

Astrogliosis in juvenile non-human primates 2 years after infant anaesthesia exposure

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Abstract

Background: Infant anaesthesia causes acute brain cell apoptosis, and later in life cognitive deficits and behavioural alterations, in non-human primates (NHPs). Various brain injuries and neurodegenerative conditions are characterised by chronic astrocyte activation (astrogliosis). Glial fibrillary acidic protein (GFAP), an astrocyte-specific protein, increases during astrogliosis and remains elevated after an injury. Whether infant anaesthesia is associated with a sustained increase in GFAP is unknown. We hypothesised that GFAP is increased in specific brain areas of NHPs 2 yr after infant anaesthesia, consistent with prior injury.

Methods: Eight 6-day-old NHPs per group were exposed to 5 h isoflurane once (1×) or three times (3×), or to room air as a control (Ctr). Two years after exposure, their brains were assessed for GFAP density changes in the primary visual cortex (V1), perirhinal cortex (PRC), hippocampal subiculum, amygdala, and orbitofrontal cortex (OFC). We also assessed concomitant microglia activation and hippocampal neurogenesis.

Results: Compared with controls, GFAP densities in V1 were increased in exposed groups (Ctr: 0.208 [0.085–0.427], 1×: 0.313 [0.108–0.533], 3×: 0.389 [0.262–0.652]), whereas the density of activated microglia was unchanged. In addition, GFAP densities were increased in the 3× group in the PRC and the subiculum, and in both exposure groups in the amygdala, but there was no increase in the OFC. There were no differences in hippocampal neurogenesis among groups.

Conclusions: Two years after infant anaesthesia, NHPs show increased GFAP without concomitant microglia activation in specific brain areas. These long-lasting structural changes in the brain caused by infant anaesthesia exposure may be associated with functional alterations at this age.

Keywords: anaesthesia; apoptosis; astrogliosis; glial fibrillary protein; isoflurane; non-human primate

Editor's key points

- Infant anaesthesia causes acute brain cell apoptosis, cognitive deficits, and behavioural alterations later in life in non-human primates.

- We hypothesised that infant anaesthesia causes chronic astrocyte activation (astrogliosis), consistent with prior injury, in specific brain areas 2 yr after exposure.
- Two years after infant anaesthesia, non-human primates show increased astrogliosis without concomitant microglia activation in specific brain areas.

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- These novel long-term structural changes occur in brain regions that showed acute increased apoptosis after exposure and that are associated with functional alterations later in life

Anaesthesia exposure of young children has been associated with impaired neurobehavioral development.^{1,2} Most clinical studies have limitations that contribute to their ambiguous outcomes. According to a recent meta-analysis,³ three well-controlled studies in the field (Pediatric Anesthesia Neurodevelopment Assessment [PANDA], Mayo Anesthesia Safety in Kids [MASK], and General Anesthesia or Awake-regional Anesthesia in Infancy [GAS])^{4–6} share the finding that there is no measurable effect on primary outcome of intelligence quotient (IQ) after relatively short exposures to anaesthesia (1–2 h) during infancy. However, secondary outcomes of these studies suggest an association between anaesthesia exposure and alterations in executive function or behaviour in school-age children as reported in questionnaires by parents and caregivers. In contrast, a large body of animal studies provides robust evidence that anaesthesia exposure during infancy causes functional alterations in the developing brain. Initial findings in neonatal rats found that anaesthesia exposure induced long-term impairments in spatial learning and memory.⁷ Importantly, studies in infant non-human primates (NHPs) exposed to anaesthesia using similar physiological monitoring as for children, reported cognitive deficits^{8–10} and behavioural alterations^{11–14} later in life.

Although the causes of the functional alterations are unknown, there is strong evidence that anaesthesia exposure of infant animals including NHPs causes an acute injury in the immature brain, initially evidenced by a widespread increase of neuronal apoptosis.^{7,15–20} More recently, *in vitro* and *in vivo* studies have reported reduced neurogenesis and impaired dendritic arborisation after anaesthesia exposure.^{21–27} Although these neuronal changes may account for long-term functional impairments, glial cells play an essential role in neuronal function, and they are also affected by infant anaesthesia exposure. For example, in neonatal NHPs, oligodendrocytes were found to undergo acute apoptosis after anaesthesia exposure, similar to neurones.^{17,28–30} In addition, infant anaesthesia exposure resulted in myelination deficits in prefrontal brain areas of mice and NHPs,³¹ and in alterations in oligodendrocyte development and myelin formation in parts of the hippocampus that were associated with cognitive deficits in exposed mice.³²

Growing evidence suggests that infant anaesthesia exposure also affects astrocyte structure and function,^{33–37} Anaesthesia-induced astrocytic dysfunction in neonatal mice is associated with behavioural alterations later in life.³⁸ Chronic activation of astrocytes, called astrogliosis, is a common response to brain insults. Astrogliosis is found in various types of brain injuries, including hypoxic or traumatic injuries, and has also been associated with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, and with neurodevelopmental disorders, including autism.^{39–46} Astrogliosis can be identified by increased levels of glial fibrillary acidic protein (GFAP),⁴⁷ a cytoskeletal protein uniquely expressed in astrocytes that plays a critical role in their activation.²⁴ In acute brain injury, GFAP expression increases and can remain elevated, representing a long-term structural change indicating a prior injury, called 'glial scar'.⁴⁸

Although acute structural changes in the developing brain caused by anaesthesia exposure such as apoptosis have been studied extensively, long-term alterations in brain structure at times when functional impairments become evident are not fully understood.^{49–52} Furthermore, it is unknown whether such structural changes are present in brain areas involved in the functional alterations found after anaesthesia exposure of infants. We therefore investigated potential long-term structural changes in juvenile NHP brains 2 yr after exposure to isoflurane during infancy by assessing astrogliosis in selected brain areas. Initially, we examined the primary visual cortex (V1) because this region has shown robust increases in brain cell apoptosis in NHPs immediately after anaesthesia exposure in previous studies,¹⁵ and functional impairments connected to this brain area have been reported.⁸ Subsequently, we studied the perirhinal cortex (PRC), the subiculum in the hippocampus, the amygdala, and the orbitofrontal cortex (OFC) as these brain areas have also shown acute apoptosis.^{15,29} These areas represent major processing centres for visual recognition memory,⁵³ social,⁵⁴ and anxiety-related^{55,56} behaviours, which are impaired or altered in juvenile NHPs after infant anaesthesia exposure.^{8,11–13} We report that anaesthesia exposure during infancy is associated with increased astrogliosis in specific brain areas of juvenile NHPs indicated by increased GFAP expression. Our results provide novel evidence for long-term structural consequences 2 yr after infant anaesthesia exposure.

Methods

Ethical approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Oregon National Primate Research Center (ONPRC) and was in compliance with all federal regulations, and the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*.⁵⁷ The ONPRC is accredited by AAALAC International.

Conditions of animal housing and euthanasia

The housing conditions of the NHPs have been described.^{13,14} Briefly, 24 rhesus macaques (*Macaca mulatta*) with an equal number of females and males were born and housed at the ONPRC (Beaverton, OR, USA). After the first year of life, the NHPs were weaned from their dams, moved from outdoors into indoor home cages, and were housed with five to six juvenile peers. Twice a day, the animals were fed with commercial monkey chow (Lab Diet Monkey Diet, St. Louis, MO, USA) supplemented with grain or produce daily and had access to water *ad libitum*. There was a 12:12 h light/dark cycle, starting with lights on at 07:00. At the age of 2 yr, the NHPs were sedated with ketamine 20 mg kg⁻¹ i.m. and transported to pathology, where basic morphometric measurements were taken. Pentobarbital 0.25 mg kg⁻¹ i.v. was administered followed by exsanguination and *in vivo* transcardial perfusion–fixation with saline followed by 4% (w/v) paraformaldehyde to prepare the brain for histopathologic analysis.

Infant anaesthesia exposure

The conditions of infant anaesthesia exposure have been described.^{13,14} Briefly, 1 day before anaesthesia exposure, the postnatal day 5 (P5) NHP neonates were separated from the

breeding group together with their dams. Each dam received a mild sedative (ketamine, i.m., 5 mg kg⁻¹), and its neonate was transferred to the operating room. After taking baseline vital parameter measurements including heart rate, respiration, temperature, blood pressure, weight, blood gas analysis, and metabolic parameters, the neonate was carefully hand restrained and exposed to isoflurane via face mask and under spontaneous ventilation. This induction technique closely resembles the method used routinely to induce anaesthesia in young children. Subsequently, the trachea was intubated and the lungs mechanically ventilated, and a surgical plane of anaesthesia was maintained for 5 h at an end-tidal isoflurane of 0.7–1.5 vol% in 30 vol% oxygen. In our previous studies, dose–response experiments revealed that 5 h exposure to these isoflurane concentrations provided anaesthesia levels that allowed the NHPs to tolerate mild to moderate mechanical stimuli, and induced neurotoxicity in the developing NHP brain.^{15,28} Maintenance of this anaesthesia level was controlled by verifying the absence of movement and <10% increase in heart rate or blood pressure in response to a mechanical stimulus.

This management using full physiologic monitoring mimicked conditions in paediatric anaesthesia. Physiological parameters were maintained within narrow ranges throughout the exposure (Supplementary Table S1) using a similar approach as reported.⁵⁸ After isoflurane exposure was stopped and the animal was awake, extubation was performed. Subsequently, animals were kept in an NHP incubator until fully recovered before they were transferred back to their dams. Animals were randomised to receive this procedure either one time (1×) on P6, three times on P6, P9, and P12 (3×), or to receive the same procedure on the same postnatal days but exposed to room air only. The NHPs in the 1× exposure group received room air on P9 and P12, after they had been exposed to isoflurane on P6. All three groups initially consisted of eight animals with equal sex distribution. One female NHP in the 3× group had to be removed from the study at about 1 yr of age because of therapy-resistant chronic diarrhoea.

Histopathology

Immediately after NHPs were euthanised, their brains were perfused with cold artificial cerebrospinal fluid by cannulation of the aorta. The left hemibrain was rapidly removed for electrophysiological studies and evaluation of dendritic morphology which required unfixed tissue. The right hemibrain was then perfused *in situ* with paraformaldehyde 4% and removed from the skull. Before further processing, the fixed hemibrains were coded to avoid bias by the investigators. The hemibrains were sliced at ~5 mm intervals, and paraffin blocks were prepared from frontal, occipital, and temporal regions, the latter including deep structures of the amygdala and the hippocampus. Several 6-µm-thick consecutive histological slices were cut from each paraffin block at three levels ~0.2 mm apart, aiming to include the brain regions of interest. Each slice was deparaffinised and stained with specific antibodies using standard immunohistochemical protocols that included antigen retrieval in citrate buffer pH 6.0. For each block, one slice was immunostained with the neuronal marker NeuN (Millipore #ABN78; Millipore, Burlington, VT, USA) to identify the brain regions of interest (i.e. V1, amygdala, hippocampus, and OFC) using the corresponding figures in a monkey brain atlas⁵⁹ that illustrate the location of the brain regions. These regions were selected because previous studies found them to

show robust apoptosis acutely^{15,29} or to be critically involved in long-term functional alterations associated with infant anaesthesia exposure.^{8,11–13} In addition, the subiculum was chosen as this hippocampal area is known to be highly vulnerable to brain injuries (e.g. hypoxia) early in life.^{60–62}

The GFAP immunoreactivity was determined by incubating slices with a rabbit monoclonal anti-GFAP antibody (Abcam #ab68428; Abcam, Cambridge, UK) diluted 1:1000 in blocking solution (phosphate-buffered saline [PBS] with 1% bovine serum albumin [BSA] and 0.3% Triton, v/v) overnight at 4°C. Slices were washed in PBS and exposed to secondary antibody by incubation in Vector ImmPRESS™ (Vector Laboratories, Inc., Burlingame, CA, USA) reagent containing anti-rabbit IgG peroxidase conjugate for 30 min, and developed with diaminobenzidine (DAB). Iba-1 immunohistochemistry was performed using a protocol the primary antibody rabbit anti-Iba-1 (Wako #019–19741; Wako Chemicals, Richmond, VA, USA) diluted 1:3000 in blocking solution. For analysis of hippocampal neurogenesis 15-µm-thick sections were cut from temporal blocks and incubated with the ki67 primary antibody (Abcam #ab15580) diluted 1:2000 in blocking solution overnight at 4°C, followed by incubation in biotinylated goat anti-rabbit secondary antibody (Vector, #BA-1000; Vector Laboratories) diluted 1:200 in blocking solution, then incubated with avidin–biotin–peroxidase complex (Vectastain Elite kit; Vector Laboratories) and developed with DAB. All immunostained slices were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). All histological slides were stored at room temperature for subsequent imaging analysis and quantification.

Quantification of GFAP area density and Iba-1 positive cells

Coded histological slides containing the brain regions of interest were scanned at high magnification using a 40× objective in an automatised slide scanner (Zeiss Axio Scan Z1 and ZEN software; Carl Zeiss, Oberkochen, Germany), and high-resolution images were stored in a hard drive for subsequent analysis. The 'ZEN 2.3 lite' software (free version) was used to create individual TIFF images containing the brain areas of interest. We measured astrogliosis and microglial activation using different units to be consistent with those used in previous studies that also reported these histopathological entities. Astrogliosis was measured as the ratio of GFAP-positive area to total area analysed, and expressed as GFAP density in percentage.^{63–65} This approach takes into consideration astrocyte hypertrophy and the accompanying increase in GFAP expression that characterises astrogliosis.⁴⁰ Microglial activation was measured as number of positive cells divided by total area analysed and expressed as Iba-1 cell density in cells per mm.^{2,66,67} Quantification of GFAP positive area and Iba-1 positive cells in each brain region was performed using the ImageJ software.

To prevent bias introduced by selecting regions of interest within specific brain areas, quantification of GFAP and Iba-1 was performed on the entire grey matter of the brain region (i.e. V1, amygdala, or OFC) present on the histological slide. GFAP-positive area and Iba-1-positive cells were automatically recognised and counted using customised macros in the ImageJ that used the plugin IHC profiler (<https://sourceforge.net/projects/ihcprofiler/>). This plugin allows unbiased quantification of area positive for immunohistochemistry (brown

areas) followed by an adjustable threshold to identify positive areas and exclude noise.⁶⁸ The analysis outcome allows quantification of the number of positive counts ('cells') along with their area. These computer-assisted quantifications of GFAP and Iba-1 in specific brain regions were performed by an investigator blinded to animal group assignment and without access to the key to identify the coded histological slides. For quantification of hippocampal ki67-positive cells, coded slides were analysed by an experienced neuropathologist. Darkly stained (brown) nuclei were counted when located either in the dentate gyrus (DG) or within a band twice the thickness of the DG into the hilum, to ensure including all relevant cells.

Statistical analysis

All statistical analysis was performed in GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). We tested for normality using the Wilk–Shapiro test. As GFAP area, Iba-1 cell density, and ki67 cell count data did not have normal distribution, descriptive statistical data for these variables are presented as median (25th–75th percentiles). Grubbs test was used to identify outliers, but no animals were excluded with this analysis. Comparison between medians was determined using Kruskal–Wallis test followed by Dunn's *post hoc* test. We used $\alpha=0.05$ for all statistical tests.

Results

GFAP densities increased in V1 after isoflurane exposure

To evaluate whether isoflurane exposure during infancy results in long-term structural alterations, we assessed the density of GFAP expression in V1 in the brains of the 2-yr-old NHPs. This brain area was selected because it shows robust apoptosis acutely after infant anaesthesia exposure.^{15,28} Guided by the rhesus monkey brain atlas⁵⁹ and strict cortical cytoarchitectural features, we identified the V1 region in our stained and scanned occipital brain slices (Fig. 1a) quantified GFAP-positive area (Fig. 1b). As shown in Fig. 1c, GFAP densities in V1 increased with exposure to isoflurane (Ctr: 0.21%

[0.085–0.43], 1×: 0.31% [0.11–0.53], 3×: 0.39% [0.26–0.65], $H_{(2)}=10.24$, $P=0.006$; Kruskal–Wallis test). *Post hoc* analysis with Dunn's multiple comparisons test showed that the differences among the control and exposed groups reached statistical significance for the 3× ($P=0.003$) but not for the 1× group ($P=0.132$).

Iba-1 cell densities were unaffected after isoflurane exposure

We analysed Iba-1 cell densities in V1 to test whether the increase in GFAP associated with isoflurane exposure during infancy was accompanied by activation of microglia in this region 2 yr after isoflurane exposure. Iba-1 positive cells (Fig. 2a) were divided by the V1 area to obtain Iba-1 cell density. In contrast to GFAP density, we did not find significant differences for Iba-1 cell densities in V1 among the groups (Fig. 2b; *n.s.*, Kruskal–Wallis test).

GFAP densities increased in the PRC, subiculum, and amygdala, but not in the OFC, after isoflurane exposure

We extended our assessment of GFAP to other brain areas that participate in cognitive functions and behaviours that are altered after infant anaesthesia exposure. We assessed this marker in the PRC and subiculum because they are critically involved in memory function, and in the amygdala and the OFC, because they play major roles in anxiety and social behaviour. In the PRC of 2-yr-old NHPs, GFAP densities differed significantly between groups (Fig. 3a: Ctr: 0.10% [0.03–0.16], 1×: 0.082% [0.049–0.154], 3×: 0.18% [0.070–0.28], $H_{(2)}=6.96$, $P=0.031$; Kruskal–Wallis test). *Post hoc* analysis with Dunn's multiple comparisons test showed that GFAP density was increased in the 3× group ($P=0.032$), but not in the 1× group ($P>0.999$) as compared with the control group. Similarly, the GFAP analysis in the subiculum revealed increased densities only for the 3× group when compared with control (Fig. 3b: Ctr: 3.07% [2.18–4.12], 1×: 1.93% [1.10–4.89], 3×: 5.16% [3.40–11.09], $H_{(2)}=13.52$, $P=0.001$, Kruskal–Wallis test, *post hoc* Dunn test

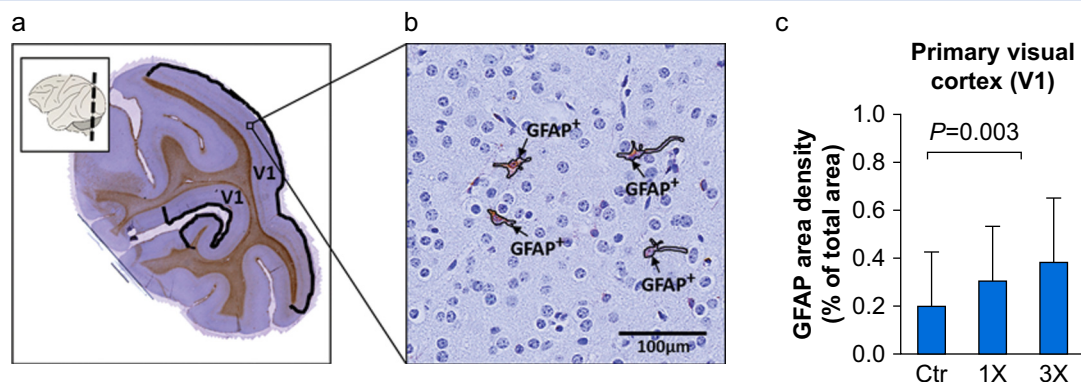


Fig 1. Glial fibrillary acidic protein (GFAP) expression in the visual cortex (V1) of juvenile non-human primates. (a) Representative image of a brain slice cut from the occipital region indicated in the inset, immunostained for GFAP (brown) and counterstained with haematoxylin (blue). The demarcated regions indicate the primary visual cortex (V1) contained in the slice. (b) High magnification image showing GFAP-positive areas (GFAP⁺) within V1. (c) GFAP area density (% of total area) in the visual cortex (V1) of juvenile non-human primates (NHPs) 2 yr after single (1×), three times (3×) or no exposure (Ctr) to isoflurane during infancy. Data are median (25th–75th percentiles), *n* (NHP)=Ctr: 8, 1×: 8, 3×: 7.

$P=0.008$). Analysis of the amygdala revealed increased GFAP densities in both exposed groups as compared with control (Fig. 3c: Ctr: 0.33% [0.19–0.48], 1 \times : 0.68% [0.46–0.82], 3 \times : 0.66% [0.434–0.81], $H_{(2)}=13.35$, $P=0.001$; Kruskal–Wallis test). Dunn's multiple comparisons test revealed statistically significant differences for both exposure groups (1 \times : $P=0.003$, 3 \times : $P=0.007$). In contrast, in the OFC there were no significant differences in GFAP area densities between groups (Fig. 3d; n.s., Kruskal–Wallis test).

Hippocampal neurogenesis is not affected after isoflurane exposure

To explore whether exposure of infants to isoflurane affects neurogenesis in the DG of the hippocampus of juvenile NHPs, we evaluated the proliferative cell marker ki67. We found that there were no significant differences for ki67 cell counts in the indicated hippocampal area between the three groups (Fig. 4; n.s., Kruskal–Wallis test).

Discussion

We report increases in GFAP-positive area in specific brain regions of juvenile NHPs 2 yr after single or multiple 5 h isoflurane exposures during infancy. These results reveal novel long-term structural changes associated with anaesthesia exposure in the developing brain. Increased GFAP densities were found in V1, the PRC, and the subiculum, regions that participate in visual recognition memory, and in the amygdala, which plays a role in anxiety-related and social behaviours. These brain functions have been shown to be altered after infant anaesthesia exposure in NHPs.^{8,11–13} Such increases in GFAP area indicate chronic astrogliosis and resemble similar changes detectable after other types of brain injury including ischaemia/hypoxia,^{41,69,70} or those associated with neurodegenerative conditions such as Alzheimer's disease⁷¹ and developmental disorders such as autism.⁴⁶ It is important to note that the long-term structural changes

reported here are accompanied by alterations in behaviours detected at the same age.¹³

The increase in GFAP indicates activation of astrocytes, which is a common and non-specific response to CNS injury. It is likely that the increase in GFAP we found 2 yr after anaesthesia exposure was in response to the acute injury caused by isoflurane exposure during infancy that is characterised by a widespread increase in apoptosis of neurones and oligodendrocytes.²⁸ Whereas mild to moderate astrogliosis is generally an acute and transient response, more severe and diffuse astrogliosis is chronic and can result in long-term tissue reorganisation. A localised increase in reactive astrocytes and GFAP density has been suggested to play a beneficial role as it represents a barrier separating the injured area from intact neuronal tissue thereby supporting restoration of tissue integrity and preventing further spread of cellular damage.⁷² However, such localised GFAP density increases also have been suggested to contribute to neuronal hyperexcitability and seizures.⁷³ In contrast, a diffuse, chronic astrogliosis has been associated with detrimental effects and inhibition of tissue recovery including attenuation of axonal regrowth after tissue lesions.^{69,74,75} The astrogliosis we found in 2-yr-old NHPs after infant isoflurane exposure appears to be of the diffuse and chronic type, and likely occurred as a response to the acute widespread apoptotic injury.²⁸ This type of gliosis can be compared with that associated with neonatal hypoxic injury, as both are chronic and diffuse in nature. However, we do not suspect that anaesthesia resulted in brain hypoxia as we were able to carefully monitor and control physiologic parameters during anaesthesia exposure, including oxygen saturation.

Astrocytes play important functions in maintaining extracellular homeostasis in the brain, modulating synaptic activity and shaping synaptogenesis (formation and pruning of synapses).⁴⁰ Astrocyte dysfunction and astrogliosis have been associated with altered synaptic structure and function.⁶⁹ Imbalances between excitatory and inhibitory synapses have been linked to behavioural alterations characteristic of autistic disorders, such as deficits in social interaction.^{76,77}

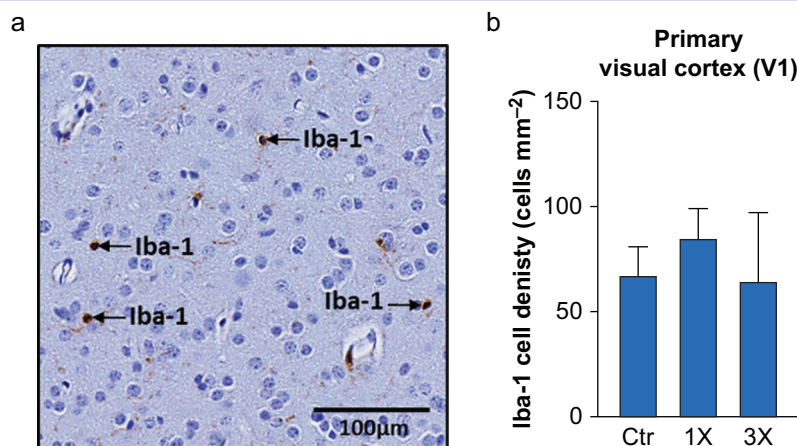


Fig 2. Iba-1 cell density in the visual cortex (V1) of juvenile non-human primates (NHPs). (a) Representative high magnification image showing Iba-1-positive cells (Iba-1) within V1. (b) Iba-1 cell density (cells mm⁻²) in the visual cortex (V1) of juvenile NHPs 2 yr after single (1 \times), three times (3 \times) or no exposure (Ctr) to isoflurane during infancy. Data are median (25th–75th percentiles), n (NHP)=Ctr: 8, 1 \times : 8, 3 \times : 7.

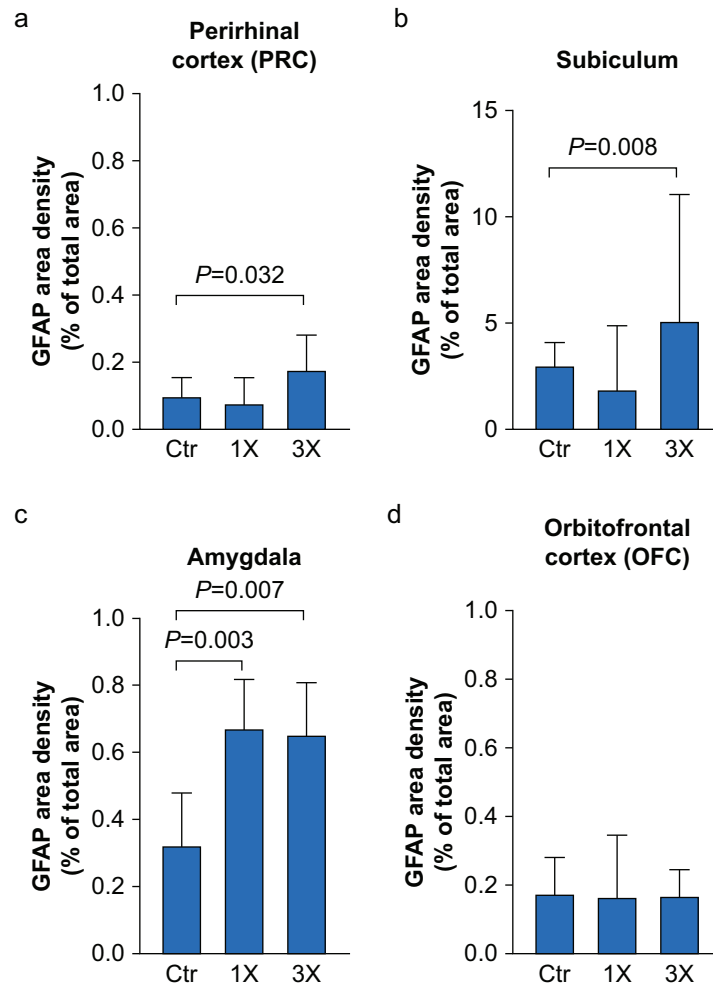


Fig 3. GFAP expression in the perirhinal cortex, subiculum, amygdala, and orbitofrontal cortex of juvenile NHPs. GFAP area density (% of total area) in (a) the PRC, (b) subiculum, (c) amygdala, and (d) OFC of juvenile NHPs 2 yr after single (1×), three times (3×) or no exposure (Ctr) to isoflurane during infancy. Data are median (25th–75th percentiles); *n* (NHP)=PRC: Ctr: 8, 1×: 7, 3×: 7; subiculum: Ctr: 8, 1×: 8, 3×: 7; amygdala: Ctr: 8, 1×: 7, 3×: 5; OFC: Ctr: 8, 1×: 8, 3×: 7. GFAP, glial fibrillary acidic protein; NHPs, non-human primates.

Thus, it is possible that the astrogliosis we found may be associated with the reduced social behaviours that we found in the same NHPs at 2 yr of age after infant anaesthesia.¹³ In support of this hypothesis, chronic astrogliosis has been associated with altered behaviour in a murine traumatic brain injury model.⁷⁸ However, further experiments in NHPs are needed to test this hypothesis.

Chronic activation of astrocytes might affect the development of critical brain areas and their connections, thereby impairing normal function.^{79,80} The astrogliosis we found in V1, the PRC, and the subiculum of NHPs 2 yr after exposure could be linked to the impaired visual recognition memory reported in similar aged NHPs.⁸ There is significant evidence that V1 could participate in visual recognition memory in rodents and NHPs. Early lesion experiments in rats indicated that the entire neocortex including V1 participates in memory storage,⁸¹ and more recent work suggests that V1 is involved in visual recognition and working memory.^{82–84} However, the critical areas for recognition memory processing are the PRC

and the hippocampus, and damage to these structures was found to impair object recognition.⁵³ Importantly, performance on the visual paired comparison task, the paradigm used in the study by Alvarado and colleagues,⁸ is dependent on PRC activity as reported in NHPs after neonatal perirhinal lesions.⁸⁵ Therefore, the increase in astrogliosis in the PRC together with V1 found in the juvenile NHPs in our study may present the structural correlate for the impairments in visual recognition memory reported after infant anaesthesia exposure.⁸ In contrast, the hippocampus including the subicular cortex plays an essential role in recognition memory that involves spatial information,^{86,87} whereas neonatal perirhinal lesions in NHPs revealed that the PRC is dispensable for spatial location.⁸⁸ We also found significant astrogliosis in the subiculum of juvenile NHPs in the 3× group, which may correlate with the lowest performance of the animals in this exposure group during the delayed response test that measures spatial working memory.¹³ Our findings of increased GFAP in temporal lobe structures (PRC and subiculum) in the brains of

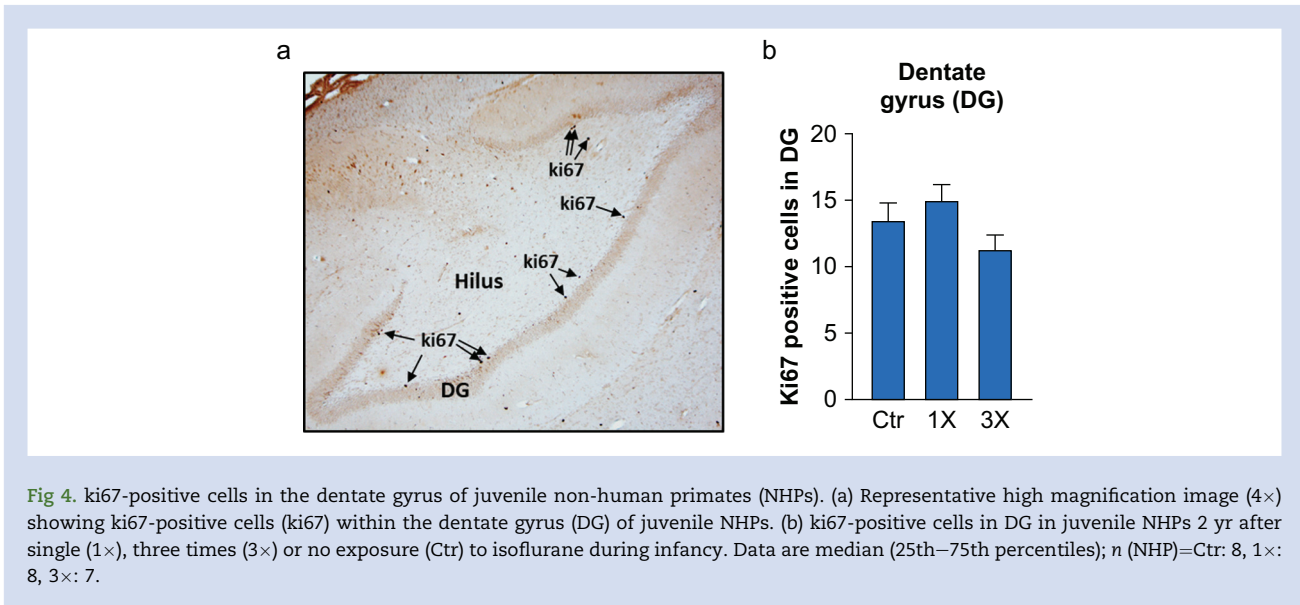


Fig 4. ki67-positive cells in the dentate gyrus of juvenile non-human primates (NHPs). (a) Representative high magnification image (4 \times) showing ki67-positive cells (ki67) within the dentate gyrus (DG) of juvenile NHPs. (b) ki67-positive cells in DG in juvenile NHPs 2 yr after single (1 \times), three times (3 \times) or no exposure (Ctr) to isoflurane during infancy. Data are median (25th–75th percentiles); n (NHP)=Ctr: 8, 1 \times : 8, 3 \times : 7.

NHPs in the 3 \times group support clinical studies showing that multiple, but not single, infant anaesthesia exposures were associated with learning disabilities and attention deficit/hyperactivity disorder later in life.^{89,90}

The astrogliosis we found in the amygdala may be associated with decreased close social behaviour and increased anxiety-related behaviours as reported in these NHPs,¹³ and in juvenile NHPs in other studies, after infant anaesthesia exposure.^{11,12,14} This component of the limbic system is substantially implicated in processing of social and anxiety-related behaviours.^{54,56,91,92} In addition, animals with ibotenic acid amygdala lesions during infancy exhibit decreased or altered social behaviour with familiar partners, and greater anxiety-related behaviours such as scratching and yawning later in life.^{93–95} Selective neurotoxic amygdala lesions in neonatal NHPs result in alterations in social behaviour, including loss of social status, decreased affiliative behaviours, and decreased social interactions.^{96–100} Hence, these alterations in social behaviours and increased anxiety-related behaviours after anaesthesia exposure during infancy could be associated with the astrogliosis that we found in the amygdala. Interestingly, we found astrogliosis in the 1 \times group only in the amygdala, suggesting that this area is more sensitive than the other areas analysed. Recent clinical studies report associations of early in life anaesthesia and alterations in behaviour that show some parallels with behavioural changes in animal studies as discussed extensively.^{3,13,101,102}

Acute brain injury activates microglia, the primary immune cells of the CNS that respond by migrating to the site of injury where they release inflammatory factors, destroy pathogens, and remove damaged cells. Microglia-mediated inflammatory responses are also considered to be key in some neurodevelopmental diseases including autistic spectrum disorders.^{103,104} We detected astrogliosis, but we did not find evidence of ongoing microglia activation by Iba-1 stain 2 yr after infant anaesthesia exposure. Although we cannot disregard microglia activation early after infant anaesthesia exposure in these NHPs, previous studies in different species support our finding as they report acute, but not long-lasting, activation of microglia after infant anaesthesia exposure.^{67,105,106} For example in neonatal mice multiple exposures

to sevoflurane induced an acute microglia activation in the hippocampus that was not evident 1 month after anaesthesia or later, suggesting that anaesthesia-induced microglia activation is acute and transient.⁶⁷ Similarly, a subsequent study using *in vivo* micro-positron emission tomography (PET)/CT imaging in NHPs found that infant sevoflurane exposure resulted in acute activation of microglia that lasted a few days but was not present after 3 weeks.¹⁰⁵ Although the molecular mechanisms of anaesthetic neurotoxicity are not completely understood, acute activation of microglia promoting inflammation and generation of reactive oxygen species (ROS) are among the proposed constituents of the underlying acute damage of the developing brain.¹⁰⁷ The lack of microglia activation 2 yr after infant exposure suggests that there is no ongoing active injury, but instead that the astrogliosis found in the juvenile brains is a result of a prior injury. Thus, we propose that the increased levels of GFAP in specific brain regions represent a long-term structural marker of anaesthesia-induced brain injury in the developing brain.

Anaesthesia exposure during infancy impairs hippocampal neurogenesis.¹⁰⁸ Several studies in neonatal rodents (P7) reported concurring findings that exposure to isoflurane, sevoflurane, propofol, or ketamine caused an acute decrease in progenitor proliferation in the DG that lasted for up to 10 days and was associated with long-term neurocognitive deficits.^{21,23–25} Another study in slightly older rodents (P14) reported persisting reductions of hippocampal neurogenesis for up to 4 weeks after exposure and deficits in memory function.²² We did not find differences in hippocampal neurogenesis 2 yr after infant anaesthesia exposure. However, we cannot discard the possibility of impaired neurogenesis at an earlier time, as this study or any other report did not investigate acute effects of anaesthesia exposure on hippocampal neurogenesis in the infant NHP.

Our study has several limitations. In the present study only a single method (GFAP staining) was used to detect astrogliosis. We chose GFAP labelling because detection of elevated GFAP levels in *post mortem* tissue is a traditional, well-established method to determine astrogliosis in various diseases.^{40,109,110} Because of its *post mortem* nature, our study can only provide information about astrogliosis at the time point

of 2 yr after infant anaesthesia exposure, and further experiments are necessary to investigate the course of astrogliosis over time. The absence of astrogliosis in the OFC does not exclude its presence in other areas that have been implicated in social and anxiety-related behaviours such as the cingulate cortex, insula, other areas of the frontal cortex, or in white matter. We do not know whether our findings of astrogliosis 2 yr after infant exposure to isoflurane for 5 h (one or three times) can be generalised to other anaesthetics or to shorter exposure durations. Further studies will be needed to examine these issues.

Our study has several strengths. The NHP brain shares the highest structural similarities to the human brain, making this a preferred model for studies of chronic structural changes after infant anaesthesia exposure. In addition, this model allowed exposure of infants under physiologically controlled conditions to closely mimic paediatric clinical settings. The physiological parameters of neonatal NHPs were maintained within narrow ranges throughout the 5 h exposures and were comparable with those published earlier using a similar approach to monitor and control physiological parameters of neonatal NHPs during exposure to several anaesthetics including isoflurane.⁵⁸ Our histopathological studies in the brains of juvenile NHPs revealed astrogliosis in several brain areas associated with anaesthesia exposure during infancy. This is the first report of this new type of structural alteration in the brain 2 yr after early-in-life anaesthesia exposure. This age in NHPs corresponds approximately to that of children aged 6–8 yr. The astrogliosis in the 2-yr-old NHPs was found concurrently with alterations in social and anxiety-related behaviours that we assessed at this age in the same animal cohort.¹³

Conclusions

We report chronic astrogliosis in juvenile non-human primates 2 yr after infant anaesthesia, providing the first neuro-anatomical evidence for long-term structural consequences after exposure. The absence of microglia activation concurrently with the long-term astrogliosis suggests that it represents an indicator of past injury caused by infant anaesthesia exposure. The astrogliosis associated with infant anaesthesia exposure could be helpful as a long-term marker of anaesthesia-induced brain injury in future research. Preventing the sustained astrogliosis may represent a novel approach to prevent functional impairments associated with infant anaesthesia exposure.

Authors' contributions

Study design/planning: AMB, MG, GAD

Study conduct: AMB, MG, LDM

Data analysis: AMB, VN, JFPZ, MG

Writing of the paper: AMB, VN, JFPZ, MG

Manuscript revision: all authors

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Declarations of interest

The authors declare that they have 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.2021.04.034>.

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