

Xenon and Hypothermia Combine to Provide Neuroprotection from Neonatal Asphyxia

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Perinatal asphyxia can result in neuronal injury with long-term neurological and behavioral consequences. Although hypothermia may provide some modest benefit, the intervention itself can produce adverse consequences. We have investigated whether xenon, an antagonist of the *N*-methyl-D-aspartate subtype of the glutamate receptor, can enhance the neuroprotection provided by mild hypothermia. Cultured neurons injured by oxygen-glucose deprivation were protected by combinations of interventions of xenon and hypothermia that, when administered alone, were not efficacious. A combination of xenon and hypothermia administered 4 hours after hypoxic-ischemic injury in neonatal rats provided synergistic neuroprotection assessed by morphological criteria, by hemispheric weight, and by functional neurological studies up to 30 days after the injury. The protective mechanism of the combination, in both *in vitro* and *in vivo* models, involved an antiapoptotic action. If applied to humans, these data suggest that low (subanesthetic) concentrations of xenon in combination with mild hypothermia may provide a safe and effective therapy for perinatal asphyxia.

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Perinatal hypoxic-ischemic brain damage remains a major cause of acute mortality and chronic neuropsychological morbidity in infants and children.¹ Given the magnitude of the problem^{2,3} and the largely ineffective current interventions (apart from possibly hypothermia⁴), strategies are needed to minimize the long-term neurological sequelae of neonatal hypoxia-ischemia.

In neonatal animal models of hypoxia-ischemia, brain injury is produced, at least in part, by apoptotic cell death^{5,6} as a consequence of excessive activation of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor. We have shown that xenon, which is a general anesthetic at about 70% of one atmosphere (atm) in humans, exhibits noncompetitive antagonism at the NMDA subtype of the glutamate receptor⁷; furthermore, we demonstrated that xenon exerts neuroprotection in a variety of models of acute neuronal injury.^{8,9} Because of xenon's safe use for anesthetic and radiological applications in humans,¹⁰ including neonates,¹¹ as well as its rapid onset of action, ease of administration,¹² and lack of toxicity,⁹ especially fetotox-

icity,¹³ we wondered whether xenon represented a viable therapeutic option for perinatal asphyxia either alone or combined with modest hypothermia.

We now report that, in both *in vitro* and *in vivo* models of asphyxial neuronal injury, xenon exerts neuroprotection through an antiapoptotic mechanism and combines synergistically with hypothermia.

Materials and Methods

The study protocol was approved by the Home Office (United Kingdom), and all efforts were made to minimize the animals' suffering and the number of animals used. BALB/c mice and Sprague–Dawley rat pups were used for *in vitro* and *in vivo* experiments, respectively. Twelve pups of either sex per dam were used and housed with a 12-hour light/dark schedule in a temperature- and humidity-controlled colony room. The pups were housed with their dams until weaning on postnatal Day 21, and then were housed in groups of four per cage.

Cell Culture

The mixed cortical glial-neuronal cocultures derived from early postnatal and fetal BALB/c mice, respectively, have

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been described previously.⁸ Briefly, whole cerebral neocortices (devoid of the hippocampal formation and basal ganglia) were prepared from early postnatal (Days 1–2) pups of BALB/c mice. After trypsination and resuspension, cells were plated at a density of 6.25×10^4 cells/cm² on 24-multiwell plates (Costar, Cambridge, MA) and cultured in a medium consisting of Eagle's minimum essential medium augmented with 20mM glucose, 26mM NaHCO₃, 10% fetal bovine serum, 10% heat-inactivated horse serum, antibiotic-antimycotic solution (Gibco, Paisley, United Kingdom), 2mM glutamine (Sigma, Poole, United Kingdom), and 10ng/ml murine epidermal growth factor (Gibco). Glial cells reached confluence about 1 week after plating. Cortical neuronal cells were obtained from fetal BALB/c mice at 14 to 16 days of gestation and were plated at a density of 1.25×10^5 cells/cm² on the confluent monolayer of glial cells derived from the corresponding genetic strain. Neuronal cells reached confluence 1 week after plating. The pure cortical neuronal cells were also obtained from fetal BALB/c mice, seeded into poly-L-lysine precoated 24-multiwell plates, and fed with neurobasal medium (Gibco) with the addition of B27 supplement (once) and glutamine (25μM). The mixed glial-neuronal cells and the pure neurons were used at 14 ± 1 and 8 ± 1 days, respectively.

Oxygen-Glucose Deprivation

Cell injury was induced using previously described methods.¹⁴ Culture medium was replaced by deoxygenated balanced salt solution (116mM NaCl, 5.4mM KCl, 0.8mM MgSO₄, 1.0mM NaH₂PO₄, 1.8mM CaCl₂, 26mM NaHCO₃, pH 7.4) in the absence of glucose and maintained in an anoxic chamber (oxygen-glucose deprivation [OGD]) at 37°C or less for 75 minutes. For the xenon experiments, solutions were prepared by bubbling pure gases (nitrogen or xenon; Air Products, Crewe, United Kingdom) through fine sintered-glass bubblers in Drechsel bottles (Pegasus, Guelph, Canada) filled with balanced salt buffer. Cells were kept in purpose-built, airtight, temperature-controlled, cell-culture chambers. These were prefilled with the desired concentration of xenon after the culture medium had been replaced by xenon-bubbled solution. In the post-OGD period, cells were returned to a normoxic incubator containing nitrogen (20% oxygen, 5% carbon dioxide, 75% nitrogen) or xenon (20% oxygen, 5% carbon dioxide, with a variable combination of xenon and nitrogen) for 24 hours at 37°C. In comparative experiments, gavestinel (Glaxo-Smith-Kline, London, United Kingdom), another NMDA antagonist, was added into the culture solution at an appropriate concentration and was present during both the injury and recovery phases of the experiments.

Measurement of Cell Injury with Lactate Dehydrogenase Assay

The neuronal injury was quantified by the amount of lactate dehydrogenase (LDH) released into the medium using a standardized colorimetric enzyme kit (Sigma). The amount of LDH released by cells that were maintained under normoxic and euglycemic conditions was subtracted to yield the LDH release that occurred in the presence of OGD. Neuronal protection provided by interventions with xenon, hypo-

thermia, or both was expressed as a fraction of the maximal LDH released without interventions.

Determination of Apoptosis and Necrosis In Vitro

The pure neuronal cells were washed with Hepes buffer, and then detached with 0.2% trypsin/EDTA 24 hours after injury by OGD, NMDA (300μM), glutamate (300μM), or staurosporine (200nM) (Sigma, Poole, UK). Thereafter, cells were stained with annexin V-FITC conjugate (0.4μg/ml), and subsequently with propidium iodide (0.8μg/ml; Sigma) in binding buffer (50mM Hepes, 150mM NaCl, 12.5 CaCl₂, and 20% bovine serum albumin; titrated to pH 7.4 with 1M NaOH). A minimum of 10,000 cells per sample was analyzed with flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, CA) to determine the population of apoptotic, necrotic, and viable cells.¹⁵

Animal Model of Hypoxic-Ischemic Injury

Seven-day-old postnatal Sprague-Dawley rats underwent right common carotid artery ligation under surgical anesthesia.¹⁶ After ligation, the animals were returned to their dams at a constant temperature (23°C) and humidity (48%) for recovery. One hour after surgery, neonatal rats were exposed to hypoxia (O₂ 8% balanced with N₂) for 90 minutes. Xenon (20–70%) was either added concurrently during hypoxia or 2 to 24 hours after the hypoxic insult for 90 minutes, using a purpose-built, closed-circle delivery system. The desired temperature (30–37°C) was achieved and maintained by submerging the exposure chambers into a water bath. On the seventh day after hypoxia-ischemia, rats were killed and their brains were removed; then the right hemisphere was separated from the left and weighed. The use of hemispheric weight as an indicator of brain damage is well validated in this model.^{17,18}

Brain Temperature Probe Implantation

To assess the brain temperature of the pups precisely, we used a telemetry system (VitalView, Mini-Mitter, OR). In each set of experiments, a temperature probe was implanted (–2mm from bregma and 2mm away from sagittal sinus; the tip of probe advanced to subcortex and fixed on the skull with glue) in a sentinel rat pup that was not further assessed for brain injury.

Determination of Apoptosis and Necrosis In Vivo

Rats were anesthetized and perfused transcardially with paraformaldehyde (4%) in phosphate buffer (PB) (0.1M) at 16, 24, or 48 hours after the intervention; brains were harvested and sectioned into 5mm-wide blocks, and then embedded in wax. The coronal sections (5μm) were harvested around –3.6mm from bregma and stained with cresyl violet. The ischemic core area in the cortex and in the dentate gyrus of the hippocampus from the ipsilateral side of each brain was analyzed using a BX60 light microscope (Olympus, Southall, United Kingdom), and the images were captured by a digital camera (Zeiss, Gottingen, Germany). A 40× objective lens with a grid was used to count the total number of cells that appeared in the grid. Cells were scored as either viable, apoptotic, or necrotic based on their morphological appear-

ance. The mean value of three sections for each brain was used per individual rat.

Assessment of Neurological Function

Thirty days after hypoxic-ischemic injury, animals underwent testing according to an established protocol that included assays of prehensile traction, strength, and performance on the balance beam, which was graded (maximal aggregate score = 9).¹⁹ Coordination was tested by placing rats on a rotarod, rotating at 30 g, and the latency to falling off the rod was assessed (maximal latency, 300 seconds). For each of the functional assays, the rat pup was tested three times with a 10-minute interval between each assessment, and the mean of the three assessments was used for each rat in the analysis. Thirty days after injury, animals were killed and perfused as stated earlier. Their brains were removed, dehydrated, embedded in wax, and sectioned coronally into 5 μ m slices. After staining the cresyl violet, six slices were selected from each animal corresponding to contiguously predefined brain regions relative to the bregma (+2, +1, 0, -2, -4, and -5mm). Each slice was photographed, and the area (measured in square millimeters) of both unlesioned and lesioned hemispheres was measured using analysis software (ImageJ v1.31, National Institutes of Health image software; Bethesda, MD). The ratio of brain matter on the lesioned hemisphere versus the unlesioned hemisphere was calculated. The ratios from each animal were plotted, and the area under the curve also was calculated.

Immunostaining for Caspase 3

Twenty-four hours after hypoxic-ischemic injury, animals were deeply anesthetized and perfused transcardially with paraformaldehyde (4%) in PB (0.1M), and the whole brain was removed. Frozen sections (35 μ m) were cut at -3.6mm from bregma, and cleaved caspase 3 (1:2,500; New England Biolab, Hitchin, United Kingdom) was stained with our established protocol.⁹

Western Blot Analysis

The ipsilateral hemispheres were harvested, lysed in lysis buffer (pH 7.5, 20mM tris(hydroxymethyl)aminomethane [Tris]-HCl, 150mM NaCl, 1mM Na₂DTA, 1mM EGTA, 1% Triton X-100 [Sigma Labs, St. Louis, MO], 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM Na₃VO₄, 2mM DL-dithiothreitol, 1mM phenylmethanesulfonyl, and 1 μ g/ml leupeptin), and centrifuged at 3,000 g for 10 minutes. Protein concentration in the supernatant was determined by Dc protein assay (Bio-Rad, Herts, United Kingdom). Protein extracts (30 μ g per sample) and a biotinylated molecular weight marker (New England Biolab) were denatured in Laemmli sample loading buffer (Bio-Rad) at 100°C for 5 minutes, separated by 10.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred in transfer buffer to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, United Kingdom). The membrane was pretreated with blocking solution (5% nonfat dry milk in Tween-containing, Tris-buffered saline [10mM Tris, 150mM NaCl, 0.1% Tween, pH 8.0]). The membranes were incubated with Bax (1:1,000; Delta Biolabs, Cambridge, United Kingdom), Bcl-x_L, or caspase 3 (1:1,000;

New England Biolab) overnight at 4°C. The horseradish peroxidase-conjugated goat antibody to rabbit or mouse IgG (1:2,000; New England Biolab) was used to detect the primary antibodies. The bands were visualized with the enhanced chemiluminescence system (ECL; Amersham Biosciences, Little Chalfont, United Kingdom), and intensities were quantified by densitometry. Results were normalized with the housekeeping protein, α -tubulin (1:2,000; Sigma), and were expressed as fractions of injured control.

Statistical Analysis

Data are given as mean \pm standard error of the mean. To investigate the nature of the interaction between xenon and hypothermia on LDH release, we compared their combined effects with those predicted assuming simple additivity. If additivity holds, then the following condition applies: $(a/\alpha_a) + (b/\alpha_b) = 1$; where a is the concentration of xenon, b is the extent of the hypothermia from 37°C measured in degrees centigrade, α_a is the concentration of xenon that reduces LDH release to 50% of its control value at 37°C, and α_b is the extent of hypothermia alone that reduces LDH release to 50% of its control value at 37°C in the absence of xenon. When xenon and hypothermia were combined, the concentration (at fixed hypothermia) or the temperature (at fixed xenon concentration) was determined to achieve 50% protection (ie, 50% of control value at 37°C in the absence of xenon) and was compared with values predicted assuming additivity. The significance of differences was determined by analysis of variance (ANOVA), followed by Student–Newman–Keuls test, unpaired t test, or Mann–Whitney U test where appropriate.

Results

Neuroprotective Effect of Xenon and Hypothermia In Vitro

An in vitro coculture of neuronal and glial cells was exposed to experimental ischemia in the form of OGD for 75 minutes, followed by a 16-hour recovery period.

Neuronal cell bodies in mixed cortical cell cultures were readily distinguishable from the underlying glial monolayer using phase-contrast microscope image capture after fixation with paraformaldehyde (2%) in PB (0.1M) and staining with cresyl violet. The control cultures demonstrated the presence of a dense network of neuronal dendrites and triangle-shaped cell bodies (Fig 1A). After intervention with 75-minute OGD, the neurons lost the dendritic network and expressed condensed cell bodies (see Fig 1B). However, in the presence of 75% xenon or temperature maintained at 20°C during OGD, the number of dead cells was considerably decreased (see Figs 1C, D). Quantifying injury by the release of LDH, the presence of xenon (12.5–75%) during OGD and recovery significantly reduced neuronal injury in a concentration-dependent manner with an IC₅₀ of $36 \pm 3\%$ atm (ANOVA followed by Student–Newman–Keuls; see Fig 1E). Application of hypothermia (33–20°C) during OGD also significantly reduced neuronal injury in a temperature-dependent

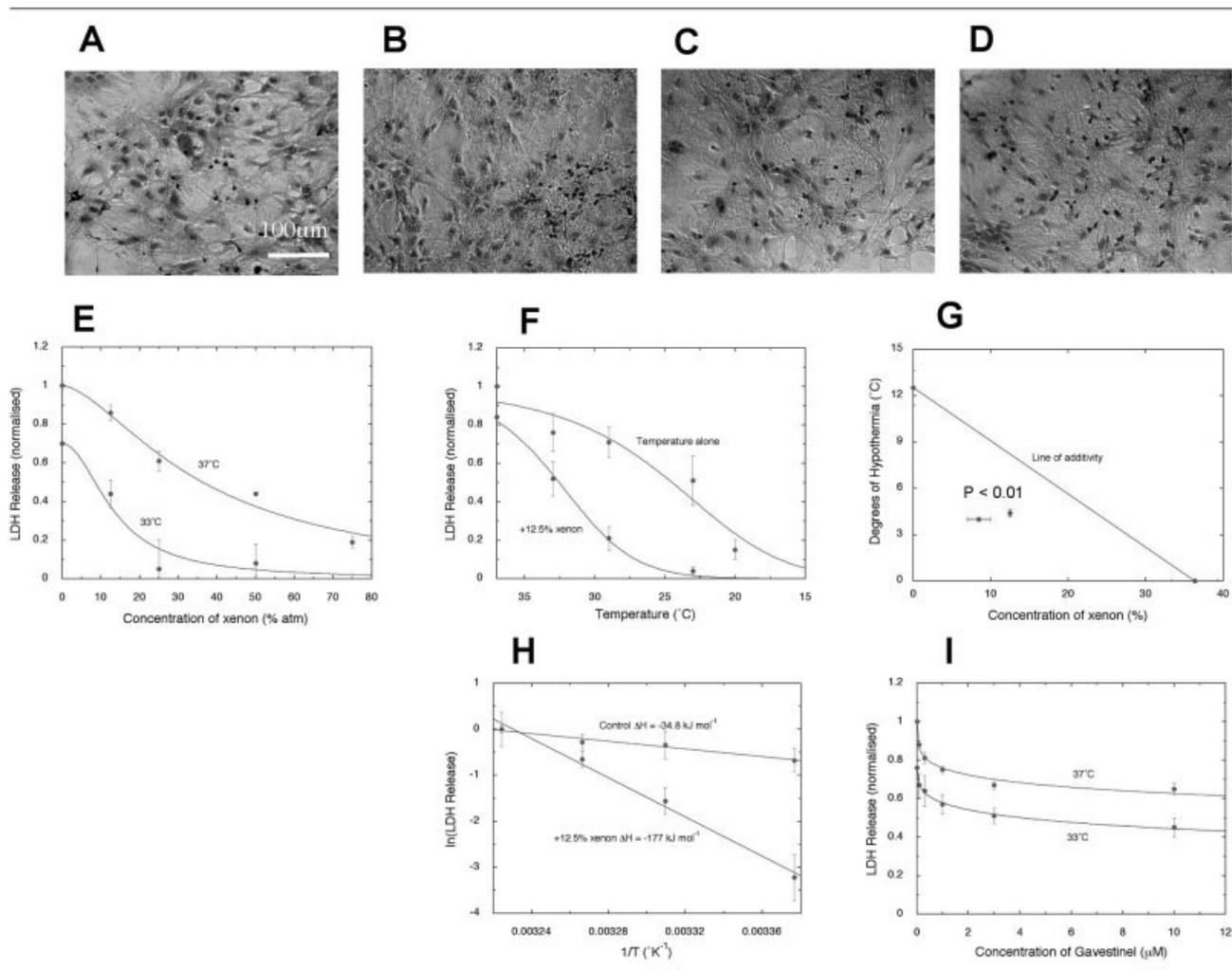


Fig 1. Neuroprotective effects of xenon and hypothermia *in vitro*. (A) Photomicrographs of normal Nissl-stained neurons, (B) after oxygen-glucose deprivation (OGD), (C) after OGD in the presence of xenon (75%), and (D) after OGD in the presence of hypothermia at 20°C. (E) Lactate dehydrogenase (LDH) release from OGD-injured cultured neurons exposed to increasing concentrations of xenon at normothermia (37°C) and hypothermia (33°C). (F) LDH release from OGD-injured cultured neurons exposed to decreasing temperature with or without xenon (12.5%). (G) Isobologram illustrating the protective efficacy of combining xenon and temperature on injured cultured neurons. Protection from damage to OGD-injured neurons is synergistically enhanced when provided in combination as reflected by that in combination the IC₅₀ values for the interventions were significantly to the left of the “line of additivity” joining the IC₅₀ of the individual interventions. (H) van’t Hoff plot showing the temperature dependence of neuroprotection for hypothermia alone and hypothermia in the presence of xenon. The enthalpy change (ΔH) is a measure of the overall heat flow during the process. (I) LDH release from OGD-injured cultured neurons exposed to increasing concentrations of gavestinel at normothermia (37°C) and hypothermia (33°C).

manner with a half-maximal effective temperature of $24 \pm 1^\circ\text{C}$ with respect to injury at 37°C (see Fig 1F).

To study the combined effects of xenon and hypothermia, we repeated these experiments, both with varying xenon concentrations at a fixed level of hypothermia (33°C) and with varying levels of hypothermia at a fixed xenon concentration (12.5%). The combination of both interventions significantly reduced neuronal injury even further (see Figs 1E, F), and the combination was found to be synergistic by isobolographic analysis ($p < 0.05$; unpaired t test; see Fig 1G).

It is convenient to plot the data in the form of a

van’t Hoff plot (in which the natural logarithm of LDH release is represented against the reciprocal of the absolute temperature; see Fig 1H) to quantify the enhanced effect of hypothermia in the presence of xenon. From the slope of the van’t Hoff plot, the standard change in enthalpy of the process (ΔH) can be calculated, and this quantifies the overall heat transfer during the process. For temperature alone, $\Delta H = 34.8 \pm 4.5 \text{ kJ mol}^{-1}$, whereas in the presence of 12.5% xenon, ΔH increased markedly to $177 \pm 12 \text{ kJ mol}^{-1}$ ($p < 0.01$; unpaired t test).

Because xenon is proposed to exert its effects

through antagonism at the NMDA subtype of the glutamate receptor,⁷ we compared its neuroprotective effects with another NMDA receptor antagonist, gavestinel (GV150526). Gavestinel is a selective glycine receptor antagonist of the NMDA receptor and previously has been shown to exert neuroprotective effects.²⁰ Like xenon⁹ it lacks the adverse effects caused by most other NMDA receptor antagonists.²¹ Application of gavestinel (0.01–100 μ M) during OGD and recovery resulted in a concentration-dependent reduction in neuronal injury with a “ceiling” effect (see Fig 1I) unlike that observed with xenon (see Fig 1E), which shows nearly complete protection at high concentrations. Hypothermia (33°C) enhanced the neuroprotection exerted by gavestinel, although, unlike xenon, the effect of modest hypothermia was roughly constant at all gavestinel concentrations tested, implying additivity rather than synergy.

Apoptosis, Necrosis, and Cell Viability In Vitro

To establish whether xenon exerted its neuroprotective effect through either the apoptotic or necrotic pathways, we stained injured cultured neurons with annexin V and propidium iodide, respectively, and sorted by flow cytometry.¹⁵ When uninjured cells were exposed to either xenon or nitrogen for 24 hours, apo-

ptosis and necrosis totalled less than 5% in both groups (Fig 2A). To provoke acute neuronal injury, we exposed cells to 300 μ M glutamate. Under these injurious conditions, xenon at 70% atm increased cell viability from 22.9 \pm 8.2% to 43.7 \pm 6.8%; whereas necrotic cell death was unaltered, xenon reduced apoptotic cell death from 56.9 \pm 4.7% to 31.1 \pm 6.8% ($p < 0.01$; ANOVA followed by Student–Newman–Keuls test; see Figs 2B–D). In further studies, xenon significantly reduced apoptotic injury produced by 300 μ M NMDA (–31%), OGD (–36%), and 200nM staurosporine (–26%) (see Figs 2E–G).

Neuroprotective Effect of Xenon and Hypothermia In Vivo

In this series of studies, a neonatal rat model of focal hypoxia-ischemia¹⁶ was used because it produces injury to the brain that shares many of the features present in brain injury seen in the full-term human neonate.²² Interventions were always conducted when the rats were in a temperature-controlled environment, and we were able to establish that the brain temperature was maintained within the desired range (Table). Because the brain damage is unilateral (Fig 3B), the ratio of right-to-left hemispheric weight (R/L ratio) can be used to quantify the extent of the injury.^{17,18} When xenon was

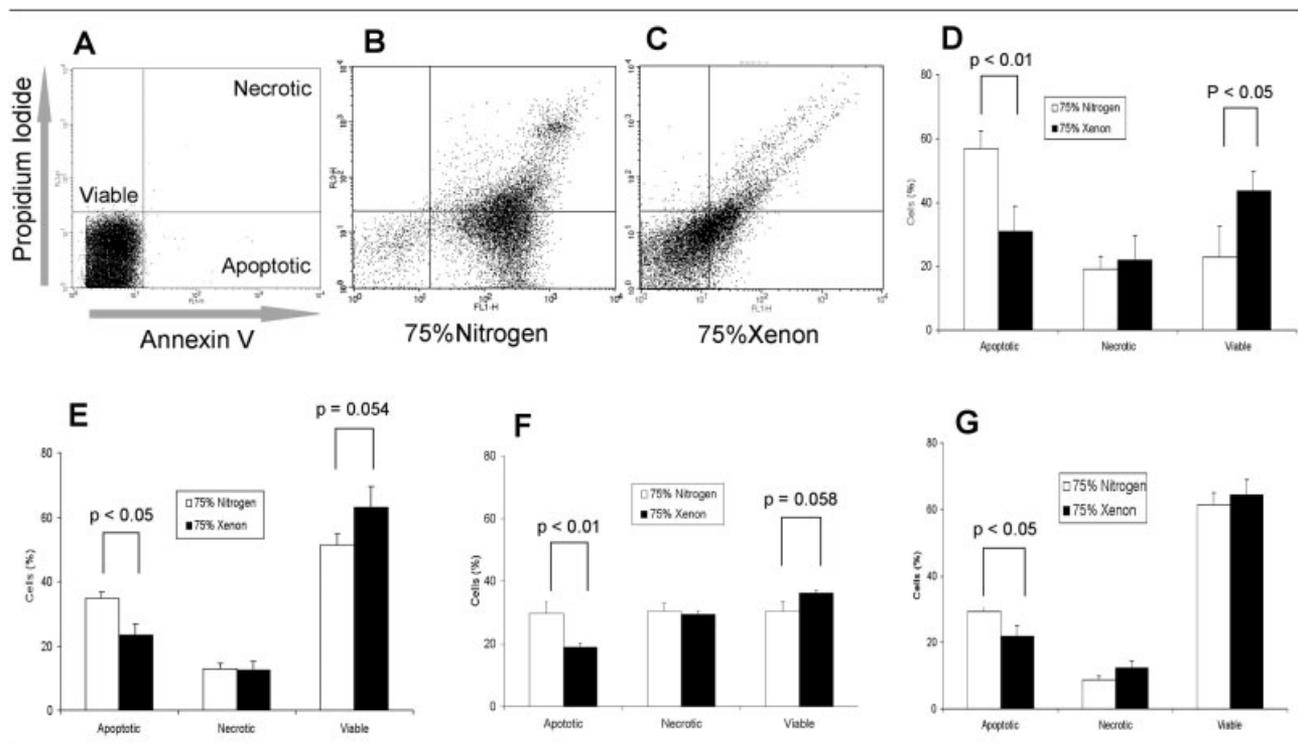


Fig 2. Xenon attenuates cell death in vitro. (A) Flow cytometry of sorted cultured neurons stained with annexin V (for apoptosis) and propidium iodide (for necrosis) under control (uninjured) conditions, (B) after treatment with the excitotoxin glutamate (300 μ M) for 10 minutes followed by N₂ (75%), or (C) where N₂ is substituted by xenon (75%). (D–G) Effects of xenon on apoptosis, necrosis, and cell viability in neurons injured with (D) glutamate (300 μ M), (E) NMDA (300 μ M), (F) oxygen glucose deprivation, or (G) staurosporine (200nM).

Table. Effect of Xe on Brain Temperature in Neonatal Rats (ambient temperature kept at 35°C), Mean (SEM), n = 4

	Air			30% O ₂ + 70% N ₂			8% O ₂ + 92% N ₂				
Time point (min)	0	10	20	0	10	20	0	10	30	40	60
	36.6 (0.13)	36.6 (0.08)	36.7 (0.09)	36.8 (0.13)	37.0 (0.09)	36.9 (0.06)	36.9 (0.14)	37 (0.11)	37 (0.08)	36.9 (0.1)	37.0 (0.08)

	Air			30% O ₂ + 70% Xe			8% O ₂ + 70% Xe + 22% N ₂				
Time point (min)	0	10	20	0	10	20	0	10	30	40	60
	36.3 (0.27)	36.5 (0.14)	36.6 (0.06)	36.9 (0.17)	37.2 (0.07)	37.2 (0.06)	37.1 (0.18)	37.2 (0.21)	37.2 (0.06)	36.9 (0.03)	37.0 (0.07)

There is no statistical significance between two groups at corresponding time point.

administered concurrently with hypoxia (see Fig 3C), it decreased brain injury, depending on concentration, with a significant effect being present at concentrations

of 40% atm and greater ($p < 0.05$; ANOVA followed by post hoc test; see Fig 3D). Xenon at 70% atm proved to be protective when administered for a 90-

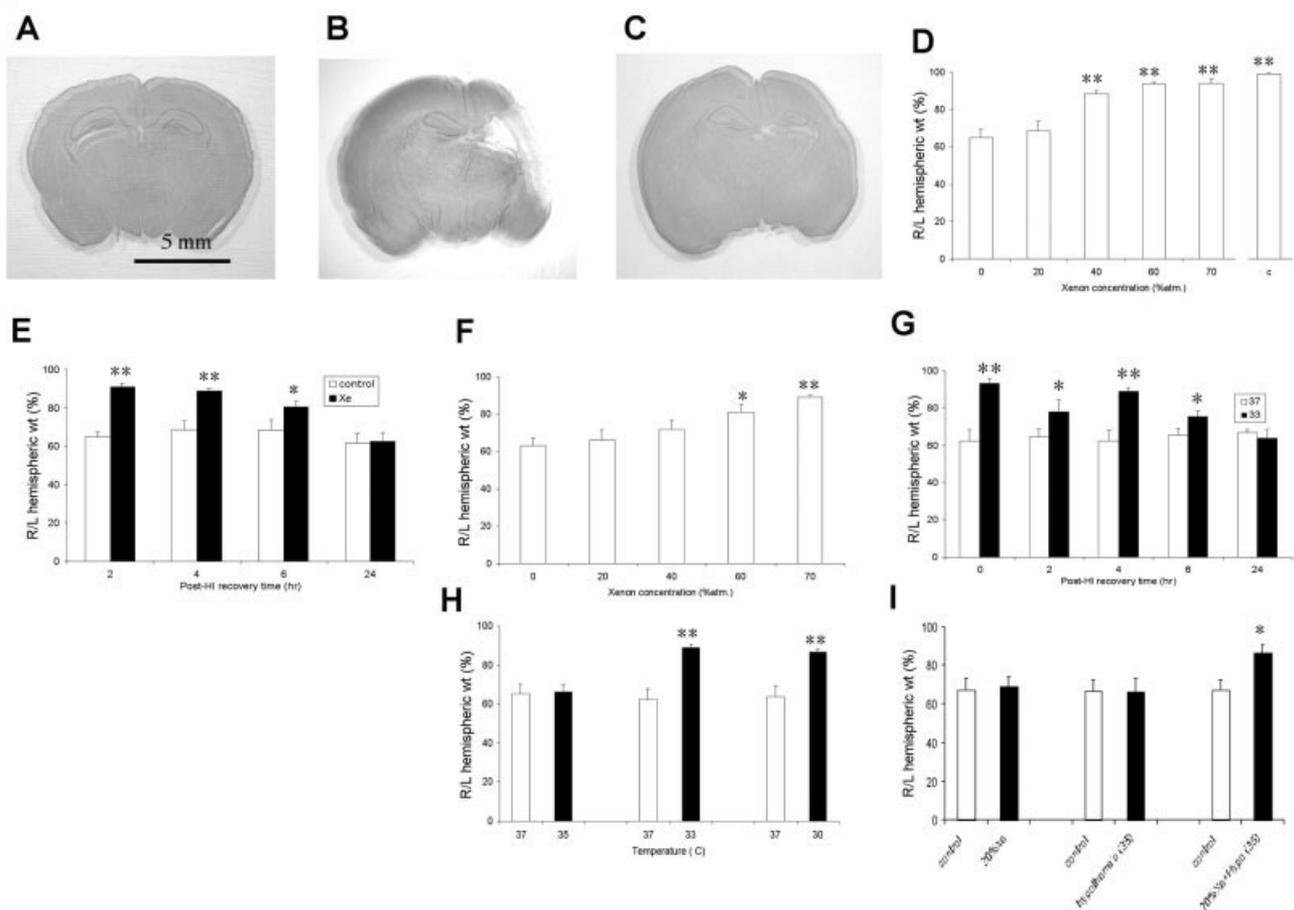


Fig 3. Neuroprotective effects of xenon and hypothermia in vivo. (A) Nissl staining of the coronal sections of brain from uninjured rats, (B) hypoxic-ischemic injured rats, and (C) xenon-protected and hypoxic-ischemic injured neonatal rats. (D) The concentration-dependent effect of xenon on the ratio of the weight of the right-to-left (injured/uninjured) cerebral hemispheres (R/L hemispheric weight) when administered concurrently with provocation hypoxic-ischemic injury. C = control or uninjured rats. (E) Effect of delaying xenon administration after hypoxic-ischemic injury on the R/L hemispheric weight ratio. (F) The concentration-dependent effect of xenon on the R/L hemispheric weight ratio administered 4 hours after hypoxic-ischemic injury. (G) Effect of delaying hypothermia (33°C) after hypoxic-ischemic injury on the R/L hemispheric weight ratio. (H) The effect of temperature, applied 4 hours after hypoxic-ischemic injury, on the R/L hemispheric weight ratio. (I) The effect of the combination of xenon (20%) and hypothermia (35°C), applied 4 hours after hypoxic-ischemic injury, on the R/L hemispheric weight ratio. * $p < 0.05$; ** $p < 0.01$ versus control (n = 6–8).

minute period up to 6 hours *after* the hypoxic-ischemic insult (see Fig 3E). When the concentration dependency of the protective effect of xenon was examined at 4 hours after the hypoxic-ischemic insult, only concentrations at or greater than 60% atm significantly increased the R/L ratio (see Fig 3F).

The time course for the protective effect of hypothermia (33°C) indicated that injury could be significantly reduced if introduced for a 90-minute period up to 6 hours *after* the hypoxic-ischemic insult (see Fig 3G). Temperature dependency of the protective effect was investigated at 4 hours after the hypoxic-ischemic insult and showed that protection occurred at 33°C and lower temperatures (see Fig 3H). Based on the xenon concentration response (see Fig 3F) and the temperature response (see Fig 3H), we then examined a combination of interventions (xenon at 20% atm and hypothermia at 35°C) that individually produced no

protection against the hypoxic-ischemic brain injury; yet, a 90-minute exposure to the combination of xenon 20% atm and hypothermia at 35°C, 4 hours after the hypoxic insult, significantly increased the R/L ratio from 0.67 ± 0.05 to 0.86 ± 0.04 ($p < 0.05$; ANOVA followed by Student–Newman–Keuls test; see Fig 3I).

Apoptosis, Necrosis, and Cell Viability In Vivo

We used the following morphological criteria to analyze apoptotic or necrotic cell death in cerebral cortex and dentate gyrus of hippocampus. Viable cells were regularly shaped with pale cytoplasm and a clearly visible, darker nucleus (Fig 4A). Apoptotic cells had dark-stained, shrunken nuclei that were spherically shaped and an intact cell membrane, often with a surrounding area of vacuolation²³ (see arrows in Figs 4B, C); two or more round apoptotic bodies (dark chromatin clumps)

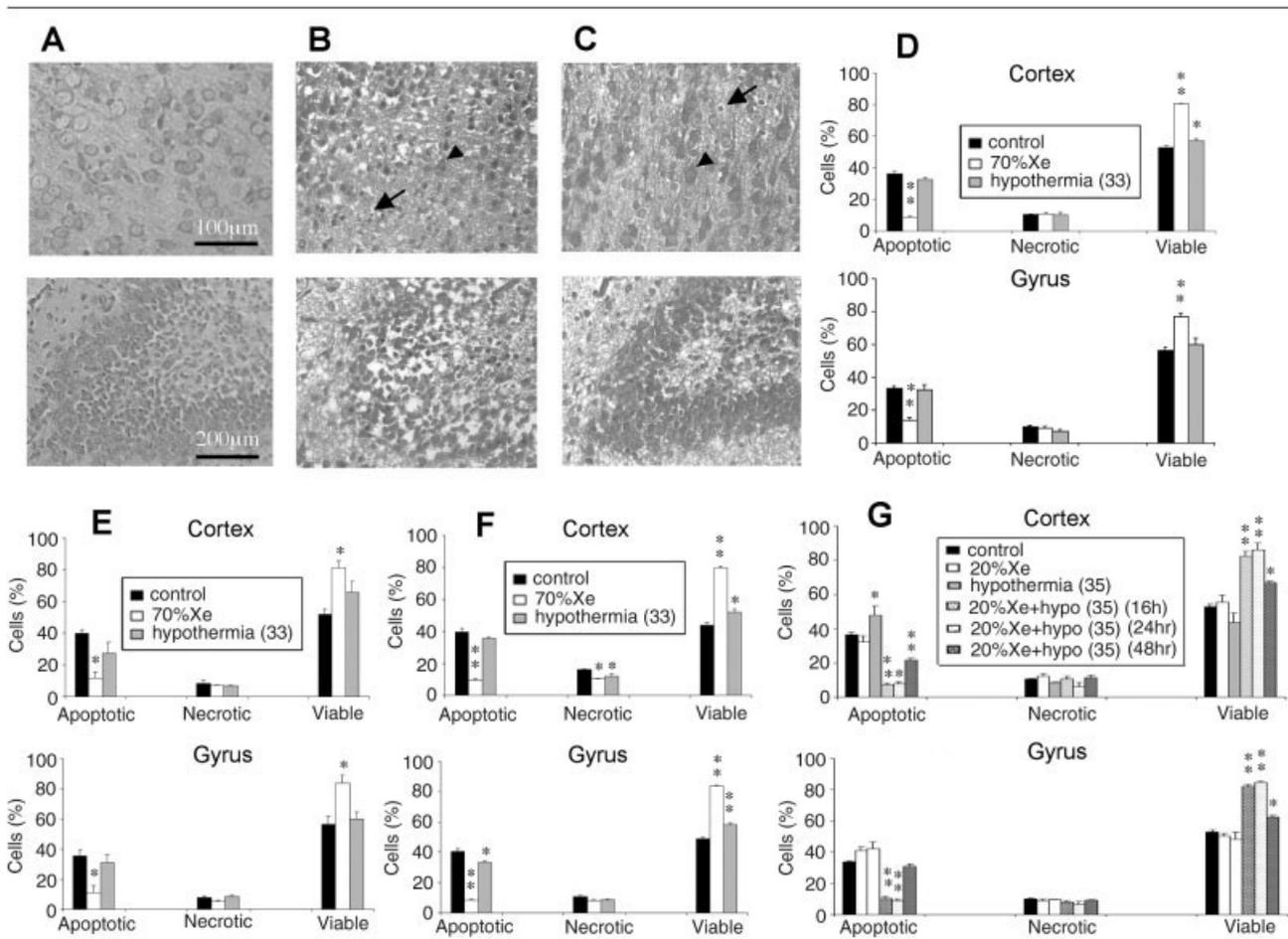


Fig 4. Xenon and hypothermia attenuates apoptotic cell death in hypoxic-ischemic injured neonatal rats. Photomicrographs of cortex (top) and hippocampal gyrus (bottom) stained with Nissl from an uninjured (A), a hypoxic-ischemic-injured (B), and a xenon-protected (70%) and hypoxic-ischemic-injured rat (C). Arrow indicates apoptotic cell; arrowhead indicates necrotic cell. The protective effect of either xenon 70% or hypothermia (33°C) are quantified in rats killed 16 (D), 24 (E), and 48 (F) hours after the injury. (G) The protective effect of a combination of subtherapeutic interventions with xenon (20%) and hypothermia is quantified. * $p < 0.05$; ** $p < 0.01$ versus control ($n = 3-4$). The histograms represent the percentage of cell death via either apoptotic or necrotic pathways and the percentage of viable cells.

were noted in apoptotic cell.²⁴ Necrotic cells were identified by intense cresyl violet staining of the cytoplasm with irregularly shaped, enlarged nuclei and loss of nuclear membrane integrity²⁴ (see Figs 4B, C, arrowhead).

Xenon at 70% atm significantly decreased apoptotic cell death and increased the viable cell count at 16, 24, and 48 hours in the cerebral cortex, as well as the hippocampal gyrus (ANOVA followed by Student–Newman–Keuls test; see Figs 4D–F). Xenon significantly decreased necrotic cell death only in the cortex at 48 hours (see Fig 4F). Although hypothermia at 33°C tended to exert an antiapoptotic effect and increased cell viability, only the latter reached statistical significance at 16 hours in the cortex and at 48 hours in both the cortex and the hippocampal gyrus (see Figs 4D, F).

Whereas neither xenon 20% atm nor hypothermia to 35°C provided an antiapoptotic effect alone, the combination of these two interventions administered at 4 hours after the hypoxic insult significantly decreased apoptosis and increased cell viability in both the cortex

and the hippocampal gyrus at each of the time points examined (see Fig 4G).

Significant neuroprotection provided by the combination of the two interventions, which are ineffective alone, demonstrates that synergy exists *in vivo* between xenon and hypothermia; these findings corroborate our *in vitro* experiments (see Figs 3I, G).

Effect on Long-Term Neurological Function

Thirty days after the injury, neurological motor function (Fig 5A) and coordination (see Fig 5B) were both significantly improved by xenon at 70% atm (Mann–Whitney *U* test). Whereas neither xenon at 20% atm nor hypothermia at 35°C significantly improved neurological function when administered alone 4 hours after the hypoxic insult, the combination restored neurological function to normal. The morphological data are expressed as the brain matter in lesioned hemisphere compared with the unlesioned hemisphere (see Fig 5C); these significantly show preservation of brain matter by 70% xenon ($p < 0.05$) and the combination

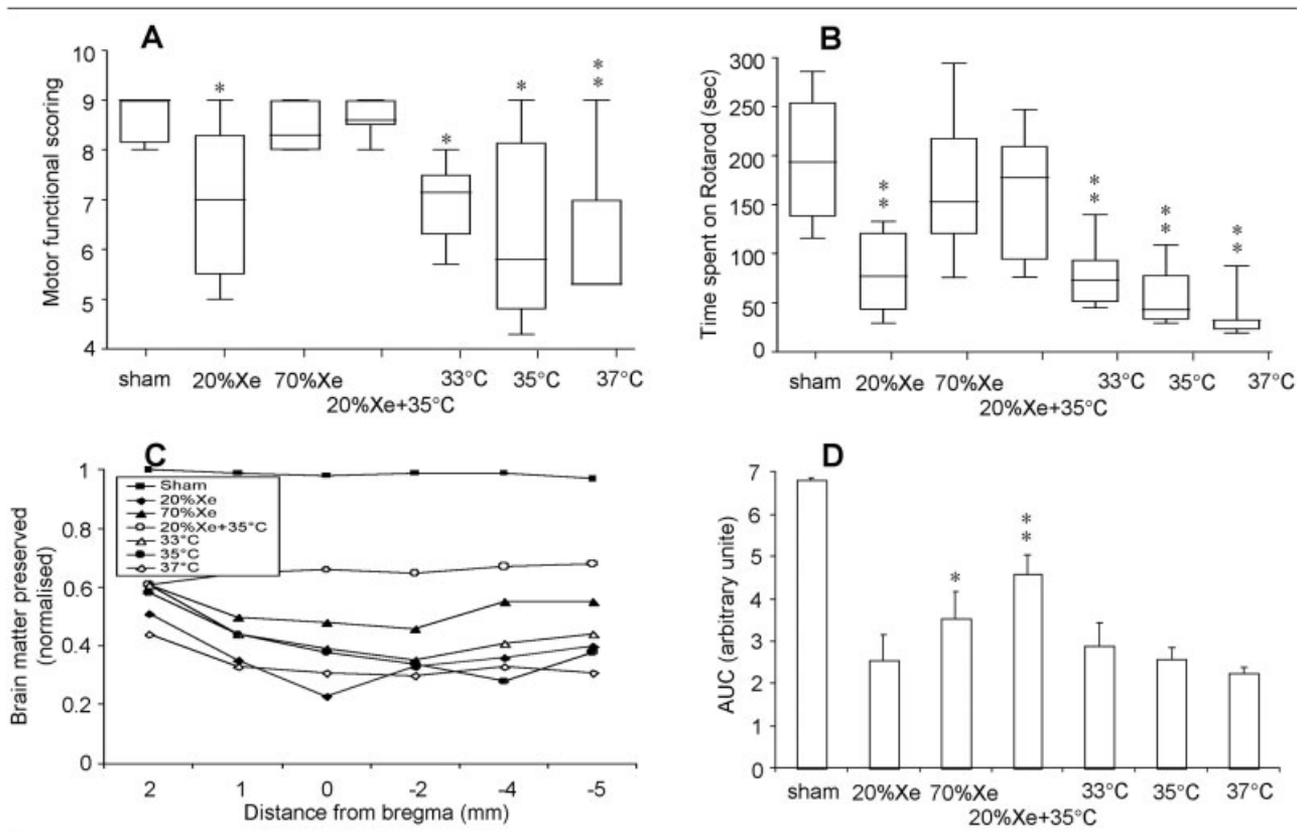


Fig 5. Xenon and hypothermia improve neurological function and attenuate brain matter loss 30 days after injury. Four hours after the hypoxic-ischemic injury, neonatal rats received either hypothermia (33 or 35°C), xenon (20 or 70%), or a combination of these two. Effect of intervention on neuromotor function (A) and time spent on a rotarod (B) are shown. * $p < 0.05$; ** $p < 0.01$ versus control ($n = 6-8$). (C) The preservation of brain matter was assessed in slices obtained from six contiguous regions referred to as distance from the bregma. Data are presented as the ratio of brain matter on the lesioned hemisphere relative to the unlesioned hemisphere. (D) Area under the curve (AUC) was derived from (C) with variations. * $p < 0.05$; ** $p < 0.01$ versus 37°C group ($n = 5$).

of xenon at 20% atm and hypothermia at 35°C ($p < 0.01$; ANOVA followed by Student–Newman–Keuls test; see Fig 5D). The long-term neurological function data corroborated the histological data, indicating that the neuroprotective effect afforded by xenon alone or xenon in combination with hypothermia was long lasting as evidenced by both sustained improvement in neurological function and attenuation of loss of brain matter.

Bax, Bcl-x_L, and Caspase 3

Apoptotic cell death induced by hypoxic-ischemia in neonates is mediated via the intrinsic (mitochondrial) pathway²⁵; therefore, we examined whether changes in protein expression of factors in this pathway were involved mechanistically in the neuroprotection provided by xenon and hypothermia. Bax and Bcl-x_L exert pivotal roles in apoptosis, promoting cell death and survival, respectively. Bax expression is enhanced from 2

to 48 hours after the hypoxic-ischemic insult, whereas Bcl-x_L expression is increased initially and then decreases 6 hours after the insult as neurons progress inexorably toward death (Figs 6A, B). Caspase 3 is one of the key executioners of apoptosis⁶; throughout the entire postinjury period, its cleaved, activated caspase 3 is increased as assessed by Western blotting (see Fig 6C), and it was confirmed by in situ immunohistochemistry to be located in the injured cerebral hemisphere (see Fig 6E). The boundary between normal and ischemic areas of brain could be identified by the immunocytochemical identification of cells expressing cleaved caspase 3 and hence undergoing apoptosis (see Figs 6F, G).

Both 70% xenon alone, as well as 20% xenon in combination with hypothermia at 35°C, significantly suppressed Bax expression (ANOVA followed by Student–Newman–Keuls test; see Fig 6H) and enhanced Bcl-x_L expression (see Fig 6I), indicating that xenon or

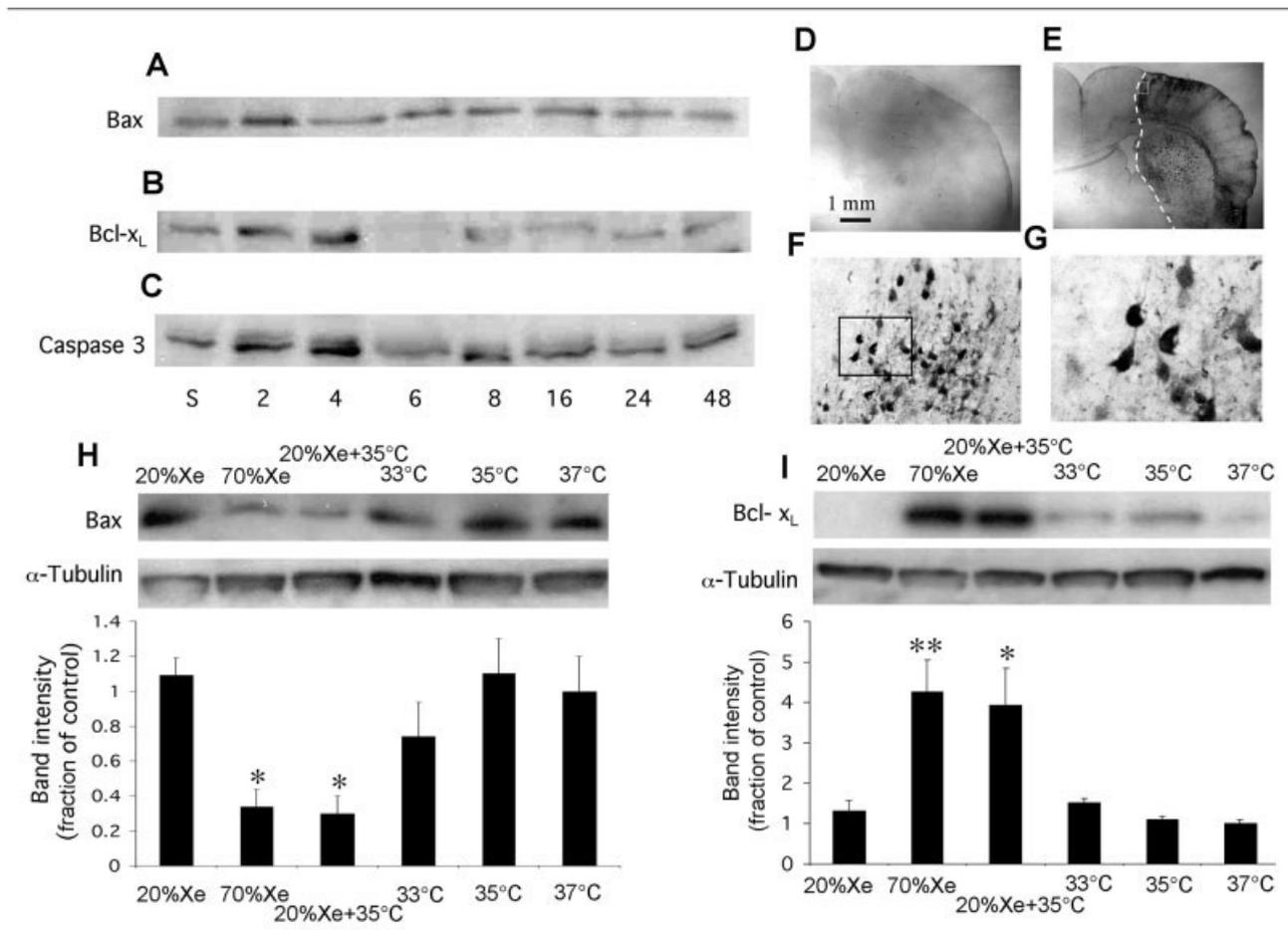


Fig 6. Effects of xenon and hypothermia on the expression of apoptotic proteins. Time course of changes in the Western blot expression of Bax (A), Bcl-x_L (B), and cleaved caspase 3 (C) in the ipsilateral hemisphere of hypoxic-ischemic-injured neonatal rats. In situ immunohistochemical expression of cleaved caspase 3 in a noninjured (D) and an injured rat (E) (objective lens $\times 1.25$) at 40 \times (F) and 100 \times magnification (G). Effect of xenon and hypothermia applied 4 hours after hypoxic-ischemic injury on expression of Bax (H) and Bcl-x_L (I) from tissue obtained from the ipsilateral cerebral hemisphere 24 hours after hypoxic-ischemic injury. * $p < 0.05$; ** $p < 0.01$ versus control ($n = 3$).

hypothermia, or both, altered the factors in the intrinsic apoptotic pathway to promote cell survival.

Discussion

By blocking the NMDA subtype of the glutamate receptor, xenon inhibits a key step in the excitotoxic pathway, and hence protects against nerve cell death²⁶ from exogenously applied NMDA, glutamate *in vitro*, as well as *N*-methyl-DL-aspartate administered *in vivo*.^{8,9} OGD *in vitro*, a model thought to closely resemble the insult caused by ischemia, also has been shown to activate excitotoxic pathways,¹³ and xenon also prevents damage from this type of injury.

Hypothermia also decreases the neuronal injury produced by OGD in cortical neuronal cells (see Figs 1C, E); these findings are consistent with previous *in vitro* experiments showing the temperature-dependent neuroprotective effect of hypothermia in rat neuronal cell cultures,²⁷ rat hippocampal brain slices,²⁸ murine neuronal-glia coculture,²⁹ as well as *in vivo* models of brain ischemia^{30,31} and traumatic brain injury.³²

Our data show that a combination of xenon and hypothermia causes a synergistic enhancement of their individual neuroprotective properties. An additive combination would suggest that the mechanisms of the two interventions are acting on two independent pathways at the cellular level, which would appear to summate when combined. Synergism implies that the two interventions converge on a single effector pathway; the combination enhances the final result more than if the two interventions were simply added. In the case of hypothermia and xenon, both exert their neuroprotective effects, at least in part, by actions on the excitotoxic pathway of neuronal damage. Hypothermia reduces glutamate release^{33,34} and also reduces the release of glycine, an important promoter of glutamate action on the NMDA receptor^{34,35}; xenon acts as an NMDA receptor antagonist. Thus, these two interventions converge on the excitotoxic pathway by decreasing neurotransmitter release and blocking the receptor for neurotransmitters that may have been released. Although excitotoxicity is not the only mechanism of damage after hypoxic-ischemia, and both interventions may have other protective effects on different pathways, it appears that reduction of excitotoxic death can explain the beneficial synergistic effects. These mechanistic studies were performed in two different species (*in vitro* mouse and *in vivo* rat models of neuronal injury); nonetheless, the synergistic interaction between xenon and hypothermia was observed in both species, indicating a preserved effect in two different mammals that may respond differently to this type of injury.

The large increase in the enthalpy associated with the reduction in LDH release that occurs when xenon is present (see Fig 1H) suggests a marked synergistic interaction that is confirmed by the isobolographic

analysis (see Fig 1G). The increase in enthalpy is considerably larger than could plausibly be attributed to the enthalpy of binding of xenon to its putative site(s) on the NMDA receptor, and the basis of the enhanced temperature dependence currently is unknown. This is in contrast with the effects we observed with another NMDA receptor antagonist, gvestinel, which showed only an additive interaction with hypothermia (see Fig 1I), which was similar to effects reported previously with MK801.³⁶

Apoptotic neuronal cell death can result from an increase in intracellular free calcium concentration as a consequence of excessive release of glutamate and activation of the NMDA receptor.³⁷ Proapoptotic factors such as Bax translocate from the cytosol into the mitochondrion,³⁸ thereby enhancing its permeability. This deleterious effect of Bax within the mitochondrion can be counteracted by being bound by the antiapoptotic protein Bcl-x_L³⁹; however, with prolonged ischemia and the subsequent reperfusion insult, Bcl-x_L becomes depleted (see Fig 6B), and the now permeable mitochondrial membrane facilitates release of cytochrome *c* into the cytosol.^{40,41} Within the cytosolic compartment, caspase 3, the key executioner of apoptosis, is cleaved and irreversibly activated (see Figs 6B–G). We found that xenon, at high concentrations (ie, 70% atm) or at a much lower concentration (ie, 20% atm) in combination with mild hypothermia (35°C), decreases expression of Bax whereas enhancing Bcl-x_L expression. Consequently, cleavage of caspase 3, and hence its activation, may not occur, and apoptosis could be interrupted. Thus, these data suggest that the mechanism(s) for the synergistic neuroprotective interaction of the combination is “upstream” of the altered expression of Bax and Bcl-x_L.

Apart from its blocking effect on NMDA receptors, xenon is a potent activator of the two-pore domain K⁺ channel⁴²; the two-pore domain K⁺ channel recently has been shown to play an important role in neuroprotection.⁴³ In addition, xenon causes a Ca²⁺-dependent metaphase arrest in astrocytes⁴⁴ that is thought to be caused by an inhibitory effect on calcium/calmodulin-dependent protein kinase-II.⁴⁵ Because calcium/calmodulin-dependent protein kinase-II inhibition protects against excitotoxin and OGD injury *in vitro*,⁴⁶ xenon may exert its intracellular neuroprotective actions through this mechanism. However, whether each of these molecular entities combine to produce the synergistic neuroprotection provided by the combination of xenon and hypothermia is not yet known.

In humans, xenon has a safety profile unequaled among general anesthetics, and only its high cost precludes its routine use for surgical procedures. Because its neuroprotective concentration is roughly 30% of the concentration required for anesthesia in humans, we speculate that the synergy provided by combining xe-

non and hypothermia, together with the safety of these interventions, can be translated into long-term clinical benefit (see Fig 5) in patients with acute neuronal injury. Furthermore, a reduction in the concentration of xenon needed for neuroprotection would favorably affect the cost of the intervention. That these interventions were efficacious even when administered several hours after the injury (see Figs 3E–I) makes this an even more attractive strategy because, in most settings, acute neuronal injury is an event that cannot be anticipated (eg, stroke, perinatal asphyxia, and so forth). Because most births, and hence asphyxial injuries, occur at some distance from a tertiary referral center, we propose that mild temperature reduction can be initiated at the primary care center where the injury occurred and maintained during transport to a tertiary referral center; if relocation can be accomplished within the 6-hour “window of opportunity” (see Fig 3), the putative benefits from the hypothermia–xenon neuroprotectant combination may still be realized.

In summary, our results indicate that the combination of xenon with mild hypothermia has a significant neuroprotective effect against hypoxic-ischemia-induced brain injury in neonates through an antiapoptotic mechanism. This synergistic combination could be exploited for the clinical treatment of acute neuronal injury including neonatal asphyxia.

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