III. Research plan

A. Introduction

1. Objectives

Neonatal anesthesia causes widespread neurodegeneration in rats and a cognitive deficit 28 days later (1). Usually neuronal loss increases the formation of new neurons from neural (stem-) progenitor cells (neurogenesis) (2-4), but our preliminary data shows decreased neural progenitor proliferation in neonatal rats following a 4-hour isoflurane anesthetic, and a cognitive deficit 5 months and 8 months later. In light of recent reports of anesthesia-induced neuronal apoptosis in many animal species, including monkeys (5,6), the FDA has become increasingly concerned about the effects of anesthetics on the developing human brain. Prior to re-evaluating any particular anesthetic or a group of anesthetics for deregulation in human neonates and infants several important questions must be answered:

A) What is the safest single anesthetic agent for anesthesia in neonates / infants? So far anesthetic cocktails(1,6), midazolam(6) and ketamine(5,7,8) have been studied.

B) What is a safe duration of an anesthetic at clinically relevant concentrations (e.g. 1 minimum alveolar concentration (MAC))? So far the shortest duration of anesthesia systematically studied in neonates has been 4 h. It is possible that some shorter duration of anesthesia will not affect neurocognitive outcome.

C) Can post-anesthesia cognitive decline be prevented by reducing neuronal apoptosis and / or stimulating neurogenesis? Erythropoietin (epo), which is critically important for neonatal brain development, enhances neurogenesis in rats (9,10). In addition epo rescues neurons from apoptotic cell death when given prior to (11) or early after an otherwise lethal insult (12,13). Preventing anesthetic induced changes in neurodegeneration, neurogenesis, and cognitive outcome with an agent as safe as epo (12) represents an attractive potential solution to the clinical problems posed by neonatal anesthetic toxicity, as well as a potential methodology for determining whether it exists in humans, as described in potential problems.

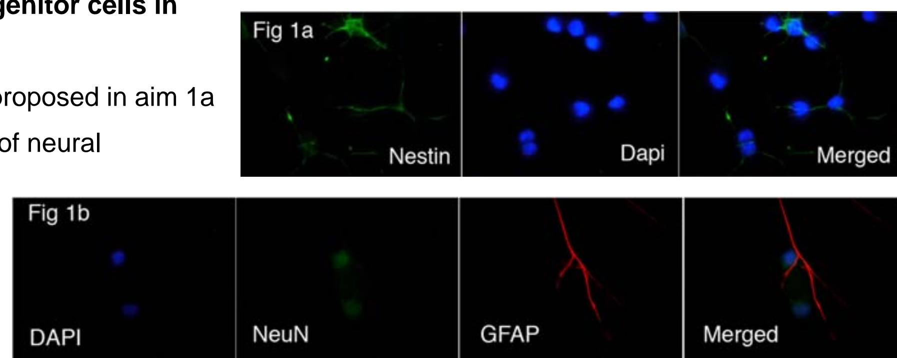
We propose to address each of these important issues. First, to answer question A) we will identify the anesthetic agent with the least toxicity on neural progenitors by assessing death in cell cultures exposed to a wide variety of agents *in vitro.* We will confirm the result by assessing neurodegeneration, neural progenitor cell proliferation, neurogenesis, and long-term cognitive outcome *in vivo*. Next, we will use this same comprehensive approach to address question B) and define the safe duration for 1 MAC of isoflurane (iso) *in vitro*, and then *in vivo*. Finally, to answer question C) we will test whether permanent neurocognitive decline due to neonatal anesthesia can be prevented by administration of epo 4h prior to anesthesia. Together the proposed studies will test the effectiveness of three easily applicable interventions aimed at improving the safety of neonatal anesthesia and thus provide the scientific foundation on which to base future human studies.

2. Background

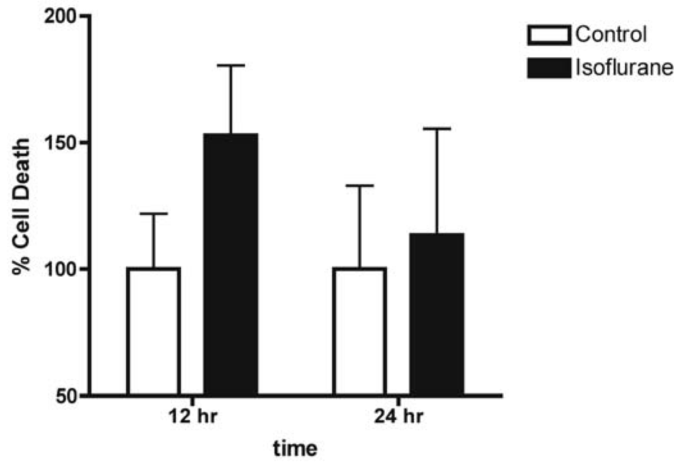
Neonatal rats develop neuronal apoptosis following anesthesia, which is associated with a neurocognitive deficit 28 days later(1,14). These findings were presented by John Olney at the 2003 Annual Meeting of the Society for Neurosurgical Anesthesia and Critical Care, and their implications caused irritation and anxiety in the anesthesia community and the lay press alike, culminating in a heated open debate in *Anesthesiology* (7,8,15,16). Since then, one key argument of clinicians, that Olney's findings in rats had not been duplicated in other species, has been slowly eroded by the demonstration of anesthesia-induced neuronal apoptosis in many animal species, including monkeys (5,6). Another key argument of clinicians is that Olney and coworkers did not study the combination of anesthesia and surgery and that anesthesia without surgery may be as deleterious to the brain as surgery without anesthesia. Our preliminary data are not subject to this argument because we found a permanent neurocognitive deficit following anesthesia during application of tail-clamping, a supramaximal pain stimulus simulating surgery, which is used to measure anesthetic depth. There is now little doubt that neurodegeneration due to neonatal anesthesia occurs. However, the relative safety of different anesthetic agents and durations is not known. Neither is the effect of epo, which in rats reduces apoptosis(11-13) and stimulates neurogenesis(9,10). Epo is a very safe drug that crosses the blood brain barrier, causes transcription of genes encoding for antiapoptotic and neurotrophic proteins, an effect which takes 4 hours to fully develop and which lasts for 3 days(11,17). In rats, epo improves motor function (18) and neurocognitive outcome 5 months after stroke (13). In an initial small clinical trial epo improved outcome following stroke (12). This is despite the fact that strokes cannot be predicted and therefore epo could only be administered following the stroke. The temporal predictability of the insult by anesthesia, on the other hand, is high. Therefore, it seems reasonable to hypothesize that epo will be effective in improving outcome following neonatal anesthesia. Understanding the range of effects of commonly used anesthetics and how those effects can be reduced is a crucial prerequisite to designing an effective and ethical human trial. We have chosen to test the efficacy of three easily translatable approaches to minimize the ill effects of anesthesia and maximize capacity of the brain to respond to an insult.

Preliminary data

Figure 1: Isoflurane is toxic to neural progenitor cells in culture.



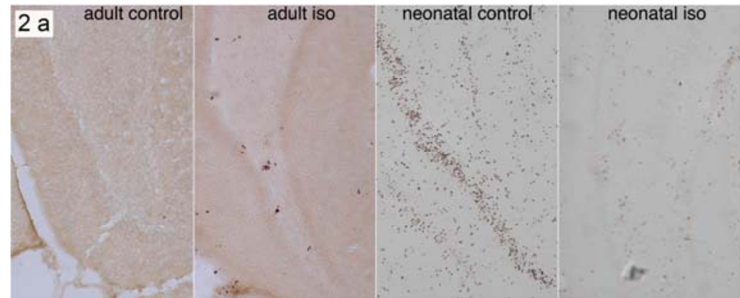
We performed experiments similar to those proposed in aim 1a and 2a. **1a)** Fluorescent microscopic image of neural progenitor cells. Nestin is a marker for undifferentiated neural (stem-) progenitor cells, which can differentiate into neurons. Approximately 80% of undifferentiated cells express this marker. Similar results (not shown) were obtained with SOX2, another marker of precursor/stem cells. Cells were counterstained with Dapi, a nuclear marker.



1b) Cells were differentiated by removal of bFGF and addition of serum containing media. NeuN is a nuclear protein seen in neurons and GFAP (glial fibrillary acidic protein) is found in astrocytes. Similar results (not shown) were seen with MAP2, another neuronal marker and O1 a marker of oligodendrocytes.

1c) The proportion of PI+ (dead) neural progenitors is significantly increased 12h following isoflurane administration.

Figure 2: Isoflurane decreases progenitor proliferation in neonatal but not in adult rats. We performed experiments similar to those proposed in aims 1b, 2b and 3. Seven-day-old and 60 day-old male Sprague Dawley rats received 4h iso or no iso (control). BrdU labeling was done on day 4 with 50mg/kg x 2 every 12 h. Rats were perfused 24h later followed by immunocytochemistry and cell-counting procedures as described in detailed methods. Microscopic image **(2a)** of the dentate gyrus of the hippocampus at 100x magnification. BrdU labeled cells appear dark. The number of BrdU+ cells is decreased significantly in neonatal **(2c)** but not in adult **(2b)** isoflurane-treated animals ($p<0.05$, MWU).



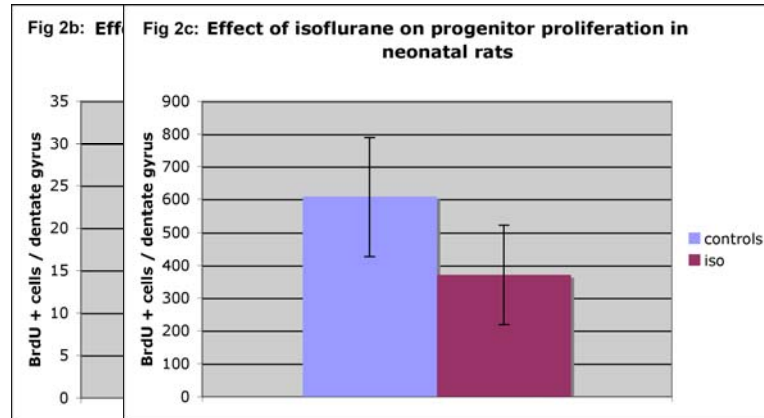
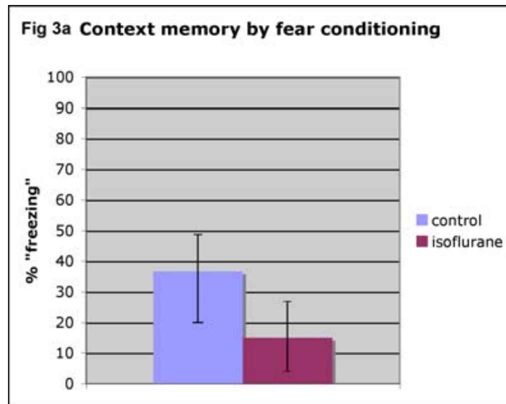


Figure 3: Neonatal isoflurane anesthesia causes a permanent neurocognitive deficit. We performed experiments similar to those described in aims 1b, 2b and 3. Fear conditioning and Morris water maze testing was performed 5 months and 8 months after a 4 h isoflurane anesthetic given to 7 day-old rats (see detailed methods). Freezing scores **(3a)** of the hippocampal-dependent portion (context memory) of fear conditioning were significantly lower in 5 month-old rats that had undergone a 4h isoflurane anesthetic as neonates than in controls ($p<0.05$ MWU-test), indicating that isoflurane caused a hippocampal-dependent but not a hippocampal independent cognitive deficit. The data for the hippocampal-independent

portion of the fear conditioning test are not shown.



Path lengths of Morris water maze trial **(3b)** in the same animals at 8 months of age. While the platform was visible, no difference in performance exists between groups. When the platform was hidden (trials 5 -10), the latencies of the iso group to reach the escape platform increased to a significantly greater degree than those of controls. ($p<0.05$, Mixed model analysis, SAS).

3. Specific aims

Anesthesia causes neurodegeneration and a decrease in progenitor proliferation resulting in a permanent impairment in cognitive development of neonatal rats. To test three strategies with high clinical applicability aimed at improving cognitive outcome of neonatal anesthesia we propose to answer the following questions.

Aim 1: What is the safest anesthetic agent commonly used in neonatal anesthesia?

Aim 1a: To determine a hierarchy of toxicity of commonly used anesthetic agents on cultured neural progenitor cells *in vitro*.

Aim 1b: We will test the effect of the most and least toxic anesthetic agents determined in aim 1a on neurodegeneration, neurogenesis and cognitive outcome *in vivo.*

Aim 2: What is a safe duration of 1 MAC of iso?

Aim 2a: To define a duration of 1 MAC of iso that is not toxic to progenitor cells *in vitro.*

Aim 2b: We will determine if the duration of iso that was safe in aim 1b is devoid of effects on neuronal apoptosis, neurogenesis and cognitive outcome *in vivo*.

Aim 3: To test the hypothesis that epo can decrease neurodegeneration, increase neurogenesis and normalize neurocognitive outcome after a 4h anesthetic with the most toxic agent identified in aim 1.

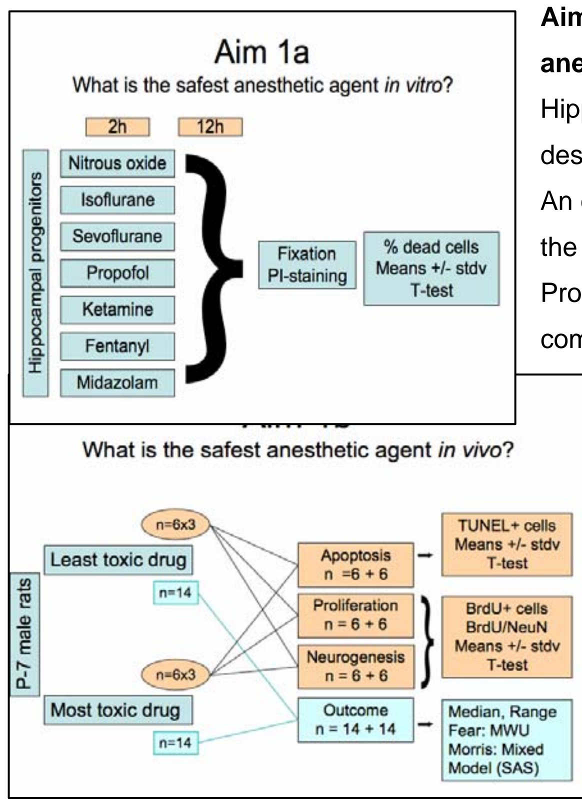
4. Significance and applicability

Roughly 1.5 million anesthetics are administered to neonates each year(7). Recent data suggest that anesthesia is toxic to neonatal neurons(1). The heated debate in the scientific(7,8,15,16) and the lay press (19-22) caused by these findings illustrates the public importance of this topic. It is now clear that the initial rat data are applicable to

a number of species including monkeys(5). Clinicians are currently caught between a rock – withholding anesthesia from neonates undergoing surgery - and a hard place – administering a neurotoxic substance. We hope to be able to help tip the balance one way or another. We think that no study of anesthetic brain damage is complete without also studying brain repair (neurogenesis) including a sufficiently long waiting period until neurocognitive testing to allow for full maturation of new neurons. While we showed that anesthesia is also toxic to neural progenitor cells *in vitro* and *in vivo*, it is unclear if this translates to a decrease in neurogenesis. An anesthesia-related decrease in neurogenesis in neonates is plausible since we also found a permanent neurocognitive defect in 5-8 month old rats that received an anesthetic as neonates, and since the rate of neurogenesis determines cognitive function (23-27). If neurogenesis plays a role in the post-anesthetic cognitive decline following neonatal anesthesia then the problem may be subject to therapeutic strategies by limiting the degree of toxicity to progenitor cells and by stimulating neurogenesis pharmacologically. The proposed research will assess the anesthetic effect on neonatal neurogenesis and link it to cognitive outcome. Further, and regardless of whether neurogenesis is affected by anesthesia, we will test three clinically applicable strategies to modify anesthetic outcome: We will 1) define a hierarchy of toxicity on neural stem cells *in vitro* and *in vivo*, 2) determine the safe duration for 1 MAC of iso *in vitro* and *in vivo* and 3) test the effectiveness of epo in improving cognitive outcome.

A clinical study of the likely underestimated and serious problem of permanent cognitive impairment from neonatal / infant anesthesia is much needed. One reason why none has been conducted is the lack of animal data on which to base a meaningful and ethical clinical trial. Any of a number of possible results of the proposed study constitutes a testable hypothesis for a human trial. In addition to the obvious benefit to patients and society of improving anesthetic safety of neonates / infants there is a potential benefit to the clinician that is highly motivating - the knowledge that humane perioperative care does not have to cause harm.

B. Methods



Aim 1a: To determine a hierarchy of toxicity of commonly used anesthetic agents on cultured neural progenitor cells *in vitro*.

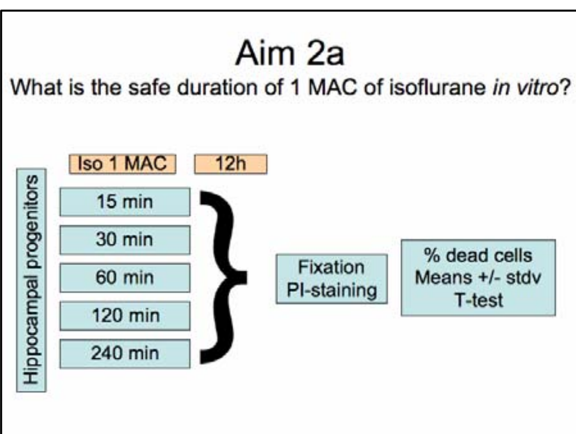
Hippocampal neural progenitor cell lines will be established as described in specific aims from 8-10 liters of neonatal rats per year. An established cell line can be used for testing different agents over the course of several months.

Progenitors will be exposed to 2h of seven anesthetic agents commonly used in pediatric anesthesia. Cells will be fixed 12 h later and cell death will be assessed by counting propidium iodide (PI) stained progenitors and performing LDH release assays.

Aim 1b: We will test the effect of the most and least toxic anesthetic agents determined in aim 1a on

neurodegeneration, neurogenesis and cognitive outcome *in vivo.*

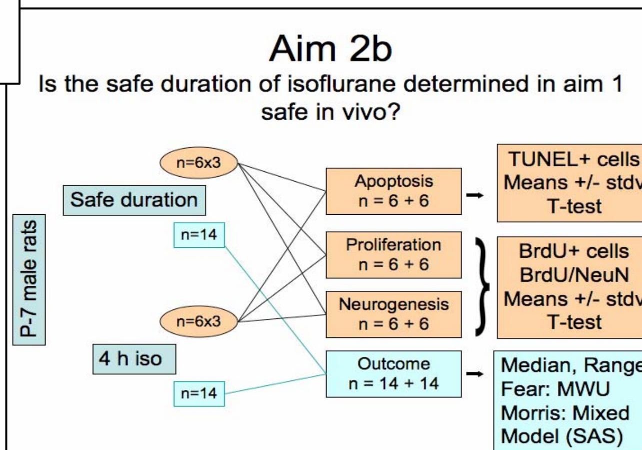
BrdU 50mg/kg will be injected intraperitoneally at the end of the anesthetic. Based on our preliminary data and Jevtovic-Todorovic's experience(28), rats will be killed after 18 h (apoptosis), 4d (progenitor proliferation), 32d (neurogenesis) and 128d (cognitive outcome). Apoptosis will be assessed by performing TUNEL staining. Progenitor proliferation will be assessed by performing immunohistochemical staining and counting of BrdU+ cells using unbiased stereology. Neurogenesis will be assessed on day 32 by counting the proportion of BrdU+ cells that are co-labeled with the neuronal marker NeuN. Neurocognitive function will be assessed by fear conditioning and Morris water maze testing starting on day 120. 64 rats namely n=6/group for immunohistochemistry to quantify apoptosis, progenitor proliferation, neurogenesis, and n=14/group for neurocognitive testing plus 11 cardiorespiratory control rats (detailed methods) are required for this aim. For statistical methodology including sample size calculations please see detailed methods.



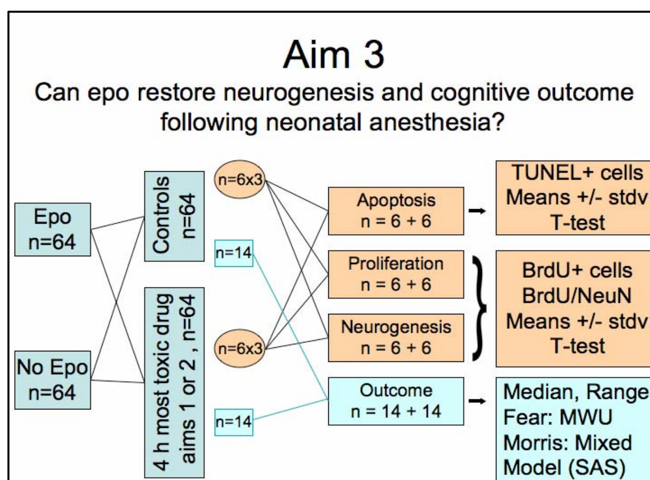
Aim 2a: To define the duration of 1 MAC of iso that is not toxic to progenitor cells *in vitro.*

Hippocampal neural progenitor cells will be exposed to isoflurane 1 MAC (1.8% per our preliminary data) for varying durations. PI staining and LDH release assays will be performed to assess the proportion of dead progenitors 12 h later.

The duration determined to be safe in vitro will later be tested in vivo (aim 2b).



Aim 2b: We will determine if the duration of isoflurane that was safe in aim 2a is devoid of effects on neuronal apoptosis, neurogenesis and cognitive outcome *in vivo.* The dose-related impact of iso on neurodegeneration, progenitor cell proliferation, neurogenesis and cognitive outcome will be determined by comparing the safe duration of 1 MAC iso from aim 2a to 4h of 1 MAC iso in P-7 rats using a similar design as in aim 1b. The rationale for varying the duration of iso rather than the concentration is to provide controlled physiologic variables (hemodynamics and oxygenation) as described in the detailed methods. 11 cardiorespiratory control rats will be required in addition to 64 rats for data collection. For sample size calculation and statistical methodology please see detailed methods.



Aim 3: To test the hypothesis that epo can decrease neurodegeneration, increase neurogenesis and normalize neurocognitive

outcome after a 4h anesthetic with the most toxic agent identified in aims 1.

7 day-old male Sprague Dawley rats will be assigned to one of four groups: 1) no anesthetic / no epo, 2) no anesthetic plus epo, 3) 4 h anesthesia / no epo or 4) 4 h anesthesia plus epo. The most toxic agent determined in aim 1 will be used for anesthesia. Epo (5 U/g, Procrit, Johnson & Johnson) will be injected ip 4h prior to the anesthetic, which should affect both apoptosis(11) and neurogenesis (9,10) as well as cognitive outcome after anesthesia (13,17). Apoptosis, progenitor proliferation, neurogenesis and neurocognitive outcome will be assessed as in aims 1b and 2b. A total of 128 animals will be needed for data collection plus an additional 22 for cardiorespiratory control (for statistical methods and sample size calculation please see detailed methods).

Detailed methods

Preparation of neural progenitor cell cultures: Progenitor cells are collected and cultured using previously described methods (29). Briefly, after decapitation during 7% isoflurane anesthesia, hippocampi are rapidly removed and placed in ice-cold Hanks BSS. Hippocampi are pooled from 6 to 10 pups, washed and triturated using a razor blade, p200 pipette, and 25G needle attached to a 3cc syringe. Cells are then grown in bFGF containing media. Non-adherent cells are collected and media is changed completely on day 1 after isolation of hippocampi. Subsequently 80% of media is replaced every 2 days. Each pooled isolation of hippocampi produces approximately 4 x 10^6 total cells yielding roughly 2 x 10^5 intact progenitor cells after 2 to 4 days in proliferation media.

Anesthetic exposure of progenitor cells: After 4 passages, cells will be exposed in a Billups-Rothenburg chamber at 37 °C to volatile anesthetics or in culture dishes at 37 °C to iv anesthetics. Anesthetic exposures times will be from 15 min to 4 hours to identify a possible safe duration of exposure. The concentrations of agents are as follows: isoflurane 1.8%(30), sevoflurane 3.3%(30) propofol 0.80 μM (31,32), ketamine 10μM (33), fentanyl 100ng/ml (34,35), midazolam 300ng/ml (34,36) and nitrous oxide 70%.

Evaluation of anesthetic toxicity in vitro: Anesthetic toxicity will be evaluated by counting total and PI positive cells to determine a ratio. Counting the total number of cells allows for a determination of proliferation rate in the exposed and unexposed groups. Anesthetic toxicity will also be evaluated using an LDH release assay (Promega Colorimeteric Cytotoxicity Kit), performed in 96 well plates. A standard curve will be run with each assay by plating and lysing different known numbers of cells. Media will be removed from cells and incubated with substrate for 30 to 45 minutes at room temperature. Stop solution will be added and absorbance at 490nm will be read using a spectrophotometer.

Rat-anesthesia: When testing a volatile anesthetic, animals will be anesthetized in a plexiglas cylinder by insufflation of the target concentration of the volatile anesthetic (iso 1.8%, sevo 3.3%, nitrous oxide 70%). Maintenance is with 1 MAC of the agent which will be determined by tail clamping experiments in all animals as previously described (37). When using nitrous oxide, maintenance is with 70% and tail clamping will be performed to simulate surgery. Nitrous oxide cannot be administered at 1 MAC without a hyperbaric environment. We consider it more important that the drug be administered in a clinically relevant context (subanesthetic, at 1 atmosphere) than to pursue a concentration that is equipotent to the other anesthetics.1 of 7 animals will serve as

a cardiorespiratory control. A P10 tubing will be inserted percutaneously into the tail artery using an operating microscope. Arterial blood pressure will be measured continuously and arterial blood gas analysis will be performed every 15 - 30 minutes. pH, PaO2, PaCO2 and blood glucose will be controlled within the physiologic ranges using an i-stat machine and, if necessary, intra-arterial injection of 5% dextrose solution and intubation of the trachea f/b mechanical ventilation using a small rodent ventilator will be performed. Pericranial temperature will be measured and controlled within a tight range of 37.5 – 38.5 °C using a computer-controlled heat exchanger placed under a copper pad that rats lie on when inside the anesthesia chamber.

If an intravenous anesthetic is tested in aim 2, i.e. if either the least or the most toxic anesthetic agent determined in aim 1 is an intravenous agent, the agent will be administered intraperitoneally until insertion of an intravenous (iv) cannula into the internal jugular vein is feasible. Maintenance for 4h will be by iv infusion of the agent to be tested. We will determine the infusion rate necessary to achieve and maintain 1 MAC-equivalent over 4 hours by tail clamping experiments as previously described(37). The anesthetic dose so determined will be administered over 4 hours followed by closure of the neck incision.

BrdU labeling and immunohistochemistry BrdU (50 mg/kg; Sigma, St. Louis, MO) will be administered intraperitoneally twice per day for 1 day (progenitor proliferation) or for 4 days (neurogenesis) starting 4 days after anesthesia. This is the peak of the anethetic effect on progenitor cell proliferation after anesthesia in neonatal rats (data not shown). Animals will be anesthetized with isoflurane and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 (PB). The brains will be removed, post-fixed for 6 hours in 4% PFA/PB, and placed in 40% sucrose overnight. 40-mm coronal sections will be cut on a microtome and stored in PB. For immunocytochemical detection of BrdU-labeled nuclei, DNA will be denatured to expose the antigen. For immunohistochemical detection of NeuN, Ki67, GFAP, and O-4 similar procedures will be applied without the denaturation steps described(2,38).

Double immunofluorescence staining, detection and quantification For phenotype determination, sections will be double labeled with anti-BrdU, and anti-NeuN, or anti-GFAP or anti-O-4 antibodies sequentially as described (2,38). Fluorescence signals will be detected using a Zeiss LSM 510 confocal imaging system with a sequential scanning mode for Alexa 488 and 594. Stacks of images (1024 x 1024 pixels) from consecutive slices of 0.7 mm in thickness will be obtained by averaging four scans per slice and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA). The number of BrdU/NeuN, BrdU/NG-2, and BrdU/GFAP double-labeled cells will be estimated by multiplying the percentages of co-localization (determined by confocal microscopy) to the total number of BrdU-labeled cells (determined by Zeiss Axioskope 2 plus epifluorescent microscope, Zeiss, New York)(2,38) **Quantification of BrdU+ cells**: Unbiased stereology (Stereo Investigator; MicroBrightField, Williston, VT) will be used to assess the volume of the hippocampus at the various time points. Every 10th slice will be counted. Counting frames (15 x 15 x 20 μm) will be placed at the intersection of a matrix (30 x 30 μm for Ki67 and 200 x 200 μm for NeuN and BrdU estimation) randomly superimposed onto the region of interest by the program. Ki67 and BrdU immunoreactive cells will be counted in the subgranular zone and SVZ in the same fashion. The volume of the dentate granule layer will be determined using Cavalieri's principal with a 60 x 60 μm grid matrix(38,39).

Detection of neurodegeneration (neuronal apoptosis) TUNEL will be performed as described previously(40) using ApopTag kit (ApopTag, Intergen Company, Purchase, NY). Frozen sections will be fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS), pH 7.4 for 5 minutes followed by quenching endogenous peroxidase activity using 3% hydrogen peroxide in 0.1 mol/L PBS for 5 minutes. Sections will then be incubated with equilibration buffer for 30 seconds followed by TdT enzyme for 1 hour. Anti-digoxigenin peroxidase conjugate will be applied to slides and incubated for 30 minutes. 3,3'-diaminobenzidine-tetrachoride (DAB; Sigma Chemical Co, St. Louis, MO) will be applied for 6 minutes. Sections will be counterstained with 0.5% methyl green for 10 minutes.

Behavioral testing:

a). Fear conditioning: This is a simple and sensitive test of hippocampal-dependent and hippocampal-independent learning(41). Rats will be individually placed in the following context: square box, white fluorescent light, chlorox smell. They will receive an unconditioned electrical stimulus of 1 mA for 2 sec to the floor grid of a box three times every 90 sec. The conditioned stimulus is a 30sec 90dB 2000hz tone. The shock will be applied during the last 2 sec of the tone. On the following day associative learning of the context (hippopcampal-dependent) and the tone (hippocampal-independent) will be assessed by recording fear behavior (“freezing”) every 8 sec for 8 min. Data will be expressed as medians and ranges. The Mann Whitney U test will be used to test for between group differences of freezing scores.

b). Water maze learning In the water maze test, the ability of the mice to locate a hidden submerged platform in a circular pool (180 cm in diameter, 50 cm deep) filled with warm (24°C) opaque water will be assessed. The rats are trained first to locate a visible platform (cued training, day 1-2), and then to locate a hidden platform (day 3-5); in a subsequent trial the hidden platform is removed (probe trial, day 5). The rats receive two training sessions per day for five consecutive days. Each session consists of three trials with a 60-minute inter-trial interval. The interval between the two daily sessions is 5 hours. Once the rats locate the platform they will be allowed to remain on it for 20 seconds. Time to reach the platform (latency), path length, and swimming speed will be recorded with a Noldus Instruments EthoVision video tracking system set to analyze two samples per second. Because the time required to locate the hidden platform is determined by both path length and swim speed we will, in addition to latency, also analyze these parameters (38).

Statistical methods If data are normally distributed they will be expressed as means +/- standard deviations. Within group comparisons will be made using ANOVA and between group comparisons will be made using Student’s T-test. If data are not normally distributed, they will be expressed as medians and ranges. Within group comparisons will be made using the Kruskall-Wallis test and between group comparisons will be made using the Mann-Whitney U test. Water maze data will be analyzed by "mixed model regression" using SAS Version 9 (SAS Institute, Cary, NC) Proc MIXED. Post hoc tests will be used to compare the least squares means and Bonferroni method will be used to adjust for multiple comparisons. A p-value of <0.05 will be considered significant.

Sample size calculation: For immunocytochemistry, a minimal group size of 6 animals is required to reach statistical power (power analysis: alpha=0.05, beta=0.8, delta= 2.9, effect size d=2.22)

For neurocognitive testing, a minimal group size of 12 is required to reach statistical power (power analysis: alpha=0.05, beta=0.8, delta 2.77, effect size d=1.60). We will use 14 animals to include a margin for error.

3. Possible outcomes, potential problems, limitations and alternative strategies

The proposed trial is not a human trial. However, given the immense potential impact of the proposed study and the fact that a human trial is currently not feasible (see below) conducting a series of well designed animal trials seems like a worthwhile undertaking. While assessing neurocognitive function following anesthesia is possible in adults, it is almost impossible in neonates for lack of the patient's own baseline and the inability to randomize neonates to anesthesia or no anesthesia. However, if meaningful evidence of a permanent cognitive deficit were established a human trial such as the one outlined below may be feasible. We therefore chose 4 months, the equivalent of the beginning of college age as our study interval in order to provide these important preliminary data. If the data from the proposed project were promising, an initial human trial might assess the effect of epo on cognitive outcome in neonates with three groups: neonatal anesthesia with or without a single dose of epo prior to anesthesia and an epo control group.

Aim 1) One possible outcome is that progenitor proliferation is decreased by anesthesia but neurogenesis is not affected. This would require increased survival or differentiation of the progeny either by an anesthetic effect or by some compensatory mechanism that the brain activates in response to reduction of its progenitor pool. If neurogenesis were not affected by anesthesia, while neurocognitive function is permanently decreased, as we have demonstrated, then neurodegeneration would be the mechanism responsible. This would be a valuable finding, with respect to development of therapeutic strategies to prevent post-anesthesia cognitive decline.

Aim 1) It is possible that the least toxic agent tested still has significant toxicity. We did not include a control group in the design of aim 1b because we will compare the results of aim 1b with the results of the control group (no epo / no anesthesia) of aim 3.

Aim 2) Likewise, it is possible that even the shortest duration of 1 MAC iso tested in aim 2 causes either apoptosis or decreased neurogenesis or both when compared to the control group of aim 3.

In this unlikely case we would have to declare the "anesthetic-choice strategy" and the "reduce-duration-of-anesthesia-strategy" a failure and defer to aim 3 for a viable strategy to improve anesthetic safety.

Aim 2) We expect that there is a safe duration of anesthesia that causes neither neurodegeneration nor decrease in neurogenesis. If the anesthetic effect on neurodegeneration is different from that on neurogenesis, such that for example a short duration of exposure causes neurodegeneration but a long duration is required to causes a decrease in neurogenesis then the effect of both threshold durations will be tested on neurocognitive outcome. This may allow comments on the relative impact of either pathophysiologic process on cognitive outcome, which would be important from a mechanistic standpoint.

4. Animal approval

The request for approval of the study protocols has been submitted to the Institutional Animal Care and Use Committee.