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Brain regional vulnerability to anaesthesia-induced neuroapoptosis shifts with age at exposure and extends into adulthood for some regions

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Editor's key points

- Pre-clinical studies of various species demonstrate anaesthetic-induced neonatal neurotoxicity.
- In the current study, mice of different ages were anaesthetized with isoflurane.
- Thereafter, neuroapoptosis was quantified in brain regions which have different age-related time courses of neurogenesis.
- Susceptibility to apoptosis appeared to parallel peaks of neurogenesis, and continued into adulthood in some areas.

Background. General anaesthesia facilitates surgical operations and painful interventions in millions of patients every year. Recent observations of anaesthetic-induced neuronal cell death in newborn animals have raised substantial concerns for young children undergoing anaesthesia. However, it remains unclear why some brain regions are more affected than others, why certain neurones are eliminated while neighbouring cells are seemingly unaffected, and what renders the developing brain exquisitely vulnerable, while the adult brain apparently remains resistant to the phenomenon.

Methods. Neonatal (P7), juvenile (P21), and young adult mice (P49) were anaesthetized with 1.5% isoflurane. At the conclusion of anaesthesia, activated cleaved caspase 3 (AC3), a marker of apoptotic cell death, was quantified in the neocortex (RSA), caudoputamen (CPu), hippocampal CA1 and dentate gyrus (DG), cerebellum (Cb), and olfactory bulb (GrO) and compared with that found in unanaesthetized littermates.

Results. After anaesthetic exposure, increased AC3 was detected in neonatal mice in RSA (11-fold, compared with controls), CPu (10-fold), CA1 (three-fold), Cb (four-fold), and GrO (four-fold). Surprisingly, AC3 continued to be elevated in the DG and GrO of juvenile (15- and 12-fold, respectively) and young adult mice (two- and four-fold, respectively).

Conclusions. The present study confirms the findings of previous studies showing peak vulnerability to anaesthesia-induced neuronal cell death in the newborn forebrain. It also shows sustained susceptibility into adulthood in areas of continued neurogenesis, substantially expanding the previously observed age of vulnerability. The differential windows of vulnerability among brain regions, which closely follow regional peaks in neurogenesis, may explain the heightened vulnerability of the developing brain because of its increased number of immature neurones.

Keywords: anaesthesia, paediatric; anaesthetics volatile, isoflurane; brain, injury; safety, drug; toxicity

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For more than one-and-a-half centuries, general anaesthesia has facilitated surgical operations and painful interventions in millions of patients, from premature infants into old age.[1] While hypnosis, analgesia, and immobility are transient effects of anaesthesia, recent findings from newborn and ageing animals have raised concerns that some anaesthetic effects may be undesired and longer lasting. One of the most concerning discoveries has been a widespread neuronal cell death observed in newborn animals after exposure to all common clinically used anaesthetics (reviewed in Istaphanous and colleagues[2] and Jevtovic-Todorovic).[3]

Neurones die via a process known as programmed cell death or apoptosis. Neuroapoptosis is a normal part of brain development in both animals and humans, and is important for eliminating excess and inappropriately integrated neurones. Exposure to anaesthetics, however, dramatically increases the rate of neuroapoptosis in developing animals, and subsequent neurocognitive impairment in these animals has heightened concerns for human patients. Reports of learning abnormalities and language impairments in children after surgery with anaesthesia early in life support these concerns.[4–6] However, whether similar neuronal cell death

occurs during clinical anaesthesia practice in humans remains uncertain.

Anaesthetic neuroapoptosis has been previously thought to be limited to animals before 14 days of life, best characterized in small rodents for the cerebral cortex and thalamus.[7 8] Moreover, studies in adult animals have thus far failed to detect any measurable cell death during anaesthetic exposure,[9] which has led to the interpretation that the phenomenon represents an inherent vulnerability of the developing brain.

However, it remains unclear why anaesthesia-induced neuroapoptosis is limited to this very early postnatal age and whether anaesthetic exposure affects all neurones similarly, regardless of their location in the brain. Brain regions develop on distinct trajectories of maturation and neurogenesis peaks at separate time points, relative to gestation, for different regions. Accordingly, we hypothesized that peak vulnerability to anaesthesia-induced neuroapoptosis among brain regions varies by age during exposure. This would predict that neuronal populations developing earlier in life are also more vulnerable to anaesthesia-induced neurodegeneration at an earlier developmental stage, whereas delayed vulnerability would be expected for brain regions with peaks of neurogenesis later in development. Accordingly, the present study quantified the expression of cleaved caspase 3, a marker of apoptotic cell death, in several brain regions experiencing peaks in neurogenesis early in development, such as retrosplenial cortex, caudoputamen, and hippocampal cornu ammonis layer I, and also regions with later peaks, such as cerebellum, dentate gyrus, and olfactory bulb in newborn, juvenile, and young adult mice after a 6 h anaesthetic exposure to isoflurane, comparing them with their respective, unanaesthetized littermates.

Methods

Animals

C57BL6/J mouse breeding pairs were housed on a 14/10 h light/dark cycle at 22°C and allowed free access to food and water. Offspring either remained with the parents until treatment on P7 (n=18) or P21 (n=17), or were weaned from the dam on P28, housed as gender-matched pairs and allowed to spontaneously exercise in running wheels for 3 weeks before treatment on P49 (n=28). P0 was considered as the day of birth. Immediately after anaesthetic exposure or fasting in room air, animals were euthanized and brains removed for further analysis. All experiments followed the National Institutes of Health guidelines, were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation, and aimed to minimize the number of animals used.

Anaesthesia exposure

On P7, P21, or P49, mice were randomly assigned to either a 6 h exposure to 1.5% isoflurane in 30% oxygen (Anaesthesia) or to fasting in room air (No Anaesthesia). Both female and male littermates were used, since preliminary experiments did not demonstrate any sex differences in neuroapoptosis (data not shown). Anaesthetic and oxygen concentrations were monitored using a gas analyser (RGM 5250, Datex-Ohmeda, Inc., Louisville, CO, USA). For treatment, animals were placed in padded acrylic containers inside incubators warmed to 35.5°C. Immediately after treatment, mice were euthanized with an intraperitoneal injection of ketamine (20 mg kg^{-1}), acepromazine (0.5 mg kg^{-1}), and xylazine (1 mg kg^{-1}). Animals were then transcardially perfused by using phosphate-buffered saline with 5% glycerol and 5% sucrose, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; pH = 7.4), containing 5% glycerol and 5% sucrose (PFA-GS). Brains were removed, fixed in PFA, and cryoprotected in 20% and 30% sucrose solutions in PBS.

Histology

Brains were cryosectioned in the sagittal plane at 40 μm using a Cryotome-SME (Thermo Electronics, Kalamazoo, MI, USA). Sections were mounted to charged slides and stored at −80°C until use. Sections between 0.60 and 0.84 mm lateral to the midline corresponding to figures 106–108 in Paxinos and Franklin's Mouse Brain Atlas[10] were then stained for activated, cleaved caspase 3 (AC3), neuronal nuclei (NeuN), and propidium iodide (PI). AC3 is the executioner caspase and a marker of commitment to apoptotic cell death. NeuN represents a neurone-specific protein signifying a post-mitotic stage of development. PI is a red-fluorescent nuclear and chromosomal counterstain, and labels all cells.

All sections were incubated for 5 min at 100°C in a 1:10 dilution of sodium citrate buffer pH 6.0 (CB910 m; Biocare, Concorde, CA, USA) in Coplin jars for antigen retrieval. After this step, slides were incubated in 0.025% Trypsin for 3 min and then in 2 M HCl at room temperature for 30 min. Then, the slides were washed twice in phosphate buffer (pH=8.5), after which sections were blocked in 5% donkey serum, 0.5% glycine, 0.5% non-fat dry milk, and 5% Tween-20 in PBS for 1 h at room temperature before primary antibodies were added.

Slide-mounted sections were incubated overnight at room temperature with two primary antibodies to accommodate combinations of compatible secondary antibodies. Sections were stained with a 1:200 dilution of rabbit cleaved caspase 3 antibody (9661L; Cell Signaling Technology, Danvers, MA, USA) and 1:200 mouse anti-NeuN monoclonal antibodies (MAB377; Millipore, Temecula, CA, USA) in blocker. After primary antibody incubation, slides were rinsed with 5% Tween-20 in PBS followed by a 4 h incubation in 1:250 dilutions of Alexa Fluor 488 donkey anti-rabbit (A21206; Invitrogen, Eugene, OR, USA) and 647 donkey anti-mouse (A31571; Invitrogen), as appropriate for primary antibody species. Slides were then incubated in coplin jars filled with 0.01% propidium iodide (P3566; Invitrogen) in PBS for 5 min. After immunostaining, sections were dehydrated in an ascending ethanol series, washed in PBS, and mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA, USA).

Quantification of neuroapoptosis

To quantify neuronal apoptosis, three-channel confocal image stacks of AC3, PI, and NeuN immunostaining were collected through the z depth of the tissue from the cerebral cortex,