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Effect of dexmedetomidine on sevoflurane-induced neurodegeneration in neonatal rats

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Abstract

Background: Structural brain abnormalities in newborn animals after prolonged exposure to all routinely used general anaesthetics have raised substantial concerns for similar effects occurring in millions of children undergoing surgeries annually. Combining a general anaesthetic with non-injurious sedatives may provide a safer anaesthetic technique. We tested dexmedetomidine as a mitigating therapy in a sevoflurane dose-sparing approach.

Methods: Neonatal rats were randomised to 6 h of sevoflurane 2.5%, sevoflurane 1% with or without three injections of dexmedetomidine every 2 h (resulting in 2.5, 5, 10, 25, 37.5, or 50 μg kg^{-1} h^{-1}), or fasting in room air. Heart rate, oxygen saturation, level of hypnosis, and response to pain were measured during exposure. Neuronal cell death was quantified histologically after exposure.

Results: Sevoflurane at 2.5% was more injurious than at 1% in the hippocampal cornu ammonis (CA)1 and CA2/3 subfields; ventral posterior and lateral dorsal thalamic nuclei; prefrontal, retrosplenial, and somatosensory cortices; and subiculum. Although sevoflurane 1% did not provide complete anaesthesia, supplementation with dexmedetomidine dose dependently increased depth of anaesthesia and diminished responses to pain. The combination of sevoflurane 1% and dexmedetomidine did not reliably reduce neuronal apoptosis relative to an equianaesthetic dose of sevoflurane 2.5%.

Conclusions: A sub-anaesthetic dose of sevoflurane combined with dexmedetomidine achieved a level of anaesthesia comparable with that of sevoflurane 2.5%. Similar levels of anaesthesia caused comparable programmed cell death in several developing brain regions. Depth of anaesthesia may be an important factor when comparing the neurotoxic effects of different anaesthetic regimens.

Keywords: apoptosis; brain injury; dexmedetomidine; neuroprotection; neurotoxicity; sevoflurane; volatile anaesthetics

Editor's key points

- Numerous animal studies have demonstrated brain structural and cognitive abnormalities after anaesthetic exposure early in life.
- Dexmedetomidine was tested as a mitigating therapy against sevoflurane-induced neurotoxicity in a rat model.
- The combination of a sub-anaesthetic dose of sevoflurane plus dexmedetomidine did not reliably reduce

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neuronal apoptosis relative to an equianaesthetic dose of sevoflurane alone.
- Equivalent levels of anaesthesia produced comparable apoptosis in developing brain, indicating that depth of anaesthesia may be important in determining the neurotoxic effects of anaesthesia in neonates.

Over the past two decades, numerous animal studies, including in non-human primates, have demonstrated brain structural and cognitive abnormalities after prolonged anaesthetic exposure early in life.[1] This has raised tremendous concerns for the safe practice of paediatric anaesthesia,[2,3] culminating in a warning by the US Food & Drug Administration (FDA) regarding prolonged or repeated exposures in infants and toddlers.[4]

No routinely used general anaesthetic is devoid of injurious effects in developing animals. Prolonged exposure to the most frequently utilised paediatric anaesthetic agent sevoflurane at doses between 2.5% and 4% leads to brain structural injury. Subsequently, cognitive and behavioural abnormalities are evident.[5–11] Shorter exposure times are less injurious,[12] suggesting a dose-related toxicity. In support of this, a clinical trial and two ambidirectional human studies have not found measurable cognitive deficits in children for exposures of less than 2 h,[13–16] whereas prolonged or repeated anaesthetic exposures have been associated with functional and structural abnormalities in children.[17–20]

It is therefore imperative to test alternative anaesthetic regimens and protective strategies. Attractive initial targets represent compounds already utilised in paediatric medicine, which can be studied economically and ethically in small animals before recommending strategies for humans.[21] The goal of this study was to compare two strategies providing complete anaesthesia with immediate clinical translatability. A dose-finding approach was taken to confirm similar depths of anaesthesia amongst regimens, and to avoid exacerbation of anaesthesia-induced hypoventilation, we did not include opioids. We compared sevoflurane alone at a dose previously found to be both anaesthetising and injurious with a combination of a sub-anaesthetic dose of sevoflurane supplemented with dexmedetomidine. Dexmedetomidine is an α_2-adrenergic receptor agonist chosen to increase the combined level of anaesthesia and to provide neuroprotection, as previously shown for brain ischaemia and for ketamine, isoflurane, and sevoflurane anaesthesia.[22–26]

Accordingly, the present study tested the hypotheses that a combination of dexmedetomidine plus a 0.3 minimum alveolar concentration (MAC) of sevoflurane (1%)[27] (i) provides a level of anaesthesia similar to sevoflurane 2.5% (0.75 MAC) without causing cardiorespiratory lethality, (ii) is associated with reduced neuronal injury compared with sevoflurane 2.5%, and (iii) ameliorates the injurious effects of sevoflurane 1%. Findings will guide future cognitive assessments in animals and inform non-human primate studies, and may help devise clinical trials to develop safer anaesthetic techniques for children.

Methods

Animals

Wistar rat breeding pairs (Charles River Laboratories, Inc., Wilmington, MA, USA) were housed in a 14/10 h light/dark cycle (lights on at 07:00) at 22°C with free access to water and chow. Nine litters were raised by their parents until Postnatal day 7 (P7, with P0 representing the date of birth) when they were randomised and blocked by mating pair; at least one animal from every litter was assigned to one of the nine treatment groups. Animals of both sexes were utilised, as we have observed no sex differences in the effects of anaesthetic exposure at this age[28] and the number of treatment groups did not allow for blocking by sex and litter. A total of 87 animals were used, allowing for group sizes of n=8–10. All experiments followed the National Institutes of Health and Animal Research: Reporting of In Vivo Experiments guidelines[29] to minimise the number of animals utilised, and were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation.

Exposure protocol

On P7, rats were randomly allocated to one of nine 6-h exposure protocols: sevoflurane 2.5% (Sevo 2.5%); sevoflurane 1% (Sevo 1%); sevoflurane 1% plus three i.p. dexmedetomidine injections every 2 h of 5 μg kg^{-1} for an hourly dose of 2.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 2.5), 10 μg kg^{-1} every 2 h (Sevo 1%+Dex 5 μg kg^{-1} h^{-1}), 20 μg kg^{-1} every 2 h (Sevo 1%+Dex 10), 50 μg kg^{-1} every 2 h (Sevo 1%+Dex 25), 75 μg kg^{-1} every 2 h (Sevo 1%+Dex 37.5), and 100 μg kg^{-1} every 2 h (Sevo 1%+Dex 50); or three 2-hourly injections of normal saline 0.1 ml (control). Doses were injected i.p. every 2 h, but are averaged per hour to facilitate comparisons. Our previous studies showed that doses of at least 12.5 μg kg^{-1} h^{-1} of dexmedetomidine were required to induce loss of righting reflex (LORR) in 7-day-old rats.[30] The dexmedetomidine injectate was prepared fresh before experiments by diluting a dexmedetomidine stock solution (100 μg ml^{-1}; Precedex™; Pfizer, New York, NY, USA) with preservative-free normal saline to a final concentration between 1 and 20 μg ml^{-1} to limit the maximum volume of injectate to 0.1 ml. All animals were kept in a 30% oxygen environment in an acrylic chamber within a heated incubator set to 35°C to maintain rectal temperatures of 36.5–37.5°C. Inhaled anaesthetic and oxygen concentrations were measured (RGM 5250; Datex-Ohmeda, Inc., Louisville, CO, USA) and adjusted according to protocol. Every 30 min, the animals were briefly (<1 min) removed from the experimental chamber for injections and measurement of heart rate and peripheral oxygen saturation (MouseSTAT®; Kent Scientific, Torrington, CT, USA), rectal temperature (Yellow Springs Instrument Co., Yellow Springs, OH, USA), and pain responses. Because of motion artifacts, baseline data were not obtainable; comparisons were made with unanaesthetised animals whose movements diminished during the study period. LORR, a behavioural surrogate for level of hypnosis in rodents, was graded from 0 to 4: 0, no response to being placed supine; 1, delayed attempt to right itself, but failing; 2, delayed, uncoordinated return to upright position; 3, sedated, but righting in a coordinated fashion; or 4, awake and not remaining supine. Pain responses were assessed by applying a mechanical force of 40 g to the medial portion of the plantar surfaces of the hind paw (Randall Selitto Paw Pressure Test Apparatus; IITC Life Science, Inc., Woodland Hills, CA, USA) and graded from 0 to 4: 0, no response; 1, single limb movement to stimulus; 2, delayed response, but generalised movement; 3, delayed motor response and subsequent increase in motor activity; or 4, immediate, vigorous response with or without vocalisation. The investigator measuring vital signs and assessing depth of anaesthesia and pain was unaware of group assignment and anaesthetic doses, but was aware whether or not the animals were exposed to an inhalational anaesthetic.

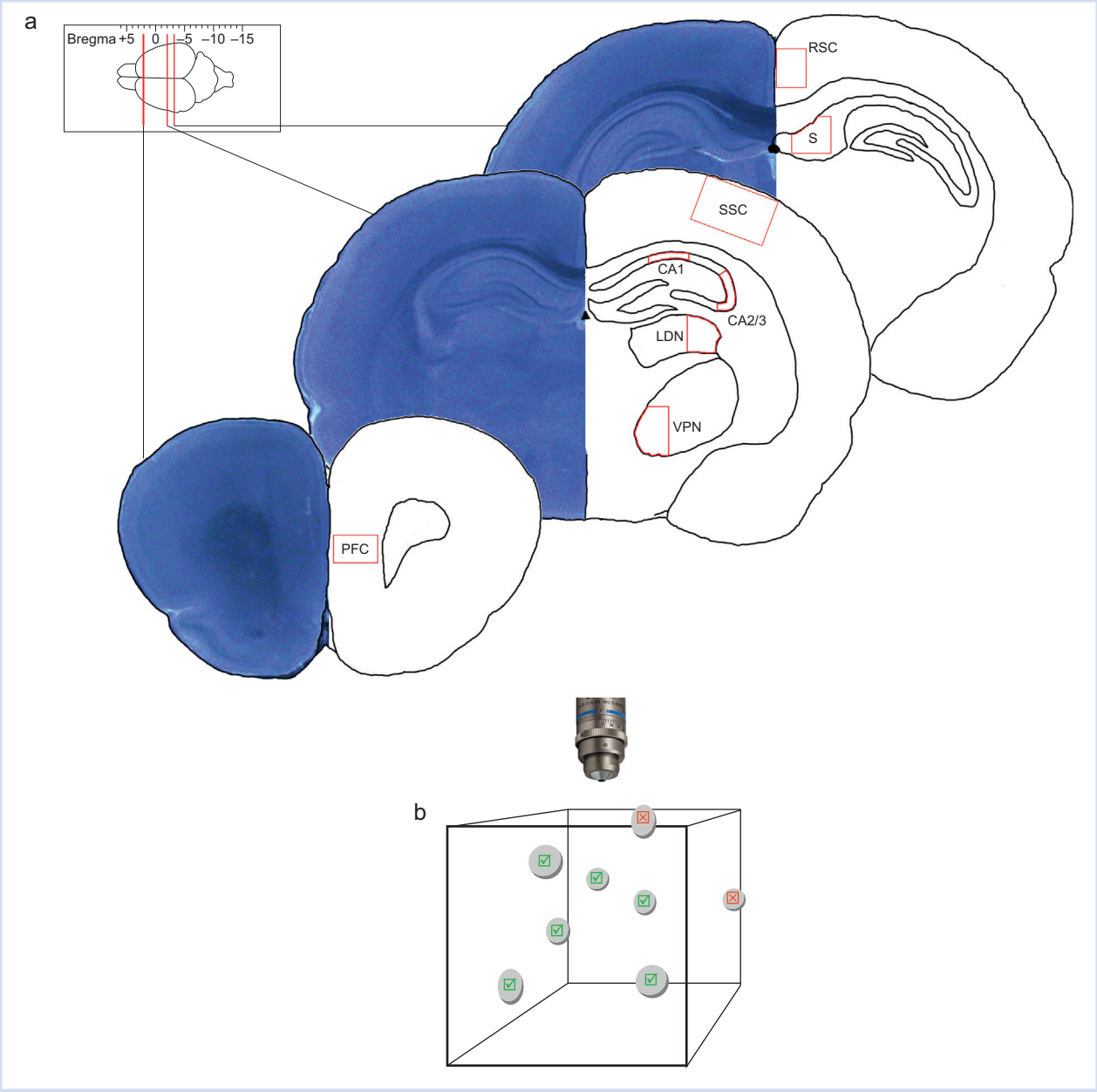


Fig 1. Schematic depiction of methods used for quantification of apoptotic neurones in representative brain regions. Regions defined according to the developing rat brain atlas.[31] (a) Sections were cut coronally to analyse prefrontal cortex (PFC), hippocampal cornu ammonis 1 and 2/3 (CA1 and CA2/3), thalamic laterodorsal nucleus (LDN), thalamic ventroposterior nucleus (VPN), somatosensory cortex (SSC), subiculum (S), and retrosplenial cortex (RSC), as outlined in red. (b) Degenerating cells co-expressing activated caspase 3, a marker for apoptotic cell death; the neuronal marker neuronal nuclei; and the nuclear marker 4′,6-diamidino-2-phenylindole were quantified by unbiased stereology throughout entire tissue sections using the optical disector method and confocal microscopy. All triple-labelled cells within the imaging z-stacks were counted (labelled with green check marks in schematic), except for those transecting the three exclusion planes (top, back, and right side, represented by a red 'x' in the schematic). Density counts were calculated using the tissue volume determined in Neurolucida utilising the counting dimensions and width of the tissue, and were averaged by brain region for each experimental group. Figure modified from Khazipov and colleagues.[31].

Histological studies

After the 6-h exposure, the animals were recovered for 30 min, and then injected i.p. with ketamine (20 mg kg^{-1}), acepromazine (0.5 mg kg^{-1}), and xylazine (1 mg kg^{-1}) before a sternotomy for transcardial perfusion with phosphate-buffered saline (PBS) 10 ml with sucrose 5% and glycerol 5%, followed by paraformaldehyde (PFA) 4%, 10 ml with sucrose 5% and glycerol 5%. Brains were removed, immersed in PFA 4% for fixation, cryoprotected serially in 20% and 30% sucrose in PBS, and frozen at −80°C. For immunohistochemical study, brains were cryosectioned in the coronal plane at 50 μm using a Cryotome

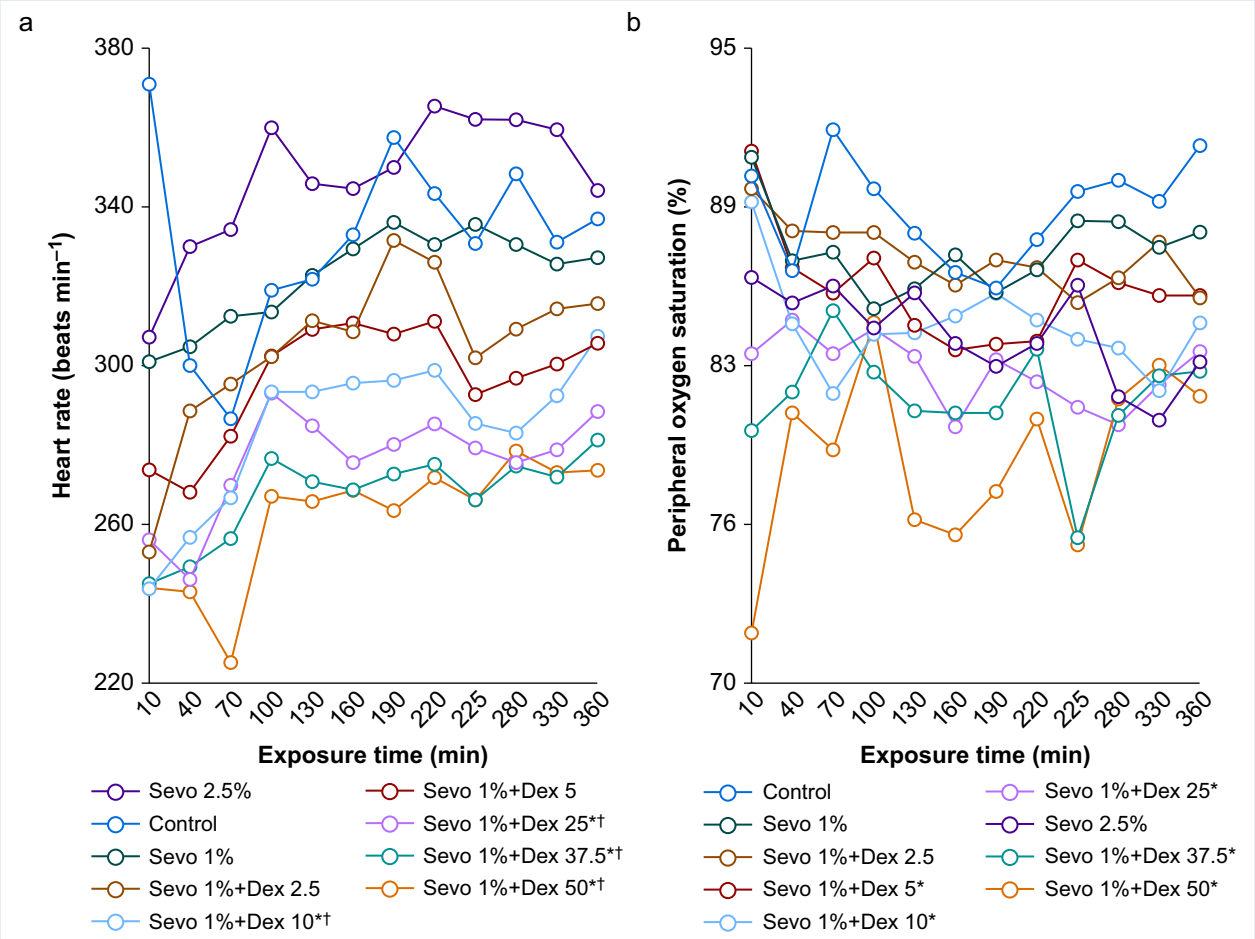


Fig 2. Heart rate and peripheral oxygen saturation. Vital signs were measured every 30 min during anaesthetic exposure in 7-day-old rats. Data represent group averages for animals exposed to sevoflurane 2.5% (Sevo 2.5%; n=10), or to sevoflurane 1% without (Sevo 1%; n=10) or with dexmedetomidine at 2.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 2.5; n=10), 5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 5; n=10), 10 μg kg^{-1} h^{-1} (Sevo 1%+Dex 10; n=9), 25 μg kg^{-1} h^{-1} (Sevo 1%+Dex 25; n=10), 37.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 37.5; n=10), or 50 μg kg^{-1} h^{-1} (Sevo 1%+Dex 50; n=10), or to room air (control; n=8). (a) Heart rate was highest in animals exposed to sevoflurane 2.5% and decreased significantly with dexmedetomidine at 10 μg kg^{-1} h^{-1} or greater plus sevoflurane 1%. Similarly, compared with control animals. (b) Peripheral oxygen saturation was significantly lower in all anaesthetised groups with dexmedetomidine at 5 μg kg^{-1} h^{-1} or greater. Oxygen saturations observed in animals anaesthetised with sevoflurane 1% plus dexmedetomidine at 10 or 25 μg kg^{-1} h^{-1} were comparable with animals exposed to sevoflurane 2.5%. Statistical analysis using mixed-effects linear model with Bonferroni correction for multiple comparisons. P<0.00138 signifies statistical significance. *Statistical significance compared with unanaesthetised controls. †Statistical significance compared with Sevo 2.5%.

SME (Thermo Fisher Scientific, Waltham, MA, USA). Sections were mounted on positively charged slides (Gorilla Scientific, Gainesville, VA, USA) and stored at −80°C until use. Neuroapoptosis was quantified by an investigator unaware of group assignment in sections located at bregma +2.2, −1.8, and −2.8 mm, corresponding to figures 23, 43, and 48, respectively, in the developing rat brain atlas,[31] for prefrontal cortex, retrosplenial cortex, somatosensory cortex, cornu ammonis (CA)1, CA2/3, laterodorsal and ventral thalamic nuclei, and subiculum (Fig. 1a).[30] Slides containing the selected sections were stained for activated cleaved caspase 3 (AC3), the executioner caspase and a marker of commitment to apoptotic cell death; neuronal nuclei (NeuN), which represents a neuron-specific protein signifying a post-mitotic stage of development; and 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to adenine- and thymine-rich regions in DNA, labelling cellular nuclei of all brain cells. For antigen retrieval, sections were treated with PBS with Tween-20 5%, Triton X-100 5%, sodium dodecyl sulfate 1%, and Tris (2-carboxyethyl) phosphine 0.1% for 16–18 h at room temperature, followed by incubation for 20 min at 100°C in a 1:10 dilution of sodium citrate buffer at pH 6.0 (CB910 m; Biocare, Concord, CA, USA) in Coplin jars. After these steps, slides were incubated in 2M HCl at room temperature for 1 h and washed twice in phosphate buffer (pH 8.5). Sections were blocked in donkey serum 5%, glycine 0.5%, and casein 0.75% in PBS for 2 h at room temperature before two conjugated antibodies in blocking solution were added: 1:200 dilution of rabbit cleaved caspase 3 antibody (9661L; Cell Signaling Technology, Danvers, MA, USA) and 1:200 anti-NeuN (MAB377; Millipore, Temecula, CA, USA). After incubation at room temperature, sections were rinsed four times with PBS for 5 min, and then dehydrated in an ascending

ethanol series, washed with PBS, followed by Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA, USA), and coverslipped.

Quantification of apoptosis

Quantification of cellular apoptosis was performed after collection of three-channel confocal image stacks of triple-immunostained sections through the *z*-dimension from regions of interest using a Nikon Eclipse Ti inverted confocal microscope with an A1R controller (Nikon Instruments, Inc., Melville, NY, USA) and a 20×, 1.2 numerical aperture objective. Regions were outlined in the DAPI channel, as shown in Fig 1a, and images were collected with $\alpha \times 1$ optical zoom, in 0.5-μm steps, format 1024 * 1024. DAPI was excited using the 405 nm laser line, and emission wavelengths were captured between 358 and 461 nm. Activated cleaved caspase 3 immunostaining was excited using 488 nm laser line, and emission wavelengths between 495 and 550 nm were used. Neuronal nuclei immunostaining was excited using the 633 nm laser line while collecting emission wavelengths between 650 and 710 nm. Resulting image stacks were transferred to Neurolucida image analysis software (version 10.31; MBF Bioscience, Williston, VT, USA) and assessed by three investigators unaware of group assignment. The number of $AC3^{+}$ neurones with NeuN co-staining was quantified using the optical disector method.[8,30,32,33] Densities of apoptotic cells were quantified as cells per cubic millimetre of examined tissue for each animal, and group averages were calculated for the examined brain regions (Fig. 1b). To reduce bias, sections were de-identified to keep the assessor unaware of group assignments, and outlined for all animals with the channel displaying caspase expression hidden. Several regions were counted by two investigators to provide external validity for the unbiased nature of the quantification process.

Statistical analysis

Group sizes were determined *a priori*; based on previous immunohistochemical experiments and because of inter-individual variability, seven to eight animals per group were deemed required to reach a significance level of 5% with 80% power on a two-sample z-test. Data were tested for normality using Wilk–Shapiro test, but are presented as mean (standard error of the mean) or percentage, as applicable, to facilitate visual comparisons. Neuronal apoptosis was compared between groups using Kruskal–Wallis rank test with Dunn's test for multiple comparisons. Group comparisons of vital signs, including heart rate and peripheral oxygen saturation, were conducted using three-level mixed-effect linear regression models accounting for clustering of rats within the same litters and for repeated measures of the same rats over time. Time and treatment group were included as categorical variables in the fixed effects, and random intercepts of litter ID and rats ID were allowed. We calculated pairwise contrasts for each of the nine treatment groups, compared them with the other eight groups, and applied Bonferroni corrections to adjust for multiple comparisons (9 * 8/2=36). Simultaneously, 95% confidence intervals were calculated, resulting in (1–0.05/36)=99.86% confidence intervals for each individual comparison. Accordingly, $P<0.00138$ (0.05/36) was considered statistically significant. Ordinal data, such as LORR and pain response, were analysed using Friedman test with Benjamini–Hochberg correction for multiple comparisons. Analyses were performed using Stata version 10.1 (StataCorp LLC, College Station, TX, USA), and significance was accepted at $P<0.05$, except as noted above.

Results

All animals survived the 6-h fasting or anaesthetic exposures. Heart rate ranged between 250 and 350 min^{-1} throughout the 6-h experimental period (Fig. 2a); average heart rates were highest in control animals and those exposed to sevoflurane 2.5% or to sevoflurane 1% plus dexmedetomidine in doses up to 5 μg kg^{-1} h^{-1}. Significantly lower heart rates were observed for dexmedetomidine 10 μg kg^{-1} h^{-1} or higher (Fig. 2a; Table 1).

Peripheral oxygen saturations averaged 80–92% in all animals and were highest in controls and animals exposed to sevoflurane 1% alone or in combination with dexmedetomidine 2.5 μg kg^{-1} h^{-1}. Animals anaesthetised with sevoflurane 1% and supplemented with dexmedetomidine 5 μg kg^{-1} h^{-1} or above exhibited lower oxygen saturations (Fig. 2b; Table 1).

Hypnosis and pain, measured by repeated assessments of the righting reflex and paw clamp response, respectively, were significantly different in all anaesthetised groups compared with control animals. Dexmedetomidine supplementation, even in the lowest dose of 2.5 μg kg^{-1} h^{-1}, significantly decreased LORR scores (Fig. 3a) and the response to pain (Fig. 3b) compared with sevoflurane 1% alone. The LORR scores in animals exposed to a combination of sevoflurane 1% and dexmedetomidine 10 μg kg^{-1} h^{-1} or above reached those of animals anaesthetised with sevoflurane 2.5%, whereas pain scores of those supplemented with dexmedetomidine 25 μg kg^{-1} h^{-1} were indistinguishable from sevoflurane 2.5%.

Histological analysis of neuronal apoptotic cell death after a 6-h exposure to sevoflurane 2.5% compared with unanaesthetised littermates showed significant increases in activated caspase 3 expression in all brain regions examined (i.e. somatosensory cortex, hippocampal CA2/3, subiculum, CA1, retrosplenial and prefrontal cortices, and ventral posterior and lateral dorsal thalamic nuclei) (Fig. 4). Neurodegeneration caused by the combination of sevoflurane 1% and dexmedetomidine 25 μg kg^{-1} h^{-1}, the lowest dose achieving comparable levels of anaesthesia and analgesia as sevoflurane 2.5%, was significantly increased compared with controls and indistinguishable from that caused by sevoflurane 2.5%. Somatosensory cortex appeared to be most vulnerable to sevoflurane exposure, as all anaesthetised animals, regardless of sevoflurane or dexmedetomidine doses, experienced significantly increased neuronal cell death, followed by hippocampal CA2/3 and prefrontal cortex. Conversely, CA1 and the ventroposterior thalamic nucleus required sevoflurane 1% with dexmedetomidine supplementation of 25 μg kg^{-1} h^{-1} or sevoflurane 2.5% to exceed the natural apoptotic cell death observed in unanesthetised animals. Figure 5 depicts representative histological sections.

Discussion

Anaesthetic exposure causes structural abnormalities in the developing mammalian brain. Neuronal cell death, dendritic alterations, and synaptic abnormalities have been demonstrated for all general anaesthetics in a variety of animal species,[1] including after 3 h of isoflurane in non-human primates.[34] As this phenomenon spans many mammalian

Table 1 Mixed-effect linear regression model contrast results for heart rate and peripheral oxygen saturation (SpO_2) comparing experimental groups. Heart rates in animals exposed to sevoflurane (Sevo) 1% were statistically significantly lower compared with control animals once dexmedetomidine (Dex) supplementation exceeded 10 μg kg^{-1} h^{-1}. Oxygen saturation statistically significantly differed from controls for dexmedetomidine supplementation starting at 5 μg kg^{-1} h^{-1}. Data cells display point differences (standard error); (Bonferroni 95% family-wise confidence intervals); and *P*-values, calculated between each treatment group at the top of each column and all reference groups in the first cell of each row. *Statistical significance following Bonferroni correction $P<0.00138$ (0.05/36).

	Sevo 1%	Sevo 1% + Dex 2.5	Sevo 1% + Dex 5	Sevo 1% + Dex 10	Sevo 1% + Dex 25	Sevo 1% + Dex 37.5	Sevo 1% + Dex 50	Sevo 2.5%
Heart rate								
Control	5.3 (6.7) (−17.5; 8.0) P=0.437	−7.1 (6.8) (−30.3; 16.0) P=0.299	−14.4 (6.8) (−37.7; 8.8) P=0.040	−31.1 (6.8) (−54.3; −7.9) P<0.0001*	−40.8 (6.8) (−63.9; −17.7) P<0.0001*	−48.1 (6.8) (−71.2; −24.9) P<0.0001*	−49.9 (6.9) (−73/4; −26.4) P<0.0001*	25.3 (12.6) (−17.8; 68.4) P=0.052
Sevo 1%		−12.4 (5.7) (−31.9; 7.1) P=0.036	−19.7 (5.8) (−39.3; −0.1) P=0.0013*	−36.3 (5.8) (−55.9; −16.7) P<0.0001*	−46.0 (5.8) (−65.7; −26.3) P<0.0001*	−53.3 (5.8) (−73.0; −33.6) P<0.0001*	−55.2 (5.9) (−62.9; −22.6) P<0.0001*	20.1 (12.1) (−8.9; 73.8) P=0.105
Sevo 1%+Dex 2.5			−7.3 (5.7) (−26.7; 12.1) P=0.208	−23.9 (5.7) (−43.4; −4.5) P=0.0001*	−33.6 (5.8) (−53.4; −13.9) P<0.0001*	−40.9 (5.9) (−60.9; −20.9) P<0.0001*	−42.8 (5.9) (−62.9; −22.6) P<0.0001*	32.5 (12.2) (−8.9; 73.8) P=0.011
Sevo 1%+Dex 5				−16.6 (5.7) (−36.1; 2.9) P=0.006	−26.3 (5.8) (−46.1; −6.5) P<0.0001*	−33.6 (5.9) (−53.7; −13.6) P<0.0001*	−35.5 (5.9) (−55.7; −15.2) P<0.0001*	39.8 (−1.7; 81.2) P=0.002
Sevo 1%+Dex 10					−9.7 (5.8) (−29.5; 10.1) P=0.102	−17.0 (5.9) (−37.0; 3.1) P=0.006	−18.8 (5.9) (−39.1; 1.4) P=0.003	56.4 (12.2) (15.0; 97.8) P<0.0001*
Sevo 1%+Dex 25						−7.3 (5.9) (−27.4; 12.8) P=0.224	−9.1 (6.0) (−29.7; 11.4) P=0.137	66.1 (12.0) (25.1; 107.1) P<0.0001*
Sevo 1%+Dex 37.5							−1.9 (5.9) (−21.8; 18.11) P=0.754	73.4 (12.2) (31.9; 114.9) P<0.0001*
Sevo 1%+Dex 50								75.2 (12.4) (33.1; 117.3) P<0.0001*
SpO_2								
Control	−2.2 (1.0) (−5.7; 1.2) P=0.032	−2.7 (1.0) (−6.2; 0.8) P=0.011	−3.6 (1.0) (−7.2; −0.1) P=0.001*	−5.5 (1.0) (−9.0; −1.9) P<0.0001*	−6.8 (1.0) (−10.3; −3.3) P<0.0001*	−8.5 (1.0) (−12.0; −5.0) P<0.0001*	−10.4 (1.0) (−14.0; −3.3) P<0.0001*	−5.1 (1.7) (−10.8; 0.6) P=0.004
Sevo 1%		−0.5 (0.9) (−3.4; 2.4) P=0.579	−1.4 (0.9) (−4.3; 1.5) P=0.107	−3.2 (0.9) (−6.1; −0.3) P=0.0005*	−4.6 (0.9) (−7.5; −1.6) P<0.0001*	−6.3 (0.9) (−9.2; −3.3) P<0.0001*	−8.2 (0.9) (−11.2; −5.2) P<0.0001*	−2.9 (1.6) (−8.3; 2.5) P=0.077
Sevo 1%+Dex 2.5			−0.9 (0.9) (−3.8; 2.0) P=0.28	−2.7 (0.9) (−5.6; 0.2) P=0.002	−4.1 (0.9) (−7.0; −1.1) P<0.0001*	−5.8 (0.9) (−8.8; −2.8) P<0.0001*	−7.7 (0.9) (−10.7; −4.7) P<0.0001*	−2.4 (1.6) (−7.8; 3.0) P=0.139
Sevo 1%+Dex 5				−1.8 (0.9) (−4.7; 1.1) P=0.04	−3.1 (0.9) (−6.1; −0.2) P=0.0007*	−4.8 (0.9) (−7.8; −1.8) P<0.0001*	−6.8 (0.9) (−9.8; −3.7) P<0.0001*	−1.5 (1.6) (−6.9; 4.0) P=0.364
Sevo 1%+Dex 10					−1.3 (0.9) (−4.3; 1.6) P=0.132	−3.0 (0.9) (−6.0; −0.03) P=0.0013*	−4.9 (0.9) (−8.0; −1.9) P<0.0001*	0.4 (1.6) (−5.1; 5.8) P=0.825
Sevo 1%+Dex 25						−1.7 (0.9) (−4.7; 1.3) P=0.060	−3.6 (0.9) (−6.7; −0.5) P=0.0002*	1.7 (1.6) (−3.7; 7.0) P=0.291
Sevo 1%+Dex 37.5							−1.9 (0.9) (−4.9; 1.1) P=0.036	3.4 (1.6) (−2.0; 8.8) P=0.040
Sevo 1%+Dex 50								5.3 (1.6) (−0.2; 10.8) P=0.002

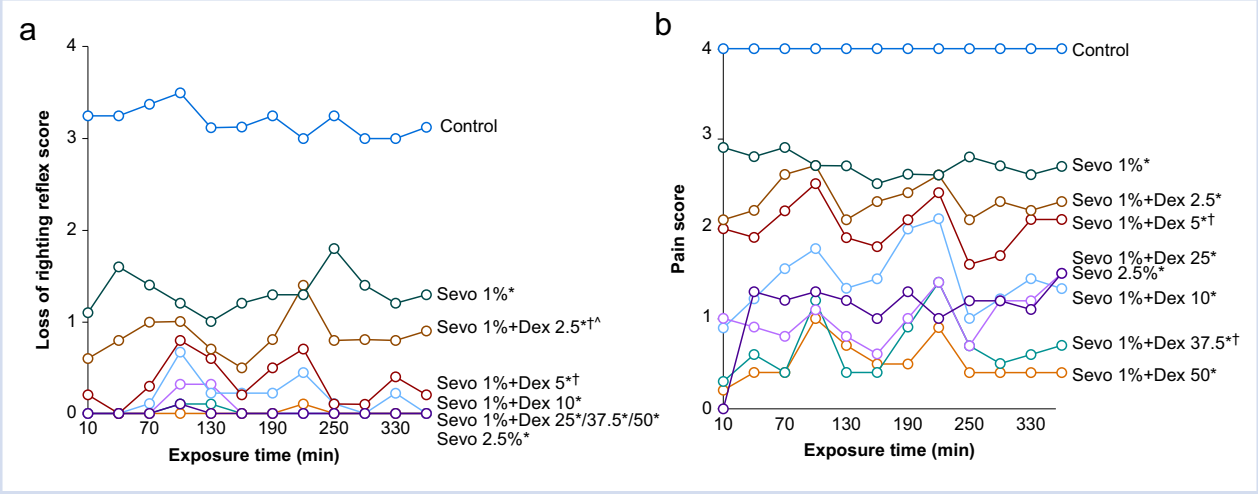


Fig 3. Loss of righting reflex and response to pain. These were measured every 30 min during anaesthetic exposure in 7-day-old rats. Data represent group averages for animals exposed to sevoflurane 2.5% (Sevo 2.5%; n=10), or to sevoflurane 1% without (Sevo 1%; n=10) or with dexmedetomidine at 2.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 2.5; n=10), 5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 5; n=10), 10 μg kg^{-1} h^{-1} (Sevo 1%+Dex 10; n=9), 25 μg kg^{-1} h^{-1} (Sevo 1%+Dex 25; n=10), 37.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 37.5; n=10), or 50 μg kg^{-1} h^{-1} (Sevo 1%+Dex 50; n=10), or to room air (control; n=8). (a) Loss of righting reflex, a measure for sedation/anaesthesia, was maintained in control animals. Animals exposed to sevoflurane 1% alone were modestly sedated and reached concentrations of anaesthesia comparable with animals exposed to sevoflurane 2.5% with dexmedetomidine at 10 μg kg^{-1} h^{-1} or more. (b) Pain responses were strongest in controls, whereas animals anaesthetised with sevoflurane 1% with dexmedetomidine 25 μg kg^{-1} h^{-1} reached pain responses comparable with animals exposed to sevoflurane 2.5%. *P<0.001 compared with unanaesthetised controls, †P<0.001 compared with Sevo 2.5%, and ^P<0.001 compared with Sevo 1%, using Friedman's test with Benjamini–Hochberg correction for multiple comparisons.

species, it is unlikely that human infants and toddlers are uniquely immune to these structural changes. To address these concerns,[35,36] the US FDA and the International Anesthesia Research Society formed SmartTots in a public–private partnership to support research into this clinical question. Although the long-term effects of anaesthetic exposure in children may not entirely match the findings obtained in animals,[21,37,38] as lasting cognitive abnormalities have not been detected after anaesthetic exposures of under 2 h in young children,[13–15] the potentially serious personal and societal ramifications of a widely used medical treatment utilized in millions of children worldwide require further research into the mechanisms and potentially mitigating strategies and alternative anaesthetic techniques.

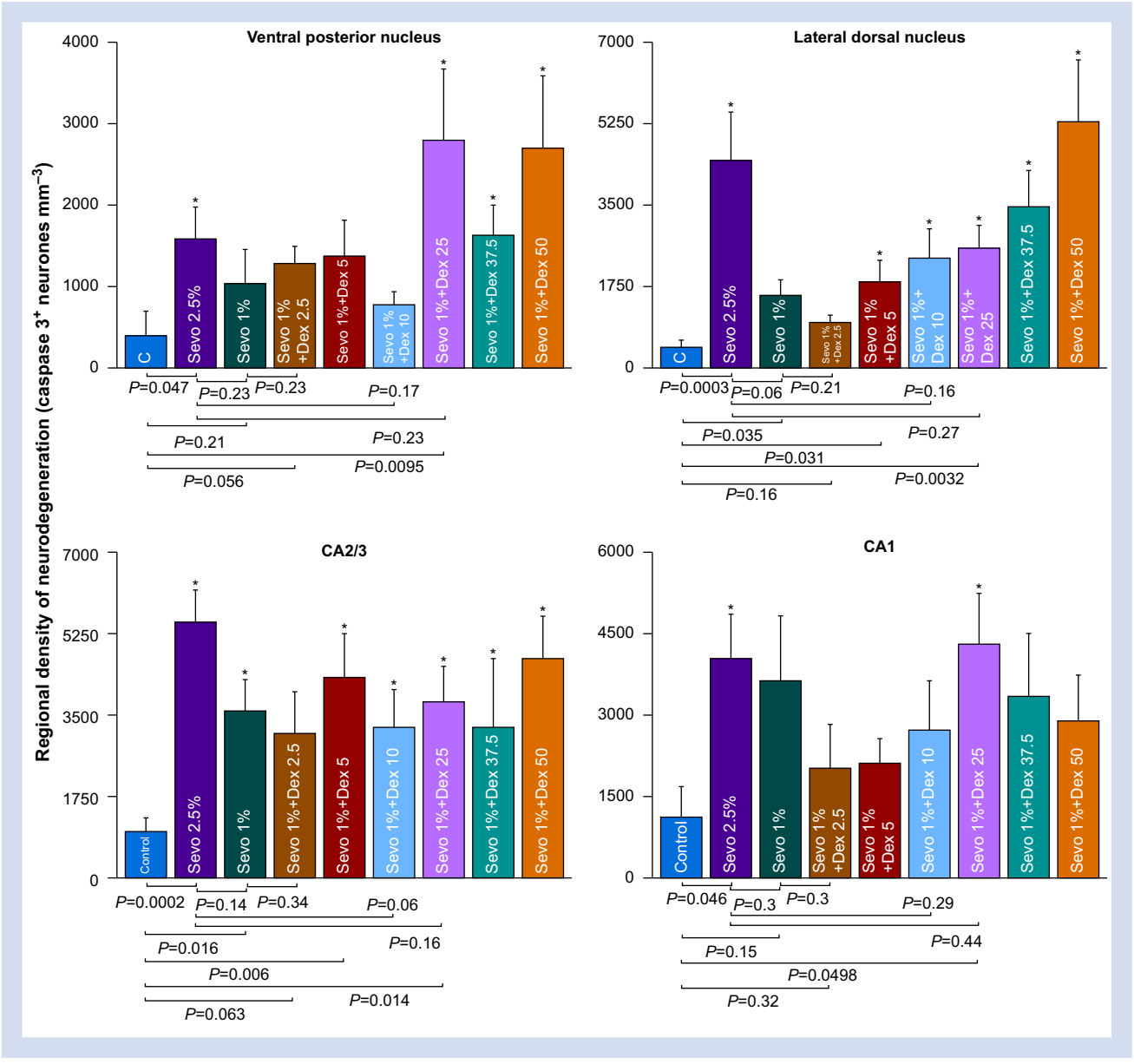
One such mitigating strategy is to incorporate the α_2-adrenergic agonist dexmedetomidine, which lacks affinity for gamma-aminobutyric acid type A and N-methyl-D-aspartate receptors that have been implicated in the mechanism for anaesthetic neurotoxicity, in an approach to reduce dose requirements for the injurious general anaesthetic sevoflurane. Dexmedetomidine has been found to diminish neuronal injury during brain ischaemia,[22] ketamine[23] and isoflurane exposure,[24,25] and sevoflurane-induced neuroapoptosis.[26]

When evaluating different anaesthetic regimens, it becomes critically important not only to match exposure times, but also to compare regimens at similar depths of anaesthesia. Dexmedetomidine administered alone for 6 h at 2.5–50 μg kg^{-1} h^{-1} causes significantly less apoptotic neuronal cell death than sevoflurane 2.5%. Moreover, we have previously found that the addition of dexmedetomidine to sevoflurane 2.5% did not protect, but rather exacerbated the deleterious effects of sevoflurane.[30] An explanation for this unexpected result could be the greater depth of anaesthesia provided by the drug combination, causing cardiorespiratory depression, which resulted in brain injury.[39] Accordingly, the present study was designed to compare two anaesthetic regimens, sevoflurane alone or a lower dose of sevoflurane plus dexmedetomidine, at similar concentrations of anaesthesia and analgesia, as determined in a dose-finding approach, in their propensity to induce neuroapoptotic cell death.

The potencies of inhaled anaesthetics are comparable in humans and small rodents, with sevoflurane 1% equating to around 0.3 MAC and 2.5% to approximately 0.6–0.7 MAC.[27,40] Importantly, however, small animals require substantially higher weight-based doses of injectable anaesthetics and sedatives.[41] Accordingly, we studied supplemental doses of dexmedetomidine that were appropriate for small rodents, ranging from 2.5 to 50 μg kg^{-1} h^{-1}, and found that the level of anaesthesia and pain relief from sevoflurane 2.5% by itself was comparable with sevoflurane 1% plus 25 μg kg^{-1} h^{-1} dexmedetomidine.

In contrast to a study that reported protective effects of adding dexmedetomidine to 0.3 MAC of isoflurane in P7 Sprague–Dawley rats,[24] we did not find any protective effect of adding dexmedetomidine to 0.3 MAC of sevoflurane, for any of the examined brain regions in Wistar rats. Although the genetic background is unlikely to explain the observed discrepancy, our focus on a wider range of brain regions,[33 42] methodological differences in the type of anaesthetic (isoflurane *vs* sevoflurane), sampling, staining, or quantification could explain the dissimilarities.

Interestingly, we have shown previously similar levels of neurodegeneration comparing equipotent doses of desflurane, isoflurane, and sevoflurane in newborn mice.[8]



These results, combined with the current findings, suggest that depth of anaesthesia, rather than any specific receptor or drug target, determines the degree of neuronal degeneration. However, recent findings of a lack of neuroapoptosis and cognitive impairment after a general anaesthetic with a neurosteroid analogue argue against universally deleterious effects of anaesthesia *per se*.[43] Accordingly, the effect on depth of anaesthesia on neuroapoptosis requires further investigation.

It is uncertain whether regional neuroapoptosis immediately after exposure represents a correlate for specific long-term structural and cognitive abnormalities.[10,44] Accordingly, it remains possible that dexmedetomidine can preserve long-term neurocognitive function, despite not ameliorating post-exposure neuroapoptosis. Because of our dose-escalation schema and our blocking paradigm by mating pair to minimise inter-litter variability, we were unable to generate adequate numbers of animals for both immediate histological and long-term neurocognitive assessments. Clinical investigations into the efficacy of dexmedetomidine supplementation during infant surgery on long-term cognitive outcomes are ongoing and might answer this question.[45]

Our study has several limitations related to current constraints in monitoring arterial pressure or electroencephalogram in neonatal rodents, and technical challenges precluding routine tracheal intubation and mechanical ventilation, as utilised during paediatric anaesthesia.[46] Anaesthetic exposures can lead to arterial blood gas abnormalities and mortality in neonatal rodents,[28] which could result in mixed neuronal injuries of hypoxaemia–ischaemia and anaesthetic neurotoxicity. However, we did not observe any mortality, contrasting with previous studies from our laboratory utilising higher anaesthetic doses.[28] Moreover, despite limitations, heart rate and oxygen saturation were clinically comparable with control subjects. Our monitoring showed limited effects of dexmedetomidine as high as 50 μg kg^{-1} h^{-1} on heart rate (20% reduction) similar to previous findings,[30,47] suggesting that cardiac output was likely not substantially diminished in

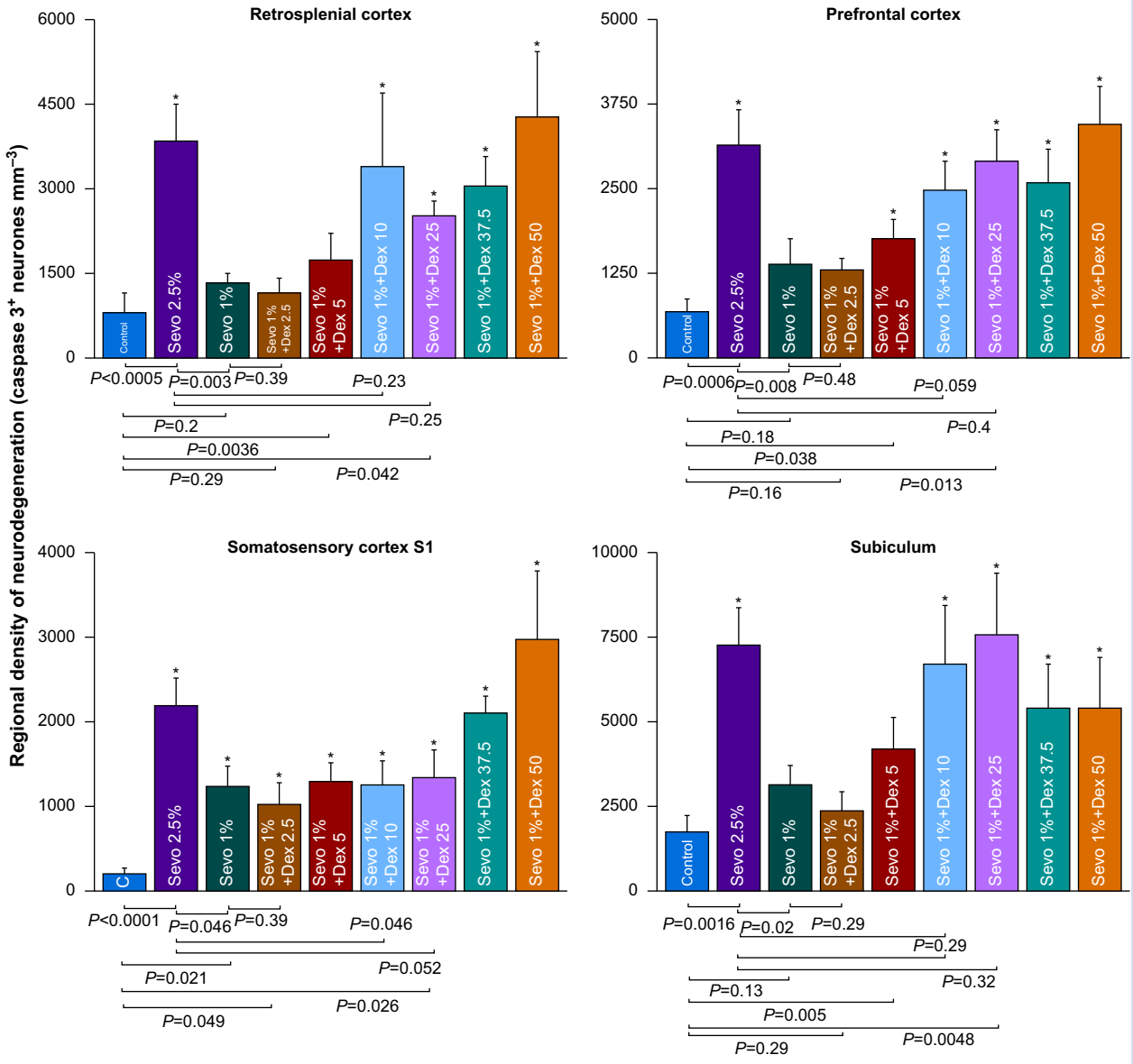


Fig 4. Prolonged sevoflurane exposure increased the density of apoptotic neurones. Bar graphs represent average density counts of dying cells as assessed by expression of the apoptotic marker activated caspase 3 in representative cortical, thalamic, and hippocampal brain regions in 7-day-old rats exposed to sevoflurane 2.5% (Sevo 2.5%; n=10), or to sevoflurane 1% without (Sevo 1%; n=10) or with dexmedetomidine at 2.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 2.5; n=10), 5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 5; n=10), 10 μg kg^{-1} h^{-1} (Sevo 1%+Dex 10; n=9), 25 μg kg^{-1} h^{-1} (Sevo 1%+Dex 25; n=10), 37.5 μg kg^{-1} h^{-1} (Sevo 1%+Dex 37.5; n=10), or 50 μg kg^{-1} h^{-1} (Sevo 1%+Dex 50; n=10), or to room air (control; n=8). C represents the control group (n=8), which was injected with normal saline. Exposure to sevoflurane 1% led to significant increases in activated caspase 3 expression in somatosensory cortex, hippocampal cornu ammonis (CA)2/3, and the lateral dorsal thalamic nucleus compared with unanaesthetised control animals, whereas sevoflurane 2.5% caused significant neuroapoptosis in all examined brain regions. Dexmedetomidine did not diminish the apoptotic effects of sevoflurane 1% in any regions. Data are represented as bar graphs to facilitate comparisons; however, non-parametric statistics were used for group comparisons, as several data cells violated normality criteria. *P<0.05 compared with control, using Kruskal–Wallis test with Dunn's test for multiple comparisons.

our animals. The combination of sub-anaesthetic sevoflurane plus dexmedetomidine to produce similar depths of anaesthesia and analgesia as sevoflurane 2.5% also caused comparable levels of desaturation. However, as the depth of anaesthesia and the level of desaturation coincided, it was not possible to determine their respective contributions to the observed neurodegeneration. Although oxygen saturation was significantly lower in animals anaesthetised with sevoflurane and dexmedetomidine at 5 μg kg^{-1} h^{-1} or higher compared with unanaesthetised controls, the clinical significance of

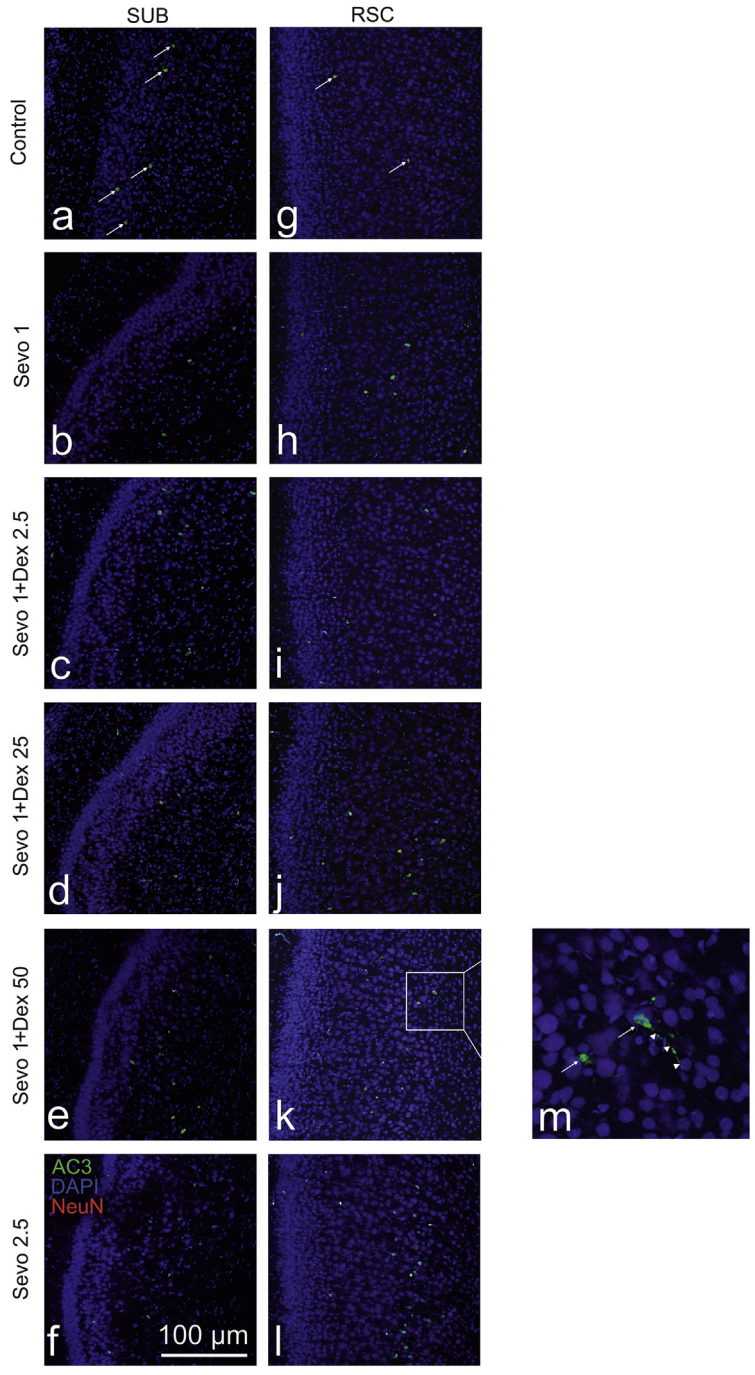


Fig 5. Prolonged sevoflurane exposure increased the number of neurones undergoing cell death labelled with the apoptotic marker activated caspase 3. Representative three-channel merged maximum projection photomicrographs obtained using confocal microscopy of brain sections from 7-day-old rat pups depicting (a–f) subiculum (SUB) and (g–l) retrosplenial cortex (RSC) at 20× magnification, after three injections of normal saline (control), sevoflurane 1% (Sevo 1%), sevoflurane 1% with dexmedetomidine at 2.5 µg kg^{-1} h^{-1} (Sevo 1%+Dex 2.5), 25 µg kg^{-1} h^{-1} (Sevo 1%+Dex 25), 50 µg kg^{-1} h^{-1} (Sevo 1%+Dex 50), or sevoflurane 2.5%. Other doses and brain regions are not shown, but are included in quantification. Tissue sections were stained for the postmitotic neuronal marker neuronal nuclei (NeuN; red), the cellular marker 4′,6-diamidino-2-phenylindole (DAPI; blue), and the apoptotic marker activated caspase 3 (AC3; green). (m) Inset shows neurones undergoing apoptosis at higher magnification; arrows represent neurones at various stages of neurodegeneration, expressing activated caspase 3; note dendritic beading as a sign for cellular disintegration (arrowheads). Degenerating neurones are surrounded by immediately adjacent, seemingly viable neurones. Scale bar: 100 µm.

diminished oxygen concentrations in the 80–90% range remains unresolved. This is especially true in light of the surprisingly low readings in control animals, which question the reliability of current oxygenation monitoring technology for neonatal rodents. The fact that neuronal injuries after anaesthetic exposures are qualitatively and regionally similar amongst spontaneously breathing small rodents with limited monitoring capabilities and stringently monitored, intubated, and mechanically ventilated non-human primates[8,48] suggests that anaesthesia-induced neurodegeneration can still be reliably studied in newborn rodents. However, particular attention needs to be paid to improving oxygenation monitoring capabilities for small animals.

Although 6 h is outside of routine paediatric anaesthetic exposures, it represents a frequent exposure time utilised in the laboratory setting,[1] facilitating comparisons with previous studies. We monitored pain responses with intermittent paw clamping, delivering repeated painful stimulation during the entire exposure. However, it remains unresolved whether such noxious stimulation during, or pain and stress after, anaesthesia exposure exacerbates,[49–51] ameliorates,[5253] or has no effect on structural or neurocognitive outcomes.[54] Moreover, we measured neuroapoptosis at its peak, and the long-term effects of the immediate loss of these neurones are unclear.[55] To avoid opioid-induced hypoventilation and further confound hypoxaemic–ischaemic and anaesthetic brain injuries, we did not include an opioid in our anaesthetic regimen, which is frequently used during perioperative care in young children.

Quantification of anaesthesia-induced apoptosis relied on stereological counts based on the well-validated optical dissector method. As regional neuronal vulnerability and injury patterns change with age,[42] dexmedetomidine could provide protection for other brain regions not examined or at older ages. Significant periods of brain development take place *in utero* in humans, but occur postnatally in small rodents. Important brain maturational events, such as regional neurogenesis, synaptogenesis, axon growth, myelin formation, brain regional development, and behavioural milestones, differ amongst species relative to gestational age and occur on dissimilar trajectories. It is therefore difficult to identify the human brain maturational stage equivalent to the P7 rat, but it is generally accepted to be equivalent to the late gestational, early neonatal period.[56] Accordingly, the observed injury pattern may not directly translate to older children undergoing anaesthesia. And lastly, although our randomisation schema did not allow for long-term neurocognitive studies or direct comparisons to animals exposed to dexmedetomidine as a single agent, we have previously shown that even dexmedetomidine as high as 50 μg kg^{-1} h^{-1} does not provide adequate levels of anaesthesia. This is consistent with clinical experience that dexmedetomidine represents a sedative and analgesic that does not produce general anaesthesia.

Conclusions

Deleterious brain structural effects observed in developing animals during prolonged exposures to inhaled anaesthetics, such as isoflurane and sevoflurane, raise substantial concerns for clinical practice. Dexmedetomidine, which does not act by the same mechanism as injurious anaesthetics, ameliorates injury during brain ischaemia and isoflurane exposure. However, we did not observe protection when combining 0.3 MAC of sevoflurane plus dexmedetomidine at doses identified to provide concentrations of anaesthesia and analgesia comparable with 0.6 MAC sevoflurane alone. Future animal studies comparing different anaesthetic regimens should include monitoring of depth of anaesthesia and continuous oxygenation throughout anaesthetic exposure.

Authors' contributions

Study design/planning: AWL
Study conduct: J-RL, BJ, RDH, BU, SYL, LE
Data analysis: J-RL, BJ, RDH, BZ, AWL
Writing of paper: J-RL, SCD, AWL
Revising of paper: AWL

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Declarations of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2021.01.033.

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