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Dexmedetomidine Attenuates Isoflurane-induced Neurocognitive Impairment in Neonatal Rats

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Background: **Neuroapoptosis is induced by the administration of anesthetic agents to the young. As α_2 adrenoceptor signaling plays a trophic role during development and is neuroprotective in several settings of neuronal injury, the authors investigated whether dexmedetomidine could provide functional protection against isoflurane-induced injury.**

Methods: **Isoflurane-induced injury was provoked in organotypic hippocampal slice cultures *in vitro* or *in vivo* in postnatal day 7 rats by a 6-h exposure to 0.75% isoflurane with or without dexmedetomidine. *In vivo*, the α_2 adrenoceptor antagonist atipamezole was used to identify if dexmedetomidine neuroprotection involved α_2 adrenoceptor activation. The γ-amino-butyric-acid type A antagonist, gabazine, was also added to the organotypic hippocampal slice cultures in the presence of isoflurane. Apoptosis was assessed using cleaved caspase-3 immunohistochemistry. Cognitive function was assessed *in vivo* on postnatal day 40 using fear conditioning.**

Results: ***In vivo*** **dexmedetomidine dose-dependently prevented isoflurane-induced injury in the hippocampus, thalamus, and cortex; this neuroprotection was attenuated by treatment with atipamezole. Although anesthetic treatment did not affect the acquisition of short-term memory, isoflurane did induce long-term memory impairment. This neurocognitive deficit was prevented by administration of dexmedetomidine, which also inhibited isoflurane-induced caspase-3 expression in organotypic hippocampal slice cultures *in vitro*; however, gabazine did not modify this neuroapoptosis.**

Conclusion: **Dexmedetomidine attenuates isoflurane-induced injury in the developing brain, providing neurocognitive protection. Isoflurane-induced injury *in vitro* appears to be independent of activation of the γ-amino-butyric-acid type A receptor. If isoflurane-induced neuroapoptosis proves to be a clinical problem, administration of dexmedetomidine may be an important adjunct to prevent isoflurane-induced neurotoxicity.**

ANESTHESIA has recently been associated with widespread apoptotic neurodegeneration in the neonatal rat brain with persistent functional neurocognitive impairment, exemplified by impaired memory formation.[1–4] This discovery has led to concern about the possible detrimental effects of anesthesia and sedation in the pediatric population. The observed apoptotic neurodegeneration mimics the neuronal injury of fetal alcohol syndrome[5] and is thought to be secondary to impaired neurotransmission during a critical period of synaptogenesis that triggers so-called neuronal suicide. Indeed, there is significant evidence that preventing synaptic neurotransmission causes deleterious long-term central nervous system changes,[6] with synaptic neurotransmission critical to avoid synaptic pruning and apoptosis of activity-deprived neurons.[7,8]

Generically, anesthetic agents are thought to inhibit synaptic neurotransmission by potentiating γ-amino-butyric-acid type A ($GABA_A$) receptors, inhibiting glutamate *N*-methyl-D-aspartate (NMDA) channels or activating two-pore potassium channels.[9] The net result leads to cellular hyperpolarization and a reduction in neuronal activity. However, during development, this artificial silencing of synapses is thought to induce an apoptotic cascade *via* disruption of the action of trophic factors, notably brain-derived neurotrophic factor,[2,3] phosphorylated extracellular signal-regulated protein kinase 1 and 2 (pERK),[2] and phosphorylated-cyclic-adenosine monophosphate (AMP) response element binding protein with subsequent stimulation of the intrinsic apoptotic cascade.[4,10] The intrinsic cascade results in cytochrome C release and Bax signaling to activate the caspase enzymes that provoke cell death by apoptosis.[4,10,11] Subsequently, extrinsic apoptotic signaling may also be activated.[10] These toxic effects have now been established after as little as 60 min of below 1 minimum alveolar concentration of isoflurane in the 7-day-old rat[12]; thus, a relationship between anesthesia, neuroapoptosis and cognitive dysfunction has been established.

The NMDA antagonist ketamine (20 mg kg^{-1} and above) and the GABAergic agonist midazolam (9 mg kg^{-1}) both induce apoptotic neurodegeneration in infant mice[13] despite having different mechanisms of anesthetic action. This has significant implications for pediatric anesthesia as these drugs are used for premedication, sedation or analgesia in several clinical settings. Furthermore, as these agents have differing mechanisms of anesthetic action, yet induce this neuroapoptosis, it has been argued that it is the anesthetic state that produces the injury.[11] To date, only one exception to this rule has been identified, the noble anesthetic gas xenon, which prevented isoflurane-induced toxicity.[4] However, xenon

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is currently not widely available; therefore, we have been seeking to identify alternative methods to ameliorate this toxicity.

Early in life, α_2 adrenoceptors are thought to play a trophic role in central nervous system signaling,[14,15] with endogenous norepinephrine activating cellular survival mechanisms such as the Ras-Raf-pERK pathway.[16,17] Activation of this Ras-Raf-pERK pathway has been associated with neuroprotection against the apoptosis induced by NMDA antagonists in the young.[2] Dexmedetomidine also increases the expression of the antiapoptotic proteins mdm2 and bcl-2 in a model of adult ischemic cerebral injury[18]; *in vitro*, it has been shown to upregulate brain-derived neurotrophic factor, phosphorylated-cyclic-AMP response element binding protein, and pERK signaling.[16,19,20] However, it is not known whether modification of these proteins represents a true antiapoptotic effect of dexmedetomidine or whether these findings were merely a correlate of increased cellular survival. Herein, we show that dexmedetomidine protects against anesthetic-induced apoptosis *in vivo* and *in vitro*, indicating that it does possess antiapoptotic qualities. Importantly, we again establish that isoflurane injury provokes a long-term neurocognitive deficit and then demonstrate that this functional deficit can be attenuated by dexmedetomidine.

Materials and Methods

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

In Vitro *Experiments*

Organotypic hippocampal slices were derived from postnatal day 8 or 9 C57Bl/6 mice pups (Harlan Laboratories, Huntingdon, United Kingdom) and cultured by the interface method[21,22] 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 McIlwain 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). 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. The culture medium was replaced the next day with fresh, temperature-equilibrated medium before exposure to gas treatments. The groups of slices (n = 15 per group) were assigned to control (air + 5% carbon dioxide), dexmedetomidine 1 μM, gabazine 50 μM, 0.75% isoflurane, 0.75% isoflurane + dexmedetomidine 1 μM, and 0.75% isoflurane + gabazine 50 μM.

All subsequent gas exposure occurred in a specially constructed exposure chamber as previously described.[23] 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, Bradford, United Kingdom). After 3–4 min of gas flow, the chambers 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.

In Vivo *Experiments*

Seven-day-old Sprague-Dawley rat pups were exposed to 6 h of 0.75% isoflurane in 25% oxygen or air in a temperature-controlled chamber (n = 6 per group). Three doses of saline or dexmedetomidine (1, 10, or 25 μg/kg) were administered by intraperitoneal injection over the 6-h exposure (at 0, 2, and 4 h). One group received 0.75% isoflurane, 25 μg/kg dexmedetomidine, and 500 μg/kg nonselective α_2 adrenoceptor antagonist atipamezole in 3 doses over the 6-h exposure (n = 4 per group). An additional three doses of 75 μg/kg dexmedetomidine in air were given to establish at extreme doses of dexmedetomidine whether apoptosis could be induced (n = 6 per group).

The animals were sacrificed (with 100 mg/kg sodium pentobarbital by intraperitoneal injection) at the end of gas exposure and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M buffer. After removal of the brain and storage overnight at 4°C in paraformaldehyde, it was transferred to 30% sucrose solution with phosphate buffer and 1% sodium azide and kept at 4°C until the brains were sectioned and stained immunohistochemically for caspase-3.

Immunohistochemistry

For the *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. Concerning

the *in vivo* experiments, the brain was sliced at 30-μm intervals beginning at −3.6 mm from the bregma, the sections were then transferred to a six-well plate containing phosphate-buffered saline. Sections were dried at 37°C for 24 h and then immunostained while adherent to the slides, before preincubation with hydrogen 0.3% peroxidase in methanol for 30 min and then rinsed in phosphate-buffered saline. The sections were then incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (1:2,500; New England Biolab, Hitchin, United Kingdom) and then washed three times in phosphate-buffered saline with 3% Triton at room temperature. Biotinylated secondary antibodies (1:200; Sigma, St. Louis, MO) and the avidin-biotin-peroxidase complex (Vector Laboratories, Orton Southgate, Peterborough, United Kingdom) were applied. The sections were again washed in phosphate-buffered saline before incubating with 0.02% 3,3′-diaminobenzidine with nickel ammonium sulfate in 0.003% hydrogen peroxide (DAB kit, Vector Laboratories). The sections were dehydrated through a gradient of ethanol solutions (70–100%) and then mounted (floating section) and covered with a cover slip.

Neurocognitive Evaluation

Seven-day-old Sprague-Dawley rat pups were exposed to 6 h of 0.75% isoflurane in 25% oxygen or air in a temperature-controlled chamber (n = 6 per group). Three doses of saline or 25 μg/kg dexmedetomidine were administered by intraperitoneal injection over the 6-h exposure (at 0, 2, and 4 h). The animals were allowed to mature until postnatal day 40 and then tested for hippocampal-dependent memory and learning function in a previously reported contextual fear-conditioning behavioral paradigm[24] in which the rats were taken from the vivarium in the behavioral room on the first test day and allowed to sit undisturbed in their homecage for 10 min. Once placed in the conditioning chamber, the rats were allowed 198 s of exploration.

The conditioning chamber was cubic (30 cm × 24 cm × 21 cm; Med Associates, Inc., St. Albans, VT) and 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. When placed in the chamber, the grid floor 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 (HEPA) filter provided background white noise of 65 db.

Afterwards, all animals received 6 cycles of 214 s of trace fear conditioning. 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 phase was 26 min. Acquisition time was defined as the time spent immobile after a shock divided by the intertrial interval. On the next day, trained rats were exposed to the same acquisition environment but received neither tone nor shock for 8 min (context test). 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). Freezing time was assessed using VideoFreeze software (Med Associates Inc., Burlington, VT); therefore, the assessment can be considered objective. The percentage of freezing time (context results) and the area under curve were derived from plots between the percentage freezing time and trial time in the tone test and were used for statistical comparison (mean ± SD, n = 6 per group).

Statistical Analyses

The number of caspase-3–positive neurons in the cortex, thalamus, and hippocampus in each brain slice were counted by an observer blinded to the experimental protocol. Four brain slices were counted per animal. The immunohistochemical and behavioral data are presented as mean ± SD. Statistical analyses was performed by ANOVA followed by *post hoc* Newman Keuls testing using the INSTAT (London, United Kingdom) program. $P < 0.05$ was set as significant.

Results

All animals survived the *in vivo* experiments. Isoflurane induced neuroapoptosis throughout the cortex, thalamus, and hippocampus reflected by the increase in the number of caspase-3–positive cells observed (fig. 1, A–C). Isoflurane (0.75% + saline) increased caspase-3 expression relative to air (+ saline) controls in the cortex from 44 ± 7 to 270 ± 34 cells ($P < 0.05$; fig. 1D), in the hippocampus from 8 ± 3 to 80 ± 11 cells ($P < 0.05$; fig. 1E), and in the thalamus from 4 ± 2 to 62 ± 15 cells ($P < 0.05$; fig. 1F). In contrast dexmedetomidine in the presence of air did not increase cellular caspase-3 expression relative to controls (fig. 1, E–F).

Dexmedetomidine (1–25 μg/kg) provided dose-dependent neuroprotection reducing isoflurane-induced caspase-3 expression in the cortex (186 ± 23 to 129 ± 29 cells; $P < 0.05$) relative to isoflurane (270 ± 34 cells), hippocampus (28 ± 11 to 15 ± 5 cells; $P < 0.05$) relative to isoflurane (80 ± 11 cells), and thalamus (21 ± 6 to 9 ± 4 cells; $P < 0.05$) relative to isoflurane (62 ± 15 cells). The addition of 25 μg/kg dexmedetomidine provided the most potent protection that was significantly better than 1 or 10 μg/kg dexmedetomidine ($P < 0.05$) in each

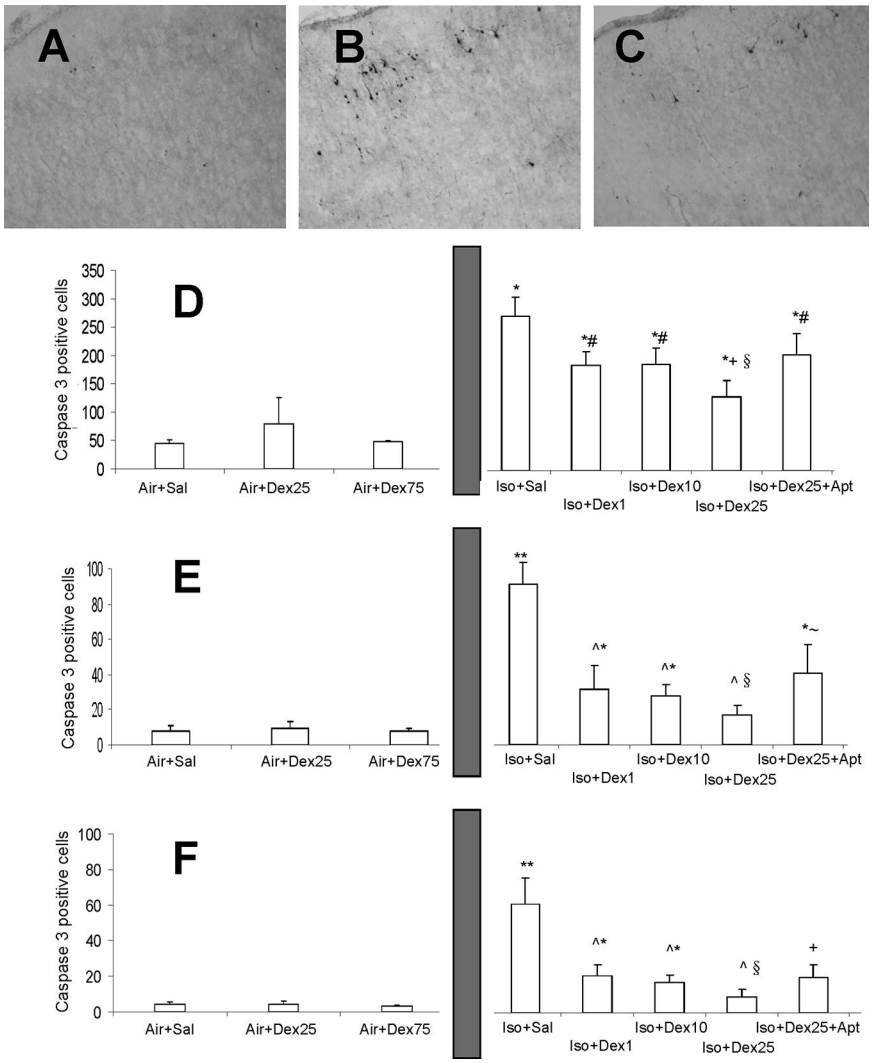

Fig. 1. Dexmedetomidine (Dex) inhibits isoflurane-induced neuroapoptosis *in vivo*. Seven-day-old rats were exposed to air or isoflurane (0.75%) for 6 h with intraperitoneal injections of saline or dexmedetomidine given three times at 0, 2, and 4 h. (*A*) Photomicrograph of a cortical section from an animal exposed to air and three doses of saline over 6 h stained immunohistochemically for caspase-3. (*B*) A similar photomicrograph of a cortical section from an animal exposed to isoflurane and three doses of saline over 6 h. (*C*) Photomicrograph of a cortical section from an animal exposed to isoflurane and three doses of 1 μg/kg dexmedetomidine over 6 h stained immunohistochemically for caspase-3. (*D*) Histogram showing the number of caspase-3–positive cortical neurons against intervention. (*E*) Histogram showing the number of caspase-3–positive hippocampal neurons against intervention. (*F*) Histogram showing the number of caspase-3–positive thalamic neurons against intervention. The interventions include: Air + Saline intraperitoneal (Air/Sal), Air + Dex 25 μg/kg (Air/Dex25), Air + Dex 75 μg/kg (Iso/Dex75), Isoflurane + saline (Iso/Sal), Iso + Dex 1 μg/kg (Iso/Dex1), Iso + Dex 10 μg/kg (Iso/Dex10), Iso + Dex 25 μg/kg (Iso/Dex25), Iso + Dex 25 μg/kg + Atipamezole 500 μg/kg (Iso/Dex25/Atp); n = 4–6 per group. * = *P* < 0.05 *versus* Air + Sal; ** = *P* < 0.001 *versus* Air + Sal; # = *P* < 0.05 *versus* Iso + Sal; + = *P* < 0.01 *versus* Iso + Sal; ^ = *P* < 0.001 *versus* Iso + Sal; § = *P* < 0.05 *versus* Iso + Dex 25; ~ = *P* < 0.05 *versus* Iso + Dex1 or Iso + Dex10.

brain area. In the hippocampus and thalamus, but not the cortex, 25 μg/kg dexmedetomidine reduced the injury induced by isoflurane to baseline ($P > 0.05$ *vs.* Air + Saline). Reversal of dexmedetomidine neuroprotection by the α_2 adrenoceptor antagonist, atipamezole, in the hippocampus ($P < 0.05$; fig. 1E), thalamus, and cortex (nonsignificant) indicates that this effect is at least partly mediated by α_2 adrenoceptors in these regions.

Consistent with previous data,[4] 6 hours of 0.75% isoflurane also induced neuroapoptosis in organotypic hippocampal slice cultures, increasing caspase-3 expression by 44% (control 18 ± 6 *vs.* isoflurane 26 ± 11 cells; $P < 0.01$; fig. 2, A and B). This effect was reversed by addition of 1 μM dexmedetomidine (20 ± 7 cells; $P < 0.05$, fig. 2C), reducing caspase-3 expression to within 10% of controls. As reported previously,[25] gabazine itself was nontoxic (showing caspase-3 expression 92% of air-saline treated controls; $P > 0.05$); it did not attenuate isoflurane-induced apoptosis ($P > 0.05$ *vs.*. isoflurane; fig. 2, D and E).

At postnatal day 40, neonatal treatment with isoflurane-saline, but not air-dexmedetomidine, induced neurocognitive impairment as assessed by context fear conditioning (a marker of long-term memory); however, none of the groups exhibited any deficit in the acquisition phase (indicating no deficit in short-term memory; fig. 3A). The percentage freezing time in the contextual fear-conditioning experiment was 48 ± 5% in controls (air-saline), 45 ± 11% with air-dexmedetomidine–treated animals, and 29 ± 7% with isoflurane-saline–treated animals (fig. 3B). Dexmedetomidine ameliorated the neurocognitive impairment induced by isoflurane; percentage freezing time 46 ± 9% with isoflurane-dexmedetomidine–treated animals.

Discussion

Isoflurane induced widespread cerebral neuroapoptosis in neonatal rat pups with subsequent long-term neurocognitive impairment of the animals. As the injury occurred in the neonatal period and animal training and testing followed this injury, this indicates impairment in learning and memory consistent with a significant hip-

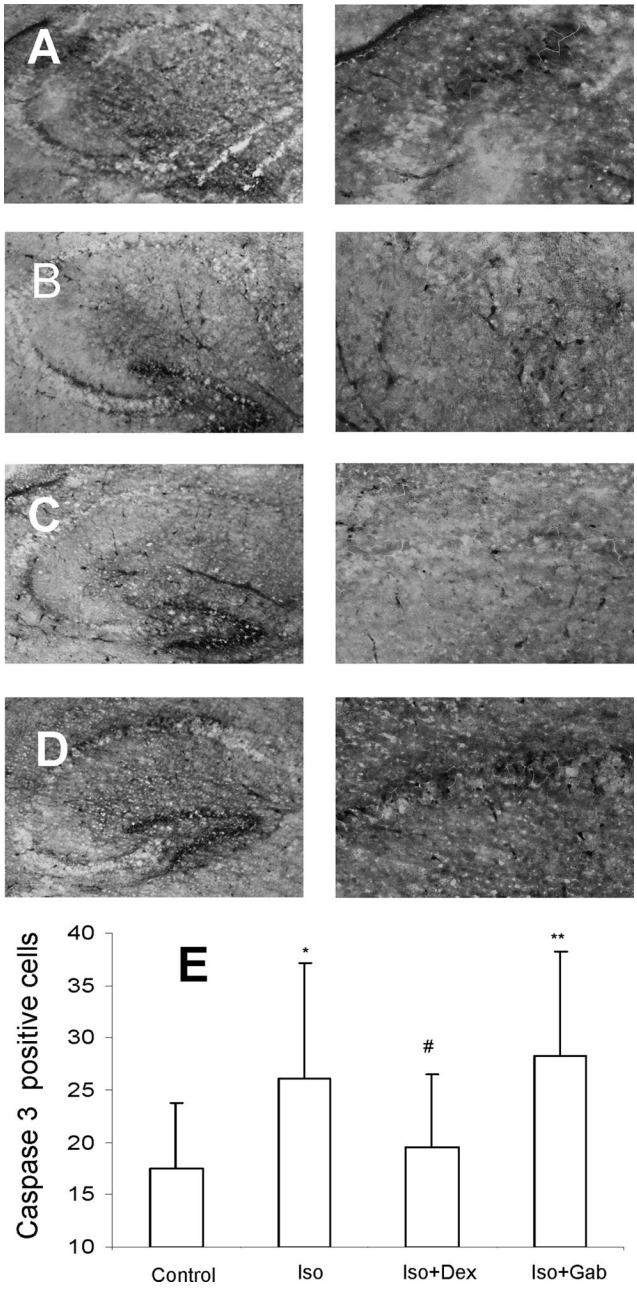

Fig. 2. Dexmedetomidine (Dex) inhibits isoflurane-induced neuroapoptosis *in vitro*. C57Bl/6 mice pup organotypic hippocampal cultures were exposed to (*A*) air + 5% carbon dioxide (control), (*B*) isoflurane 0.75% (Iso), (*C*) isoflurane 0.75% + Dex 1 μM (Iso + Dex), and (*D*) isoflurane + gabazine 50 μM (Iso + Gab) for 6 h and then stained for caspase-3 using immunohistochemistry. Quantified data are presented in section *E*. * = *P* < 0.01 *versus* Control; ** = *P* < 0.001 *versus* Control; # = *P* < 0.05 *versus* Iso and *P* < 0.01 *versus* Iso + Gab.

pocampal lesion.[24,26] These data support previous experiments showing a significant hippocampal injury after anesthetic treatment.[1] Dexmedetomidine provided neuroprotection against isoflurane-induced neuroapoptosis in a dose-dependent manner, acting *via* activation of α_2 adrenoceptors (as atipamezole reversed dexmedetomidine's neuroprotective effect). Crucially, dexmedetomidine prevented the neurocognitive sequelae of isoflurane treatment. Importantly, in contrast to isoflurane (and other agents such as midazolam and ketamine[13]), dexmedetomidine itself lacks neurotoxicity even at extremely high doses such as 75 μg/kg dexmedetomidine (a dose that is 75 times the ED_{50} for hypnosis[27]). Dexmedetomidine also did not induce neurocognitive impairment at the clinically relevant dose of 25 μg/kg. Although dexmedetomidine could also attenuate isoflurane-induced neuroapoptosis in organotypic hippocampal slice cultures, gabazine did not reverse this effect, suggesting that isoflurane's neuroapoptotic effect is not mediated by $GABA_A$ receptors.

Caveats

These results indicate that dexmedetomidine can inhibit neuroapoptosis provoked by isoflurane *in vitro* and *in vivo*; however, several caveats need to be raised before further interpretation of our data. Previous reports have shown that the apoptosis involved neurons, and the injured cells in our study morphologically appear to be neurons. Therefore we assume the dying cells are neurons; this is supported by our data showing a neurocognitive deficit induced by isoflurane. In addition, our marker of apoptosis and cell death, caspase-3 expression, has been previously validated in this model of anesthetic-injury.[1–4] Although we (and others[1]) have correlated the apoptosis observed with the neurocognitive deficits induced by isoflurane in neonatal rats, we still cannot exclude that other mechanisms (such as effects on neurogenesis or synaptic function) do not contribute to the pathogenesis or the protection afforded by dexmedetomidine.

Despite data showing that the hypnotic, analgesic, and neuroprotective effects of dexmedetomidine primarily relate to activation of the α_{2A} adrenoceptor,[28,29] atipamezole significantly inhibited dexmedetomidine's neuroprotective effect only in the hippocampus. Although there was a trend to a reversal in effect in the thalamus and cortex, atipamezole did not significantly alter dexmedetomidine protection in these regions. This may indicate alternate receptor targets in these regions, such as imidazoline receptors,[30] but we suspect a type II error may also account for these findings.

Minimal disturbances in arterial blood gases[1,3] have been reported in previous studies; however, the potential to induce hypoglycemia in these animals during the anesthetic period is of concern,[31] but this has also been shown not to occur.[12] Indeed, it is possible that the addition of dexmedetomidine to isoflurane could exacerbate both the cardiovascular and respiratory depression of the anesthetic state; however, it is also conceivable that high doses of dexmedetomidine may have increased either blood pressure (*via* activation of α_{2B} adrenoceptors) or glucose (*via* α_{2A} adrenoceptors). Therefore, we also conducted an *in vitro* experiment to control for the potential confounding effects of hypoxia,

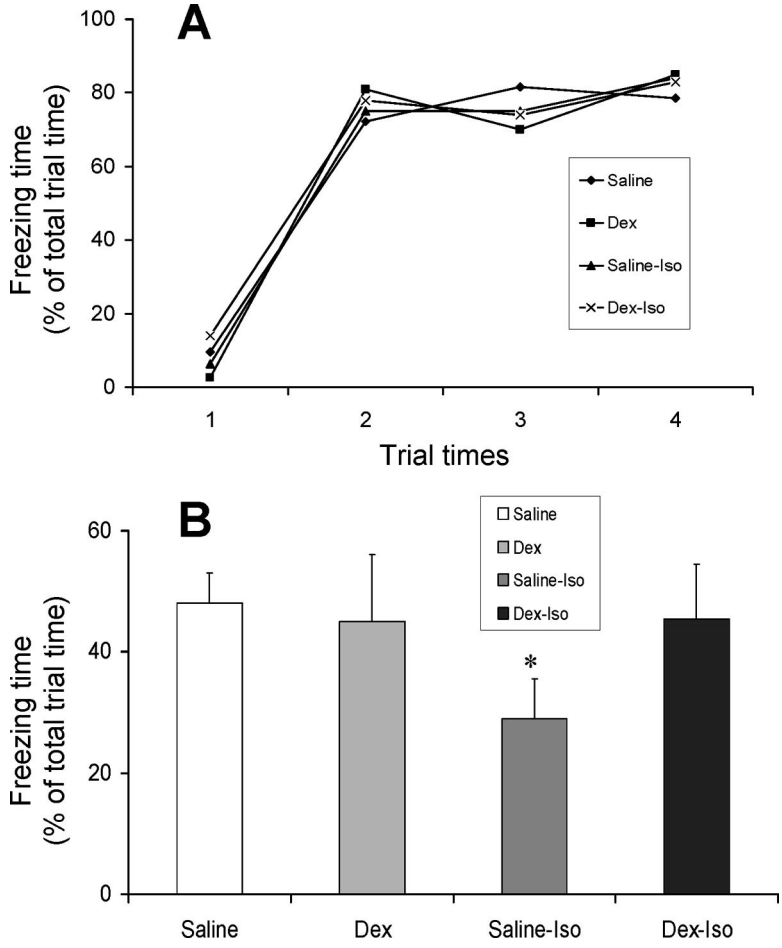

Fig. 3. Cognitive function assessed by trace fear conditioning. Seven-day-old Sprague-Dawley rat pups were exposed with air or 0.75% isoflurane (Iso) in oxygen with or without saline or dexmedetomidine (Dex) treatment for 6 h. They were allowed to live up to 40 days and then tested for hippocampal-dependent memory and learning function. (*A*) The plot of the mean percentage of freezing time of acquisition against six test trials of trace fear conditioning (day 1). (*B*) The mean of the percentage of freezing time (context results) obtained from trace fear conditioning (day 2). Mean ± SD (n = 6); * = $P < 0.05$ *versus* other groups.

glucose, and temperature dysregulation using the organotypic hippocampal slice culture model. We employed the latter experimental paradigm because synapses remain intact, which is imperative because inhibition of synaptic neurotransmission is hypothesized as critical to the injury. It is known that isoflurane is directly neurotoxic in the organotypic hippocampal slice culture model.[4,32] We used a single dose of dexmedetomidine (1 μM) in our organotypic hippocampal slice culture studies and did not corroborate the extensive dose response curve that was obtained *in vivo* because the aim in this experiment was to identify whether dexmedetomidine was acting *via* a direct or physiologic mechanism. These data suggest that dexmedetomidine can prevent the isoflurane injury by direct action within the central nervous system.

It should also be noted that dexmedetomidine, although neuroprotective, does not entirely reverse the isoflurane injury in the cortex (despite prominent neuroprotection of the thalamus and hippocampus being observed). However, our neurocognitive tests did not uncover an isoflurane-associated deficit in memory acquisition that typically depends on a functional prefrontal cortex.[33] Therefore, despite significant apoptosis in the cortex, our study suggests the cortex is not functionally impaired after isoflurane treatment (0.75% for 6 h), but we do suggest that the effects of other doses of isoflurane still require investigation. In monkeys, ketamine injury is primarily involved the cortex rather than subcortical structures[34]; it is possible that, if observed in humans, cortical apoptosis induced by anesthetics may be the predominant injury. Further tests of cortex-based neurocognitive function in rodents and primates should be conducted before this injury is dismissed.

A final difficulty plaguing all preclinical studies is the ability to extrapolate across species; in this regard, differential interspecies vulnerability to isoflurane injury may be apparent. Indeed, recent data have suggested that monkey brains may be less vulnerable to ketamine injury than rodent brains.[34] However, isoflurane may be more potent at inducing apoptosis than ketamine, especially because the injury is apparent after subanesthetic isoflurane concentrations lasting only 1 h.[12] Whether anesthetic-induced neurotoxicity is a clinical problem requires further investigation, including studies involving monkeys and ultimately humans; while we await these answers, we need to strive to obtain a safe anesthetic therapy.

Mechanism of Dexmedetomidine Neuroprotection

In these studies, we have explored whether dexmedetomidine is antiapoptotic (as suggested, but not di-

rectly investigated by many preclinical studies). We propose that administration of an α_2 adrenoceptor agonist during the critical phase of synaptogenesis activates the endogenous postsynaptic norepinephrine-mediated trophic system, which couples to a pERK-Bcl-2 pathway to produce its antiapoptotic effect.[19,20,21] Further studies will probe the involvement of this pathway *in vivo*. Despite our data showing a role for α_2 adrenoceptor activation in dexmedetomidine neuroprotection, other potential receptor subtypes, such as the imidazoline receptors, can upregulate pERK and are activated by dexmedetomidine,[30] thus providing an alternative mechanism for dexmedetomidine's neuroprotective effect.

Mechanism of Isoflurane Neurotoxicity

Interestingly, gabazine could not reverse the isoflurane-induced neurodegeneration despite the hypothesis that, by potentiating $GABA_A$ receptor activity, isoflurane inhibits neurotransmission detrimentally during the critical period of synaptogenesis.[11] Thus, $GABA_A$ receptor activation may not be critical for isoflurane-induced neurotoxicity, although it is of interest that $GABA_A$ receptor antagonists can attenuate isoflurane's neuroprotective effect.[25,35] While $GABA_A$ receptor activation remains an important target for anesthesia, especially for intravenous anesthetics such as propofol, its role in halogenated volatile anesthesia is less clear.[36,37] Therefore, it would appear that activation of the $GABA_A$ receptor is important for isoflurane neuroprotection but not necessarily critical for toxicity. Whether $GABA_A$ receptor antagonism can attenuate propofol-induced neurotoxicity[38] will be of interest because it reverses the propofol anesthetic state.[39] However, $GABA_A$ receptors are not involved in the neurotoxicity; therefore, it may be possible to design a safe anesthetic agent for use in the young. Interestingly, a difference in the ability of sevoflurane and isoflurane to induce apoptosis has also been observed previously,[40] although preliminary evidence suggests that sevoflurane, similar to isoflurane, also induces neuroapoptosis in the neonatal rat brain.[41]

Another receptor target that may be responsible for the isoflurane injury is the NMDA receptor, which plays a critical role in neurodevelopment.[8] Each of the neuroapoptotic-inducing anesthetics, including isoflurane, ketamine, and MK-801, inhibit the NMDA receptor subtype of the glutamate receptor.[1–5,10–13] An exception to this rule is xenon, another NMDA receptor antagonist, which produces protection against isoflurane-induced injury rather than neuroapoptosis in the neonatal rat brain.[4] We consider it likely that xenon exerts an antiapoptotic effect independent of its action at the NMDA receptor. It is of interest that both α_2 adrenoceptor agonists and xenon can attenuate the injury produced by NMDA antagonists in the adult brain[42,43]; therefore, despite differences in the morphology of the adult and neonatal toxicity, we cannot discount overlapping mechanisms of injury. In addition, we have not as yet evaluated whether a neuroprotective cocktail of xenon and dexmedetomidine can be employed to further reduce isoflurane toxicity because they provide synergistic protection against neonatal hypoxic-ischemic injury.[44]

Neurocognitive Effects

Our results from our fear conditioning paradigm support the previous reports of neurocognitive impairment in adult rats after neonatal anesthesia.[1,45] Fear conditioning consists of placing a rat or a mouse in a chamber and giving one or more mild electric foot shocks. After a shock, the animal becomes immobile (freezing), a natural response to fear that can be used as an indication of memory formation. Complex neuronal circuitry involving the frontal cortex, hippocampus, periaqueductal gray, and rostral ventral medulla underlie the acquisition and retention of fear conditioning.[24] Notably, a damaged hippocampus is unable to process the incoming stimuli producing a memory deficit.[26]

Interestingly, all groups displayed normal learning assessed by the acquisition of memory; the animals showed increasing levels of freezing across the training tones, with freezing levels post-shock on sixth pairing approximately 70%. This indicates a normal short-term memory, a function predominantly involving the prefrontal cortex.[31] In the context assessment, 24 h after acquisition training, animals exposed to isoflurane and saline displayed less freezing when compared to naive controls, indicating a neurocognitive deficit. Thus isoflurane-treated animals showed an abnormal response to contextual fear conditioning, indicating a severe hippocampal lesion[24,26] consistent with previous reports.[1] However, our experiments employed a much lower dose of anesthetic than in the previous studies (0.75% isoflurane *vs.* 0.75% isoflurane plus 75% nitrous oxide and 9 mg/kg midazolam). Even with subanesthetic dosing, the potential for functional neurocognitive deficit is apparent. In contrast, dexmedetomidine alone did not induce any memory deficit. Furthermore, the addition of dexmedetomidine to isoflurane reversed the neurocognitive compromise induced by isoflurane. This is of critical importance because dexmedetomidine is the first agent to be shown to reverse the neurocognitive dysfunction provoked by isoflurane.

Dexmedetomidine is widely available and has an expanding role in pediatric clinical practice; therefore, if anesthetic-induced neurodegeneration is proven to be a clinical problem, we may already have available a therapeutic intervention that can be employed in this setting where necessary. In situations where dexmedetomidine is not available, another α_2 agonist, clonidine, could be a candidate, although further studies are warranted because, although atipamezole significantly reversed dexmedetomidine's neuroprotective effect in the hippocampus, the protection afforded in the thalamus and cortex were not sig-

nificantly attenuated; we cannot be sure that all α_2 adrenoceptor agonists will afford this protection.

Clinical Implications

Clinically, no information has detailed the extent of anesthetic-induced neonatal neurodegeneration in humans. In terms of neurodevelopment, a 7-day-old rat pup represents the peak of the synaptogenic period, but this period extends from birth to up to 2–3 yr in humans; therefore, the window of vulnerability may be greater in humans.[46,47] One cannot advocate withholding anesthesia or analgesia during early human life on the basis of these findings because of the harm that this can do.[47–50] However, if anesthetic-induced neurodegeneration is revealed as a clinical problem for pediatric anesthesia, administration of an α_2 adrenoceptor agonist during the anesthesia maybe prudent. Thus, this study has uncovered a plausible and promising novel application of a widely available class of drugs that may significantly affect the safety of clinical practice.

The use of α_2 adrenoceptor agonists in pediatric practice is expanding as a result of their potent sedative/hypnotic qualities, analgesic action, potential organ-protective effects, reduction in postoperative nausea and vomiting and delirium, and relative lack of respiratory side effects.[51,52] Their use in neonatal practice requires evaluation based on these factors.[51] In the future, their organ-protective, including neuroprotective, effects may be of importance to the provision of safe, balanced pediatric anesthesia.[47]

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