British Journal of Anaesthesia **110** (S1): i19–i28 (2013)
doi:10.1093/bja/aet169

BJA

Isoflurane affects the cytoskeleton but not survival, proliferation, or synaptogenic properties of rat astrocytes *in vitro*

D. J. Culley[1]*, E. K. Cotran[1], E. Karlsson[1], A. Palanisamy[1], J. D. Boyd[2], Z. Xie[3] and G. Crosby[1]

[1] Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
[2] Laboratory for Drug Discovery in Neurodegeneration (LDDN), Harvard NeuroDiscovery Center, Harvard Medical School, Brigham and Women's Hospital, Cambridge, MA 02139, USA
[3] Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, MA 02114, USA

* Corresponding author. E-mail: dculley@zeus.bwh.harvard.edu

Editor's key points

- General anaesthetics can have toxic effects on neurones, but their effects on glia are unclear.
- Isoflurane reduced astrocyte cytoskeletal protein expression, but did not reduce their survival, proliferation, or motility.
- The neurotoxic effects of isoflurane are unlikely due to disruption of astrocytes, which are not seriously impaired despite their many shared signalling mechanisms with neurones.

Background. More than half of the cells in the brain are glia and yet the impact of general anaesthetics on these cells is largely unexamined. We hypothesized that astroglia, which are strongly implicated in neuronal well-being and synapse formation and function, are vulnerable to adverse effects of isoflurane.

Methods. Cultured rat astrocytes were treated with 1.4% isoflurane in air or air alone for 4 h. Viability, proliferation, and cytoskeleton were assessed by colorimetric assay, immunocytochemistry, or a migration assay at the end of treatment or 2 days later. Also, primary rat cortical neurones were treated for 4 days with conditioned medium from control [astrocyte-conditioned media (ACM)], or isoflurane-exposed astrocytes (Iso-ACM) and synaptic puncta were assessed by synapsin 1 and PSD-95 immunostaining.

Results. By several measures, isoflurane did not kill astrocytes. Nor, based on incorporation of a thymidine analogue, did it inhibit proliferation. Isoflurane had no effect on F-actin but reduced expression of α-tubulin and glial fibrillary acidic protein both during exposure ($P<0.05$ and $P<0.001$, respectively) and 2 days later ($P<0.01$), but did not impair astrocyte motility. ACM increased formation of PSD-95 but not synapsin 1 positive puncta in neuronal cultures, and Iso-ACM was equally effective.

Conclusions. Isoflurane decreased expression of microtubule and intermediate filament proteins in astrocytes *in vitro*, but did not affect their viability, proliferation, motility, and ability to support synapses.

Keywords: astrocytes; isoflurane

Accepted for publication: 21 March 2013

There is growing evidence from rodents and primates that exposure to commonly used general anaesthetics can lead to structural and functional changes in the brain. During key periods of brain development these effects include apoptotic neurodegeneration, synapse loss or gain, decreased neurogenesis, and cognitive and behavioural deficits that last into adulthood.[1–8] Likewise, in adult or senescent animals general anaesthetic exposure can result in altered mitochondrial energetics, activation of pro-apoptotic caspases, and long-lasting learning deficits.[9–11]

In these situations, the target of the anaesthetics is presumed to be neurones. However, glia outnumber neurones in the brain and are vital for its integrity and function.[12 13] Astroglia in particular play major metabolic, structural, and functional roles in supporting neurones and synapses and protecting them from injury[12 14 15] and astrocyte dysfunction is implicated in a variety of neurologic and neurodevelopmental disorders, including those marked by learning and memory defects.[16 17] Nonetheless, the impact of general anaesthetics on astrocytes is largely unexamined. Most general anaesthetics are γ-amminobutyric acid (GABA) or *N*-methyl-D-aspartate (NMDA) receptor modulators or both,[18] and astrocytes express abundant GABA and NMDA receptors, which have properties similar to those of neurones.[16 19] Indeed, isoflurane produces more profound suppression of astrocyte than neural activity in the adult rat brain *in vivo*.[20] Accordingly, we hypothesized that adverse effects of these agents on the central nervous system (CNS) might reflect astrocyte dysfunction. We tested this possibility *in vitro* by investigating the impact of the commonly used general anaesthetic agent isoflurane on astrocyte viability and proliferation, motility and cytoskeleton, and ability to support synapses.

Methods

The experimental protocol was approved by the Harvard Medical Area Standing Committee on Animals. Astrocytes were exposed *in vitro* to 1.4% isoflurane in air or air alone for 4 h and assayed at the conclusion of exposure or 2 days later. We have described most of the procedures and methods elsewhere.[21]

Astrocyte harvest and culture

Pregnant female rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were killed on embryonic day 18 by CO_2 intoxication, the embryos harvested and placed in ice-cold phosphate-buffered saline plus penicillin-streptomycin [(PBS+), Invitrogen, Carlsbad, CA, USA] and the cerebral cortex removed. The cortices were washed three times by centrifugation in PBS+ and then incubated in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) with 4% papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), Dispase II (Roche Diagnostics, Indianapolis, IN, USA) and Dnase 1 (Recominbant Rnase-free Dnase 1, Roche Diagnostics). Samples were triturated, washed and then suspended in DMEM/fetal bovine serum (FBS) [Advanced DMEM with glutamine and penicillin-streptomycin (Invitrogen)]. Five million cells were plated in tissue culture flasks (BD Biosciences, Bedford, MA, USA) containing DMEM/FBS and placed in a humidified cell culture incubator at 37°C with 5% CO_2. Five millilitres of fresh media were added to each flask three times per week. When cells reached 75–80% confluence, they were passaged, subjected to a partial media exchange, and re-plated at a density 5×10^6 cells. At Passage 2 [day *in vitro* (DIV) 14], cells were plated into 96-well tissue culture plates at 10^3 cells per well. Matched plates were prepared for each assay from the same batch of cells, and then were placed in the incubator for 2 days before randomization to isoflurane or control treatment. To reduce variability, matched plates were treated, fixed, stained, and imaged at the same time. Compared with the *in vivo* state, the age of cells cultured under these conditions is indeterminate. However, the cells stain prominently with vimentin, a marker of immature astroglia (data not shown), suggesting they maintain an immature phenotype.

Isoflurane exposure

Matched 96-well plates were placed in identical air-tight, humidified chambers (Billups-Rothenberg, Del Mar, CA, USA) flushed with 21% oxygen—5% CO_2—74% nitrogen (control) or the control gas mixed with 1.4% isoflurane. The entire experiment was performed in a room maintained at 37°C and the chambers and gas content-certified canisters (Airgas, Hingham, MA, USA) were temperature equilibrated in the room overnight. Isoflurane, oxygen, and carbon dioxide concentrations were measured every 30 min with an agent analyzer (Ohmeda 5250 RGM, Louisville, CO, USA). Treatment was terminated when the plates were removed from the chambers.

Cell viability

Cell viability and death were assessed at the end of isoflurane exposure or 48 h later by propidium iodide (PI) staining, lactate dehydrogenase (LDH) release, and fluorescence immunocytochemistry for cleaved caspase 3 and cytochrome C.[21] Hoechst 33 342, a nuclear stain, was used to determine cell number. PI is a fluorescent molecule that binds DNA; as it is membrane impermeant, PI labels only non-viable cells. LDH is released into the culture medium as cells die and is a measure of necrotic cell death. Translocation of cyctochrome C from the cytoplasm to the nucleus and cleavage and activation of caspase 3 are indices of apoptotic cell death. Cells treated with staurosporine (3 μM), a caspase 3 activator, served as a positive control. LDH was measured in the supernatant with a commercially available colorimetric cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany) and a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions.[21] For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and then incubated overnight with primary antibodies for the apoptotic markers activated caspase 3 (Abcam, Inc., Cambridge, MA, USA; 1:500 dilution) or cytochrome C (Abcam, Inc., Cambridge, MA, USA; 1:650 dilution). After application of a secondary antibody (as appropriate) and washing, Hoechst 33 342 was applied. Cells were washed again and stored in the dark at 4°C until image acquisition and cell number and protein expression were measured with a high-throughput fluorescence microscope, as outlined below.

Cell division

Cell proliferation was assessed during and 48 h after exposure to isoflurane by incorporation of 5-ethynyl-2′-deoxyuridine (EdU) using a commercially available kit [Click-iT™ EdU Alexa Fluor® High-Throughput Imaging (HCS) Assay, Invitrogen] according to the manufacturer's instructions.[21] EdU is a thymidine analogue that is incorporated into cells only during S-phase of cell division and hence is used to assess proliferation.[22] Briefly, EdU (final concentration 10 μM) was added to 20 wells of a 96-well plate containing cells in DMEM/FBS media either immediately before treatment or 44 h later. Cells were incubated with EdU for 4 h and then fixed with 4% paraformaldehyde in PBS and treated with 0.5% Triton® X-100. After washing, cells were incubated with Click-iT™ reaction cocktail, washed, and blocked with bovine serum albumin in PBS. To identify astrocytes, cells were incubated with an anti-glial fibrillary acidic protein (GFAP) antibody (Millipore, Billerica, MA, USA) and labelled with Hoechst 33 342 to determine cell number. Specimens were stored in the dark at 4°C until image acquisition.

Cytoskeletal protein expression

Control and isoflurane-exposed cells were processed at the end of treatment or 48 h later for immunocytochemistry for the intermediate filament protein GFAP (Millipore; 1:500 dilution); the microtubule protein α-tubulin (Abcam, Inc.; 1:1000