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Xenon Mitigates Isoflurane-induced Neuronal Apoptosis in the Developing Rodent Brain

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Background: **Anesthetics, including isoflurane and nitrous oxide, an antagonist of the *N*-methyl-D-aspartate subtype of the glutamate receptor, have been demonstrated to induce apoptotic neurodegeneration when administered during neurodevelopment. Xenon, also an *N*-methyl-D-aspartate antagonist, not only lacks the characteristic toxicity produced by other *N*-methyl-D-aspartate antagonists, but also attenuates the neurotoxicity produced by this class of agent. Therefore, the current study sought to investigate xenon's putative protective properties against anesthetic-induced neuronal apoptosis.**

Method: **Separate cohorts (n = 5 or 6 per group) of 7-day-old rats were randomly assigned and exposed to eight gas mixtures: air, 75% nitrous oxide, 75% xenon, 0.75% isoflurane, 0.75% isoflurane plus 35% or 75% nitrous oxide, 0.75% isoflurane plus 30% or 60% xenon for 6 h. Rats were killed, and cortical and hippocampal apoptosis was assessed using caspase-3 immunostaining. In separate cohorts, cortices were isolated for immunoblotting of caspase 3, caspase 8, caspase 9, and cytochrome *c*. Organotypic hippocampal slices of postnatal mice pups were derived and cultured for 24 h before similar gas exposures, as above, and subsequently processed for caspase-3 immunostaining.**

Results: ***In vivo*** **administration of isoflurane enhances neuronal apoptosis. When combined with isoflurane, nitrous oxide significantly increases whereas xenon significantly reduces apoptosis to a value no different from that of controls. *In vitro* studies corroborate the ability of xenon to attenuate isoflurane-induced apoptosis. Isoflurane enhanced expression of indicators of the intrinsic and common apoptotic pathways; this enhancement was increased by nitrous oxide but attenuated by xenon.**

Conclusions: **The current study demonstrates that xenon prevents isoflurane-induced neonatal neuronal apoptosis.**

SYNAPTOGENESIS is a highly regulated period of brain development that is exquisitely sensitive to environmental influences. The processes involved in neurodevelopment are conserved among species; in rodents, synaptogenesis occurs predominantly as a postnatal event, extending from approximately 2 days before birth to 2 weeks after birth, whereas in humans, it begins during the third trimester of pregnancy and lasts until a few years after birth.[1] Also known as a "brain growth spurt," synaptogenesis involves cellular proliferation, differentiation, and neuronal migration to target zones where formation of synapses result in the establishment of functional neuronal circuits. Neurotransmitters (including γ-aminobutyric acid and glutamate) and their receptors exert fundamental roles in neuronal migration,[2] dendritic filopodia stabilization, and synapse development and stabilization,[3] although their role in morphologic neurodevelopment is not definitively known.[4]

General anesthetics modulate specific ligand-gated ion channels, principally γ-aminobutyric acid type A receptor and the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor, thereby altering synaptic function.[5] The possibility that these synaptic actions could result in long-term consequences for the developing brain was pursued by the same group of investigators that had originally identified the deleterious effects of alcohol during neurodevelopment.[6] Therefore, postnatal exposure to anesthetics, which are similar to alcohol in their behavioral and molecular effects, were demonstrated to exhibit comparable morphologic and functional impairments.[7–9] Published evidence has shown that NMDA receptor antagonists in neonatal rats produced specific patterns of degeneration on neurons (in contrast to ameliorative actions on the glia)[10]; on electron microscopy, the degeneration that was noted was identical to apoptotic cell death.[11]

Xenon has been used in clinical anesthetic practice for more than 50 yr[12]; we reported that xenon was a noncompetitive NMDA receptor antagonist.[13] Because of the strong correlation between activation of NMDA receptors and neuronal injury, we surmised that xenon may act as a neuroprotectant, which we subsequently demonstrated in both *in vitro* and *in vivo* models of acute neuronal injury[14–19] and in a preconditioning setting where xenon attenuates brain damage induced by hypoxic–ischemic injury in neonates.[20] Exposure to xenon induces phosphorylation of cyclic AMP response element–binding protein (pCREB), which recruits CREB-binding protein to induce transcription of several prosurvival genes, including brain-derived neurotrophic factor and the prosurvival protein Bcl-2, whose expression is increased after xenon exposure.[20–22] In contrast, unlike xenon, nitrous oxide, another anesthetic gas that antagonizes the NMDA receptor subtype, inhibits protein kinase C activity[23] and does not increase either the

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transcription factor pCREB or the expression of prosurvival proteins.[20–22] These divergent molecular actions of xenon and nitrous oxide encouraged us to investigate possible differences that these anesthetic gases would have on isoflurane-induced neurodegeneration in the developing brain. We hypothesized that nitrous oxide enhances whereas xenon mitigates isoflurane-induced neuronal apoptosis; also, we sought to explore the possible cellular signaling pathways involved in their putative injurious and protective actions during brain development.

Materials and Methods

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

In Vivo *Experiments*

Postnatal day 7 Sprague-Dawley rat pups were used because previous work has established that NMDA receptor antagonists have their maximal neurodegenerative effect 7 days postpartum.[10] Separate cohorts of rat pups (n = 5 or 6) were exposed to one of the following gas combinations for 6 h: air, 75% xenon, 75% nitrous oxide, 0.75% isoflurane, 30% xenon plus 0.75% isoflurane, 60% xenon plus 0.75% isoflurane, 35% nitrous oxide plus 0.75% isoflurane, and 75% nitrous oxide plus 0.75% isoflurane. The remaining constituents were 25% oxygen in all groups and balanced with nitrogen where necessary. In a pilot study, we had confirmed that 60% xenon plus 0.75% isoflurane is equiantinociceptive to 75% nitrous oxide plus 0.75% isoflurane assessed by equivalent inhibition of formalin-induced c-Fos expression in the spinal cord (42 ± 4 *vs.* 48 ± 5, n = 4; $P > 0.05$). The high cost of xenon precludes its use in an open circuit; consequently, xenon and xenon plus isoflurane groups received gases using a customized closed-circuit system, in which carbon dioxide is eliminated with soda lime and water vapor with silica gel as previously described.[20] Xenon concentration was measured continuously by an in-line gas analyzer. Gas concentrations for oxygen, isoflurane, and nitrous oxide were monitored with an S/5 spirometry module (Datex-Ohmeda, Bradford, United Kingdom); gases were delivered by calibrated flowmeters.

To precisely control the body temperature of the pups, the individual exposure chambers were partially submerged in a water bath, and the water temperature was adjusted to obtain a desired brain temperature (37°C) as measured with a telemetry temperature monitoring system (VitalView; Mini-Mitter, Sunriver, OR). One hour before the exposure experiment, a temperature probe was implanted (−2 mm from bregma and 2 mm away from sagittal sinus with the tip of probe advanced to subcortex) in a single sentinel rat/experimental cohort and fixed to the skull with glue during a brief isoflurane anesthetic. (Sentinel rats were not further analyzed.)

Rats were killed with a 100-mg/kg intraperitoneal sodium pentobarbital injection at the end of gas exposure and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and kept at 4°C overnight within paraformaldehyde. The fixed brains were then transferred to a solution of 30% sucrose with phosphate buffer and 1% sodium azide and were kept refrigerated until the brains were stained for immunohistochemistry. Other cohorts were killed by an overdose of sodium pentobarbital after gas exposure, and the cortex was harvested for immunoblotting.

In Vitro *Experiments*

Organotypic hippocampal slices (OHCs) were derived from postnatal day 8 or 9 C57Bl/6 mice pups (Harlon) and cultured by the interface method[24,25] with some modifications. In brief, the brain was quickly dissected and placed in ice-cooled (4°C) dissection solution. All stages of slice preparation were performed under sterile and ice-cooled conditions. Excess tissue (including the cerebellum, olfactory bulbs, and meninges) was removed, and the brain was cut into 400-μm sagittal slices using a McIllwain Tissue Chopper (Mickle Laboratory, Cambridge, United Kingdom). Under a dissecting microscope and avoiding contact with the hippocampus, the slices were separated using fine forceps. Slices containing the intact hippocampus were selected and positioned onto 30-mm-diameter semiporous cell culture inserts (five slices per insert) (Falcon; Becton Dickinson Labware, Millipore, Bedford, MA) and placed in a six-well tissue culture tray (Multiwell; Falcon, Becton Dickinson Labware, Bedford, MA). Eagle minimum essential medium enhanced with heat-inactivated horse serum (1.5 ml) was then transferred to each well.

The slices were incubated for 24 h in humidified air at 37°C, enriched with 5% carbon dioxide, to allow for recovery from the preparative trauma. The culture medium was replaced the following day with fresh, temperature-equilibrated medium before exposure to gas treatments. The groups of slices (n = 15) were assigned to six of the gas exposures previously mentioned (omitting the lower xenon and nitrous oxide concentrations in combination with isoflurane).

All subsequent gas exposure occurred in a specially constructed exposure chamber as previously described.[15] The gases, warmed by a water bath, were delivered in the headspace above the slices by a standard anesthetic machine at 2–3 l/min, and concentrations were monitored with an S/5 spirometry module (Datex-Ohmeda) and a 439XE monitor (Air Products, London, United Kingdom). After 3–4 min of gas flow, the cham-

bers were sealed and placed in a 37°C incubator for 6 h (Galaxy R Carbon Dioxide Chamber; Wolf Laboratories, Pocklington, York, United Kingdom). After exposure, the slices were returned to the incubator for a further 12 h of culture to allow for suitable caspase-3 expression and then fixed overnight in 4% paraformaldehyde and subsequently immersed in 30% sucrose for a further 24 h at 4°C before slicing with a cryostat.

Caspase-3 Immunohistochemistry

Caspase 3 triggers the execution phase of apoptosis. The relation between activation of caspase 3 (by immunoreactivity) and apoptotic cell death (by both morphologic criteria on Nissl-stained sections as well as ultrastructural features on electron microscopy) has been established in a neonatal model of acute neuronal injury[26]; also, we have recently reported on the relation between activation of caspase 3 (within 6 h by both immunohistochemistry and immunoblotting), apoptotic cell death (at 24 and 48 h by Nissl staining), and neurologic functional deficit (at 30 days) in a neonatal model of hypoxic–ischemic injury.[27] Therefore, we consider activated caspase 3 to be a relevant surrogate marker of impending apoptotic cell death.

For *in vivo* experiments, the brain was sliced at 30-μm intervals beginning at −3.6 mm from the bregma. The cut sections were transferred to a six-well plate containing phosphate-buffered saline (PBS) for cleaved caspase 3 using the floating method of immunostaining. For *in vitro* experiments, the slices were sectioned at 25-μm intervals using a cryostat, and the inner sections were mounted onto Super Plus–coated glass slides (VWR International, Lutterworth, United Kingdom). The sections were allowed to dry at 37°C for 24 h and then immunostained while adherent to the slides. Briefly, after preincubation with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, the sections were rinsed in PBS. After incubation overnight at 4°C with rabbit anticleaved caspase 3 (1:2,500; New England Biolab, Hitchin, United Kingdom), the sections were washed three times in PBS with Triton 3% at room temperature. The biotinylated secondary antibodies (1:200; Sigma, St. Louis, MO) and the avidin–biotin–peroxidase complex (Vector Lab, Orton Southgate, Peterborough, United Kingdom) were applied. After the sections were washed with PBS, the peroxidase reaction was developed by incubating the section in 0.02% 3,3′diaminobenzidine tetrahydrochloride (Sigma) solution containing 0.003% hydrogen peroxide. Finally, the sections were dehydrated through a gradient of ethanol solutions (70–100%) and then mounted (floating section) and covered with a cover slip. Control sections were processed identically and in parallel; however, they were incubated with PBS instead of the primary antibodies. No labeling was detected in these controls.

Immunoblotting

After gas exposure, Sprague-Dawley rat pups (n = 5 or 6 per group) were killed; the brains were immediately removed; and the cortex (an area particularly prone to neuronal apoptosis) was separated, harvested, and frozen at −80°C. The samples were then homogenized (Polytron homogenizer by Kinematica, Bethlehem, PA) in ice-cooled lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2DTA, 1 mM EGTA, 1% Triton, 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; pH 7.5) and centrifuged at 3,000*g* for 10 min at 4°C to remove cellular debris. The supernatant was further centrifuged twice, initially at 12,000*g* for 15 min at 4°C and a second time at 20,000*g* for 45 min at 4°C, which separated cytosolic and mitochondrial cytochrome *c* protein fractions. The supernatant, stored at −80°C, and subsequently used to blot caspase 3, caspase 8, caspase 9, and cytosolic cytochrome *c*. The protein concentration of lysates was determined with the Bradford protein assay (Bio-Rad, Herts, United Kingdom). Protein extracts (10 μg per sample) were denaturated in NuPAGE LDS Sample buffer (Invitrogen, Paisley, United Kingdom) at 70°C for 10 min and then were loaded on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Buckinghamshire, United Kingdom) and incubated with a blocking solution composed of 5% fat dry milk in Tween-containing Tris-buffered saline (pH 8.0, 10 mM Tris, 150 mM NaCl, 0.1% Tween). The "blocked" membrane was incubated overnight at 4°C with the following antibodies: rabbit anticleaved caspase 3, rabbit anticleaved caspase 9 (New England Biolab), chicken anticleaved caspase 8 (Abcam plc, Cambridge, United Kingdom), and mouse anti–cytochrome *c* (BD Biosciences Pharmingen, Oxford, United Kingdom). After washing the membrane for 20 min with four changes of Tween-containing Tris-buffered saline, it was incubated for 1 h at room temperature with the appropriate horseradish peroxidase–conjugated secondary antibody directed at the primary antibody. The bands were then visualized with enhanced chemiluminescence (New England Biolab) and exposed onto Hyperfilm ECL film (Amersham Biosciences). The band density was analyzed densitometrically and then presented as protein expression relative to the control and normalized with the housekeeping protein α-tubulin used in the linear portion of the densitometry curve.

Data Analysis

Photomicrographs were taken for the whole hemisphere and hippocampus for *in vivo* and the hippocampus CA area for *in vitro* experiments with a BX-60 light

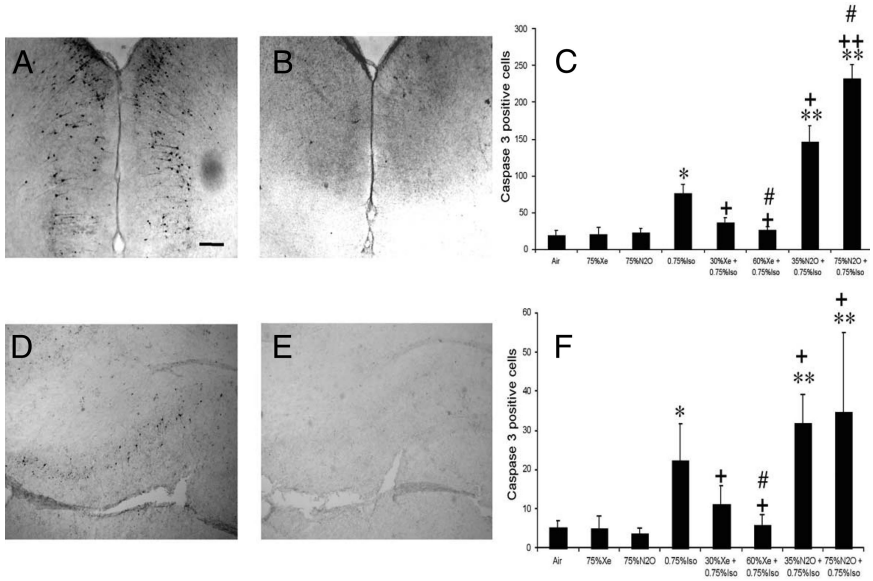

Fig. 1. Apoptotic neurodegeneration induced by anesthetics (75% nitrous oxide [N_2O], 75% xenon [Xe], 0.75% isoflurane [Iso], 0.75% Iso plus 75% or 35% N_2O, 0.75% Iso plus 60% or 30% Xe) as measured with cleaved caspase-3 immunostaining in the cortex and the hippocampus of day 7 neonatal rats. (*A*) Photomicrograph of the cortex of a neonatal rat exposed to 0.75% Iso plus 75% N_2O. (*B*) Photomicrograph of the cortex of a neonatal rat exposed to 0.75% Iso plus 60% Xe. (*C*) Data (mean ± SD, n = 5 or 6) of caspase 3–positive cells in the cortex from all treatment groups. (*D*) Photomicrograph of the hippocampus of a neonatal rat exposed to 0.75% Iso plus 75% N_2O. (*E*) Photomicrograph of the hippocampus of a neonatal rat exposed to 0.75% Iso plus 60% Xe. (*F*) Data (mean ± SD, n = 5 or 6) of caspase 3–positive cells in the hippocampus from all treatment groups. * $P < 0.01$, ** $P < 0.001$ *versus* air. + $P < 0.05$, ++ $P < 0.01$ *versus* 0.75% Iso. # $P < 0.05$ *versus* 30% Xe plus 0.75% Iso or 35% N_2O plus 0.75% Iso. *Scale bar* = 200 μm.

microscope (Olympus, Southall, United Kingdom) and an Axiocam digital camera (Zeiss, Göttingen, Germany). The images were then printed on paper, and an investigator blinded to the experimental cohort counted the number of caspase 3 positive cells in the desired areas. Counting was performed on slices from the cortex and the hippocampus for *in vivo* experiments and from the hippocampal CA1–CA3 subregions for *in vitro* experiments. For Western blotting, the band intensities were quantified by densitometry and then normalized with α-tubulin and expressed as fractions of control (non-exposed) sample in the same gel. All results are expressed as mean ± SD. Statistical analysis was performed by analysis of variance followed by *post hoc* Newman-Keuls test for comparison where appropriate. A *P* value of less than 0.05 was considered statistically significant.

Results

In Vivo *Experiments*

Neuronal cells exhibiting caspase-3 activation were readily distinguishable as a black cell body with dendritic staining (fig. 1A). The background level of cortical activated capase-3 staining was 19 ± 6. When rat pups were exposed to either 75% nitrous oxide or 75% xenon, there was no significant increase in caspase 3–positive cells compared with controls (22.5 ± 5.9 and 20 ± 10, respectively); however, administration of 0.75% isoflurane alone produced a moderate increase of activated caspase-3 staining (77 ± 11; $P < 0.05$ *vs.* air). When combined with 0.75% isoflurane, both low (35%) and high (75%) concentrations of nitrous oxide significantly enhanced isoflurane-induced apoptosis to 146 ± 22 and 232 ± 20 ($P < 0.01$ *vs.* air), respectively. Contrastingly, exposure to a combination of either the low (30%) or the high (60%) concentration of xenon with 0.75% isoflurane resulted in neuronal apoptosis (37 ± 6 and 27 ± 4, respectively) that was no different from that seen with exposure to air ($P > 0.05$). The reduction of apoptosis induced by 60% xenon plus 0.75% isoflurane is statistically greater than that induced by 30% xenon plus 0.75% isoflurane ($P < 0.05$). There was a statistically significant difference in apoptosis between 0.75% isoflurane alone *versus* 0.75% isoflurane in combination with either concentration of xenon ($P < 0.05$), or with 35% ($P < 0.05$) or 75% nitrous oxide ($P < 0.01$) (figs. 1A–C).

Neither 75% nitrous oxide nor 75% xenon increased caspase 3–positive cells in the hippocampus above that seen with air exposure (4 ± 1 and 5 ± 3, respectively, *vs.* 5 ± 2 with air; $P > 0.05$). Isoflurane at 0.75% alone significantly increased the number of apoptotic neurons in the hippocampus (22 ± 10 *vs.* 5 ± 2 in air controls; $P < 0.05$), as did the combination of 0.75% isoflurane with both a low (35%; 32 ± 7) and a high (75%; 35 ± 20) concentration of nitrous oxide ($P < 0.01$ *vs.* air) (fig. 1D). The combination of 0.75% isoflurane with either a low (30%) or a high (60%) concentration of xenon (fig. 1E) resulted in neuronal apoptosis (11 ± 5 and 6 ± 3, respectively) that was no different from that seen with exposure to air ($P > 0.05$) (fig. 1F). The reduction of apoptosis caused by 60% xenon plus 0.75% isoflurane is statistically greater than that induced by 30% xenon plus 0.75% isoflurane ($P < 0.05$). There was a statistically significant difference in apoptosis between 0.75% isoflurane alone *versus* 0.75% isoflurane in combination with both concentrations of either xenon or nitrous oxide ($P < 0.05$ or 0.01) (fig. 1F).

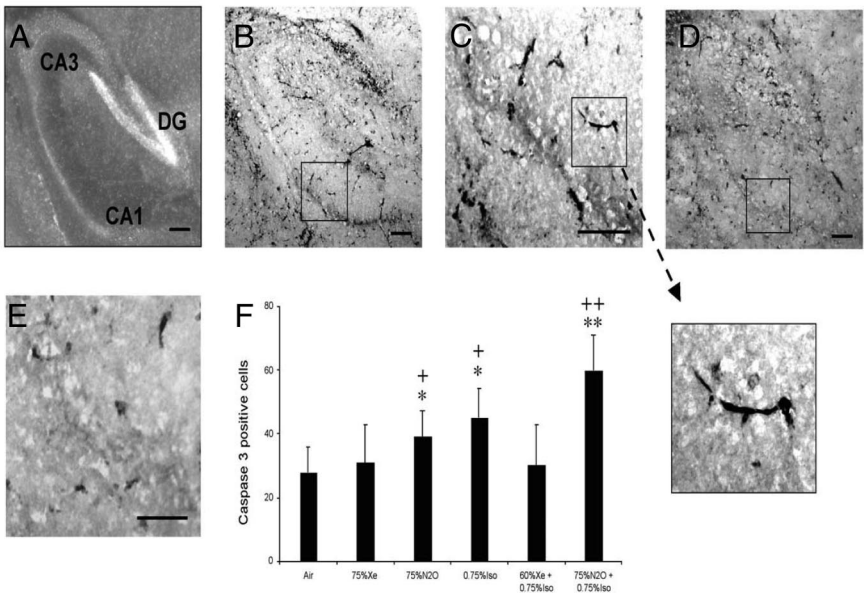

Fig. 2. Cleaved caspase-3 expression in the hippocampus of organotypic hippocampal slice culture derived from C57BL6 neonatal mice pups after anesthetic gas treatment. (*A*) Representative living culture was killed by exposure to glutamate (50 μM), and the image was enhanced with propidium iodide staining, acting as a guide to outline the CA area of the hippocampus in *B* and *D*. DG = dentate gyrus. (*B*) Representative photomicrograph of hippocampal culture treated with 0.75% isoflurane (Iso) plus 75% nitrous oxide (N_2O) followed by caspase-3 immunostaining. (*C*) High-magnification image derived from the highlighted area of *B*, indicating that cleaved caspase 3–positive cells are identifiable by black cell body and dendritic staining. (*D*) Representative photomicrograph of hippocampal culture treated with 0.75% Iso plus 60% xenon (Xe) followed by caspase-3 immunostaining. (*E*) High-magnification image derived from the highlighted area of *D*. (*F*) Data (mean ± SD, n = 15) of cleaved caspase 3–positive cells counted from whole CA area of hippocampus after 0.75% Iso, 75% N_2O, 60% Xe, or 0.75% Iso combined with either 75% N_2O or 60% Xe. * $P < 0.05$, ** $P < 0.01$ *versus* control. + $P < 0.05$, ++$P < 0.01$ *versus* Iso plus Xe. *Scale bar* in *A*, *B*, and *D* = 200 μm; *scale bar* in *C* and *E* = 100 μm.

In Vitro *Experiments in Cultured Organotypic Hippocampal Slices*

The structure of hippocampal slice culture was readily identifiable when a living culture was killed by exposure to glutamate (50 μM) and the image was enhanced with propidium iodide fluorescent staining (fig. 2A). Neuronal cells expressing cleaved caspase 3 in hippocampal slices were readily distinguishable (figs. 2B–E). Because of the high level of cleaved caspase 3–positive cells in the dentate gyrus (probably because of the artifact introduced by slicing the hippocampus), this region of the hippocampus was not further considered. The background level of apoptosis in the whole CA area in air-exposed slices was 28 ± 8. Exposure to each of 0.75% isoflurane, 75% nitrous oxide, and the combination of 0.75% isoflurane and 75% nitrous oxide resulted in a statistically significant increase in neuronal apoptosis to values of 45 ± 9 ($P < 0.05$), 39 ± 8 ($P < 0.05$), and 65 ± 11 ($P < 0.01$), respectively (figs. 2B, C, and F). The administration of 75% xenon did not statistically increase caspase 3–positive cells (31 ± 9) ($P > 0.05$); combining 60% xenon with 0.75% isoflurane resulted in a level of apoptosis (30 ± 12) that was indistinguishable from air-exposed slices ($P > 0.05$) (figs. 2D–F).

Immunoblotting

For caspase 8, there was no change of protein expression between the treatment groups, suggesting that the extrinsic apoptotic pathway is not activated after 6 h of exposure to any of the regimens of anesthetic gases (fig. 3A). Xenon at 75% did not increase the activation of caspase 9, cytochrome *c*, or caspase 3 when compared with control; however, 75% nitrous oxide increased signaling within the intrinsic apoptotic pathway, assessed by cytochrome *c* activity, 2.2-fold relative to air ($P < 0.01$). Isoflurane at 0.75% activates the common apoptotic pathway, assessed with caspase-3 activity, 1.8-fold relative to air ($P < 0.05$) (figs. 3B and C). Nitrous oxide at 75% in combination with 0.75% isoflurane significantly enhanced expression of caspase 9 (1.9-fold), cytochrome *c* (1.7-fold), and caspase 3 (2.3-fold) relative to air ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). Combining 60% xenon with 0.75% isoflurane resulted in no difference in expression of any component of the intrinsic or common apoptotic pathways when compared with air exposure (figs. 3B–D).

Discussion

This study demonstrates that exposure of neonatal rats to xenon, unlike nitrous oxide, does not enhance isoflurane-induced neuronal apoptosis as reflected by caspase-3 immunostaining; remarkably, xenon *protected* against isoflurane-induced apoptosis in a concentration-dependent manner. The qualitative difference between the *in vivo* effects of nitrous oxide and xenon on isoflurane-induced apoptosis was confirmed in an *in vitro* hippocampal slice culture model; we interpret this to mean that the deleterious effects of nitrous oxide and the ameliorative effects of xenon on isoflurane-induced apoptosis are not due to possible different physiologic perturbations induced by these anesthetic combinations in the *in vivo* paradigm. Immunoblotting data from the *in vivo* experiments suggest that the mechanism for isoflurane-induced apoptosis seems to involve activation

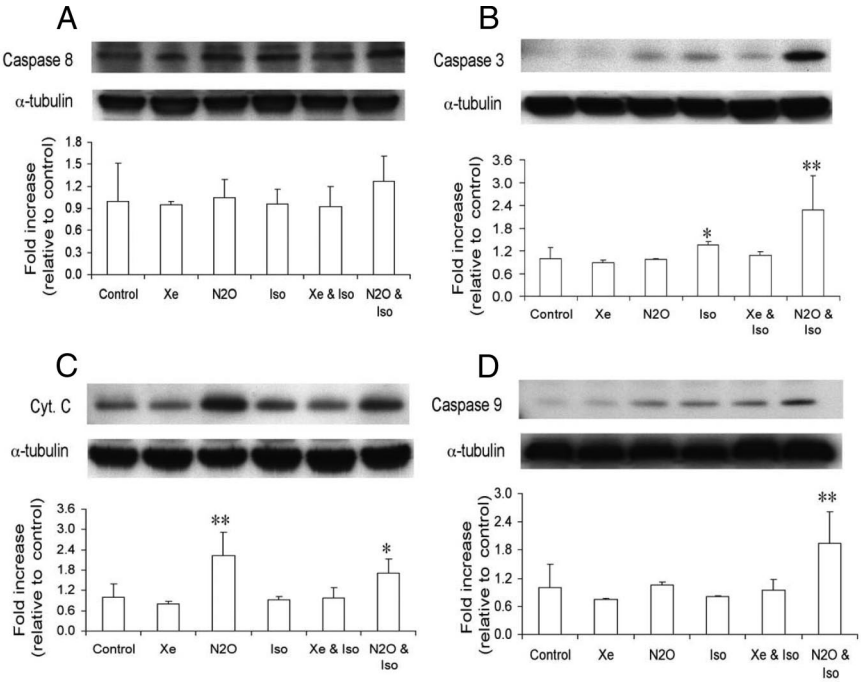

Fig. 3. Effects of anesthetic exposure (75% nitrous oxide [N_2O], 75% xenon [Xe], 0.75% isoflurane [Iso], 0.75% Iso plus 75% N_2O, or 0.75% Iso plus 60% Xe) for 6 h in day 7 rats on expression of signaling components of apoptotic pathways. Representative immunoblotting images and mean ± SD (n = 4) of expression of caspase 8 (*A*), caspase 3 (*B*), cytosolic cytochrome *c* (*C*), and caspase 9 (*D*) induced by each anesthetic exposure as reflected by the band density normalized with α-tubulin. Control = air exposure; Cyt. C = cytochrome *c*. * $P < 0.05$, ** $P < 0.01$ *versus* control.

of the intrinsic apoptotic pathways, an effect that is enhanced by nitrous oxide but attenuated by xenon.

The hallmark of human brain development in fetal and early postnatal life is the extremely rapid turnover of synapses (as high as 20% per day)[28] with a correspondingly high level of physiologic apoptosis, as neurons that do not reach their synaptic targets are eliminated.[29] Modulation of the NMDA and/or γ-aminobutyric acid receptors induces a robust apoptotic response during synaptogenesis,[6,10] and this is of particular interest because it is these receptors that are perturbed by drugs used to effect sedation, anticonvulsion, and anesthesia.[10,30,31] Exposure of neonatal rats to a "standard" anesthetic regimen of isoflurane, nitrous oxide, and midazolam produced a greater than 50-fold increase in the number of degenerating neurons in the laterodorsal and anteroventral thalamic nuclei (and to some extent layer II of the parietal cortex)[10]; these morphologic changes were associated with a functional neurologic deficit in behavior tests later in life.

In light of concern that these findings generated, the current study aimed to establish whether xenon, an anesthetic with neuroprotective properties, triggers similar neuronal apoptosis in the neonatal rat. Although confirming that exposure to certain anesthetic agents during the critical stage of synaptogenesis causes apoptosis, our data indicate that this is not present for all anesthetic combinations, because xenon not only does not enhance but even protects against isoflurane-induced apoptosis.

Despite the earlier *in vivo* demonstration that anesthetics can provoke neurodegeneration, the concept that the anesthetic itself is directly cytotoxic has been questioned.[32] After *in vivo* exposure to anesthetics, pathophysiologic changes associated with the anesthetic state may have been induced in these relatively unmonitored neonatal rodent models that may have, indirectly, produced neurodegeneration. Metabolic (*e.g.*, hyperglycemia), respiratory (*e.g.*, hypoxemia and hypercapnia), and cardiovascular changes induced by general anesthetics are difficult to monitor, and subsequently control, in neonatal rodent models. By using an *in vitro* model of cultured OHCs, we sought to mitigate these concerns associated with the *in vivo* models; this brain slice model served as a surrogate system in which to investigate the direct effects of anesthetics on neuronal apoptosis. Because the OHCs continue to exhibit physiologic synaptic connections,[33] which together enable the neurons to continue to mature and differentiate,[34] these are functionally similar to the hippocampus within the intact brain and also exhibit sensitivity to anoxia, hypoglycemia, and glutamate-induced injury.[35] Within the OHC model, isoflurane[36] and nitrous oxide, either individually or in combination, are directly toxic during early brain development (fig. 2). Remarkably, xenon did not increase neuronal apoptosis in this setting; rather, the addition of xenon to isoflurane significantly decreased isoflurane-induced apoptosis to a level that is indistinguishable from apoptosis seen with air exposure. When clinically relevant and equivalent anesthetic regimens are compared, *i.e.*, isoflurane and nitrous oxide *versus* isoflurane and xenon, there is a statistically significant difference in the degree of neuronal apoptosis ($P < 0.01$). From these findings, we deduce that the apoptotic effect that is observed when anesthetics are administered during synaptogenesis *in vivo* is due to the anes-

thetic itself and not due to possible differences in the pathophysiologic changes induced by the different anesthetic regimens.

It is worth commenting on the differences that were noted between the *in vivo* and *in vitro* experiments. In our *in vitro* model, nitrous oxide alone induced apoptosis. This may be accounted for by differential sensitivities to anesthetics between rodent species (because the *in vitro* model was derived from neonatal mice) or the inability of the *in vitro* model to reproduce, in totality, the conditions seen *in vivo*. Although the ability to tightly control physiologic variables is a key advantage of the OHC model, limitations of the OHC model include the fact that it cannot take into consideration humoral factors that may influence (both positively and negatively) the putative injurious effects of anesthetics.

In a further attempt to dissect the molecular mechanisms involved, immunoblotting was used to establish the pattern of apoptosis among the different treatment groups, whereby xenon attenuated and nitrous oxide increased isoflurane-induced apoptosis. After 6 h of anesthetic exposure, the cytotoxic property of anesthetics seems to occur *via* the intrinsic apoptotic pathway as evidenced by the release of cytochrome *c* into the cytosol and activation of caspase 9. Our data corroborate the findings by Yon *et al.*,[37] who previously demonstrated that early neurodegeneration induced by the triple anesthetic combination of isoflurane, midazolam, and nitrous oxide is associated with a corresponding increase in cytochrome *c* and caspase 9. Yon *et al.*[37] also suggested that the extrinsic pathway may play a later role in anesthetic-induced neurodegeneration.

It is not known at which juncture anesthetics influence the apoptotic pathway. Modulation of either NMDA or γ-aminobutyric acid receptors are capable of inducing apoptosis; possibly, these modulatory effects converge on the apoptotic mechanism upstream of the mitochondria by inducing changes in neurotrophin activation that, in turn, dysregulate the intracellular proapoptotic and antiapoptotic mechanisms, such as the Bax/Bcl-2 ratio.[22] The qualitative differences produced by xenon *versus* nitrous oxide may provide insight into the possible mechanism; xenon can up-regulate prosurvival proteins and brain-derived neurotrophic factor, whereas exposure to nitrous oxide does not and may explain the different effects that these anesthetic gases have on isoflurane-induced apoptosis.[20]

It must be appreciated that the *in vivo* rodent model is an experimental paradigm and is not directly analogous to clinical pediatric anesthetic practice. Because of the different time course of neurodevelopment in rodents and humans, the 6-h duration of exposure in this study can be likened to an excessively prolonged anesthetic exposure in humans.[38] Further work is required in different species, especially in primates, to explore whether these observations in rodents are artifactual, due to excessively long exposure times, in neurodevelopmental terms. In addition, concerns have been expressed that, although the model accurately reflects anesthetic conditions, it does not represent clinical practice due to the lack of a surgical stimulus; therefore, surgical levels of anesthesia in the absence of surgical stimuli may induce a more extreme change in neuronal excitation than is usually present clinically. It is worth remembering that neonatal anesthesia is sometimes given in the absence of surgical stimulus, *e.g.*, for neuroimaging procedures.

Our data suggest that xenon may represent an anesthetic that could mitigate anesthetic-induced apoptosis; therefore, use of xenon in pediatric anesthesia may increase the safety of current general anesthetic protocols. Currently, xenon is only registered for anesthesia in adults, and clinical studies in younger age groups will be necessary to determine whether the other salubrious properties seen in adults receiving xenon (*e.g.*, cardiorespiratory stability and rapid induction and emergence)[39–41] pertain in neonates.

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