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Maternal Hypoxia during Pregnancy Delays the Development of Motor Reflexes in Newborn Mice

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Key Words
Hypoxia-induced brain damage · Magnesium sulfate, neuroprotective function · Righting reflex

Abstract
Prenatal hypoxic-ischemic brain injury is believed to cause permanent neurological deficits in newborns. We investigated the possibility that maternal hypoxia during pregnancy leads to offspring brain damage and its prevention by i.p. administration of $MgSO_4$. Pregnant mice at gestation day 17 were exposed to hypoxia or air following pretreatment with saline or Mg. Newborn mice to mothers exposed to hypoxia demonstrated faster development of morphogenic parameters such as eyelid opening, hair growth and teeth eruption. In addition, hypoxia delayed the development of motor reflexes. Pretreatment with Mg compensates for hypoxia-induced impairment and in some cases accelerates the development of these functions. In conclusion, maternal hypoxia significantly modifies the developmental process of newborn mice. In our study, pretreatment with Mg showed significant prophylactic action against motor impairments.

Introduction

Maternal insults during pregnancy, including hypoxia, seizures, and infection, are known to be major causes of fetal brain damage. The association between hypoxia and fetal brain damage is well described and proven [1]. Hypoxia can produce temporary brain dysfunction or permanent brain injury, depending on the duration, intensity of oxygen deprivation and age of the fetus. Accumulating evidence in the last decade suggests that glutamate-mediated excitotoxicity may play an important role in neuronal injury [2]. The hypoxia/ischemia cascade leads to neuronal cell death through overstimulation of the excitatory amino acid receptors [3, 4], cellular calcium influx, and formation of free radicals and nitric oxide. The mechanisms or pathophysiology of hypoxia-induced fetal neurotoxicity are complex and not fully understood. The physiological characteristics of the glutamate N-methyl-*D*-aspartate receptor (NMDAR) changed markedly during development in a way that can also determine cell vulnerability to excitotoxicity. Indeed, the results of several studies implicate that the neurotoxicity resulting from overstimulation of the excitatory amino acid receptor is extremely active in the immature rat brain compared to the adult rat brain [5]. Studies have

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demonstrated that magnesium can protect against brain injury and NMDA-induced neurodegeneration in neonatal rats [6]. In human babies, perinatal hypoxia is a major cause of perinatal brain injury, with a particular focus on periventricular leukomalacia (PVL). PVL is the predominant form of brain injury in the premature infant and underlies the development of the spastic motor deficits of cerebral palsy as well as cognitive impairment [7]. Recent studies have shown that prenatal exposure to $MgSO_4$ in very low birth weight infants was associated with a reduction in cerebral palsy [8, 9]. An observational study showed that 7.1% of 42 infants later diagnosed with cerebral palsy had been exposed to $MgSO_4$ in utero, compared to 36% of the control survivors. The authors concluded that $MgSO_4$ might have a prophylactic effect against cerebral palsy in infants with birth weights <1,500 g. In a study of very low birth weight children at 3–5 years of age, those who had been exposed to $MgSO_4$ showed a lower prevalence of cerebral palsy or mental retardation (0.9 and 1.8%, respectively) than those not exposed (7.7 and 5.8%, respectively) [9]. They also concluded that prenatal $MgSO_4$ exposure was associated with reduced risk of cerebral palsy and possibly mental retardation in very low birth weight children [8, 9]. The protective effect of $MgSO_4$ pretreatment on embryonic brain injury in rats was studied by Hallak et al. [10]. They demonstrated reduced brain size and elevated histological brain injury in embryo brains following a hypoxic episode; both of these effects were prevented by $MgSO_4$ load. The NMDAR was shown to be the first glutamate receptor expressed, it is required for the formation of neuronal circuits. Therefore blocking NMDAR with magnesium may result in the disturbance of neurogenesis [15].

In the present study we examined whether maternal hypoxia during day 17 of gestation affects newborn brain development as well as the possibility that $MgSO_4$ could reduce the hypoxic effects.

Methods

Pregnant Jackson Black C-57 mice at gestation day 17 were randomly assigned to one of four groups: (1) saline injections (intra-peritoneal; i.p.) followed by room air exposure (S+A, n = 8); (2) $MgSO_4$ injections (i.p.) followed by room air exposure (Mg+A, n = 8); (3) saline injections (i.p.) followed by hypoxia chamber exposure (S+H, n = 8), and (4) $MgSO_4$ injections (i.p.) followed by hypoxia chamber exposure (Mg+H, n = 8). Day of delivery was monitored, and from each mother 8 newborns were kept alive and used to test developmental parameters. The maternal $MgSO_4$ injection protocol involves an i.p. loading dose of 270 mg/kg followed by 27 mg/kg every 20 min for 4 h. A second loading dose of 270 mg/kg was given at the end of the 4-hour period. Control mice were injected with saline following the same volume and schedule. The protocol was that of Hallak et al. [10]. Using this protocol, 30 min following the last injection of $MgSO_4$ or saline, magnesium values in mothers' blood were 5.6 mg/dl compared to 3.6 mg/dl in the blood of saline-injected mothers. Following $MgSO_4$ injections, pregnant mice were placed in a plexiglass chamber (20 × 10 × 10 cm) that was perfused with a gas mixture of 9% oxygen, 3% CO_2, and balanced nitrogen for a 2-hour period immediately following the 4-hour injection protocol. Animals of the control group (S+A; Mg+A) were placed in the same chambers perfused with air for a 2-hour period.

For the examination of brain weight at postnatal days 1 (P1), P7, P14 and P21, the mice were anesthetized by i.p. administration of ketamine (0.8 mg/g) and rompun (0.16 mg/g). After adequate anesthesia, the animals were perfused transcardially with 4% paraform aldehyde (PFA). After 5–10 min of perfusion, the scalp skin and bone tissues were removed and the brains were obtained intact and kept overnight in 4% PFA. The brains were washed in PBS, then weighed and stored for later analysis (not included in the current study).

Phenotype Aspects Examined in Newborn Mice

Two newborns per litter were tested daily during the first month of life for their general phenotypic and morphogenic aspects, such as body weight, hair growth, day of eyelid opening and teething.

Reflex Development in Newborn Mice

Two newborns per litter were tested daily during the first month of newborns' life for the development of several reflexes. The *righting reflex* is the measure of time required for a newborn that has been placed on his back to right himself on his 4 feet. *Bar holding* is the reflex to grip a thin bar while the bar slowly rotates (4 cycles/min). The score recorded is the largest rotation angle that the newborn can grip the bar (this test is a light version of the rotarod, adjusted to newborn mice). *Inclined climbing* tests the ability of the mice to climb on slopes of 30, 50, 70 and 90 degrees.

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 performed. χ^2 test was used to compare the time course of eyelid opening, hair growth and teeth eruption.

Results

Morphogenic Parameters

Two offsprings of each mother were examined daily during the first month. Maternal hypoxia (S+H) or maternal Mg alone (Mg+A) did not affect the newborn body weight (fig. 1a). However, maternal Mg+H significantly increased the body weight of the newborns compared to newborns from mothers of all other groups ($p < 0.04$, t test, P10–P21). This difference was observed at the end of the first week and remained prominent at the age of 14–21 days.

On examination, the brain weight of newborns from mothers of the S+H group was generally greater than the brain weight from the S+A group ($p = 0.08$, fig. 1b). No other effects on brain weight were observed in the treatment groups compared to the S+A group. The difference between the S+H and Mg+A groups was observed at P21 ($p < 0.05$). The ratio of brain/body weight was reduced with age progression. However, this ratio was significantly lower in the Mg+H group (P7–P21, $p < 0.03$, t test, n = 3–6 for each group at each day examined) in comparison to the control S+A group.

Eyelid opening in newborns began in the control group (S+A) at P13. Between P13 and P16 all newborns opened their eyes (fig. 2a). Maternal hypoxic episode (S+H) accelerated eyelid opening ($p < 0.005$, χ^2 test, McNemar). The percentage of newborns that opened their eyes on all the mentioned days was higher in the hypoxic group than in the control group. This was also reflected on the day when 100% of the hypoxic group eyelids were opened, 1 day earlier than in the control group. Maternal pretreatment with magnesium (Mg+H) significantly advanced the day of eyelid opening compared to controls ($p < 0.006$, χ^2 test, McNemar). In this group, newborns began opening their eyes 1 day earlier and also had completed opening their eyes 1 day earlier.

Body hair appearance began at the age of P10 in control group newborns and within 1 day all of the offspring presented full hair coverage (fig. 2b). Hypoxic conditions or magnesium administration alone did not affect the first day of body hair appearance. However, 1 and 2 days of delay were observed in newborns belonging to the Mg+A and S+H groups, respectively, in presenting 100% hair growth. The combined treatment, Mg+H, significantly facilitated the beginning of hair appearance without affecting the day on which 100% offspring were covered with body hair ($p < 0.05$, χ^2 test, McNemar).

Teething began in all groups at P11, except for the Mg+A group, where teeth appeared 1 day later (P12). Ma-

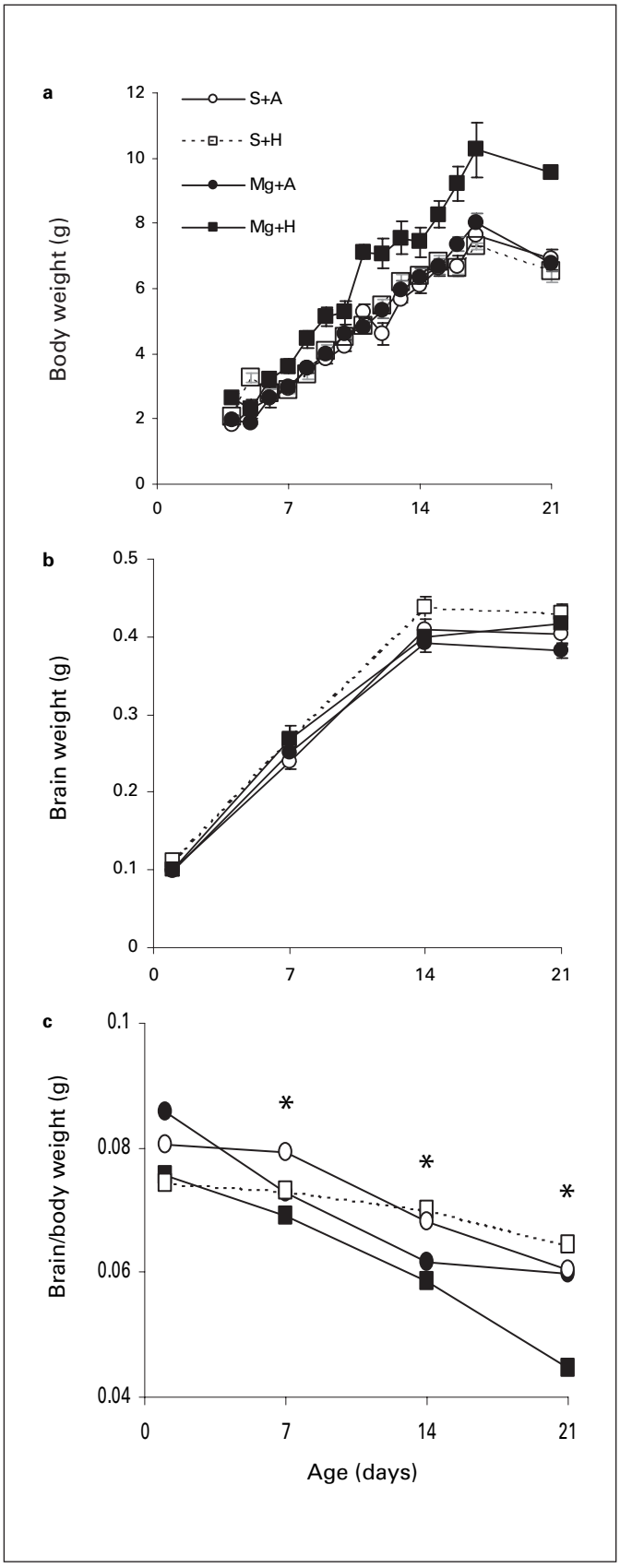


Fig. 1. Effect of hypoxia and $MgSO_4$ on newborn body and brain weight. **a** Newborns' body weight increased daily up to P17 in all tested groups; thereafter (P21), a stable weight was observed. Mg+H treatment significantly increase body weight beginning on P10 (n = 12–17 in all groups, all days). **b** Brain weight of all neonates increased until P14 in all tested groups; thereafter (P21), a stable weight was observed. Similar brain weight was observed in all groups (n = 4–7 for each data point). **c** The ratio of brain/body weight was reduced in the Mg+H group compared to controls (* $p < 0.03$, n = 4–7 for each data point).

ternal exposure to hypoxia (in the S+H group) did not affect the developmental profile. All newborns completed teething on P14 except newborns of the Mg+H group, which exhibited acceleration of this process by P12, i.e. 2 days earlier compared with controls (fig. 2c; $p < 0.003$, χ^2 test, McNemar).

Reflex Development

Figure 3a demonstrates that all control mice successfully righted themselves between P4 and P11. Exposure to hypoxia, with and without magnesium pretreatment, accelerated the righting reflex development. This acceleration effect mainly occurred in the combined treatment group (Mg+H), all other newborns showed righting reflex by P7 ($p < 0.02$, χ^2 test, McNemar).

An analysis of the time the newborns needed to right themselves showed that newborns of the Mg+H group were faster in reflex performance than the control group ($p < 0.06$, t test). In contrast, newborns treated with either Mg+A or S+H were slightly slower than the control group ($p < 0.06$, t test).

Motor coordination and muscle strength were examined by the ability of newborns to walk on an inclined slope. Mice were examined on 70- and 90-degree slopes. The angle all newborns in the group could climb was averaged and is presented in figure 4a. Maternal hypoxia (S+H) significantly reduced the ability of newborns to perform this task between P7 and P11 ($p < 0.02$, t test). Locomotion reflex was presented in all groups, however, newborns belonging to the S+H group failed to grip the surface of the inclining slope.

Coordination and muscle strength were also examined by a light version of the rotarod test. Newborns of the S+H and Mg+A groups failed to grip the rotating bar until P13. Thereafter, their performance was similar to that of the control group. Newborns belonging to the Mg+H group were able to grip a full cycle as early as P13/14, earlier than the control group.

Summarizing the results of the different tasks that examined coordination and muscle strength, newborns belonging to the hypoxia group exhibited inferior perfor-

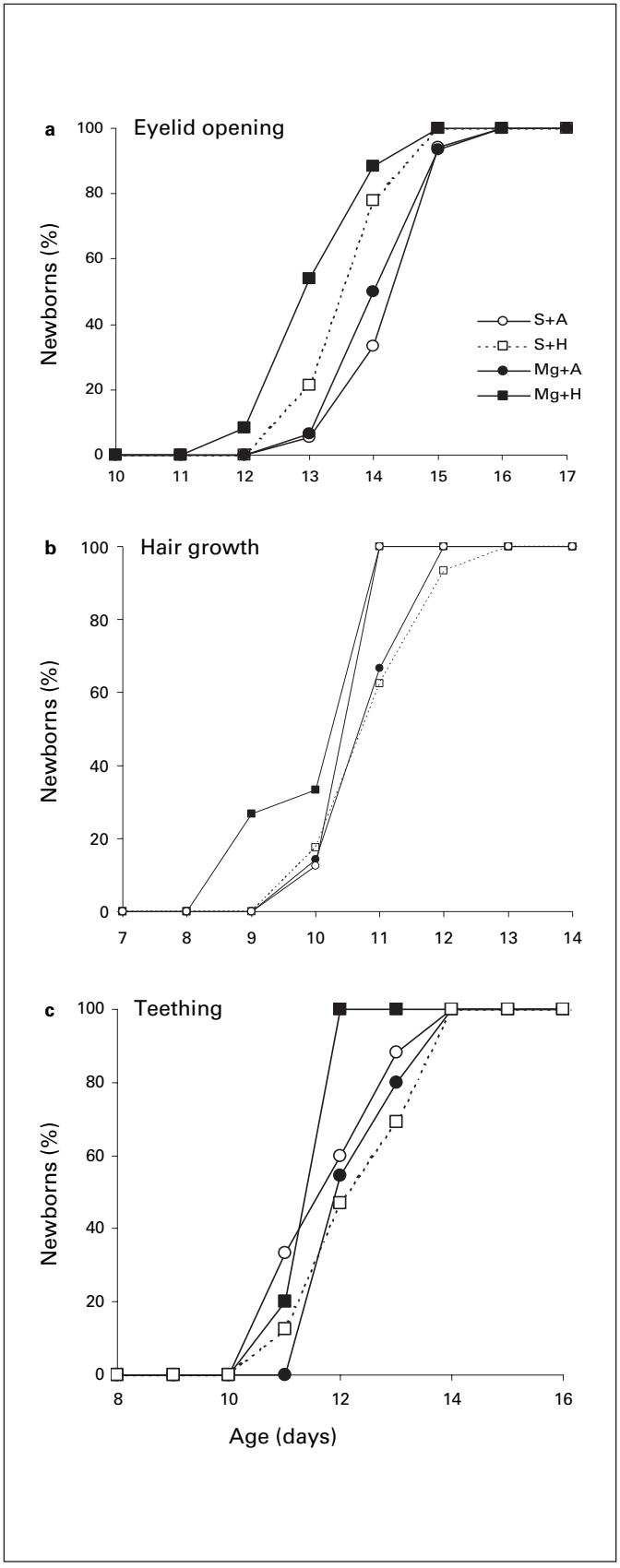
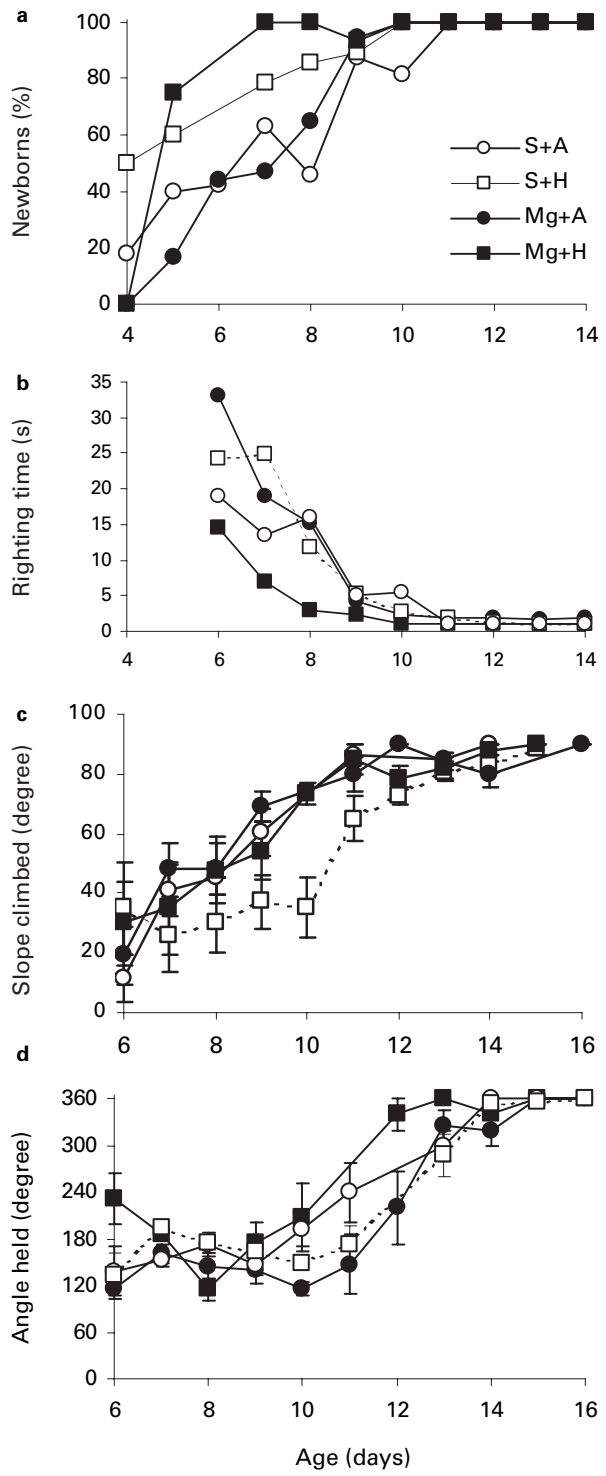


Fig. 2. Effect of hypoxia and $MgSO_4$ on morphogenic parameters. **a** The percentage of neonates that open their eyes was higher in the two groups exposed to hypoxia during embryonic stage. **b** Body hair growth was slightly delayed by a separate hypoxia or $MgSO_4$ treatment. However, when both treatments were given, accelerated hair growth was observed. **c** Teething was faster in the Mg+H group than in all other groups (n = 13–18 for all data points).



mance in two of these tests. Maternal pretreatment with Mg (Mg+H) either prevented the damage or improved the newborns' performance.

Discussion

The development of morphogenic parameters was significantly affected by all treatments. However, only the group treated with both $MgSO_4$ and hypoxia showed a significant effect on the body weight of the newborn, indicating that these two treatments do not simply offset each other. In addition, the difference in weight was not observed on the first day. The difference gradually developed with age, suggesting long-lasting modulation of the developmental processes induced by the treatment. Differences in body weight were not correlated with brain weight.

Eyelid opening is a general index used to indicate possible developmental difficulties. Both groups of newborns belonging to mothers that were exposed to hypoxia opened their eyelids earlier than newborns belonging to the control or Mg+A groups. A tendency for facilitated development was reported following prenatal stress that was induced by transitory and variable living conditions [11]. A similar tendency for early maturation was also shown for the development of body hair.

At birth, newborn mice already move their limbs and have the ability to coordinate limb function, which supports the search for the mother and her nipples. However, most of the motor circuits are not as fully developed as the muscle mass; both gradually develop postnatally. Therefore the effect of all the treatments of the present study on the development of motor function and coordination suggest that hypoxic episodes perturb the developmental plan of the neuronal circuits and/or musculature required for the performance of motor reflexes. Since all newborns were able to walk on a flat plane, we suggest

Fig. 3. Effect of hypoxia and $MgSO_4$ on the development of motor reflexes. The development of the righting reflex was measured as percent of newborns succeeding to right themselves (**a**) and the time they needed to do so (**b**). The percentage of newborns righting themselves was higher in the Mg+H group compared to controls, this group also required less time for righting. **c** Locomotion on inclining slope: the average angle that newborns could climb was lower in the S+H group than in all other groups. **d** Maternal hypoxia and $MgSO_4$ treatments (separately) reduced the ability of offspring to hold onto a rotating bar. Combined treatment (Mg+H) neutralized this effect.

that maternal hypoxia mainly affected newborn motor strength. In our experiments, maternal pretreatment with $MgSO_4$ significantly protected against the hypoxic effect (cf. the slope climbing results) or the accelerated rate of development (righting reflex and bar holding). It was suggested by Hallak et al. [10] that the protective effect of $MgSO_4$ could be due to the protection against histologic damage. Though they did not examine motor areas in the brain in their study, a protective effect was found in fetal hippocampus and thalamus. A similar effect may be present in other brain areas. We observed a reduced width of the secondary motor cortex in the A+H group at age P7 and P14; $MgSO_4$ (Mg+H) prevented this damage (unpublished data).

It was previously suggested that glutamate toxicity mediates hypoxia-induced brain damage [3, 6]. It is believed that the protective effect of $MgSO_4$ blocks NMDAR and the attenuation of calcium ion currents across the NMDA-associated ionic channel. However, the special properties of this receptor, which facilitate input integration from different neurons as its cardinal role in synaptic plasticity, make it of central importance during activity-dependent neurogenesis [12]. Blocking the NMDAR by APV affected the protein synthesis at developing synapses [13] and the establishment of innervations to certain brain regions [14]. Accordingly, we hypothesized that the $MgSO_4$ load may have a larger effect than that observed in our data. $MgSO_4$ load on embryonic day 17 had no effect on most parameters examined; an influence of this treatment was observed only in the rotarod test and it was similar to the hypoxic effect on the performance in this task. We conclude that 4 h of exposure to high levels of $MgSO_4$ caused only minor long-term destruction. On the other hand, there is only a limited number of functions that can be examined in the young mice, since more complicated behaviors develop later in life. The effect of this treatment in older mice is now under consideration in our laboratories.

Overall, a maternal hypoxic episode for 2 h during gestation day 17 severely impaired the development of morphogenic parameters as well as motor strength in newborns during the first month. These effects could be related to modifications in the developmental process, which were revealed 7–14 days following the event itself. Pretreatment with $MgSO_4$ not only protected against the motor impairment but it also accelerated reflex development as well as some morphogenic parameters. $MgSO_4$ load during embryonic development did not show significant consequences on the parameters examined in the present study.

In conclusion, hypoxic newborn mice pretreated with $MgSO_4$ showed normal or accelerated development of motor reflex, which may suggest $MgSO_4$ as a possible future prophylactic agent in cases of prenatal hypoxia.

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