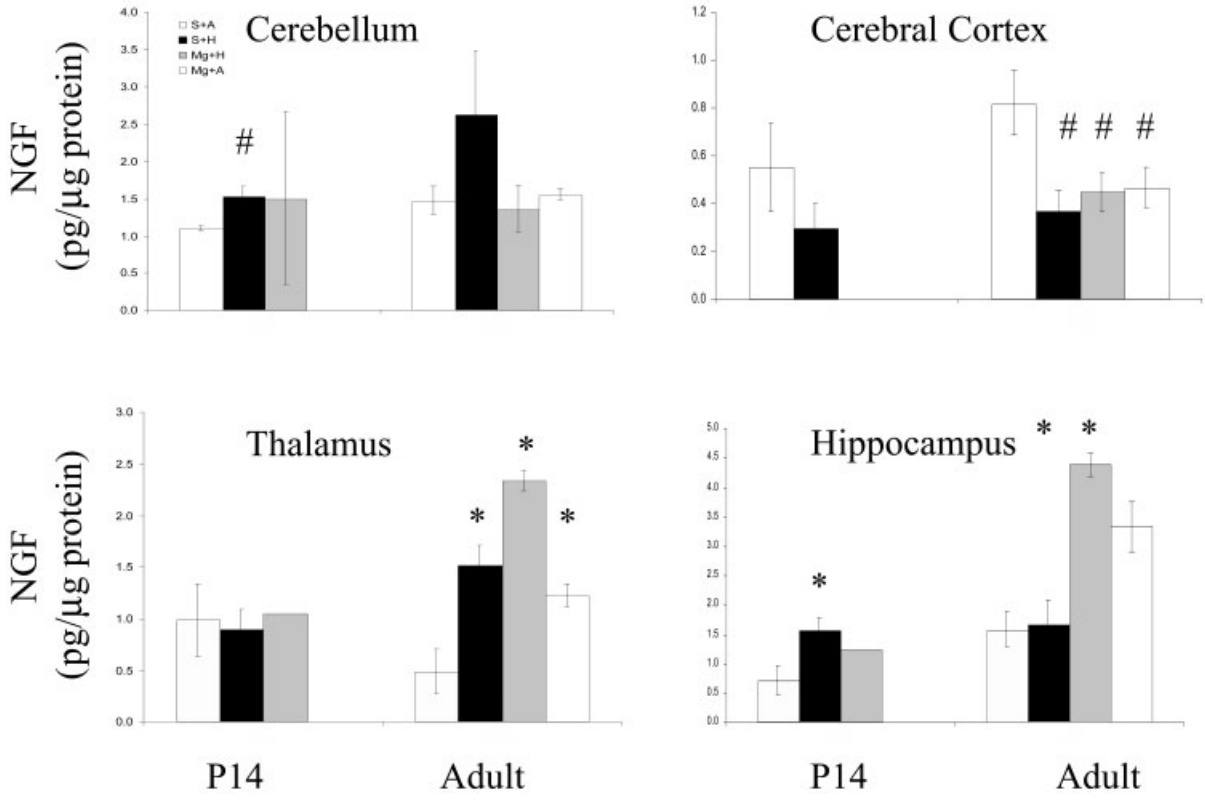


Fig. 7. The effect of maternal hypoxia and Mg on NGF levels in newborn brains. NGF expression was examined in offspring at P14 and in the adult brain in cerebellum (**A**), cerebral cortex (**B**), thalamus (**C**), and hippocampus (**D**). $n = 4-8$ samples at each point, apart from Mg + H P14 cerebral cortex, thalamus, and hippocampus, in which $n = 1$. $\star P < 0.05$, $\#P < 0.08$.

memory, however, was impaired in all hypoxia and Mg groups. An evaluation of histologic correlates in the hippocampus, supporting this behavior, indicated damage to CA1 region in both developing and adult offspring in groups treated with Mg, with or without hypoxia. At the age of embryonic day 17 (E17), neurons in the CA1 region underwent extensive maturation, dendrite formation, synaptogenesis (Bayer, 1980a,b; Altman and Bayer, 1990; Tyzio et al., 1999), and spinogenesis (Sorra and Harris, 2000). This stage in neuron development depends primarily on neuronal activity. A reduction of neuronal activity and blockade of the NMDA receptor by a high Mg concentration may therefore play a critical role in accurate development of this brain region. In contrast, in the DG region, Mg + H induced a 30% increase in granular cell layer width in adult offspring. In the DG, the addition of new granular cells is a continuing process in developing and adult hippocampus (Gould et al., 1998; Van Praagh et al., 2002). It is possible that elevated levels of NGF and BDNF in the adult offspring pretreated with Mg (Fig. 6 and 7) induced cell proliferation and thus increased the DG width.

Mg Effect

Although the major question of the present study was whether Mg pretreatment causes a prophylactic action preventing hypoxia damage in the developing brain, another important issue may have been answered partially by our observations: What is the effect of an Mg load on E17 brain development? In terms of neuronal activity, Mg may function through several mechanisms: (1) an Mg load may change the threshold for action potential firing by changing the ionic strength in CSF; (2) it may reduce neurotransmitter release from presynaptic terminals (Wernig, 1972); and (3) it may partially block the NMDAR (Monaghan et al., 1989). All the above may reduce neuronal activity and thus affect activity-dependent development (Kleinschmidt et al., 1987; Simon et al., 1992; Katz and Shatz, 1996). During development, NMDA receptors undergo extensive changes in their subunit composition and their sensitivity to Mg blockade (Monyer et al., 1992, 1994; Kuner and Schoepfer, 1996). Moreover, differences in NMDAR composition were also observed in different brain regions (Monyer et al., 1994). Differences in the response to Mg pretreatment were also observed in our present study; major damage was observed in the hippocampus and cerebral cortex. Mg treatments caused significant modifications in neurotrophic factor levels in these two brain regions as well. On the behavioral level,

Mg treatments partially impaired motor function (ataxia) and spatial learning (short- and long-term memory). These observations corroborate studies indicating the major role of glutamate, and specifically the NMDAR, in neuronal circuit formation (Cline and Constantine-Paton, 1989; Simon et al., 1992).

Deficient spatial learning in offspring of both hypoxia- and Mg-treated mice may result from histologic damage in the hippocampal and cerebral cortex regions. Histologic damage was demonstrated in both regions after Mg treatment.

In conclusion, maternal hypoxia impaired various behavioral aspects in adult offspring. Blocking of the NMDAR was suggested as a tool to prevent excitotoxicity, due to elevated activation of the NMDAR. Based on our results, it seems that maternal hypoxia impaired different types of behavior in adult mice. Mg treatment also caused such an effect. It is possible that damage observed after hypoxia or Mg treatment is a result of both hyper- and hypoactivation of the same receptor. When both treatments were combined, however, the damage was prevented and some types of behavior, mainly motor, remained normal.

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