Magnesium induces neuronal apoptosis by suppressing excitability

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Magnesium induces neuronal apoptosis by suppressing excitability

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In clinical obstetrics, magnesium sulfate (MgSO\(_4\)) use is widespread, but effects on brain development are unknown. Many agents that depress neuronal excitability increase developmental neuroapoptosis. In this study, we used dissociated cultures of rodent hippocampus to examine the effects of Mg\(^{++}\) on excitability and survival. Mg\(^{++}\)-induced caspase-3-associated cell loss at clinically relevant concentrations. Whole-cell patch-clamp techniques measured Mg\(^{++}\) effects on action potential threshold, action potential peak amplitude, spike number and changes in resting membrane potential. Mg\(^{--}\) depolarized action potential threshold, presumably from surface charge screening effects on voltage-gated sodium channels. Mg\(^{++}\) also decreased the number of action potentials in response to fixed current injection without affecting action potential peak amplitude. Surprisingly, Mg\(^{++}\) also depolarized neuronal resting potential in a concentration-dependent manner with a +5.2 mV shift at 10 mM. Voltage ramps suggested that Mg\(^{++}\) blocked a potassium conductance contributing to the resting potential. In spite of this depolarizing effect of Mg\(^{++}\), the net inhibitory effect of Mg\(^{++}\) nearly completely silenced neuronal network activity measured with multielectrode array recordings. We conclude that although Mg\(^{++}\) has complex effects on cellular excitability, the overall inhibitory influence of Mg\(^{++}\) decreases neuronal survival. Taken together with recent in vivo evidence, our results suggest that caution may be warranted in the use of Mg\(^{++}\) in clinical obstetrics and neonatology.

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Magnesium sulfate (MgSO\(_4\)) has been used in clinical obstetrics for over 70 years to treat pre-eclampsia/eclampsia and preterm labor, conditions that complicate approximately 3 and 12.4% pregnancies, respectively, in the United States each year.\(^1\) Currently, MgSO\(_4\) is the standard of care for preventing and treating eclamptic seizures, and although its safety and effectiveness as a tocolytic is controversial,\(^2,3\) it is estimated that over 150,000 women in the United States receive tocolytic therapy at or before 34 weeks of gestation yearly, with MgSO\(_4\) used as a first line of treatment in the majority of cases.\(^4,5\) Although MgSO\(_4\) has generally been accepted as being safe with no adverse effects in neonates, many studies have addressed whether the exposure of normal developing brains to high concentrations of Mg\(^{++}\) has any deleterious effects or consequences.

In contrast, Mg\(^{++}\) has been studied extensively as a potential neuroprotectant to prevent or improve outcomes of brain injury resulting from perinatal hypoxia/ischemia such as encephalopathy, intraventricular hemorrhage, periventricular leukomalacia and cerebral palsy.\(^6,7\) However, the safety and neuroprotective efficacy of Mg\(^{++}\) for premature, at-risk neonates remains controversial.\(^8\) Recent reviews evaluating the use of MgSO\(_4\) for neuroprotection of the fetus or use as a tocolytic in women at risk for preterm birth concluded that neuroprotective effects have not been established; MgSO\(_4\) is ineffective at delaying or preventing preterm birth and may actually increase neonatal morbidity and mortality.\(^2,9\)

Magnesium is second only to potassium in abundance as an intracellular cation in the human body, and Mg\(^{++}\) participates in many cellular functions, including energy production, synaptic neurotransmission and intracellular signaling. Mg\(^{++}\) blocks N-methyl-D-aspartate (NMDA) glutamate receptor ion channels, preventing ion flow at typical neuronal resting potentials.\(^10\) Divalent cations, including Mg\(^{++}\) at mM concentrations, decrease the activation of voltage-gated channels through surface charge screening effects,\(^11–14\) thereby reducing neuronal excitability. Mg\(^{++}\) at high extracellular concentrations also blocks Ca\(^{++}\) influx and diminishes synaptic transmission.\(^15\) Tocolytic actions apparently result from reduced myometrial contractility through extra- and intracellular Mg\(^{++}\) actions.\(^16\) However, supra-physiological Mg\(^{++}\) also blocks potassium conductances and g-aminobutyric acid (GABA)\(_A\) receptors.\(^17–19\) Recent evidence suggests that relief from long-term Mg\(^{++}\)-induced inhibition might result in homeostatic increases in long-term potentiation and plasticity.\(^20\) Therefore, at supraphysiological concentrations, Mg\(^{++}\) may have complex actions on neuronal excitability, and it is not clear whether net excitation or inhibition will prevail during prolonged incubation.

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Abbreviations: AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; GABA, \(\gamma\)-aminobutyric acid; ANOVA, analysis of variance; ASDR, array-wide spike detection rate; \(V_m\), membrane potential; MEA, multielectrode array; NMDA, N-methyl-D-aspartate; ITTX, tetrodotoxin

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Compounds that inhibit neuronal activity, including NMDA antagonists and calcium channel blockers, trigger widespread apoptotic neurodegeneration in the developing brain during the window of vulnerability (mice and rats 0–14 days postnatally).21–23 In rodents, this critical period roughly corresponds to the third trimester of human development, when MgSO₄ exposure typically occurs in humans.24 If Mg⁺⁺ sufficiently inhibits neurons, Mg⁺⁺ administration to young animals might increase developmental neuroapoptosis. Indeed, we showed that in vivo MgSO₄ induces widespread central nervous system neuroapoptosis at postnatal day 3 and 7, but not postnatal day 14.25 This work did not explicitly investigate whether Mg⁺⁺ acted directly or indirectly on susceptible target neurons, a question to which in vitro preparations are particularly well suited.

Given the complex cellular actions of Mg⁺⁺ on excitability noted above and questions regarding whether Mg⁺⁺ directly causes neuronal death, we examined survival in vitro, coupled with electrophysiological studies to test effects of Mg⁺⁺ on excitability of neurons. For these mechanistic studies, we exploited a primary culture preparation of rodent hippocampal neurons.

Results

Survival effects/activated caspase-3 neurons. We found that both MgSO₄ and MgCl₂ at concentrations of 5 and 10 mM induced death of hippocampal neurons relative to control cultures (Figures 1a and b). Fewer neurons were observed in treated cultures versus controls, and cellular profiles of the dying neurons were often visible and characterized by condensed, fragmented nuclei, as is often associated with apoptotic cell death.21 MgSO₄ and MgCl₂ both had strong negative effects on survival at 5 mM and at 10 mM added Mg⁺⁺ (Figure 1b). This places the concentration at which Mg⁺⁺ kills neurons in the range in which it has important effects on ion channels and is clinically relevant.26 On the basis of the significant degree of cell death produced at concentrations of 5 and 10 mM of MgSO₄, we examined survival in a concentration-response study comparing 1, 2.5 and 5 mM. In these studies (Figure 1c), a trend of neuronal loss occurred with concentrations as low as 1 mM of added MgSO₄ (P = 0.07). This trend became statistically significant at 2.5 and 5 mM above control conditions (Figure 1c).

MgSO₄ induces a wave of apoptotic neurodegeneration in the neonatal mouse brain.25 In part defined by activation of caspase-3, an effector caspase in many types of neuronal apoptosis,27 To determine if Mg⁺⁺ exposed neurons in vitro undergo apoptotic changes and to positively identify dying neurons, we immunostained the hippocampal cultures for activated caspase-3. Immunostaining for activated caspase-3 neurons, we immunostained the hippocampal cultures for activated caspase-3. Immunostaining for activated caspase-3 was positive in susceptible cells, a question to which in vitro preparations are particularly well suited.

Apoptosis in response to activity blockade in vitro shows significantly increased caspase-3 staining in susceptible cells, and Mg⁺⁺ produced a concentration-dependent increase in the percentage of immunoreactive neurons in cultures (Figure 2), similar to a variety of treatments that inhibit neuronal activity.28,29 The absolute mean numbers of healthy cells in the control versus treated groups were similar (68.8 ± 22.8 cells per control; 64.1 ± 20.5 cells per 5 mM; and 64.4 ± 26.7 cells per 10 mM). This similarity resulted from the early evaluation of the cultures at 3 days after MgSO₄ treatment (Figure 2) versus the 6 days after treatment evaluation in Figure 1 (see also Moulder et al.28). Both the 5 and 10 mM MgSO₄ groups showed significantly higher percentages of caspase-3-positive cells relative to sibling
controls (Figures 2c and d). The low absolute percentages of caspase-3 immunoreactive neurons reflect the slow overall loss of neurons, combined with transient caspase-3 immunoreactivity in individual neurons. A snapshot of caspase-3 immunoreactivity at any time point during the progressive loss of neurons produces only a minority of positive cells.28,29 Chronic incubation of neurons with elevated concentrations of KCl in the culture medium has been shown to protect neurons from apoptotic cell death resulting from over-inhibition and from neurotrophic factor deprivation.23,28–31 KCl depolarization is thought to prevent apoptosis by moderately increasing intracellular Ca\(^{2+}\) concentration.30 If Mg\(^{2+}\) acts by decreasing neuronal activity, we would hypothesize that treatment with KCl would counteract the effect of Mg\(^{2+}\). In contrast, if Mg\(^{2+}\) acts by other mechanisms, such as overexcitation, we might expect that KCl depolarization should exacerbate the death. Therefore, to help further clarify the nature of Mg\(^{2+}\)-induced neuronal death in vitro, we tested the effect of 30 mM KCl on caspase-associated cell death induced by Mg\(^{2+}\). We found that KCl significantly protected against both spontaneous neuronal death and against cell death induced by MgSO\(_4\) (Figure 2d). As shown in the previous experiments, comparison of control versus MgSO\(_4\) showed significantly increased activated caspase-3 presence. Comparison of Figures 2c and d shows considerable variability in the amount of attrition-related apoptosis among control cultures of different platings (~8% in Figure 2c versus ~2% in Figure 2d). Although all the factors participating in the level of background apoptosis are not fully understood, the Mg\(^{2+}\)-induced increase in caspase-3 immunoreactivity was robust in the context of both lower and higher attritional loss.

**Electrophysiology.** To determine the effects of Mg\(^{2+}\) on the excitability of hippocampal neurons, electrophysiology experiments were performed with the same concentrations of MgCl\(_2\) as the survival experiments (2.5, 5, and 10 mM). MgCl\(_2\), rather than MgSO\(_4\), was used in the electrophysiology experiments because chloride salts are routinely used in culture electrophysiology. As we predicted a net reduction of excitability by Mg\(^{2+}\) based on our survival studies, we compared the effects of escalating concentrations of tetrodotoxin (TTX), a highly selective antagonist of voltage-gated sodium channels with well defined effects on excitability.32 TTX silencing kills neurons in hippocampal culture through apoptosis over a similar time course as that of Mg\(^{2+}\),28,29 so it served as a relevant comparator.
In current-clamp mode, a 20 ms current pulse of +70 to +220 pA was used to initiate action potentials. Current amplitudes well beyond the rheobase of the cell were used to help ensure that spiking was elicited by a fixed current amplitude, in spite of the reduced excitability anticipated with Mg²⁺ and TTX application. Traces were recorded and analyzed for action potential threshold, action potential peak amplitude, spike number and changes in resting membrane potential ($V_m$).

Figure 3 shows that there was a concentration-dependent increase in action potential thresholds with Mg²⁺ treatment (Figures 3a and b), which resulted in a decrease in excitability, measured as the number of spikes elicited by the fixed current injection (Figure 3c). The effect on spike initiation became so severe at 10 mM Mg²⁺ that in two cells we were unable to evoke action potentials at this concentration. TTX (30 nM) also significantly depolarized threshold (Figure 4a). Quantitatively, the effect of 30 nM TTX on action potential threshold was similar to the effect of 2.5 and 5 mM Mg²⁺, with stronger effects of 60 nM and 90 nM TTX ($P<0.001$) compared with 30 nM.

Mg²⁺ did not significantly affect the peak amplitude of action potentials except at the highest concentration (10 mM). However, the change in maximum slope and peak amplitude of the first derivative of the action potential (Figures 3b and 4) is consistent with a decreased availability and activation of Na⁺ channels and subsequent shift in threshold. In contrast, TTX at concentrations of 30 nM ($P<0.01$) and higher ($P<0.001$) significantly decreased the amplitude of action potentials (Figure 4c). This was expected because of the direct blockade of sodium channels by TTX. Mg²⁺ also induced a concentration-dependent decrease in the number of action potentials evoked by the fixed-amplitude current injection (Figure 4b). Like Mg²⁺, TTX at 30, 60 and 90 nM decreased the number of spikes relative to baseline (Figure 4).

In spite of these inhibitory effects of Mg²⁺, we also observed an unanticipated excitatory effect of Mg²⁺. Mg²⁺ depolarized neuronal resting $V_m$ in a concentration-dependent manner (Figures 5a and b). This effect was associated with an increased cellular input resistance (Figure 5c). To define the approximate reversal potential of the current

Figure 3 Mg²⁺ increases voltage threshold for action potential initiation and decreases the number of spikes in response to prolonged current injection. (a) Representative examples of action potentials elicited by a fixed-amplitude current injection (+120 pA) in whole-cell patch-clamp recordings. Action potential thresholds are given for each trace, representing from left to right: 1 (baseline), 2.5, 5 and 10 mM additional MgCl₂. See Figure 4 for summary data. (b) First derivative traces from the examples shown in (a). Mg²⁺ reduced the maximum slope of action potentials (peak amplitude of the first derivative), consistent with a reduction in availability of Na⁺ channels. (c) Effects of Mg²⁺ on the number of action potentials elicited in response to a fixed current injection. The examples are from the same recordings as in panels (a) and (b). See Figure 4 for summary data.
involved, we voltage-clamped cells and performed voltage ramp protocols (Figure 5c). The change in slope of the current response to the voltage ramp highlights the decrease in membrane conductance in response to Mg\(^{2+}\) application. Digital subtraction of the baseline response from the response in the presence of Mg\(^{2+}\) suggested a strongly negative reversal potential for the Mg\(^{2+}\)-sensitive current (\(-84.8 \pm 14.3\) mV, \(n = 6\) cells). In one cell, we verified that reversal was unaffected by increasing the pipette chloride concentration to 140 mM (reversal potential for chloride (\(E_{Cl}^-\)) = 0 mV). In most cells, the Mg\(^{2+}\)-sensitive current showed apparent inward rectification (see example in Figure 5c). This could suggest that Mg\(^{2+}\) blocks an inwardly rectifying current, but we cannot exclude a voltage-dependent block of an Ohmic conductance. As expected, TTX did not produce any consistent change in resting \(V_m\) (\(-63.4 \pm 2.4\) mV versus 30 nM TTX: \(-63.7 \pm 3.7\) mV). In summary, these experiments suggest that Mg\(^{2+}\) influences resting potential primarily by blocking a potassium conductance open at rest, possibly an inwardly rectifying conductance.

In the context of our current-clamped cells bathed in postsynaptic receptor antagonists, the inhibitory effects of Mg\(^{2+}\) seemed to dominate the excitatory effect on resting potential. However, the mixed effects of Mg\(^{2+}\) raise the question of which effects would predominate in a spontaneously active network, such as in our survival studies. To answer this question, we turned to multielectrode array (MEA) recordings from our cultures (Figure 6). We used 30 nM TTX
as a comparator because it approximated the increased threshold of action potentials and decreased excitability observed with the intermediate concentration of Mg$^{++}$. When the MEA cultures were bathed in recording saline containing an additional 2.5 or 5 mM Mg$^{++}$, a nearly complete silencing of the neuronal network was observed (Figure 6). TTX at 30 nM similarly depressed network activity, with an almost complete suppression of activity (Figure 6). In all cases, treatment effects were compared against the average activity from control and washout conditions to account for potential loss of activity because of neuronal damage from repeated solution exchanges. Activity levels in Mg$^{++}$ 2.5 mM, Mg$^{++}$ 5 mM and TTX 30 nM were not significantly different from each other and all strongly and nearly completely suppressed network activity. These results clearly suggest that inhibition is the predominant effect of elevated extracellular Mg$^{++}$ in the intact network.

Discussion

Although Mg$^{++}$ has complex effects on cellular excitability, including both excitatory and inhibitory influences, our data indicate that the overall inhibitory influence of Mg$^{++}$ accounts for decreased neuronal survival with prolonged exposure. In spite of a surprising depolarization of resting potential, the net effect of Mg$^{++}$ is inhibitory, as shown by recordings of network activity. Decreased excitability triggers neuronal death, and depolarization with potassium protects against death. These findings suggest that Mg$^{++}$-induced neuronal apoptosis in vivo likely results directly from the effects of Mg$^{++}$ on susceptible neurons rather than through secondary consequences of systemic Mg$^{++}$ administration to animals. In clinical obstetrics, Mg$^{++}$ administration is typically titrated to maintain maternal serum levels of approximately 4–8 mg per 100 ml (1.6–3.3 mM). These concentrations are near the lowest concentrations that induced significant neuronal toxicity in our experiments.

Our present observations are also consistent with our previous in vitro studies showing that depression of spontaneous activity of hippocampal neurons in vitro produces apoptotic cell loss. Although the culture model has been useful and generally correlates well with in vivo results, there is at least one difference with in vivo neonatal studies that merits mention. Activity-dependent cell loss in vitro has a characteristically slower time course than observed in vivo. In vitro loss takes >3 days, whereas markers of apoptosis in vivo are observed within a few hours of treatment. Nevertheless, the in vitro model has been useful for identifying and studying mechanistic details of apoptosis induced by agents that suppress neuronal excitability in vivo.

Endogenous magnesium maintains cell function, integrity, and energy production, and extracellular Mg$^{++}$ modulates a variety of different ion channels and receptors, including NMDA receptors, Ca$^{++}$ channels, GABA$_A$ receptors and voltage-gated channels. Manipulation of each of these channel types has been shown to affect cell survival during developmental synaptogenesis, when many neurons undergo physiological cell death. The common theme with apoptotic neuroactive agents, including Mg$^{++}$, is that neuronal activity is strongly suppressed. In susceptible,
developing neurons activity suppression activates an apoptotic ‘trigger’. This common trigger could be reduced intracellular Ca\(^{2+}\) but the trigger set-point can be influenced by environmental factors.\(^{39}\)

In spite of the variety of Mg\(^{2+}\)-sensitive targets that could be relevant to effects on survival, our results suggest that altered action potential initiation and sodium channel gating are likely key to explaining survival effects. Our comparisons with the selective sodium channel blocker TTX are the basis for this conclusion. We have previously shown that TTX silencing of neurons is sufficient to cause apoptosis over a similar time course to that observed here with Mg\(^{2+}\). As Mg\(^{2+}\) raises action potential threshold to values similar to those achieved by TTX, and both have similar effects on network excitability in MEA recordings, it is reasonable to conclude that effects on Na\(^{+}\) channel gating and action potential threshold primarily explain Mg\(^{2+}\) effects on excitability and survival, with little need to invoke mechanistic explanations involving other channels (e.g., NMDA receptors or Ca\(^{2+}\) channels). If cells rarely reach action potential threshold because of altered voltage-gated channel gating, voltage-gated Ca\(^{2+}\) channels will not open. Similarly, the direct effect of Mg\(^{2+}\) on NMDA receptors will be largely irrelevant in the absence of action potentials to drive glutamate release. However, contributions from these other inhibitory mechanisms cannot be completely excluded.

The effects on action potential threshold and network excitability almost certainly result from classically described effects of divalent cations on voltage-gated channels. Surface charge screening effects by Mg\(^{2+}\) and by other divalent cations have been described in numerous settings.\(^{11-14,32}\) This effect likely results from binding of extracellular divalent cations to fixed negative charges at the cellular membrane.\(^{32}\) This effectively reduces the surface potential and produces a local hyperpolarizing influence on the voltage-sensitive domains of channels. This charge screening effect is detected as a depolarizing shift in voltage-dependent channel activation and inactivation. Classical work described the influence of a multitude of divalent cations on excitability in nodes of Ranvier of frog myelinated nerve fibers; a 10-fold increase in ion concentration shifts the firing threshold up to \(+25\) mV depending on the ion.\(^{12}\) Specifically, an increase in Mg\(^{2+}\) from 1 to 10 mM shifts Na\(^{+}\) channel activation by approximately \(+15\) mV, coinciding with the approximately \(+13\) mV shift in action potential threshold observed in our experiments.

We also identified a potential excitatory effect of Mg\(^{2+}\) on resting \(V_m\). A rise in Mg\(^{2+}\) concentration from 1 to 10 mM depolarized the resting \(V_m\) by 5.2 mV. To our knowledge, this effect of Mg\(^{2+}\) has not been previously reported. Candidate mechanisms based on the reversal potential of the Mg\(^{2+}\)-sensitive current include block of various ‘leak’ channels such as inward rectifying K\(^{-}\) channels and 2-pore domain K\(^{-}\) channels, such as Kir2.1 and TREV-1 K\(^{-}\) channels, respectively.\(^{18,19}\) Although Mg\(^{2+}\)-induced depolarization could, at face value, increase cell excitability, the surface charge screening effects described above would prevent significant functional effects of the depolarization on excitability. The resting depolarization was smaller ( \(+5.2\) mV) than the expected shift in gating and steady state inactivation ( \(+13\) mV) from surface charge effects (see above).

The influence of Mg\(^{2+}\) on resting \(V_m\) deserves further exploration and could provide further understanding of the contribution of ‘leak’ channels to the cellular resting \(V_m\).

The use of Mg\(^{2+}\) in clinical obstetrics and neonatology as an anticonvulsant, tocolytic and neuroprotegrant is widespread, but studies supporting its clinical efficacy are controversial, and no studies have examined or established the safety of its effects on normal, developing brains. Taken together with our recent \(in\) \textit{vivo} study,\(^{20}\) our results suggest that developing neurons and neuronal networks require an appropriate level of active input for proper development and survival. Clinically relevant Mg\(^{2+}\) concentrations kill neurons at this stage by dampening excitability and increasing apoptosis. We observed Mg\(^{2+}\)-induced apoptotic cell death in our previous \textit{in vivo} studies, and this was supported by the present \textit{in vitro} experiments. Our experiments help link Mg\(^{2+}\) to other neuro-depressive agents that promote apoptosis in young neurons. Further studies are required to assess the long-term effect of prenatal Mg\(^{2+}\) exposure on proper neurocognitive and intellectual development.

Materials and Methods

Hippocampal cultures. Cultures were prepared using previously described procedures.\(^{37}\) Briefly, hippocampal cells were harvested from 1- to 3-day-postnatal Sprague–Dawley rats. Slices (500–800 \(\mu\)m thick) of hippocampus were enzymatically treated with 1 mg/ml papa in oxygenated Leibovitz’s L-15 medium for 20–30 min. After transfer to Eagle’s minimal essential medium containing 5% horse serum, 5% fetal calf serum, 17 mM d-glucose, 400 \(\mu\)M glutamine, 50 U/ml penicillin and 50 \(\mu\)g/ml streptomycin, slices were gently triturated by passage through Pasteur pipettes of decreasing diameter until a single-cell suspension of approximately 300 000 cells/ml was obtained. Mass cultures were plated from this suspension onto a collagen substrate (0.5 mg/ml rat tail, Sigma type I, St. Louis, MO, USA), which was spread uniformly across a 35-mm plastic culture dish.\(^{38}\) At the time cells were used for experiments ( \(\sim 7–15\) days in vitro), mass culture neurons were present at a density of approximately 500 cells/mm\(^2\). After 3 days in culture, all cells were treated with 10 \(\mu\)M cytosine arabinoside to halt glial proliferation.

Survival and cell counts. To determine whether forebrain neurons are sensitive to Mg\(^{2+}\)-induced cell death, we treated cultured hippocampal neurons for 6 days beginning at 5 days in vitro with various concentrations of MgSO\(_4\) or MgCl\(_2\). Survival was assessed with counts of healthy neurons under phase-contrast optics using an inverted microscope with a 20 \(\times\) objective. Ten random fields from sibling culture dishes from the same plating were counted for surviving neurons. Cells were counted as live if somas retained a smooth, phase-bright appearance with visible processes. This is a protocol similar to that used by our group to show the neuronal death of \(\textit{in vivo}\) studies, and this was supported by the present \textit{in vitro} experiments. Our experiments help link Mg\(^{2+}\) to other neuro-depressive agents that promote apoptosis in young neurons. Further studies are required to assess the long-term effect of prenatal Mg\(^{2+}\) exposure on proper neurocognitive and intellectual development.

Immunostaining. To assess if the neuronal death observed was apoptotic, immunostaining for activated caspase-3 was performed. At 6 days in vitro, cultures were treated with various concentrations of MgSO\(_4\) alone and in combination with 30 mM KCl. After 72 h of treatment, cultures were fixed for 7 min in 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline. Cells were permeabilized with 0.1% Triton X-100, blocked with 10% serum, and incubated for 4 h with caspase-3 antibody (Cell Signaling Technology, Danvers, CA, USA) at a final dilution of 1:1000. Cultures were then incubated in secondary antibody for 30 min, reacted in the dark with ABC reagents (Vector Laboratories, Burlingame, CA, USA) for 10–15 min, then incubated in VIP peroxidase substrate for 2–4 min. Healthy neurons and caspase-3-positive neurons were counted in 10 different fields of the culture dish as described in the survival studies.

Electrophysiology. Conventional whole-cell patch clamping was employed using an Axopatch 200B patch-clamp amplifier and a Digidata 1322 acquisition board (MDS, Sunnyvale, CA, USA). Recordings were obtained using pipettes.
of 7–8 MΩ open-tip resistance when filled with standard pipette solution containing (in mM) K-glucuronate (140), NaCl (4), CaCl\(_2\) (0.5), EGTA (5) and HEPES (10), and pH adjusted to 7.25 with NaOH. At the time of experiments, culture medium was replaced with an extracellular recording saline consisting of (in mM) NaCl (138), KCl (4), CaCl\(_2\) (2.0), MgCl\(_2\) (1.0), glucose (10) and HEPES (10), and pH adjusted to 7.25 with KOH. d-2-amino-5-phosphonovalerate 50 μM, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline-dione 1 μM and bicuculline 50 μM were added to the extracellular solution to block NMDA, AMPA and GABA currents, respectively. Pipette current offset in bath solution was adjusted to zero before gigaseal formation. Pipette capacitance was compensated in the cell-attached configuration. On patch rupture, current-clamp circuitry was used. The initial resting \( V_n \) was set to near –65 mV with a small bias current (if necessary) before initiating recordings. Changes from this initial value were not subsequently compensated. Action potentials were stimulated by current injection of +70 to +220 pA. \( V_n \) was sampled at 50 kHz and low-pass filtered at 10 kHz. MgCl\(_2\) and TTX were delivered by local perfusion with a multi-barrel pipette positioned approximately 0.5 mm from the cell of interest. Data were sampled and recorded using pCLAMP9 and analyzed with Clampfit 9 software (MDS).

Multi-electrode array studies. MEAs were coated with poly-o-lysine and laminin per the manufacturer’s instructions, and dispersed cultures were grown as described above. All 7 and 10 days in vitro, one-third of the media was removed and replaced with fresh Neurobasal supplemented with B27 and glutamine. Recordings were made with the MEA-60 recording system (MultiChannel Systems, Reutlingen, Germany) with the headstage in an incubator set at 29 °C and equilibrated with 5% CO\(_2\) in room air with no additional humidity. The lower temperature was necessary because the electronics in the headstage generate ~7 °C of excess heat. The MEA itself rested on a heating plate inside the headstage to maintain the cultures at 37 °C. The media in the MEA was replaced with the above described extracellular solution to block NMDA, AMPA and GABA currents, respectively. Pipette capacitance was compensated in the cell-attached configuration. Data were sampled and recorded using pCLAMP9 and analyzed with Clampfit 9 software (MDS).

Statistical analysis. For cell survival studies, cell counts were performed on at least six independent experiments. For all experimental conditions tested, the experimental groups were sibling culture dishes from the same culture plating matched with a control dish containing baseline culture medium. A one-sample \( t \)-test was used in which the mean for each experimental condition was compared with a theoretical mean of 100% and reported as percent survival relative to control. For caspase-3 immunostaining, means for healthy cells and caspase-3-positive neurons were determined from at least five independent experiments. The experimental conditions and groups were tested as described above. Percent of caspase-3-positive to healthy cells was calculated and a one-way analysis of variance (ANOVA) with either Tukey’s or Dunnett’s post hoc testing was used to compare means of each experimental condition. For electrophysiology studies, AP thresholds were measured from first derivative waveforms derived from the original action potential trace. Action potential peak amplitude, resting \( V_n \) and number of action potential spikes per sweep were also calculated. All electrophysiology data were analyzed with a repeated-measures ANOVA, followed by Tukey’s post hoc analysis. For all experiments, the data were analyzed for statistical significance using Prism (GraphPad Software, Inc., La Jolla, CA, USA). MEA data were quantified using the array-wide spike detection rate (ASDR),\(^{40}\) defined as the number of spikes detected in all contacts of the MEA during each second of recording. The average ASDR for each 30-min data set was used as a summary measure of activity. Activity during Mg\(^{2+}\) or TTX exposure was compared with the average of the baseline and wash activity levels using a one-way ANOVA followed by a post hoc Tukey’s test with \( P < 0.05 \) considered significant. Data from the MEA experiments were analyzed in Igor Pro (Waveformics, Lake Oswego, OR, USA).

Conflict of interest

The authors declare no conflict of interest.

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