



**HAL**  
open science

## Consequences of General Anesthesia in Infancy on Behavior and Brain Structure

Jean-Philippe Salaün, Audrey Chagnot, Arnaud Cachia, Nicolas Poirel, Valérie Datin-Dorrière, Cléo Dujarrier, Eloïse Lemarchand, Marine Rolland, Lisa Delalande, Pierre Gressens, et al.

► **To cite this version:**

Jean-Philippe Salaün, Audrey Chagnot, Arnaud Cachia, Nicolas Poirel, Valérie Datin-Dorrière, et al.. Consequences of General Anesthesia in Infancy on Behavior and Brain Structure. *Anesthesia & Analgesia*, 2023, 136 (2), pp.240-250. 10.1213/ANE.0000000000006233 . hal-03957734

**HAL Id: hal-03957734**

**<https://cnrs.hal.science/hal-03957734v1>**

Submitted on 27 Apr 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## RESEARCH ARTICLE

Full title: **Consequences of general anesthesia in infancy on behavior and brain structure.**

Running head: **Pediatric anesthesia: lasting effects on brain.**

**Authors :** Jean-Philippe Salaün MD PhD<sup>1,2</sup>, Audrey Chagnot PhD<sup>1</sup>, Arnaud Cachia PhD<sup>3,8</sup>, Nicolas Poirel PhD<sup>3,5,8</sup>, Valérie Datin-Dorrière MD PhD<sup>3,4,5</sup>, Cléo Dujarrier MSc<sup>1</sup>, Eloïse Lemarchand PhD<sup>1</sup>, Marine Rolland MD<sup>1,2</sup>, Lisa Delalande PhD<sup>3</sup>, Pierre Gressens MD PhD<sup>6</sup>, Bernard Guillois MD<sup>4</sup>, Olivier Houdé PhD<sup>3,5,8</sup>, Damien Levard MSc<sup>1</sup>, Clément Gakuba MD PhD<sup>1,2</sup>, Marine Moyon PhD<sup>3</sup>, Mikael Naveau PhD<sup>9</sup>, François Orliac MD PhD<sup>3,5</sup>, Gilles Orliaguet MD PhD<sup>10</sup>, Jean-Luc Hanouz MD PhD<sup>2,7</sup>, Véronique Agin PhD<sup>1</sup>, Grégoire Borst PhD<sup>\*3,8</sup>, & Denis Vivien PhD<sup>\*1,11</sup>.

\*These authors contributed equally to this work as last authors.

### Affiliations:

1. Normandie Univ, UNICAEN, INSERM, GIP Cyceron, Institut Blood and Brain @Caen-Normandie (BB@C), UMR-S U1237, Physiopathology and Imaging of Neurological Disorders (PhIND), Caen, France.
2. CHU Caen, Department of Anesthesiology and Critical Care Medicine, Caen University Hospital, Avenue de la Côte de Nacre, Caen, 14033, France.
3. Université de Paris, LaPsyDé, CNRS, 75005, Paris, France.
4. CHU Caen, Department of Neonatology, Caen University Hospital, Avenue de la Côte de Nacre, Caen, 14033, France.
5. GIP Cyceron, Caen, France.
6. Université de Paris, NeuroDiderot, INSERM, Paris, France.
7. EA 4650, Caen Normandy University, Unicaen, Esplanade de la Paix, CS 14 032, F-14000 Caen, France.
8. Institut Universitaire de France (IUF), Paris, France.
9. CNRS, UMR-S 3408, GIP Cyceron, Normandie Université, Caen, France.
10. Department of Pediatric Anesthesia and Intensive Care, Necker-Enfants Malades University Hospital, AP-HP. Centre - Université de Paris, France; Université de Paris, EA 7323 "Pharmacologie et évaluation des thérapeutiques chez l'enfant et la femme enceinte", 75006 Paris, France.
11. CHU Caen, Department of Clinical Research, Caen University Hospital, Avenue de la Côte de Nacre, Caen, 14033, France.

**Corresponding author : Jean-Philippe Salaün**

Department of Anesthesiology and Critical Care Medicine, Caen University Hospital, Avenue de la Côte de Nacre, Caen, 14033, France.

Phone number: +33644295087

Email ID: [salaunjeanphilippe@gmail.com](mailto:salaunjeanphilippe@gmail.com)

**Key points summary**

- **Question:** Does early exposure to general anesthesia impact long-term behavior and brain structure in mice and humans?
- **Findings:** This is the first translational study that suggests lasting effects of early life exposure to anesthetics on later emotional control behaviors and brain structures, both in rodents and humans.
- **Meaning:** These considerations should stimulate the development of less brain toxic anesthetic agents and to avoid early exposure to general anesthesia whenever it is possible.

**Word count**

- **Abstract:** 267.
- **Introduction:** 274.
- **Discussion:** 965.
- **Entire body of the text:** 4000.

**Conflicts of interest/ Financial disclosures**

None.

**Funding statement**

This work was supported by the INSERM (French National Institute for Health and Medical Research), the French Ministry for Research and upper Education, the Caen-Normandy University, The Institute Blood and Brain @ Caen-Normandie (BB@C), European FEDER funds “Fonds Européens de Développement Economique et Régional Normandie” and the French National Research Agency (grant/award number: ANR-14-CE30-0014-01 APEX).

**Authors contribution**

**Jean-Philippe Salaün** : This author helped to conception and design of the study, acquisition and analysis of data, drafting a significant portion of the manuscript or figures

**Audrey Chagnot** : This author helped to acquisition and analysis of data, drafting a significant portion of the manuscript or figures

**Arnaud Cachia** : This author helped to conception and design of the study, drafting a significant portion of the manuscript or figures

**Nicolas Poirel** : This author helped to conception and design of the study

**Valérie Datin-Dorrière** : This author helped to acquisition and analysis of data

**Cléo Dujarrier** : This author helped to acquisition and analysis of data

**Eloïse Lemarchand**: This author helped to the revision process

**Marine Rolland** : This author helped to draft a significant portion of the manuscript or figures

**Lisa Delalande** : This author helped to acquisition and analysis of data

**Pierre Gressens** : This author helped to draft a significant portion of the manuscript or figures

**Bernard Guillois** : This author helped to conception and design of the study

**Olivier Houdé** : This author helped to conception and design of the study

**Damien Levard** : This author helped to acquisition and analysis of data

**Clément Gakuba** : This author helped to draft a significant portion of the manuscript or figures

**Marine Moyon** : This author helped to acquisition and analysis of data

**Mikael Naveau** : This author helped to acquisition and analysis of data

**François Orliac** : This author helped to acquisition and analysis of data

**Gilles Orliaguet** : This author helped to draft a significant portion of the manuscript or figures

**Jean-Luc Hanouz** : This author helped to draft a significant portion of the manuscript or figures

**Véronique Agin** : This author helped to acquisition and analysis of data, drafting a significant portion of the manuscript or figures

**Grégoire Borst** : This author helped to conception and design of the study

**Denis Vivien** : This author helped to conception and design of the study

## **ABSTRACT**

### **Background**

One in seven children needs general anesthesia before three years old. Brain toxicity of anesthetics is controversial. Our objective was to clarify whether exposure to general anesthesia of the developing brain could lead to lasting behavioral and structural brain changes.

### **Methods**

A first study was performed in mice. The behaviors (fear conditioning, Y-Maze and actimetry) and brain anatomy (high-resolution Magnetic Resonance Imaging) of six to eight weeks old Swiss mice exposed or not to general anesthesia from 4 to 10 days-old were evaluated. A second study was a complementary analysis from the pre-existing APEX cohort to assess the replicability of our data in humans. The behaviors (Behavior Rating Inventory of Executive Function emotional control and working memory score, Backward Digit Span and Raven 36) and brain anatomy (high-resolution Magnetic Resonance Imaging) were compared in 102 children 9 to 10 years exposed or not to a single general anesthesia (surgery) during infancy.

### **Results**

The animal study revealed chronic exacerbated fear behavior in the adult mice (95% CI: 4 to 80;  $p=0.03$ ) exposed to postnatal general anesthesia; this was associated with a 11% (95% CI: 7.5 to 14.5%) reduction of the periaqueductal grey matter ( $p=0.046$ ). The study in humans suggested lower emotional control (95% CI: 0.33 to 9.10;  $p=0.06$ ) and a 6.1% (95% CI: 4.3 to 7.8%) reduction in the posterior part of the right inferior frontal gyrus ( $p=0.019$ ) in the children who had been exposed to a single general anesthesia.

### **Conclusion**

The preclinical and clinical findings of these independent studies suggest lasting effects of early life exposure to anesthetics on later emotional control behaviors and brain structures.

**KEYWORDS**

developing brain; brain toxicity; pediatric anesthesia.

## **GLOSSARY OF TERMS**

**APEX:** APprentissages EXécutifs et cerveau chez les enfants d'âge scolaire

**BRIEF:** Behavior Rating Inventory of Executive Function

**CAT:** Computational Anatomy Toolbox

**DARTEL:** Diffeomorphic Image Registration Algorithm

**DBM:** Deformation-Based Morphometry

**FEW:** Family Wise Error rate

**GA:** General Anesthesia

**MRI:** Magnetic Resonance brain Imaging

**SPM:** Statistical Parametric Mapping software

**VBM:** Voxel-Based Morphometry

## INTRODUCTION

Increasing preclinical studies suggest that general anesthesia (GA) has negative effects on the developing brain<sup>1</sup>. Exposures to GABA agonists or NMDA antagonists resulted in structural and functional brain impairments including behavioral changes<sup>2</sup>. The issue of the clinical relevance of general anesthetics brain toxicity in children was highlighted in the last decade but gathered less consistent proofs. Some cohort studies reported an association between GA and impaired neurodevelopment<sup>3</sup> while others do not<sup>4</sup>. In one hand, preclinical studies are mandatory even though species differences may preclude any clinical translation. On the other hand, it is practically difficult to design a randomized clinical study on the effect of GA on the developing brain. Human cohort analysis remains an interesting way to examine the question raised. Although the morphologies of rodent and human brains are different, neural circuits involved in cognitive and emotional functions are beginning to be better understood<sup>5,6</sup>.

We used a translational approach. We present the result of 1) an experimental study focused on behavioral and anatomical changes which occur following postnatal exposure to GA in mice, and 2) the analysis of data recorded in an ongoing French cohort study of developing children (“APprentissages EXécutifs et cerveau chez les enfants d’âge scolaire”; APEX). The authors had access to behavior and magnetic resonance brain imaging (MRI) of 9-10 years old children exposed or not to a single GA for minor surgery during infancy.

The main objective of the study was to examine behavioral and structural brain changes in mice and children at a distance from the exposure of the developing brain to general anesthesia.



## **METHODS**

The preclinical study was in accordance with the French ethical laws (Decree 2013-118, Approval n°8962) and the European Communities Council guidelines (2010/63/EU).

The clinical study was approved by a French national ethic committee (IRB 2015-A00383-46, 21/05/2015) and carried out in line with the declaration of Helsinki (1964). Parents or legal guardians gave written consent and children agreed to participate.

The main objective of the preclinical study was to examine behavioral and structural brain changes in mice at a distance from exposure to postnatal GA.

The main objective of the APEX data analysis was to examine long-term behavioral and structural brain imaging according to the exposure or not to GA in infancy. The secondary objectives of the APEX data analysis were to examine the relationship between (1) behavioral changes and structural brain abnormalities (2) the age at exposure to anesthesia and the magnitude of behavioral and structural brain changes.

## **Preclinical investigations**

### *Animals*

Experiments were performed on 6-8 weeks Swiss male mice (Janvier Labs, Le Genest-Saint-Isle, France). The animal care facilities were the Centre Universitaire de Ressources Biologiques (CURB, Caen Normandy University, France; approval n° A14118015).

Body weight was measured before behavioral evaluations. All behavioral tests were performed between 8AM-5PM in a room with dim illumination (6 lx) by an investigator blinded to the allocation group.

Mice were randomly allocated to one of these 2 groups:

- the GA-exposed group: mice were exposed to 1.3% isoflurane (corresponding to 1 MAC in mice), in 100% O<sub>2</sub> during 90 min each day from 4 to 10 days of life).

- the control group: mice were exposed to 100% O<sub>2</sub>, during 90 min each day from P4 to P10.

The exposition occurred in a plastic anesthesia chamber of 19x19x11.5cm with a fresh gas inlet (3 L.min<sup>-1</sup>) on its lid, an aspiration on one of its sides and a humidification system. A warm pad was placed in the anesthesia chamber to maintain the rectal temperature of the neonatal mice to 37°C during the procedure. A gas analyzer was applied to monitor gas concentrations (Capnomac Ultima®, Datex Engstrom). Hypnosis for mice placed under general anesthesia was established by the control of loss of righting reflex, absence of limb withdrawal when applying pressure to the paw and monitoring of heart and respiratory rates. Monitoring of heart and respiratory rates (Small Animal Monitoring and Gating System from SA Instruments Inc®) were performed during each period of exposure to anesthetics and during each procedure of MRI. Awakening of the anesthetized mice after purging volatile anesthetic from the ventilation system was assessed by paraclinical (plethysmography) and clinical observation (responses to tactile stimulations). Finally, these pups were smeared with own bedding and sent back to their dams. At 6-8 weeks old, behavioral experiments and brain imaging were performed (**Table 1**).

### ***Behavioral assessment***

The following behavioral tests were used for motor, emotional and cognitive functions assessment: actimetry<sup>7</sup>, fear conditioning<sup>8</sup> and Y-maze<sup>9</sup>.

**Actimetry.** Spontaneous locomotor activity was quantified by using a rack of eight activity cages equipped with horizontal infrared beams located across the long axis of the cage (Imetronic®, Pessac, France). Mice were placed in individual acrylic chambers (30 x 20 x 20 cm) for 60 min. The number of horizontal and vertical movements was determined by breaks in movement-sensitive photo-beams that were then converted into locomotor activity counts. This test evaluates the hypo- or hyperactivity.

**Contextual fear conditioning.** Contextual fear memory was investigated in a conditioning chamber with white walls (67x53x55 cm, BIOSEB, Chaville, France). Signal generated by the mice movement was recorded and analyzed through a high sensitivity weight transducer system. The analogical signal was transmitted to the Freezing software module through the load cell unit for recording purposes and posterior analysis in terms of activity/immobility (Freezing). On the training day, animals were first individually placed into the conditioning chamber during a 4 min acclimatizing session. During the subsequent training (acquisition) session, two shocks (0.4 mA, 2 sec duration, 2 min inter-trial interval) were administered in the mice's feet. Thirty seconds after the final shock, the mice were returned in their home cages. Twenty-four hours or 1 month after the training session, contextual fear memory was assessed by returning the mice to the conditioning chamber and measuring freezing behavior during a 4 min recall test. The measurement of fear was performed by considering the freezing time, defined as immobility (i.e. the absence of all movements with the exception of those related to respiration) for at least a period of 500 msec.

**Y-maze.** Mice were submitted to a two-trial place recognition task in a Y-maze (34 x 8 x 15 cm; BMP Chaudronnerie, Bretteville-sur-Odon, France) based on a novelty free-choice

exploration paradigm previously developed to study spatial cognition processes in rodents. Extra-maze distal cues were suspended on the black curtains of the room walls at a distance of 123 cm from the maze (from the top of the cues to the center of the maze). During the first trial (acquisition session), one of the three arms of the maze was closed. Each mouse was then placed at the end of the starting arm (Arm 1), and was allowed to visit the accessible portions of the maze (Arm 1 / Arm 2) for 5 min. The mouse was then returned to its cage for three hours, before being tested for a second trial (recall session), in which they had free access to all three arms (Arm 1 / Arm 2 / New arm) for 5 min. Spatial performance was assessed through the comparison of the number of visits in each arm. It has been shown that control mice spend more time in the New arm than in the familiar arms (Arm 1 / Arm2) therefore indicating processing of remote spatial cues.

### ***Brain Imaging***

We used a 7T preclinical magnetic resonance imaging (Bruker, Pharmascan, Advance III) to acquire a 3D isotropic T2 weighted image of the brain of each mouse (TR=13.5s, TE=48ms, RARE acceleration factor=8, matrix=90x90x90, isotropic resolution=0.2mm<sup>3</sup>). The acquisitions were realized on mice anesthetized with isoflurane (1-2% in 100% O<sub>2</sub>) over 20 minutes. Deformation-Based Morphometry<sup>10</sup>, and not Voxel-Based Morphometry (VBM), was used to analyze mouse brain anatomy as DBM overcome the VBM brain segmentation step, which is tedious with mouse.

## **The APEX cohort**

### ***Participants***

The data for the clinical study were derived from the APEX study (2015-2019) which aimed at testing in healthy school-aged children (9-10 years) the behavioral and neural changes induced by an inhibitory control training or a metacognitive mindfulness meditation practice, compared to an active control training (details of APEX recruitment can be found in a previous report<sup>11</sup>). Only baseline data, namely before intervention, were included in the present study. We included all participants (n=102) of the APEX cohort who were right-handed as determined by the Edinburgh Handedness Inventory<sup>12</sup>, born full-term, with normal or corrected-to-normal vision, no history of neurological diseases and cerebral abnormalities. Thus, 24 children who were exposed to a single general anesthesia for non-urgent minor surgery between 1-10 years (**Fig. 1**) constituted the GA-exposed group. Exposure to GA was evaluated by a standardized data form completed by the children's parents. We did not have access to the drugs and dosage of the used during GA. The 78 children who had not been exposed to GA constituted the control group.

### ***Behavioral assessment***

The following behavioral tests were used to assess working memory ability, non-verbal intelligence and emotional control.

**Working memory.** Children working memory abilities were assessed thanks to the backward digit span task<sup>6</sup>. In this task, the children listened to series of discrete digits and were asked to recall the series of digits in the reverse order of presentation. The children first performed two series of two digits. The series of digits were incrementally increased by one digit every two trials. The task was stopped when the children failed to recall two trials with the same number

of digits. The working memory score was defined as the number of series correctly recalled. Working memory were also investigated thanks to the Behavior Rating Inventory of Executive Function (BRIEF) parent form<sup>13</sup>, in order to investigate daily working memory abilities.

**Non-verbal intelligence.** We used the Raven's progressive matrices<sup>14</sup> to assess non-verbal intelligence. This test is a nonverbal task typically used to measure abstract reasoning and estimate fluid intelligence. In each test item, children are asked to identify the missing element that completes a pattern.

**Emotional control.** Emotional control was assessed using the standard Behavior Rating Inventory of Executive Function (BRIEF) parent form<sup>13</sup>.

### ***Brain Imaging***

The APEX study included brain MRI in children aged 9-11 years old. Before the scans, children were familiarized with the MRI session noise in a fake MRI and were trained not to move during the acquisition. T1 MRI acquisitions were performed while they passively watched a mute cartoon on an MRI-compatible screen to reduce motion, provide a positive experience and decrease waiting times<sup>15</sup>. We used the APEX cohort high-resolution isotropic 3T sagittal structural T1 MRI data (TE = 3.3; TR = 7.2; flip angle = 9; 1 x 1 x 1 mm) acquired at the Cycleron biomedical imaging platform (Archieva, Philips Medical System, The Netherlands). Local grey matter volumes were automatically assessed on the whole brain based on the standard voxel-based morphometry (VBM) approach using the Statistical Parametric Mapping software (SPM12) (<http://www.fil.ion.ucl.ac.uk/spm/>) and the Computational Anatomy Toolbox (CAT12) (<http://www.neuro.uni-jena.de/cat/>), a fully automatic, toolbox for performing robust and accurate VBM analyses<sup>16</sup>. We used the CAT12 (CAT12.6) toolbox which is optimized for the segmentation of brains of children (robust initial affine registration

and sample-specific template based on a diffeomorphic image registration algorithm (DARTEL)<sup>17</sup>.

## **Statistical Analysis**

Data were analyzed using the GraphPad Prism software (GraphPad Software, San Diego, California, United States). All analyses were two-tailed.

### ***Preclinical behavioral data***

Analysis of the datasets via parametric approaches turned out to be inappropriate in some cases due to violation of normality (Shapiro-Wilk tests). Therefore, parametric or non-parametric approaches were used whenever appropriated. Behavioral performance was compared between groups using Unpaired Student's t-tests (Actimetry; Fear conditioning; Body Weight). Behavioral performance was compared within groups using Paired Student's t-tests (Y-maze: acquisition session). Friedman's tests were used for multiple comparisons (Y-maze: recall session). When an appropriate significant effect was detected by Friedman's tests, we performed post-hoc analysis using Dunn's Multiple Comparison Tests.

### ***Preclinical imaging data***

Using Advanced Normalization Tools (ANTs), an anatomical template of the control mice was built. Estimating the volumetric differences between the animals, the Jacobian determinant of the deformation field needed to warp the individuals to the template was used. Using SPM12, a second level of analysis was performed of these Jacobian determinant images to test morphological differences between groups. This second level analysis consist of voxel-wise T-tests corrected for the Family Wise Error rate (FWE).



### ***Clinical behavioral data***

Shapiro-Wilk analysis of the whole dataset revealed that the data followed a gaussian distribution, thus enabling the use of standard univariate analyze of covariance (ANCOVA) for each clinical behavioral data. The models included the following covariables: age of the child at tests and monthly parental income, as a proxy of socio-economic status.

### ***Clinical imaging data***

Voxelwise statistical analyses were performed on the whole brain using SPM12 software, within the General Linear Model (GLM) framework. Analyses were performed on the smoothed (8 mm) and modulated grey matter maps with gender, age, parental income as a proxy of socio-economic status, and weight at birth as matching variables. All analyses were performed using a voxelwise threshold at  $p < 0.05$ , corrected for multiple testing using SPM12 Family Wise Error (FWE) method. We further investigated some possible associations of grey matter deficits with behavioral scores using small volume correction (SVC) with a 10 x 10 x 10 mm search volume centered at the VBM cluster max voxel.

### ***Sample size justifications***

We decided of the number of animals per group for neuroanatomy and behavior tasks based on our previous published studies and ethical recommendations. Because the clinical study was a secondary analysis of the APEX cohort, it was not possible to calculate statistical power a priori. All statistical powers were calculated a posteriori using G\*Power.

Preclinical investigations: Post hoc power calculations were found equal at 0.59 for fear conditioning at one month recall session, 0.05 for actimetry horizontal activity, 0.05 for actimetry vertical activity and 0.24 for Y-maze recall session. Based on our group sizes for GA-

exposed mice and controls, we determined the effect size required for  $1-\beta=0.9$  and  $\alpha=0.05$  at  $d=0.65$  for fear conditioning at one month recall session,  $d=0.04$  for actimetry horizontal activity,  $d=0.04$  for actimetry vertical activity and  $d=0.37$  for Y-maze recall session. Post hoc power was found equal at 0.99 for MRI cluster. Based on our group sizes for GA- exposed mice and controls, we determined the effect size required for  $1-\beta=0.9$  and  $\alpha=0.05$  at  $d=3.86$  for MRI cluster.

The APEX cohort: Post hoc power calculations were found equal at 0.43 for BRIEF emotional control score, 0.13 for BRIEF working memory score, 0.05 for Raven's progressive matrices and 0.05 for Backward Digital Span. Based on our group sizes for GA- exposed children and controls, we determined the effect size required for  $1-\beta=0.9$  and  $\alpha=0.05$  at  $d=0.42$  for BRIEF emotional control score,  $d=0.19$  for BRIEF working memory score,  $d=0.005$  for Raven's progressive matrices and  $d=0.03$  for Backward Digital Span. Post hoc power was found equal at 0.99 for MRI cluster. Based on our group sizes for GA- exposed children and controls, we determined the effect size required for  $1-\beta=0.9$  and  $\alpha=0.05$  at  $d=2.86$  for MRI cluster.

## RESULTS

### Preclinical investigations

No mortality was reported during experiments.

Control and GA-exposed mice showed similar body weight at 6 weeks of life (control mice: weight (g) =  $39.5 \pm 2.8$ ; GA-exposed mice: weight =  $39.7 \pm 2.7$ ).

**MRI Deformation based morphometry.** There was a 11% (95% CI: 7.5% to 14.5%) reduction in the volume of the periaqueductal grey matter in the GA-exposed group (**Fig. 2**; peak-level,  $t=7.32$ ,  $dof=15$ ;  $p\text{-FWE} = 0.046$ ).

**Actimetry.** There was no significant difference between groups in horizontal locomotor activities (**Fig. 3A**) and vertical activities (**Fig. 3B**).

**Contextual fear conditioning.** There was no significant difference between groups in the percentage of freezing during the acclimatizing period (acquisition) session: all mice showed high levels of fear following the two aversive unconditioned stimuli without differences between groups (**Fig. 3C**). One month after the training session, there was a higher freezing time (ft) in the GA-exposed mice (ft =  $115 \pm 73$  sec) as compared to the control group (ft =  $73 \pm 56$  sec; 95% confidence interval: 4 to 80;  $p = 0.03$ ; **Fig. 3D**).

**Y-maze.** During the acquisition session, there was no significant difference between groups in the total number of visits in the two free access arms of the Y-maze (**Fig. 3E and 3F**). During the recall session performed three hours later, the new arm of the maze was visited similarly in GA-exposed and Control groups (**Fig. 3G and 3H**).

## **The APEX cohort**

There was no difference between groups in main children's characteristics (**Table 2**). In the GA-exposed children, mean age at anesthesia was  $4.0 \pm 2.3$  years old (29% of patients were 3 years old and under, 54% were between 4 and 6 years old and 17% were over 6 years old).

**BRIEF emotional control score.** **Figure 4A** suggests lower emotional control in the GA-exposed children ( $58 \pm 12$ ) as compared to the Control ones ( $53 \pm 11$ ; difference=5; 95% CI of the difference: 0.33 to 9.10;  $p=0.06$ ).

**BRIEF working memory score.** There was no significant difference between groups in the BRIEF working memory scores (**Fig. 4B**).

**Raven's progressive matrices.** There was no significant difference between groups in the Raven's progressive matrices (**Fig. 4C**).

**Backward Digit Span.** There was no significant difference between groups in the Backward Digit Span performance (**Fig. 4D**).

Multivariate analysis found no relationship between behavioral and memory scores and age at the time of anesthesia.

**MRI Voxel based morphometry (VB gr M) analysis.** In the GA-exposed children there was a 6.1% (95% CI: 4.3 to 7.8%) lower grey matter volume in a single cluster corresponding to the posterior part of the right inferior frontal gyrus, BA9, 'aal' atlas (MNI coordinates  $x,y,z = [51;3;27]$ ); height threshold:  $Z = 4.85$ ,  $p\text{-FWE} = 0.019$ ; **Fig. 4E, 4F, 4G, 4H**). There was no difference in local white matter volume between groups.

Furthermore, an SVC analysis centered at  $[51;3;27]$  revealed positive main effects of the BRIEF emotional control score (height threshold:  $Z = 2.65$ ,  $p\text{-FWE} = 0.047$ ) and of the age at GA (height threshold:  $Z = 2.94$ ,  $p\text{-FWE} = 0.024$ ) on the volume of grey matter in the posterior part of the right inferior frontal gyrus. Of note, the same analysis with the BRIEF working memory score did not yield any significant correlation.

## DISCUSSION

Our preclinical study revealed exacerbated fear behavior associated with an atrophy of the periaqueductal grey matter in the adult mice exposed to postnatal GA. Our clinical study provides the first evidence that exposure to GA in childhood can lead to a grey matter atrophy in the right prefrontal gyrus which is more pronounced the earlier the exposure to anesthesia.

Our study revealed a periaqueductal grey matter atrophy in mice 6-8 weeks after repeated postnatal anesthesia with 1 MAC of isoflurane (day 4-10). The periaqueductal grey matter is a key component in context fear discrimination in mice<sup>18</sup> and is involved in anxiety and depression<sup>19</sup>. These results are consistent with the literature<sup>20</sup>. The lack of difference in locomotor activity and working memory between mice neonatally exposed to isoflurane is consistent with the literature. Repeated isoflurane anesthesia exposure of mice during development (day 7 to 9) does not impact locomotor activity<sup>21</sup>. Moreover, performance in working memory was not impaired in adult mice exposed to three hours of 2.7% isoflurane (day 6)<sup>22</sup>. Human data obtained from the APEX cohort suggested that the GA-exposed children (age at anesthesia:  $4.0 \pm 2.3$  years old) presented lower grey matter volumes in the right inferior frontal gyrus, the more pronounced the earlier the anesthesia. Reductions in the inferior frontal gyrus volume have been associated with impaired emotion functions and later depression<sup>23,24</sup>. These neuroimaging results could be associated with impaired emotional control. Our results are consistent with a meta-analysis of the 3 main prospective clinical studies about anesthesia-induced brain toxicity, which showed an impaired BRIEF executive function global score (RR: 1.68 [95% CI 1.23-2.30];  $p=0.001$ ) in 841 children exposed to GA<sup>25</sup>.

## Strengths

These term born children of the APEX cohort, scheduled for minor non-urgent surgery, had no history of neurological and cerebral abnormalities that could influence the interpretation of the results<sup>26</sup>. The analyses were controlled for socio-economic status. In our study, ear nose and throat surgeries (46.0%), digestive (20.8%) and urological surgeries (16.7%) were in decreasing order of frequency the most represented surgical specialties. Similar patterns of the indications of surgery were reported in the literature<sup>27</sup>. This clinical study provides the first evidence that exposure to GA in childhood (excluding the neonatal period) can lead to grey matter atrophy. It would be interesting to re-evaluate the possible impact of GA on the parameters evaluated in the previous APEX related studies such as the cortex anatomy<sup>11</sup> and the baseline resting-state function which may be intrinsically related to the local structure<sup>28</sup>. Only one study had yet reported a grey matter decrease after GA in childhood<sup>29</sup>. The correction for multiple comparisons was applied only to the voxels within the single region of interest and not for all the voxels of the brain as in our study. This statistical method could be associated with high false positive rates. In our study, the grey matter atrophy persisted after *FWE*-correction for multiple tests on the whole brain.

The most likely mechanism to explain the atrophy detected after early GA exposure is the apoptosis of GABAergic and glutamatergic neurons<sup>2</sup>. After exposing neural stem cell to 1 MAC of sevoflurane, cell viability decreased and cytotoxicity increased in time-dependent way<sup>30</sup>. This is all the more likely because periaqueductal grey matter contains GABAergic interneurons<sup>31</sup>. Another possible mechanism is neuroinflammation<sup>32</sup>. In monkeys, the interest of a non-invasive neuroimaging evaluation of glial activation after early sevoflurane exposition was pointed out by using Positron Emission Tomography<sup>33</sup>.

## Limitations and perspectives

The first limitation is the lack of data about the drugs administered in children from the APEX study. It could be hypothesized that anesthesia involved sevoflurane, the most currently used anesthetics in pediatric anesthesia in France<sup>34</sup>. Moreover, no perioperative anesthetic complications were reported in the health records. The second limitation is that the GA-exposed children were exposed at various ages. The third limitation is that mice underwent multiple exposures to approximate concentrations of isoflurane and that their mean arterial pressure during anesthesia could not be monitored for technical reasons. However, heart and respiratory rates were monitored and there was no death in the GA-exposed mice. The fourth limitation is that if rodents are the most commonly used mammalian models for studying the neurotoxicity of anesthetics, their brain morphologies are dissimilar to humans<sup>35</sup>. However, the affected regions by general anesthesia (1) in mice and (2) in humans (periaqueductal grey matter and the right inferior frontal gyrus, respectively), although not identical, are both involved in the control of emotions. For instance, Rozeske's study has identified a subpopulation of prefrontal-periaqueductal grey-projecting neurons that control switching between different emotional states during contextual fear discrimination in mice<sup>18</sup>. Similarly, Blakemore's study has highlighted a cortico-subcortical network involving right inferior frontal gyrus and periaqueductal grey matter also implicated in emotional control in human<sup>36</sup>. Moreover, rodents brain activity during daily life situations mainly rests upon fear processes, an adaptive surviving behavior which depends largely on the periaqueductal grey matter<sup>37</sup>. Similarly, the human prefrontal part of the brain is a core region mainly necessary for daily life activity, in order to accurately deal with emotional and behavioral regulation<sup>23</sup>.

Future preclinical and clinical studies should also investigate to what extent detrimental effects of GA could be driven by side effects occurring during GA such as hypotension, hypocapnia or hypoxia<sup>38</sup>. The NECTARINE study recently highlighted difficulties to accurately monitor

neonates and small infants<sup>39</sup>. This is also difficult in newborn mice. Finally, intraoperative data are often missing in studies on anesthesia toxicity<sup>13,40</sup>.

## **Conclusion**

We present the first translational study that analyzes the behavioral and structural brain impact of childhood exposure even to a single GA, the result of a successful collaboration between preclinical and clinical researchers. It should stimulate the development of less brain toxic anesthetic agents, better monitoring anesthesia in children and setting up long-term monitoring of our cohort in order to determine whether children are prone to develop psychiatric disorders.



## **ACKNOWLEDGEMENTS**

The authors thank the children who participated in the study and their families.

## REFERENCES

1. Jevtovic-Todorovic V. Monkey business: the importance of mounting behavioural evidence for anaesthesia-induced developmental neurotoxicity. *Br J Anaesth*. 2018;120(4):617-619.
2. Jevtovic-Todorovic V. Exposure of Developing Brain to General Anesthesia: What Is the Animal Evidence? *Anesthesiology*. 2018;128(4):832-839.
3. Wilder RT, Flick RP, Sprung J, et al. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology*. 2009;110(4):796-804.
4. McCann ME, de Graaff JC, Dorris L, et al. Neurodevelopmental outcome at 5 years of age after general anaesthesia or awake-regional anaesthesia in infancy (GAS): an international, multicentre, randomised, controlled equivalence trial. *Lancet Lond Engl*. 2019;393(10172):664-677.
5. Russell LE, Dalgleish HWP, Nutbrown R, et al. All-optical interrogation of neural circuits in behaving mice. *Nat Protoc*. 2022; 17(7):1579-1620.
6. Poirel N, Borst G, Simon G, et al. Number conservation is related to children's prefrontal inhibitory control: an fMRI study of a piagetian task. *PloS One*. 2012;7(7):e40802.
7. Mannara F, Radosevic M, Planagumà J, et al. Allosteric modulation of NMDA receptors prevents the antibody effects of patients with anti-NMDAR encephalitis. *Brain J Neurol*. 2020;143(9):2709-2720.
8. Chatterjee S, Angelakos CC, Bahl E, et al. The CBP KIX domain regulates long-term memory and circadian activity. *BMC Biol*. 2020;18(1):155.
9. Dellu F, Contarino A, Simon H, Koob GF, Gold LH. Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. *Neurobiol Learn Mem*. 2000;73(1):31-48.

10. Chung MK, Worsley KJ, Paus T, et al. A unified statistical approach to deformation-based morphometry. *NeuroImage*. 2001;14(3):595-606.
11. Delalande L, Moyon M, Tissier C, et al. Complex and subtle structural changes in prefrontal cortex induced by inhibitory control training from childhood to adolescence. *Dev Sci*. Published online August 30, 2019:e12898.
12. Oldfield RC. The assessment and analysis of handedness: The Edinburgh inventory. *Neuropsychologia*. 1971;9(1):97-113.
13. Baron IS. Behavior rating inventory of executive function. *Child Neuropsychol J Norm Abnorm Dev Child Adolesc*. 2000;6(3):235-238.
14. Raven J, Raven JC, Court JH. Manual for Raven's progressive matrices and vocabulary scales. Oxford, UK: Oxford Psychologists Press; 1998.
15. Lemaire C, Moran GR, Swan H. Impact of audio/visual systems on pediatric sedation in magnetic resonance imaging. *J Magn Reson Imaging JMRI*. 2009;30(3):649-655.
16. Ashburner J. Computational anatomy with the SPM software. *Magn Reson Imaging*. 2009;27(8):1163-1174.
17. Ashburner J. A fast diffeomorphic image registration algorithm. *NeuroImage*. 2007;38(1):95-113.
18. Rozeske RR, Jercog D, Karalis N, et al. Prefrontal-Periaqueductal Gray-Projecting Neurons Mediate Context Fear Discrimination. *Neuron*. 2018;97(4):898-910.e6.
19. Ho YC, Lin TB, Hsieh MC, et al. Periaqueductal Gray Glutamatergic Transmission Governs Chronic Stress-Induced Depression. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol*. 2018;43(2):302-312.
20. Satomoto M, Sun Z, Adachi YU, Makita K. Neonatal Sevoflurane Exposure Induces Adulthood Fear-induced Learning Disability and Decreases Glutamatergic Neurons in the Basolateral Amygdala. *J Neurosurg Anesthesiol*. 2018;30(1):59-64.

21. Rosenholm M, Paro E, Antila H, Vöikar V, Rantamäki T. Repeated brief isoflurane anesthesia during early postnatal development produces negligible changes on adult behavior in male mice. *PloS One*. 2017;12(4):e0175258.
22. Kodama M, Satoh Y, Otsubo Y, et al. Neonatal desflurane exposure induces more robust neuroapoptosis than do isoflurane and sevoflurane and impairs working memory. *Anesthesiology*. 2011;115(5):979-991.
23. Zhang L, Li W, Wang L, et al. Altered functional connectivity of right inferior frontal gyrus subregions in bipolar disorder: a resting state fMRI study. *J Affect Disord*. 2020;272:58-65.
24. Luby JL, Barch D, Whalen D, Tillman R, Belden A. Association Between Early Life Adversity and Risk for Poor Emotional and Physical Health in Adolescence: A Putative Mechanistic Neurodevelopmental Pathway. *JAMA Pediatr*. 2017;171(12):1168-1175.
25. Ing C, Jackson WM, Zaccariello MJ, et al. Prospectively assessed neurodevelopmental outcomes in studies of anaesthetic neurotoxicity in children: a systematic review and meta-analysis. *Br J Anaesth*. 2021;126(2):433-444.
26. Andropoulos DB, Easley RB, Brady K, et al. Changing expectations for neurological outcomes after the neonatal arterial switch operation. *Ann Thorac Surg*. 2012;94(4):1250-1255; discussion 1255-1256.
27. Habre W, Disma N, Virag K, et al. Incidence of severe critical events in paediatric anaesthesia (APRICOT): a prospective multicentre observational study in 261 hospitals in Europe. *Lancet Respir Med*. 2017;5(5):412-425.
28. Salvia E, Tissier C, Charron S, et al. The local properties of bold signal fluctuations at rest monitor inhibitory control training in adolescents. *Dev Cogn Neurosci*. 2019;38:100664.
29. Backeljauw B, Holland SK, Altaye M, Loepke AW. Cognition and Brain Structure Following Early Childhood Surgery With Anesthesia. *Pediatrics*. 2015;136(1):e1-12.

30. Qiu J, Shi P, Mao W, Zhao Y, Liu W, Wang Y. Effect of apoptosis in neural stem cells treated with sevoflurane. *BMC Anesthesiol.* 2015;15:25.
31. Yin W, Mei L, Sun T, et al. A Central Amygdala-Ventrolateral Periaqueductal Gray Matter Pathway for Pain in a Mouse Model of Depression-like Behavior. *Anesthesiology.* 2020;132(5):1175-1196.
32. Baud O, Saint-Faust M. Neuroinflammation in the Developing Brain: Risk Factors, Involvement of Microglial Cells, and Implication for Early Anesthesia. *Anesth Analg.* 2019;128(4):718-725.
33. Zhang X, Liu S, Newport GD, et al. In Vivo Monitoring of Sevoflurane-induced Adverse Effects in Neonatal Nonhuman Primates Using Small-animal Positron Emission Tomography. *Anesthesiology.* 2016;125(1):133-146.
34. Apai C, Shah R, Tran K, Pandya Shah S. Anesthesia and the Developing Brain: A Review of Sevoflurane-induced Neurotoxicity in Pediatric Populations. *Clin Ther.* 2021;43(4):762-778.
35. Chinn GA, Pearn ML, Vutskits L, et al. Standards for preclinical research and publications in developmental anaesthetic neurotoxicity: expert opinion statement from the SmartTots preclinical working group. *Br J Anaesth.* 2020;124(5):585-593.
36. Blakemore RL, Rieger SW, Vuilleumier P. Negative emotions facilitate isometric force through activation of prefrontal cortex and periaqueductal gray. *NeuroImage.* 2016;124(Pt A):627-640.
37. Deng H, Xiao X, Wang Z. Periaqueductal Gray Neuronal Activities Underlie Different Aspects of Defensive Behaviors. *J Neurosci Off J Soc Neurosci.* 2016;36(29):7580-7588.
38. Weiss M, Vutskits L, Hansen TG, Engelhardt T. Safe Anesthesia For Every Tot - The SAFETOTS initiative. *Curr Opin Anaesthesiol.* 2015;28(3):302-307.
39. Disma N, Veyckemans F, Virag K, et al. Morbidity and mortality after anaesthesia in

early life: results of the European prospective multicentre observational study, neonate and children audit of anaesthesia practice in Europe (NECTARINE). *Br J Anaesth.* 2021;126(6):1157-1172.

40. Warner DO, Zaccariello MJ, Katusic SK, et al. Neuropsychological and Behavioral Outcomes after Exposure of Young Children to Procedures Requiring General Anesthesia: The Mayo Anesthesia Safety in Kids (MASK) Study. *Anesthesiology.* 2018;129(1):89-105.

## TABLES AND FIGURES LEGENDS

**Table 1: Number and age of mice used in the study.**

**Table 2: Human population characteristics.**

*p*-value: \* < 0.05; \*\* < 0.01; \*\*\* < 0.001.

### **Figure 1: Characteristics of the children exposed to anesthesia**

(A) Ages of the anesthetized children.

(B) Reasons for anesthesia.

### **Figure 2: Preclinical imaging**

(A) For illustration purpose and ease of visualization, representation of the cluster (*uncorrected statistics*), showing an area covering the left periaqueductal grey matter.

(B) Proportion of grey matter is lower in general anesthetized mice vs. controls in the cluster.

(C,D,E) Orthogonal slices representing the position of the cluster (*FWE-corrected*).

(F,G,H) Orthogonal slices representing the position of the extended cluster (*FWE-uncorrected*).

(I,J,K) 3D views of the extended cluster (*FWE-uncorrected*).

### **Figure 3: Preclinical behavioral testing**

(A,B) Actimetry: horizontal (A) and vertical (B) spontaneous locomotor activity. No differences in horizontal or vertical movements were observed between control and general anesthesia-subjected mice (22 control and 17 anesthesia). Boxplots show distributions with black horizontal lines indicating the median, box margins denoting the lower and upper quartiles. Whiskers show the minimum and maximum values.

(C,D) Fear conditioning: acquisition (C) and recall (D) sessions. Freezing response at 30 days after acquisition is higher in general anesthesia-subjected mice (22 control and 22 anesthesia).

Boxplots show distributions with black horizontal lines indicating the median, box margins denoting the lower and upper quartiles. Whiskers show the minimum and maximum values.

(E,F,G,H) Y-Maze: acquisition (E,F) and recall (G,H) sessions. Visit frequency in the new arm is higher than in familiar arms (1,2) at recall in both control and general anesthesia-subjected mice (24 control and 24 anesthesia). Boxplots show distributions with black horizontal lines indicating the median, box margins denoting the lower and upper quartiles. Whiskers show the minimum and maximum values.

#### **Figure 4: Clinical imaging and behavioral testing**

(A) Trend to higher BRIEF emotional control score in children subjected to a single general anesthesia ( $p$ -corrected = 0.05; controlled for gender, age, parental income as a proxy of socio-economic status, and weight at birth). Scores on the BRIEF working memory scale (B), RAVEN36 (C), and backward digit span (D) did not differ between the groups.

(E, F) Lower local grey matter volume in children who were subjected to a single general anesthesia ( $p$ -FWE < 0.05; controlled for gender, age, parental income as a proxy of socio-economic status, and weight at birth).

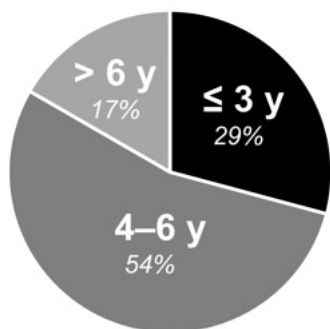
(G) Three-dimensional rendering of the cortical surface and VBM cluster in the posterior part of the right inferior frontal gyrus (BA9) (voxel maximum: Talairach's x, y, z = [51;3;27]). For illustration purpose and ease of visualization, the cluster is represented with uncorrected statistics ( $p$ -uncorrected < 0.001).

(H) Grey matter volume in the VBM cluster located in the posterior part of the right inferior frontal gyrus is lower in children subjected to a single general anesthesia ( $p$ -FWE < 0.05; controlled for gender, age, parental income as a proxy of socio-economic status, and weight at birth).

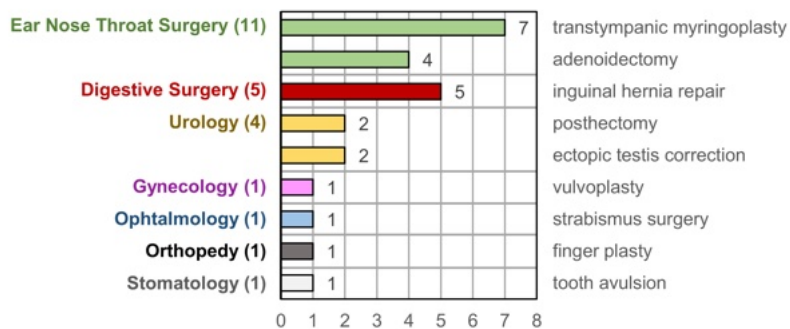


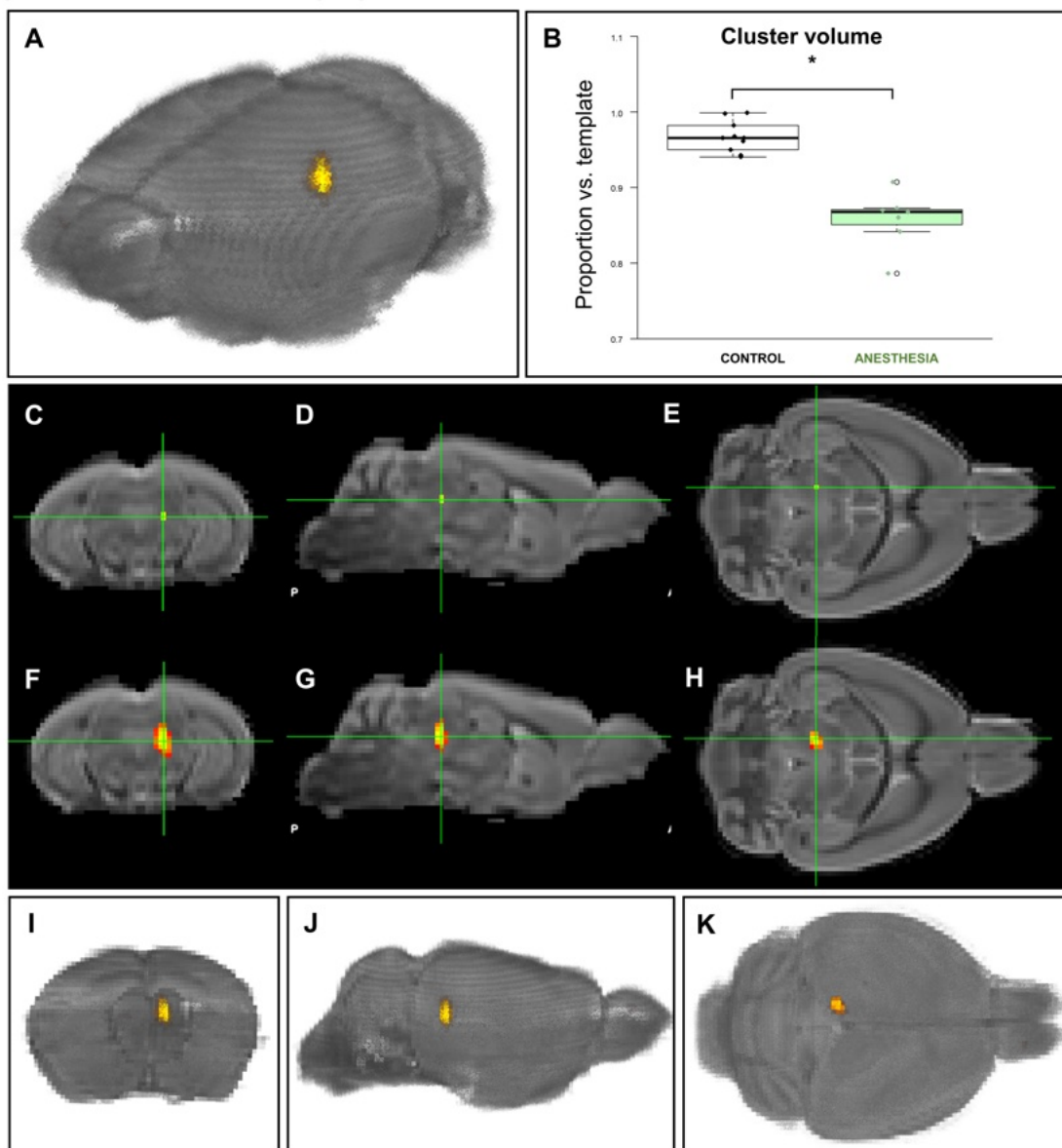
**Figure 1 : Characteristics of the children exposed to anesthesia**

**A**      **Ages of the anesthetized children**

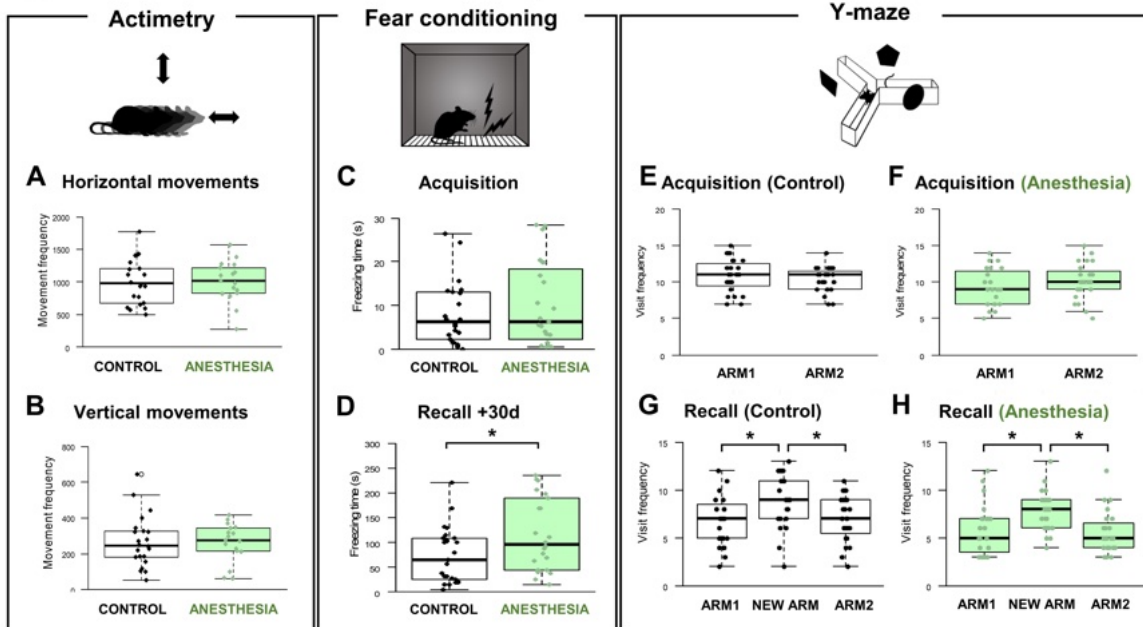


**B**      **Reasons for anesthesia**



**Figure 2 : Preclinical imaging**

**Figure 3 : Preclinical behavioral testing**



**Figure 4 : Clinical imaging and behavioral testing**

