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Xenon Pretreatment Attenuates Anesthetic-induced Apoptosis in the Developing Brain in Comparison with Nitrous Oxide and Hypoxia

Yi Shu, B.Sc.,* Shivali M. Patel, M.B.B.S., B.Sc.,† Chen Pac-Soo, M.B.Ch.B., M.D., F.R.C.A.,‡ António Rei Fidalgo, B.Sc., M.Sc.,* Yanjie Wan, M.D.,§ Mervyn Maze, M.B.B.S., F.R.C.A.,‖ Daqing Ma, M.D., Ph.D.#

ABSTRACT

Background: Administration of certain general anesthetics to rodents during the synaptogenic phase of neurodevelopment produces neuronal injury. Preconditioning (pretreatment) can reduce tissue injury caused by a severe insult; the authors investigated whether pretreatment strategies can protect the developing brain from anesthetic-induced neurotoxicity.

Methods: Seven-day-old Sprague–Dawley rats were pretreated with one of the following: 70% xenon, 70% nitrous oxide, or 8% hypoxia for 2 h; 24 h later, rats were exposed to the neurotoxic combination of 70% nitrous oxide and 0.75% isoflurane for 6 h. Cortical and hippocampal neuroapoptosis was assessed using caspase-3 immunostaining. Separate cohorts were maintained for 40 days at which time cognitive function with trace fear conditioning was performed. In other pretreated cohorts, rat cortices were isolated for immunoblotting of caspase-3, Bcl-2, cytochrome *C*, P53, and mitogen-activated protein kinases. To obviate physiologic influences, organotypic hippocampal slices harvested from postnatal rat pups were cultured for 5 days and exposed to the same conditions as obtained for the *in vivo* studies, and caspase-3 immunostaining was again the measured outcome.

Result: Xenon pretreatment prevented nitrous oxide- and isoflurane-induced neuroapoptosis (*in vivo* and *in vitro*) and cognitive deterioration (*in vivo*). Contrastingly, nitrous oxide- and isoflurane-induced neuroapoptosis was exacerbated by hypoxic pretreatment. Nitrous oxide pretreatment had no effect. Xenon pretreatment increased Bcl-2 expression and decreased both cytochrome *C* release and P53 expression; conversely, the opposite was evident after hypoxic pretreatment.

Conclusions: Although xenon pretreatment protects against nitrous oxide- and isoflurane-induced neuroapoptosis, hypoxic pretreatment exacerbates anesthetic-induced neonatal neurodegeneration.

What We Already Know about This Topic

- Preconditioning by transient hypoxia or anesthetic exposure can protect the brain and heart from subsequent ischemia
- Whether preconditioning is effective against cell death from neonatal exposure to anesthetics is not known

What This Article Tells us That Is New

- In neonatal rats, preconditioning with xenon protected against isoflurane-/N_2O-induced cell death and learning deficits, and preconditioning with transient hypoxia worsened it

THE ethanol-induced fetal alcohol syndrome provided evidence that anesthetics may be capable of damaging the brain during the synaptogeneic phase of neurodevelopment because of the similarity of their properties at the *N*-

* PhD Student, † Trainee Doctor, # Senior Lecturer, Anesthetics, Pain Medicine and Intensive Care, Division of Surgery, Department of Surgery and Cancer, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom. ‡ Consultant Anaesthetist, Anesthetics, Pain Medicine and Intensive Care, Division of Surgery, Department of Surgery and Cancer, Imperial College London, Chelsea and Westminster Hospital, and Consultant Anaesthetist, Department of Anesthetics, Wycombe Hospital, Buckinghamshire Hospitals NHS Trust, High Wycombe, Buckinghamshire, United Kingdom. § Clinical Director, Department of Anesthesiology, Gongli Hospital, Pudong, China. ‖ Professor, Chair, Department of Anesthesiology, University of California, San Francisco, San Francisco, California.

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Address correspondence to Dr. Ma: Anaesthetics, Pain Medicine and Intensive Care, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, United Kingdom. d.ma@imperial.ac.uk.

methyl-D-aspartic acid (NMDA) and γ-aminobutyric acid (GABA) A receptors.[1–5] Jevtovic-Todorovic *et al.*[6] showed that administration of a combination of midazolam, nitrous oxide, and isoflurane to 7-day-old rats produced widespread apoptotic neurodegeneration in the brain and subsequent impairment of memory or learning that persisted into early adulthood.

Drugs acting on NMDA and/or $GABA_A$ receptors during synaptogenesis, also known as brain growth spurt,[7] can have such profound effects because the cells, in the developing central nervous system, are differentiating and migrating to target zones and establishing synaptic contact with neighboring neurons, thereby producing functional neuronal circuits. Neurotransmitters, including GABA and glutamate and their receptors, play a fundamental role during this process as they are essential for neuronal migration, dendritic filopodia stabilization, synaptic development, and stabilization.[8]

In rodents, synaptogenesis is predominantly a postnatal event and is most intense just before birth and in the first 2 postnatal weeks. In humans, synaptogenesis begins at 6 months' gestation and ends a few years after birth.[7] We sought to determine whether anesthetic-induced neurotoxicity could be attenuated by preconditioning. Preconditioning (also referred to as pretreatment in this study) is a ubiquitous mechanism whereby an intervention enhances the tolerance of the organism to injury-inducing conditions. We sought to determine whether pretreatment strategies can be effective against anesthetic-induced neurotoxicity. Ischemia has been used as the stimulus in most studies of preconditioning; however, other interventions can also stimulate preconditioning, for example, hyperoxia, oxidative stress, hyperthermia, heat shock, and anesthetics including xenon.[9,10] We postulated that pretreatment may protect the brain of neonatal rats from anesthetic drug-induced apoptosis.

Materials and Methods

This study was approved by the Home Office, London, United Kingdom, and conforms to the United Kingdom Animals (Scientific Procedures) Act, 1986.

Neuronal Apoptosis Induced by Anesthetics In Vivo

Previously, we showed that pretreatment with 70% xenon for 2 h can attenuate hypoxic–ischemic-induced injury in a model of asphyxia in rats.[10] The neuroprotective properties of hypoxic pretreatment (8% oxygen for 2 h) are also well established,[11,12] which we confirmed in our pilot studies (data not shown).

Seven-day-old Sprague–Dawley rat pups were exposed to 70% xenon or 70% N_2O balanced with oxygen or 8% oxygen balanced with nitrogen for 2 h in six purpose-built multichambers (1–2 pups/chamber). To reduce the wastage of costly xenon, it was delivered in a customized closed-circuit system, in which carbon dioxide was absorbed by soda lime and water vapor with silica gel. Twenty-four hours after pretreatment, rats were exposed to the anesthetic combination of 70% N_2O + 0.75% isoflurane for 6 h. To precisely control the body temperature of the pups, all exposure chambers were partially submerged in a water bath, and the water temperature was adjusted to obtain a desired brain temperature of 37°C. This was performed with one pup brain being planted with a temperature probe, and the brain temperature was measured with a telemetry temperature monitoring system (VitalView; Mini-Mitter, Bend, OR). This pup was excluded for further data analysis. Oxygen, nitrous oxide, isoflurane, and xenon were regularly monitored with a gas monitoring system (Datex-Ohmeda, Bradford, United Kingdon; a 439XE monitor, Air Products, London, United Kingdom) *via* a sampling tube inserted into the experimental chamber.

Rats were killed with an intraperitoneal injection of 100 mg/kg^{-1} sodium pentobarbital 48 h after pretreatment and perfused with a fixative.[10] The brains were removed and kept at 4°C overnight in paraformaldehyde. The fixed brains were then transferred to a solution of 30% sucrose with phosphate buffer and 1% sodium azide and were refrigerated until they were immunostained for the apoptotic marker caspase-3.

Cognitive Function Assessment

Like the Morris water maze, trace fear conditioning is also a standard method to measure hippocampal-dependent memory,[13] and hence, this paradigm was used in the current study to assess cognitive function. Cognitive assessment was performed in a separate cohort of animals 40 days after anesthetic exposure. The conditioning chamber (30 cm × 24 cm × 21 cm; Med Associates Inc., St. Albans, VT) had a white opaque back wall, aluminum sidewalls, and a clear polycarbonate front door. The conditioning box had a removable grid floor and waste pan. Between each rat, the box was cleaned with an almond-scented solution and dried thoroughly. The grid floor contained 36 stainless steel rods (diameter, 3 mm) spaced 8 mm center to center, which made contact with a circuit board through which a scrambled shock was delivered. During training and context testing, a standard high efficiency particulate air filter provided a background noise of 65 db. On day 1 (41 days after anesthetic exposure) (acquisition), all animals received six cycles of 214 s of the trace fear conditioning paradigm. The tone was presented for 16 s (2 kHz) followed by a trace interval of 18 s and subsequent foot shock (2 s, 0.85 mA). The rats were removed from the conditioning chamber 198 s after the last shock and returned to their home cage. The total time of the acquisition protocol was 26 min. Working memory during acquisition was defined as the time spent immobile after a shock divided by the intertrial interval.

On the next day (context test), the trained rats were placed for 8 min to the same acquisition environment but without unconditional stimulus (the foot shock) or conditional stimulus (the tone). The percentage of time an animal froze during the 8-min observation periods was calculated as the number of observations judged to be

freezing divided by the total number of observations in 8 min (*i.e.*, 60 observations).

On the third day, the tone test was performed. Rats were placed in modified environment (increased lighting, flat floor, and triangular side walls were inserted along with a cinnamon-scented solution) where they were allowed to acclimatize for 192 s before being presented to three cycles of a 16-s tone followed by an intertrial interval of 240 s. Freezing time was automatically scored using VideoFreeze software (Med Associates Inc., Burlington, VT). The overall percentage of freezing time (context results) and the area under curve (tone test) were derived from plots between the percentage of freezing time and trial time in the tone test and were used for statistical comparison.

The overall percentage of freezing time was used to assess hippocampal memory during the context test, whereas for the tone test, the area under curve after tone presentation was derived from the graph, which plotted the percentage of freezing time against trial time.

Western Blotting

Rat pups were killed 30 h after pretreatment with or without anesthetic insult. The brains were removed immediately, and the cortices were separated and kept at −80°C. The samples were then lysed in cell lysis buffer (pH 7.5, 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2DTA, 1 mM ethylene glycol tetraacetic acid, 1%trition, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DL-dithiothreitol, 1 mM phenylmethanesulfonyl, and 1 μg/ml leupeptin). The homogenized sample was centrifuged at 3,000*g* for 10 min at 4°C to remove cellular debris. The supernatant was further centrifuged twice, initially at 11,000*g* for 20 min at 4°C for Western blotting of Bcl-2 and cleaved caspase-3 and a second time at 20,000*g* for 45 min at 4°C to separate cytosolic and mitochondrial material for Western blotting of cytosolic cytochrome *C*. Protein concentration of the supernatant was determined using the Bradford Protein Assay (Bio-Rad, Herts, United Kingdom) and 20 μg per sample was used for Western blot studies. After electrophoresis, proteins were electrotransferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Buckinghamshire, United Kingdom). The membrane was probed with primary antibodies for Bcl-2, cleaved caspase-3 (New England Biolabs, Hitchin, Hertfordshire, United Kingdom), cytochrome *C* (BD Biosciences Pharmingen, Oxford, United Kingdom), P53, and mitogen-activated protein kinases (New England Biolabs), followed by relevant species-derived horseradish peroxidise-conjugated secondary antibody for 1 h at room temperature. Thereafter, the membrane was reprobed with housekeeping protein α-tubulin antibody (Sigma, Poole, United Kingdom). The protein bands were visualized with enhanced chemiluminescence (New England Biolabs) with hyperfilm development (Amersham Biosciences). Densitometric analysis with normalization relative to α-tubulin was performed, and data are presented as protein expressed relative to control.

Neuronal Apoptosis Induced by Anesthetics In Vitro

Organotypic hippocampal slice preparation was reported previously.[10] Briefly, neonatal rat pups at postnatal day 8 were anesthetized using 0.5% isoflurane until quiescent and killed by decapitation. The brains were quickly dissected and placed in an ice cooled (4°C) dissection solution, and excess tissues (cerebellum, olfactory bulbs, and meninges) were removed. The brains were cut into 400-μm saggital slices using a Mclllwain Tissue Chopper (Mickle Laboratory, Cambridge, United Kingdom). The slices with hippocampus were selected and placed on 30-mm-diameter semiporous cell culture inserts (Falcon, Becton Dickinson Labware, Millipore, Bedford, MA). The tissues were then cultured for 24 h in humidified air at 37°C and enriched with 5% CO_2 to allow them to recover from the trauma induced by the extraction. All subsequent gas exposures for pretreatment and subsequent anesthetic insults were similar to the *in vivo* studies (see above, this section) and occurred in a specially constructed exposure chamber.[10] The gas mixtures consisting of one of the following: 70% xenon, 70% N_2O with 5% CO_2 and 20% O_2 balanced with nitrogen or oxygen, and 8% oxygen with 5% CO_2 balanced with nitrogen, were warmed by a water bath and delivered in the headspace above the slices by a standard anesthetic machine at 2–3 $l \cdot min^{-1}$; the concentrations were closely monitored with an S/5 spirometry module (Datex-Ohmeda, Bradford, United Kingdom) and a 439XE monitor (Air Products, London, United Kingdom). The gases were delivered for 3–4 min; thereafter, the chambers were sealed and placed in an incubator at 37°C for 2 h (Galaxy R Carbon Dioxide Chamber, Wolf Laboratories, York, United Kingdom). After exposure, the slices were returned to the incubator in humidified air at 37°C, enriched with 5% CO_2, for a further 12 h to allow for caspase-3 expression and were then fixed overnight in 4% paraformaldehyde.

Caspase-3 Immunohistochemistry

The brain samples from *in vivo* experiments or cultured slices from *in vitro* experiments were immersed in 30% sucrose with phosphate buffer and 1% sodium azide and refrigerated until they shrunk. They were then frozen and thereafter sliced in a coronal axis into 30-μm-thick sections using a cryostat (Bright Instrument Company Ltd., Huntingdon, United Kingdom). The sections relative to bregma-2 *in vivo* and the inner sections *in vitro* were stained *in situ* with cleaved caspase-3, a well-established marker of neuronal apoptosis in the developing brain.[14,15] Briefly, the slices were quenched in a solution of 70% methanol and 0.3% hydrogen peroxide for 30 min and then incubated in 3% blocking phosphate-buffered saline solution containing 0.3% Triton-X (Promega Corporation, Madison, WI) and 1% goat serum (Vector Laboratories Inc., Burlingame, CA) for 1 h at room temperature. After overnight incubation at 4°C with rabbit anticleaved caspase-3 antibody (1:1500) (New England Biolabs, Hertfordshire, United Kingdom), the sections were incubated with biotinylated goat antirabbit IgG (1:

200; Chemicon International, Temecula, CA) for 1 h at room temperature followed by Avidin-Biotin-peroxidase complex (Vector Laboratories Inc.) for 1 h. The positive caspase-3 cells were visualized with 3,3′-diamino-benzidine solution (Vector Laboratories Inc.). Thereafter, the slices were mounted onto Super Plus-coated glass slides and dried in a dark area. They were then dehydrated with alcohol, cleared with xylene, and covered with glass before light microscopy.

Photomicrographs were taken of the whole hemisphere and hippocampus for *in vivo* experiments and of the hippocampal cornu ammonis area for *in vitro* experiments with a BX-60 light microscope (Olympus, Southall, United Kingdom) and an Axiocam digital camera (Zeiss, Göttingen, Germany). The images were then printed, and an investigator blinded to the experimental protocol counted the number of 3,3′-diaminobenzidine stained (black) cell bodies in the desired areas. Counting was performed on slices from the whole cortex and and from the hippocampal cornu ammonis 1–3 subregions for *in vitro* experiments.

Statistical Analysis

All results are continuous change data, and the normal distribution can be assumed albeit a relatively small sample size. Thus, the results were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance followed by *post hoc* Student–Newman–Keuls test for comparison where appropriate using an interactive statistical package (London, United Kingdom). $P < 0.05$ was considered statistically significant.

Results

Neuronal Apoptosis Induced by Anesthetics In Vivo

There were some casapse-3-positive cells in the cortex in the naive control (fig. 1A). However, the combined administration of N_2O and isoflurane produced a significant increase in the number of caspase-3-positive cells (fig. 1B). Xenon pretreatment significantly decreased the number of caspase-3 cells induced by the administration of the combination of N_2O + isoflurane (fig. 1C); N_2O pretreatment did not have any significant effect (fig. 1D). In contrast, hypoxic pretreatment significantly enhanced the cellular apoptosis induced by the combination of N_2O + isoflurane (fig. 1E). The pretreatment regimens had no significant effect on baseline cellular apotosis (fig. 1F). Quantitative data analysis of the immunohistochemically stained brain slices showed that hypoxic pretreatment of the rats followed by exposure to N_2O + isoflurane produced a significant increase in the number of caspase 3-positive cells in the cortex to 220 ± 22 from 177 ± 18 as observed in the group receiving a combination of N_2O + isoflurane only ($P < 0.05$). Xenon pretreatment significantly decreased it to 61 ± 6 ($P < 0.01$), whereas N_2O pretreatment produced no significant change. A similar pattern of change in cellular apoptosis was found in the hippocampus (fig. 1G). These findings were also confirmed by Western blot analysis (figs. 1H and I).

Neuronal Apoptosis Induced by Anesthetics In Vitro

The neuronal apoptosis seen in the *in vivo* experiments could have been due to either the indirect effect of the physiologic disturbances or the inherent effect of anesthetics or to both. To obviate the possible physiologic effects, organotypic hippocampal slices was used, and the data are presented in figure 2. The number of caspase-3 cells was significantly increased to 53 ± 3 after N_2O + isoflurane exposure from a baseline level of 29 ± 2 ($P < 0.05$) (figs. 2A, B, and F). The increase in caspase-3 cells was significantly reduced to 33 ± 4 by xenon pretreatment ($P < 0.05$) (figs. 2C and F); apoptosis was unchanged by N_2O preconditioning (figs. 2D and F). Remarkably, apoptosis was significantly increased by hypoxic pretreatment to 78 ± 7 ($P < 0.05$) (figs. 2E and F).

Molecular Species

Pretreatment with 70% xenon for 2 h followed 24 h later by exposure to N_2O + isoflurane triggered a relative up-regulation in the expression of Bcl-2 (1.29 ± 0.05; $P < 0.05$), measured 24 h after event, compared with the control, which was treated with N_2O + isoflurane alone. Pretreatment with either 70% N_2O in oxygen (1.15 ± 0.1) or 8% oxygen in nitrogen (1.21 ± 0.39) without exposure to N_2O + isoflurane caused no significant change in the expression of Bcl-2 ($P > 0.05$), but pretreatment with 8% hypoxia followed by exposure to the anesthetic gas mixture caused a significant decrease in the expression of Bcl-2 (0.7 ± 0.03), ($P > 0.05$) (fig. 3A). Cytochrome *C* immunoreactivity was analyzed by Western blotting of the cytosolic fraction of the cortical extracts of rats pups subjected to preconditioning. Pretreatment with 8% oxygen in nitrogen and 70% N_2O caused a significant increase in the expression of cytochrome *C* to 2.23 ± 0.2 and 1.8 ± 0.1, respectively, relative to the N_2O + isoflurane control ($P < 0.05$ or 0.01) (fig. 3B). Xenon pretreatment caused a decrease in the expression of p53 to 0.79 ± 0.05 relative to N_2O + isoflurane control, whereas hypoxic pretreatment induced a relative increase to 1.37 ± 0.1 ($P < 0.05$), and N_2O pretreatment produced no significant change (fig. 3C). There were no significant changes in mitogen-activated protein kinase phosphorylation after any of the above treatments (fig. 3D).

Cognitive Function Assessment

After pretreatment and exposure to the anesthetic gas mixture, cohorts of rats were allowed to live up to the age of 40 days for the assessment of cognitive function using trace fear conditioning. Hypoxia pretreatment followed by N_2O + isoflurane administration (11 ± 1; $P < 0.05$) and N_2O + isoflurane administration alone (15 ± 2; $P < 0.05$) resulted in shorter hippocampal dependent-freezing response time in the context test than xenon pretreatment followed by N_2O + isoflurane treatment (24 ± 1). The latter was similar to that observed in naive controls (27 ± 7; $P > 0.05$) (fig. 4A). The results of the tone test, mainly used to assess amygdala function, showed that the area under curve (arbitrary unit) was higher in the xenon preconditioned group exposed to N_2O + isoflurane (243 ± 27) than in the hypoxic preconditioned group exposed

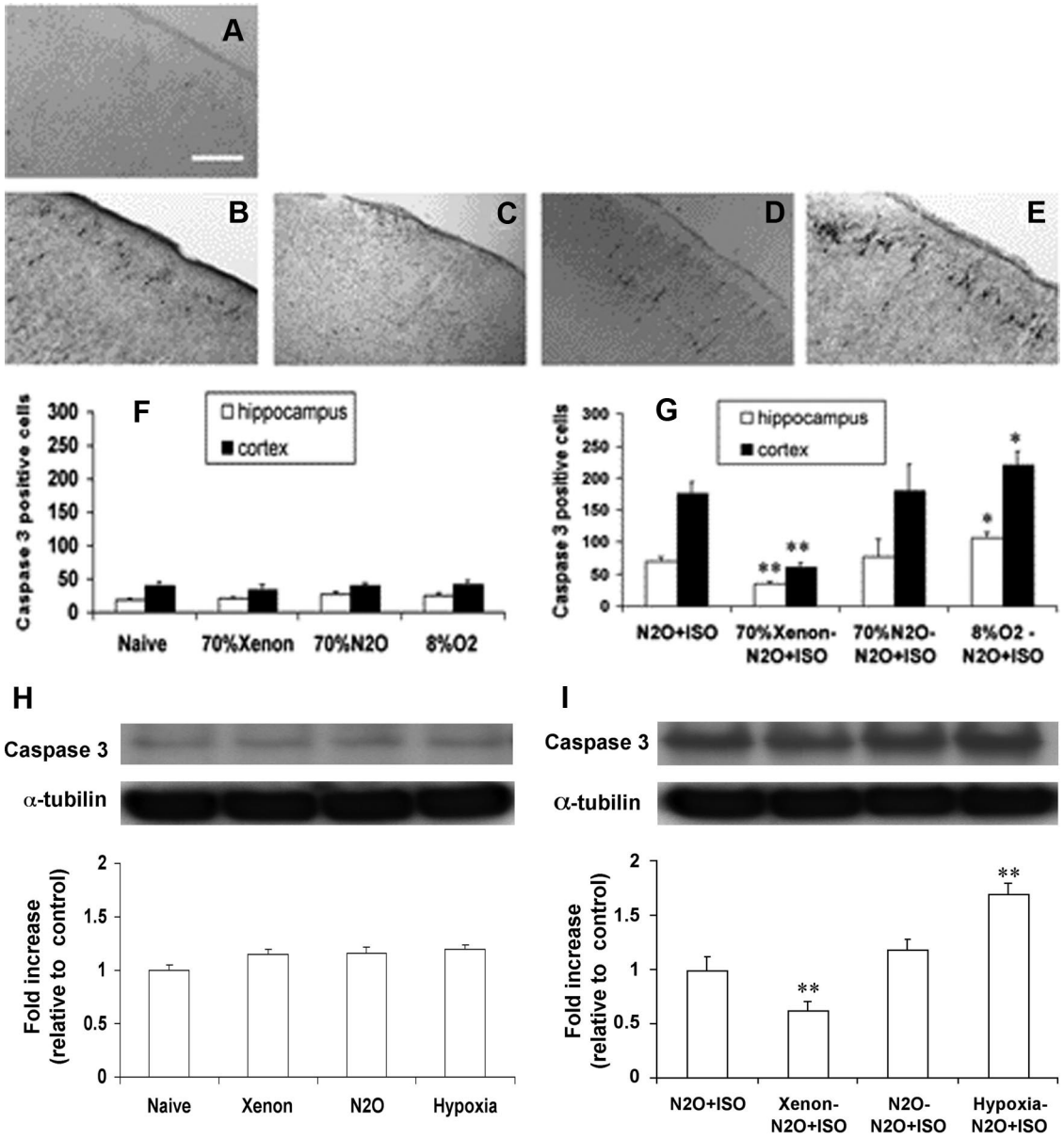

Fig. 1. Caspase-3-positive cells in the cortex and hippocampus *in vivo*. A photomicrograph from the cortex of a 7-day-old rat pup, which received no treatment (naive control) (*A*), 70% N_2O + 0.75% isoflurane (N_2O + ISO) (*B*), 70% xenon (*C*), 70% N_2O (*D*), and 8% oxygen (hypoxia) preconditioning (*E*) followed by N_2O + ISO. (*F*) Mean data of caspase-3-postive cells in both the hippocampus and cortex from pups, which received no treatment (naive control) or which were preconditioned with either 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) only. (*G*) Mean data of caspase-3-positive cells in both the hippocampus and cortex from 7-day-old rat pups, which received N_2O + ISO alone or which were preconditioned with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) followed by N_2O + ISO. (*H*) *Top*: example bands from 7-day-old rat pups that received no treatment (naive control) or pretreated with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) only; *bottom*: mean data of immunoreactivity from pups that received preconditioning with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia). (*I*) *Top*: Example bands from 7-day-old rat pups that received N_2O + ISO alone or preconditioning with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) followed by N_2O + ISO; *bottom*: mean data of immunoreactivity from pups that received N_2O + ISO or preconditioning with 70% xenon, 70% N_2O, or 8% oxygen in nitrogen (hypoxia) followed by N_2O + ISO. Mean ± SD, n = 5–6. * $P < 0.05$, ** $P < 0.01$ *versus* N_2O + ISO. Bar = 100 μm.

to the neurotoxic anesthetic combination (157 ± 25; $P < 0.05$) and the positive control group that received N_2O + isoflurane only (200 ± 26; $P < 0.05$). The area under curve in the xenon pretreatment followed by N_2O + isoflurane group was not significantly different from that of the naive group (261 ± 26) (figs. 4B and C).

Discussion

In this study, we observed significant increases in caspase-3 levels, both *in vitro* and *in vivo*, in the hippocampus and cortex of 7-day-old rats exposed to 0.7% isoflurane + 70% N_2O in oxygen for 6 h at 1 atmospheric ambient pressure compared with naive controls. The observed increase in

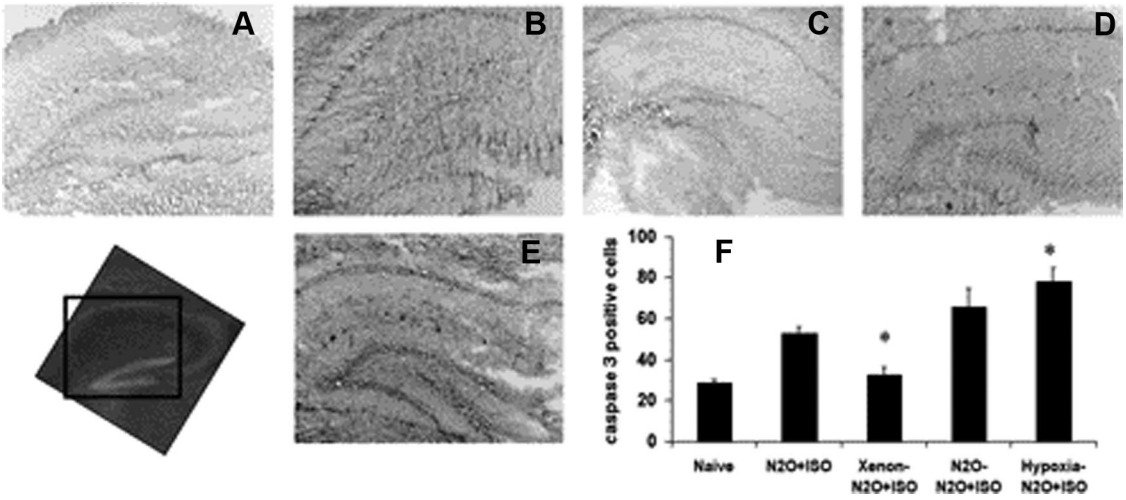

Fig. 2. Caspase-3-positive cells in the hippocampus *in vitro*. A photomicrograph of an organotypic hippocampal slice harvested from postnatal day 8 rat pups, cultured for 5 days before no treatment (naive control) (*A*), 70% N_2O + 0.75% isoflurane (N_2O + ISO) (*B*), 70% xenon (*C*), 70% N_2O (*D*), and 8% oxygen (hypoxia) preconditioning followed by N_2O + ISO (*E*). (*F*) Mean data of caspase 3-positive cells in the hippocampus from 7-day-old rat pups that received no treatment (naive control), N_2O + ISO alone or preconditioning with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) followed by N_2O + ISO. Mean ± SD, n = 5–6. * $P <$ 0.05 *versus* N_2O + ISO. The inserted image: a representative living culture was killed by exposure to glutamate (50 μM), and the image was enhanced with propidium iodide staining, acting as a guide to outline the hippocampal area being examined.

caspase-3 levels is due to the direct effects of the anesthetic drugs on the neural tissues and not because of altered physiology in neonatal rats, for example, hypoperfusion of the brain or hypoglycemia, because similar results were observed in the organotypic hippocampal slices. In both *in vitro* and *in vivo* studies, xenon was effective at suppressing N_2O + isoflurane-induced apoptosis and subsequent deterioration in cognitive function. Conversely, hypoxic pretreat-

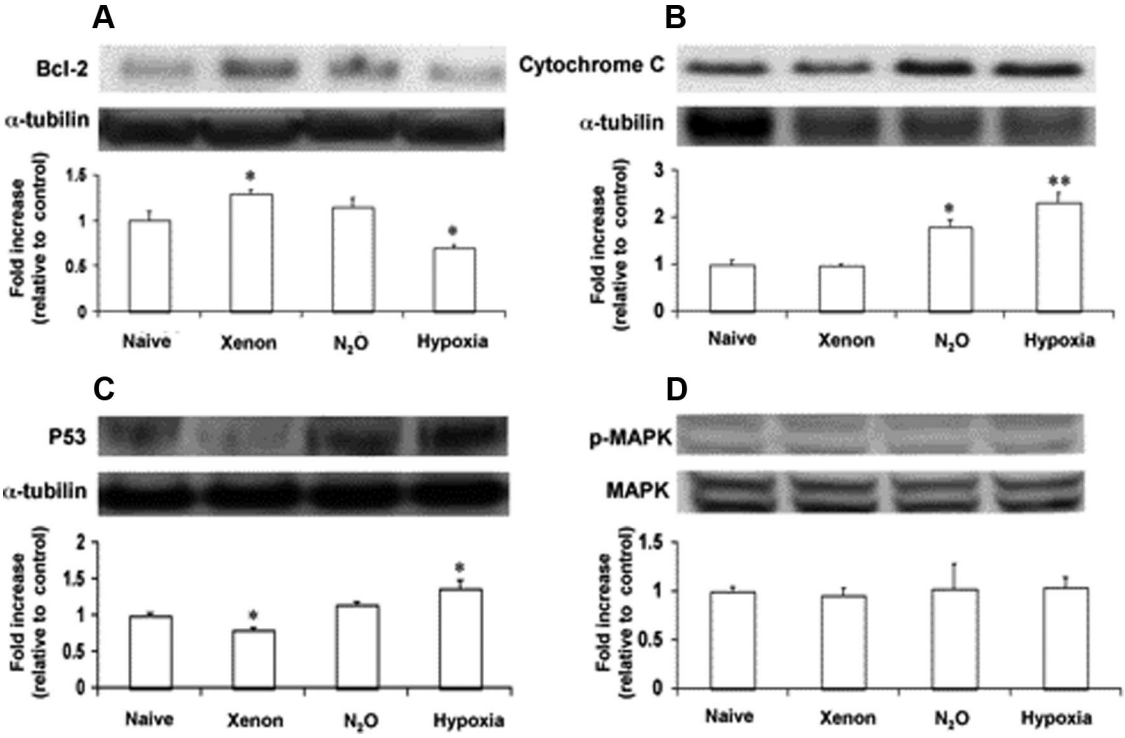

Fig. 3. Changes of protein immunoreactivity in the cerebral cortex of pups. Seven-day-old rat pups that received no pretreatment or were pretreated with 70% xenon, 70% N_2O, or 8% oxygen (hypoxia) followed by 70% N_2O + 0.75% isoflurane 24 h later, and their cortices were harvested for immunoblotting. (*A*) Bcl-2; (*B*) cytochrome *C*; (*C*) P53; (*D*) phosphorylated mitogen-activated protein kinases (pMAPK). In each section, *top*, example bands; *bottom*, mean data ± SD (n = 4–5). * $P <$ 0.05, ** $P <$ 0.01 *versus* control (being treated only with 70% N_2O + 0.75% isoflurane without pretreatment).

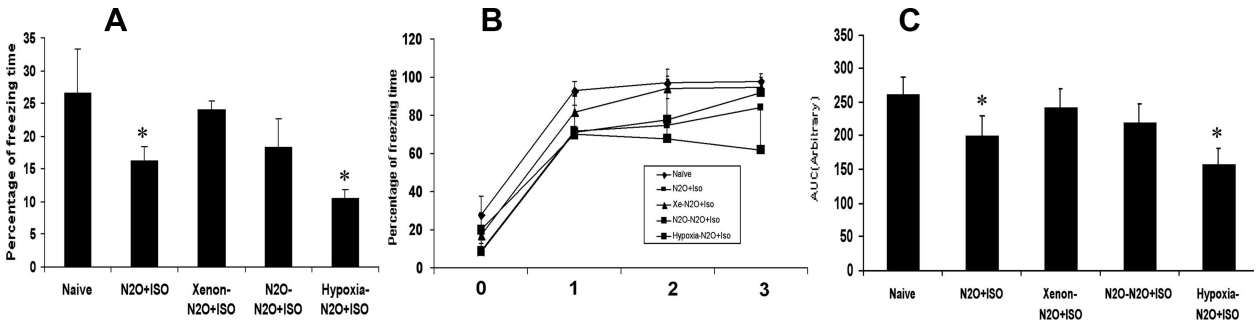

Fig. 4. Cognitive function assessed with trace fear conditioning. Seven-day-old rat pups that received no treatment (naive control), 70% N_2O + 0.75% isoflurane (N_2O + ISO), 70% xenon, 70% N_2O, and 8% oxygen (hypoxia) preconditioning followed by N_2O + ISO, and they were then allowed to live up to the age of 40 days for cognitive function test. (*A*) The mean data of percentage of freezing time after context testing. (*B*) The mean data of percentage of freezing time after tone testing. (*C*) The average data of the area under curve (AUC) derived from (*B*). Mean ± SD, n = 6. * $P < 0.05$ *versus* naive.

ment exacerbated apoptotic neurodegeneration and worsened cognitive impairment in the rats when they reached adulthood.

The mechanism responsible for the increase in caspase-3 levels in the developing brain during synaptogenesis after exposure to anesthetic drugs remains elusive. Drugs that block NMDA receptors or promote $GABA_A$ receptor function have been shown to produce neurodegeneration in neonatal animal models.[3,16], $GABA_A$ and NMDA receptors and their agonists play a fundamental role during synaptogenesis as they are essential for migration of neurons to the appropriate regions of the nervous system, dendritic filopodia stabilization, synaptic development, and stabilization.[2] Any interventions including pharmacologic agents, for example, anesthetics affecting $GABA_A$- and NMDA-mediated synaptic transmission, are not favorable for normal neuronal development. This hypothesis is supported by a study conducted by Wang and coworkers in cortical neuronal cultures derived from 3-day-old monkeys.[17] They showed that blockade of NMDA receptors by ketamine for 24 h was associated with an increase in neuronal apoptosis, and when the transcription and translation of NMDA receptors were inhibited by the addition of NMDA receptor NR1subunit antisense oligodeoxy nucleotide to the cultured neurons, this effect was abolished.[17] Furthermore, cellular ionic and neurotrophin homeostases are also crucial for normal neuronal survival or development. This has been clearly demonstrated by previous elegant studies, showing that excessive calcium release induced cell apoptosis is due to the activation of the endoplasmic reticulum membrane 1,4,5-trisphosphate receptor by isoflurane.[18] Also, isoflurane reduces tissue plasminogen activator release and subsequently enhances pro-brain-derived neurotrophic factor/p75NTR-mediated apoptosis.[19]

In this study, we investigated the therapeutic potential of pretreatment to prevent neuronal apoptosis produced by anesthetic drugs during synaptogenesis. The protective effect of pretreatment with xenon against N_2O + isoflurane-induced neuronal apoptosis in neonatal rats could be partly attributed to the drug-inhibiting mitochondria-induced activation of the caspase-3 pathway as xenon up-regulated the expression of antiapoptotic Bcl-2 and down-regulated proapoptotic tumor suppressor transcription factor P53. These were associated with no significant changes at the cytochrome *C* level. Xenon pretreatment also caused an increase in the phosphorylation of cyclic adenosine monophosphate response element binding protein transcription factor and brain-derived neurotrophic factor expression.[10] In contrast to xenon pretreatment, hypoxic pretreatment exacerbated N_2O + isoflurane-induced neuronal apoptosis by decreasing the expression of antiapoptotic factor Bcl-2 and increasing the expression of proapoptotic transcription factor p53, resulting in an increase in cytochrome *C* release from the mitochondria. However, it should be pointed out that similar to isoflurane, xenon may also have dual effects, that is, neuroprotection and neurotoxicity, depending on the duration of exposure.[20] Indeed, exposure to xenon for a short period of 2 h, as in our current study, is protective, whereas longer periods of exposure produced mild toxicity to the developing brain.[21] It was noted in our study that N_2O increased both Bcl-2 and cytochrome *C*. This may be one of the reasons why N_2O failed to induce a pretreatment state and also failed to protect anesthetic-induced neurotoxicity although similar to xenon, N_2O is also an NMDA receptor antagonist. Interestingly, isoflurane was not neurotoxic to rat fetal brain if it was delivered at the late gestational stage,[22] whereas isoflurane exposed to postnatal rats even at lower concentrations was still toxic.[14] Moreover, isoflurane has been shown to protect the brain, heart, lungs, and kidney from ischemic insult at pretreatment settings.[23–26] It may be postulated that the subject's age and experimental conditions are crucial factors for isoflurane to be protective or toxic. Nevertheless, our result is in keeping with a previous study on rats, which showed that pretreatment with nitrous oxide failed to reduce the extent of myocardial infarction after 25 min of coronary occlusion.[27]

The results from trace fear conditioning testing are in line with previous reports of cognitive impairment in rats after exposure to general anesthesia during the neonatal period.[6,28,29] Although the frontal cortex, hippocampus,

periaqueductal gray, and rostral ventral medulla mainly underlie the acquisition of fear memories, hippocampal damage is causally linked to long-term memory deficit.[30] In our study, rats displayed a normal acquisition response to the several tone-shock pairings using a trace fear conditioning paradigm. The main difference seen between the several treatments was during the context test, which is related to hippocampal function.[13] Our data showed that the rats pretreated with either nitrous oxide or hypoxia displayed less freezing compared with naive controls and xenon-pretreated animals (fig. 4). These changes were compatible with damage to hippocampus as is shown that neuroapoptosis was increased in the hippocampus of rats (fig. 1).

The relevance to the human species of the aforementioned experimental findings in neonatal rats and of those in many other studies conducted in neonatal animals is being questioned,[31] the main argument being the lack of strong evidence of its toxicity in neonatal humans. However, Wilder *et al.*[32] in a retrospective study of the medical and educational records of children in Olmsted County showed, using multivariate analysis, that children who had repeated general anesthetics before the age of 4 yr had a higher incidence of learning difficulties compared with those who received only one general anesthetic or no anesthetic. In another retrospective study, Kalkman *et al.*[33] showed that children under 24 months undergoing urologic procedures under general anesthesia tended to show more behavioral problems than those operated after 24 months of age under general anesthesia.

Our experimental model showed that a period of hypoxia followed soon afterward by general anesthesia with an inhalational agent and nitrous oxide during the neonatal period resulted in neuronal death and subsequent cognitive impairment. This unexpected finding may have important clinical implications. For example, neonates born with transposition of great vessels are hypoxic shortly after birth; they require urgent surgery to allow oxygenated blood from the pulmonary circulation to reach the systemic circulation. Babies born with gastroschisis or severe exomphalos also require urgent surgery to repair the abdominal defect. If they are unfortunate enough to experience fetal distress during childbirth, they may be at an increased risk of brain injury after surgical correction during anesthesia with the offending agents. If this finding can be extrapolated to clinical practice, then pediatric anesthesiologists dealing with similar situations need to choose carefully the anesthetic regimen to minimize or even prevent brain injury.

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