Title: Isoflurane-induced neuroinflammation in children with hydrocephalus: A bench-to-bedside, translational study of molecular pathways and therapeutic approaches.

Overall Hypothesis. Children with hydrocephalus (HC) exposed to isoflurane anesthesia show increased neuroinflammatory markers in serum and cerebrospinal fluid when compared to children not exposed to isoflurane. Moreover, children undergoing isoflurane anesthesia for diagnostic procedures demonstrate increases in serum inflammatory markers despite lack of neuropathology or surgically-induced stress.

Innovation.

Evaluation of anesthetic risks in human children.

Clinical, prospective pediatric studies evaluating effects of anesthetic exposure on the central nervous system (CNS) are inexistent due to ethical and methodological considerations. 78% of children with HC, once treated, go on to suffer neurologic deficits.[1-4] Neuroinflammation and neurotoxic effects during anesthesia might be in cause.[5]

The role of inflammation in HC remains poorly understood. Adult animal models show that active inflammation correlates with severity and can be measured in CSF.[6] This has not been explored in humans. It is critical to quantify HC-induced neuroinflammation, evaluate how anesthesia modulates this inflammation, and measure its contributions to HC-related morbidity. Children with HC undergoing ventriculoperitoneal shunt (VPS) insertion have been selected as our study population due to a number of circumstances making them ideal for this study. While obtaining CSF and serum for research in healthy children is ethically inappropriate and methodologically challenging, CSF sampling is routine care during VPS insertion. CSF cytokine and micro-RNA (miRNA) levels are used to measure the severity of neuroinflammation. Healthy children scheduled for MRI with anesthesia for non-neurologic disease will serve as control.

CSF cytokine levels are markers of CNS inflammation, and are reported elevated in adult patients undergoing VPS insertion after subarachnoid hemorrhage.[7] Few studies have evaluated inflammation in children with HC, with or without anesthesia.[8,9] CSF biomarkers are likely to reflect these changes. An increase in CSF cytokine levels in certain types of HC has been reported, but the elevation was not statistically significant.[19] We plan to stratify the results by age to avoid bias while allowing comparison with previous investigations.

miRNAs represent a novel method to quantify inflammation in biological systems. miRNAs are small (18-25 nucleotides) non-coding nucleic acid molecules. They regulate (usually in an inhibitory fashion) gene expression at the post-transcriptional level.[10-12] 500 human miRNA sequences are known, however, specific targets of individual miRNAs remain elusive. Although a mystery, miRNA targets may potentially lead to therapeutic intervention. miRNAs have a critical role in the development of the CNS, including neural induction and differentiation, neural patterning, cell specification, axonal pathfinding, and neural pruning (apoptosis).[10-16]

Our model is a novel use of four miRNAs in quantifying neuroinflammatory response in children with HC undergoing anesthesia. Elevated CSF miRNAs, particularly the four molecules we plan to measure, have been reported in neuroinflammatory conditions.[17-19]

The present study will demonstrate neuroinflammation in patients with HC anesthetized with isoflurane and determine pathways involved in the neuroinflammatory process. These observations could lead to specific targets for therapeutic interventions and contribute to decreasing morbidity and improving quality of life of these children. We intend to identify the relationship between neuroinflammation and severity of disease and hope to make future therapeutic recommendations.

This novel, clinical, human model will help elucidate the role of inflammation in human HC and examine the effects of one anesthetic agent. Results will help expand our understanding of anesthesia-induced neuroinflammation. If neuroinflammation is demonstrated, the potential for treatment innovations could dramatically enhance the quality of life of children with this devastating disease.

Translational Significance

Each year, millions of children receive general anesthesia. Isoflurane, a GABA type A ($GABA_A$) receptor agonist, is an inhaled anesthetic commonly used in clinical anesthesia worldwide. Anesthetics have traditionally been assumed to be safe as long as severe hypotension and hypoxia are avoided. Anesthesia may induce neuroinflammation and alter the formation of normal synaptic connections. These changes could lead to excessive neuronal loss or disruption of synaptogenesis at critical times during brain development and

consequently cause cognitive dysfunction. Recent reports of neurotoxicity induced with isoflurane and other anesthetics in animal models have triggered significant concerns about the safety of these agents.[20]

Basic research data on the effects of isoflurane on brain development originate mainly from rodent studies[21-23] with very limited work in animals with gyrencephalic brains, such as swine and non-human primates.[24,25] These studies have significant limitations and vagaries in interpretation, having used solely an antibody-based immunohistochemical (IHC) technique to identify the presence of triggered "apoptotic" cells. Unequivocal end-stage apoptosis and cell death were not clearly demonstrated histologically in these studies.[24,25] Many previous studies showing anesthesia-induced neurotoxicity may have overestimated the true degree of neuronal injury and its true clinical implications. Most importantly, no known study has examined the effects of anesthetics on neuroinflammation, an arguably more important process when considering lasting neurodegeneration that leads to later neurocognitive deficits. CNS inflammatory response in children after anesthesia exposure has become our primary focus in an attempt to elucidate the true consequence of these agents on child brain development and cognitive function.

Specific Aims

1. ***To establish the neuroinflammation profile in positive-control (lipopolysaccharide (LPS) treated) and isoflurane-treated neonatal piglets.***

   Hypothesis: Piglets treated with LPS (an *E. coli* endotoxin) have increased peripheral blood and CSF proinflammatory cytokine levels; increased peripheral blood and CSF proinflammatory microRNA (miRNA) concentrations; and increased brain microglial activation when compared to controls. This "positive control" will allow comparison with piglets exposed to isoflurane.

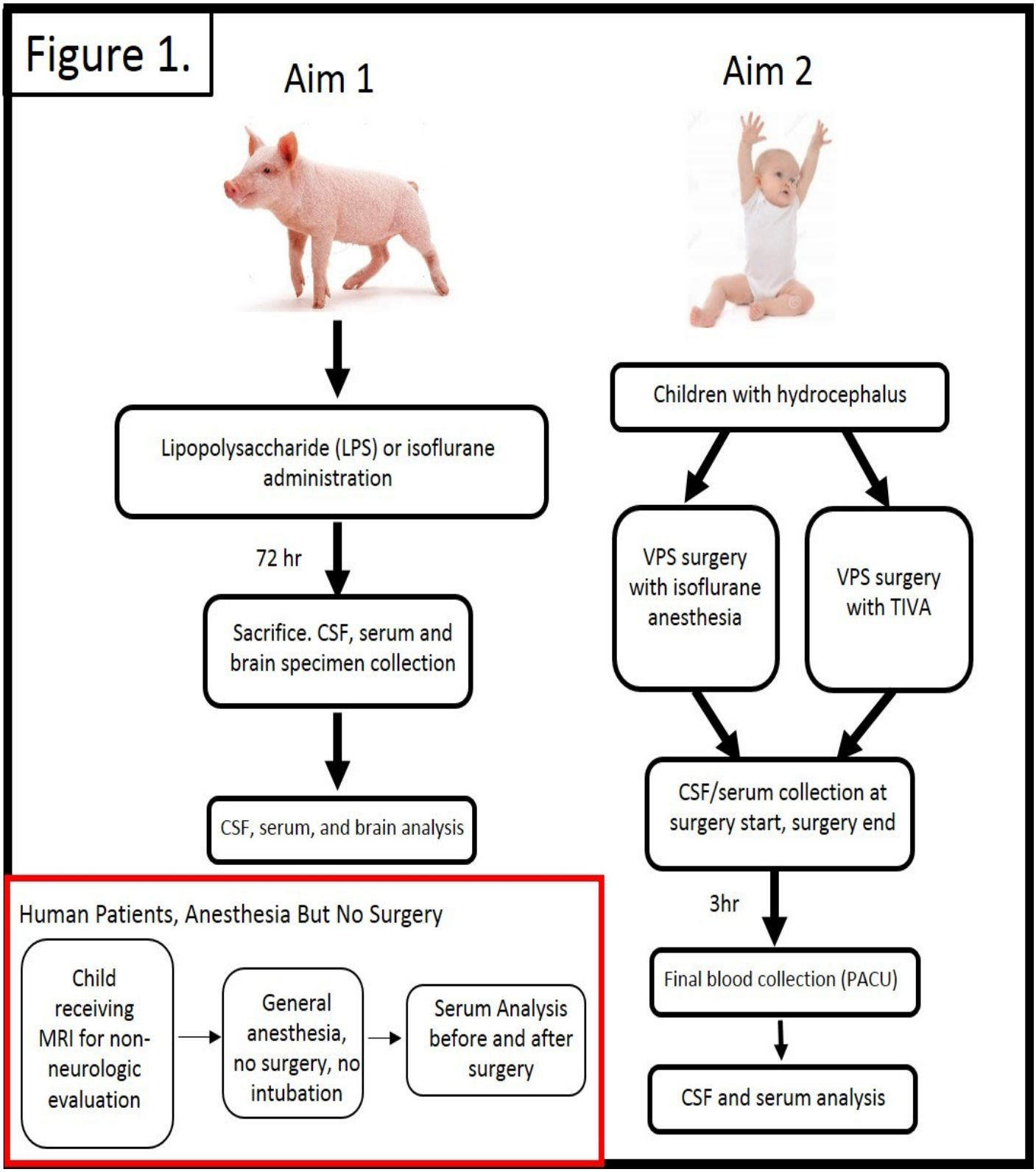
   Further, piglets administered isoflurane anesthesia show increased peripheral blood and CSF cytokine levels; increased peripheral blood and CSF proinflammatory miRNA concentrations; and increased brain microglial activation when compared to controls.

2. ***To establish the neuroinflammation profile in human children with hydrocephalus before and after VPS insertion with isoflurane anesthesia.***

   Hypothesis: Children with hydrocephalus have increased peripheral blood and CSF cytokine and proinflammatory miRNA levels after isoflurane anesthesia when compared with blood and CSF sampled before anesthesia and surgery, despite surgical relief of hydrocephalus. This exacerbation of neuroinflammation shall persist for 24 hours and longer.

   It is further hypothesized that anesthesia-induced neuroinflammation will be reduced or eliminated using a total intravenous anesthesia (TIVA) technique with dexmedetomidine and remifentanil instead of isoflurane. This suggests a direct role of isoflurane in the neuroinflammatory process. Children undergoing inhaled anesthesia for magnetic resonance imaging (MRI) without tracheal intubation or surgery will serve as a control.

Summary Illustration. Please see **Figure 1** for a graphic representation of the experiment.



Preliminary Data. Two years have been invested in developing a valid piglet model to study anesthesia-induced neurotoxicity. We can now safely and effectively use this model to easily test a number of different conditions and anesthetic regimens. We are confident it is the most clinically relevant animal model for neuro-inflammation investigations. It reproduces exactly the same surgical and anesthetic conditions children undergo in the operating room. The equipment includes a standard anesthesia machine used clinically for the care of children at NCH (**Figure 2**). All routine physiological monitoring devices are used. Piglets receive an inhalational induction of anesthesia, a peripheral intravenous catheter, and tracheal intubation. Their lungs are mechanically ventilated and they are monitored throughout the experimental and recovery period. An infusion of dextrose-containing isotonic fluid is given. Arterial blood gases and chemistries are monitored every hour to prevent acid-base abnormalities and electrolyte disturbances. Normothermia is actively maintained. Perioperative antibiotics are used at an appropriate per kilogram human dose to prevent wound infection. The piglets are demographically similar. Care is taken to prevent confounders that could make interpretation of data problematic. Please see **Table 1** for a summary of average vital signs and selected laboratory values for this animal study.

Figure 2. An animal undergoing an isoflurane exposure experiment. Piglets are intubated, mechanically ventilated, and fully monitored. They also receive an infusion of dextrose-containing intravenous fluid. They are actively warmed and temperature is monitored throughout the procedure. Arterial blood pressure is measured through a femoral arterial arterial catheter and basic laboratory values are checked every hour. Pictured are Tanner Koppert (senior lab associate) and Christopher Zhang (OSU Medical Student and MDSR Research Fellowship Candidate).

Average Vital Signs and Lab Values in Isoflurane-Treated Animals (n=9)		
Parameter	Average Value	Normal Range
Age (Days)	11	n/a
Weight (kg)	3.2	3-4
Arterial Blood Pressure (Systolicm mmHg)	80	65-95
Arterial Blood Pressure (Diastolic)	44	35-55
Respiratory Rate (Breaths per Minute)	49	30-60
Heart Rate (Beats per Minute)	154	120-200
Arterial Oxygen Saturation (%)	99	95-100
Arterial pH	7.47	7.45-7.55
Serum Sodium (mEq/L)	131	139-153
Serum Potassium (mEq/L)	5	4.4-6.5
Serum Glucose (mg/gL)	168	66-116

Table 1. Isoflurane-treated piglets have normal vital signs and laboratory values throughout the experimental period.

To date, 27 animals have been studied. Microscopic evaluation of the brains of these animals was performed by a pediatric neuropathologist at NCH, and has yielded several exciting findings. Surprisingly, the typical “footprint” of neuroinflammation was not observed either with anesthesia or LPS. One may speculate that brain tissue was harvested too early after the insult, i.e., 48 hours. It is possible that when harvesting brain tissue after 48 hours of convalescence, manifestations of acute inflammation are not yet apparent. In one rodent study, hallmarks of inflammation and neurodegeneration were not seen until 72 hours had elapsed, and in some cases it took as long as 14 days.[26] Cytokines, however, have been shown to be significantly elevated a few hours after the initial insult.[27] Therefore, to identify early inflammation, performing ELISA for inflammatory cytokines (TNF-α, IL-1β, MCP-1, PGE2, and NF-κB p65), and qPCR for proinflammatory miRNAs will be essential. Immunofluorescent labeling will be used to identify activated inflammatory cells in the brain tissue of these animals. It is most likely to support early inflammation, which will guide in making further improvements to our research model. For this reason, we have elected to allow 72 hours for convalescence after experimental exposure.

The most concerning findings on microscopic examination of piglets’ brains were twofold: 1) a significant morphological and structural change in the isoflurane and LPS treated animals when compared to controls, and 2) an acute ischemic infarct in one of three isoflurane-treated animals that was not present in either of the other groups.

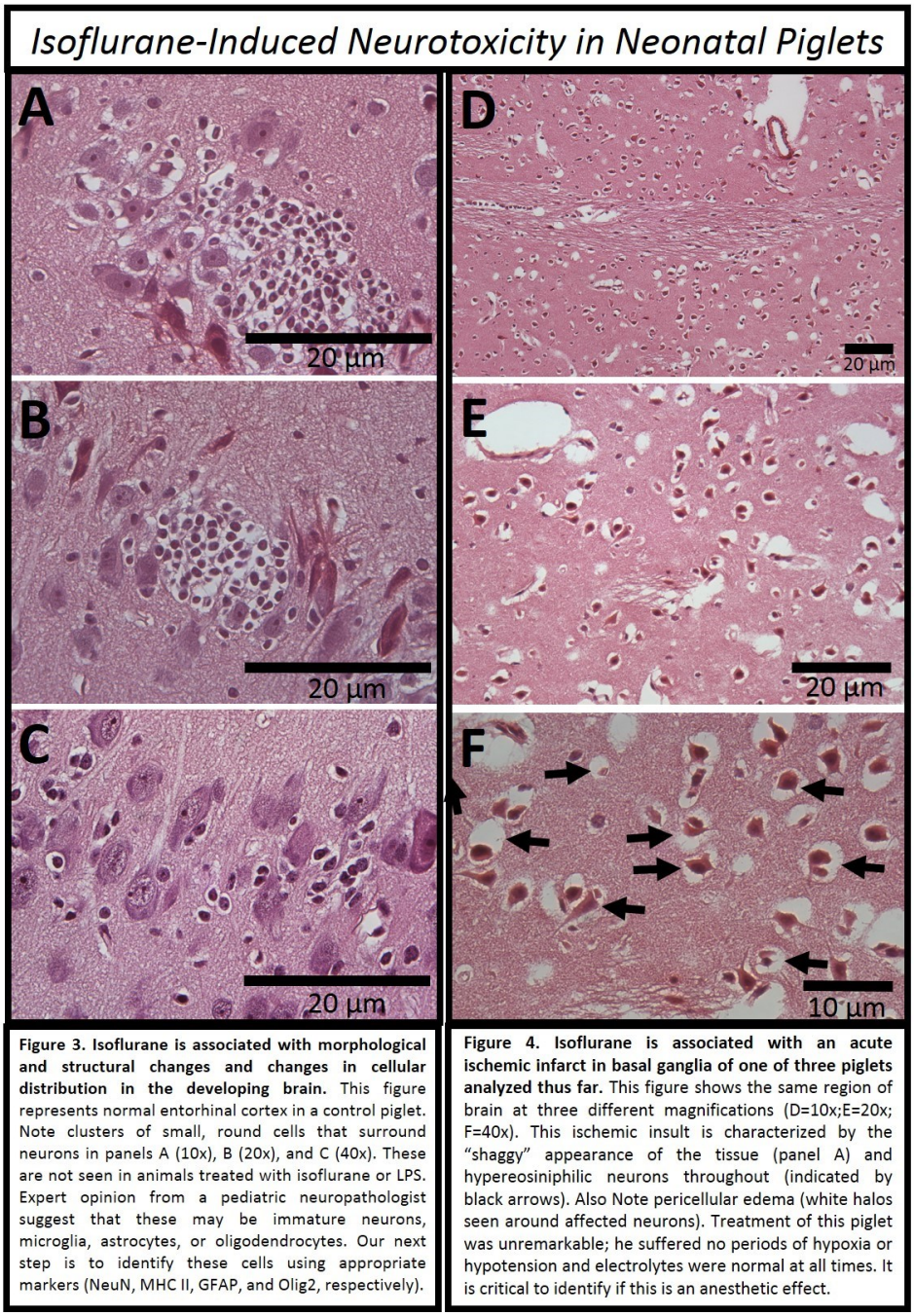


Figure 3. Isoflurane is associated with morphological and structural changes and changes in cellular distribution in the developing brain. This figure represents normal entorhinal cortex in a control piglet. Note clusters of small, round cells that surround neurons in panels A (10x), B (20x), and C (40x). These are not seen in animals treated with isoflurane or LPS. Expert opinion from a pediatric neuropathologist suggest that these may be immature neurons, microglia, astrocytes, or oligodendrocytes. Our next step is to identify these cells using appropriate markers (NeuN, MHC II, GFAP, and Olig2, respectively).

Figure 4. Isoflurane is associated with an acute ischemic infarct in basal ganglia of one of three piglets analyzed thus far. This figure shows the same region of brain at three different magnifications (D=10x;E=20x; F=40x). This ischemic insult is characterized by the "shaggy" appearance of the tissue (panel A) and hypereosiniphilic neurons throughout (indicated by black arrows). Also Note pericellular edema (white halos seen around affected neurons). Treatment of this piglet was unremarkable; he suffered no periods of hypoxia or hypotension and electrolytes were normal at all times. It is critical to identify if this is an anesthetic effect.

The first concerning finding is related to a significant change in the morphology and structure in the entorhinal cortex and CA1 hippocampus. In control animals, prevalent clusters of small, round cells in the neuronal layers of both areas were noted (**Figure 3**, panels A, B, and C). Based on review of the literature and consultation with a pediatric neuropathologist, they are most likely immature neurons. They could also represent microglial cells, astrocytes, or oligodendrocytes in development. *These cells were virtually obliterated in isoflurane and LPS-treated animals.* The loss of these cells was seen in every LPS or isoflurane-treated animal studied to date. We hypothesize that the cells may be dying as a result of neuroinflammation, ischemia, or both when animals are treated with isoflurane or LPS. Identifying their implications and the mechanisms involved in their loss will be essential to determine the significance of this observation. To achieve this goal, immunohistochemical labeling will be utilized for immature neurons (NeuN), astrocytes (GFAP), microglia (MHC II), and oligodendrocytes (Olig2).

The second and most concerning finding in the isoflurane-treated animals was an acute basal ganglia ischemic infarct. Our colleague pediatric neuropathologist is of the opinion that the infarct, which was seen in the basal ganglia, is 12-24 hours old (**Figure 4**, panels D, E, and F). This is extremely concerning given the fact that brain infarcts in children are often "subclinical" and found incidentally when cerebral imaging is performed later for another reason. Kim et al. reported that 21% of children had abnormalities, some of which were described as "unspecified white matter abnormality of unclear etiology".[28] So-called "silent" strokes are shockingly common in children with sickle cell disease.[29,30] It is not impossible that anesthetics, particularly when repeated, may be causing areas of ischemic infarction that are never detected because they do not cause typical stigmata of stroke, such as weakness, facial droop, dysarthria, or gait disturbance. We are concerned that if subclinical infarcts are occurring, they may be partially responsible for the neurocognitive deficits that have been reported in children after multiple anesthetics.[31] It is vital to learn whether this is a common effect of anesthesia in animals with complex (gyrencephalic) brains in order to make anesthesia safer for infants and children.

Materials and Methods

Aim 1 (Tzagournis Medical Research Facility (OSU)): All neonatal piglet (10-14 days old, 2-3 kg) experimentations will be performed following The OSU Institutional Animal Care and Use Committee (IACUC) policy (protocol number 2014A00000018, already underway). Animals will arrive in the vivarium 24 hours before experimentation for acclimation to the environment.

The positive control (LPS) arm receives *E. coli* endotoxin intraperitoneally (100 mcg/kg). This induces acute inflammation with minimal morbidity.[32] **For the experimental (isoflurane) arm**, animals are anesthetized with 5% isoflurane in 100% O_2 via face cone. Tracheal intubation and mechanical ventilation to ensure normocapnia and normoxia are used. Isoflurane 2% in 50% oxygen/50% air is administered during vascular cannulation and throughout the study period. The femoral vein and artery catheters are placed via surgical cut-down, allowing medication and fluid administration, continuous arterial blood pressure monitoring, and blood sampling. End-tidal CO_2 and rectal temperature are monitored continuously. Arterial blood gases are measured hourly throughout. Anesthesia exposure lasts 3 hours after which vascular catheters are removed. The surgical incision is closed and infiltrated with local anesthetic. The piglet is awakened, the trachea extubated, and the animal is returned to its cage. Subcutaneous buprenorphine is given every three hours for pain management, supervised by experienced animal care personnel. **For both arms:** based on our preliminary observations (see above), piglets are allowed to convalesce for 72 hours to ensure better pathological identification of the presence of neuroinflammation. The piglets convalesce in temperature-controlled cages and receive commercial piglet milk replacer. After 72 hours, anesthesia with 5% isoflurane in 100% oxygen is given for non-survival surgery. Peripheral blood is obtained via venipuncture and CSF collected via lumbar puncture. The CSF and serum are stored at -80^{o}C. Animals receive transaortic heparinized phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) and the brains harvested and saved for later analysis.

Serum and CSF analysis. Serum and CSF are analyzed for miRNA (miRNA) and inflammatory cytokine levels. miR-155, miR-146a, miR-146b, and miR-142-3p are quantified using qPCR. The following cytokines are measured using enzyme-linked immunosorbent assay (ELISA): TNF-α, IL-1β, MCP-1, PGE2, and NF-κBp65.

Immunohistochemistry. The hippocampus is dissected from the brain and fixed. Hippocampal tissues are sectioned to 40μm thickness, mounted on slides, and stained according to established procedures for MHC II, GFAP, S100β, and anti-ionized calcium binding adaptor molecule 1.

Aim 2 (Nationwide Children's Hospital): Human Subjects.

VPS Patients. Children aged 1 month to 16 years with hydrocephalus undergoing VPS insertion will be randomized to one of two standardized general anesthetics necessitating tracheal intubation. Clinician's discretion will be used to determine doses and rates of infusion.

Conventional Anesthetic Group:

Induction:
- Propofol 2-3 mg/kg
- Fentanyl§ 1-2 μg/kg when indicated
- Rocuronium dose at provider discretion

Maintenance:
- Isoflurane 1-2% in 50% O_2 in air or sevoflurane (2-4%) in 50% O2 in air
- Rocuronium if needed
- Isotonic fluid 10-30 mL/kg (lactated Ringer's or normal saline)
- Neuromuscular blockade reversal: glycopyrrolate 0.01 mg/kg / neostigmine 0.07 mg/kg or sugammadex 2-4 mg/kg
- Fentanyl§ at provider discretion

Total intravenous anesthesia (TIVA) Group:

Induction:
- Nitrous oxide for IV start (if needed)
- Fentanyl§ 1-2 μg/kg
- Dexmedetomidine§ 1 μg/kg (over 10 minutes)
- Rocuronium dose at provider discretion

Maintenance:
- FiO_2: 50% (in 50% air)

- Dexmedetomidine§ infusion at a dose of 1-2 µg/kg/hr } **titrated to a BIS of ≤60**
- Remifentanil infusion at 0.2-0.8 µg/kg/min }
- Rocuronium if needed
- Isotonic fluid 10-30 mL/kg (lactated Ringer's or normal saline)
- Reversal of neuromuscular blockade as in isoflurane group
- Fentanyl§ at provider discretion

***Rocuronium 1.2 mg/kg for rapid sequence induction if indicated**

§Dexmedetomidine and fentanyl are not approved by the FDA for use in children <18 years, but have been routinely used safely in pediatric anesthesia of all ages for years.[33-35] An Investigational New Drug (IND) application for each drug has been submitted and is pending.

Rocuronium will not be administered until BIS is <60.

This dose of dexmedetomidine has been used for many types of procedures in children. At our institution, we use a dose range of 0.3 mcg/kg/hr – 3 mcg/kg/hr depending on surgical need. Per our usual clinical practice, all doses are titrated based on clinical response. A dose of 1 mcg/kg/hour was used in one study for sedation for MRI scans.[49] Another study reported doses in the 1-2 mcg/kg/hour range for oral surgery.[50] Finally, high-dose dexmedetomidine infusions have been reported to be safe in children. In fact, one report describes an infant who received sixty times the intended dose with no serious sequelae.[51,52]

MRI Patients. Children 6 months to 10 years undergoing MRI under general anesthesia for non-CNS pathology. Standard general anesthesia will include:

Induction:
- Sevoflurane induction
- Peripheral intravenous catheter
- Placement of an LMA

Maintenance:
- FiO_2: 50% in air
- Isoflurane 1-2% in 50% O_2 in air
- Isotonic fluid (lactated Ringer's or normal saline) 10-30 mL/kg
- Infants <10kg: D5LR titrated to deliver glucose at 5 mg/kg/min

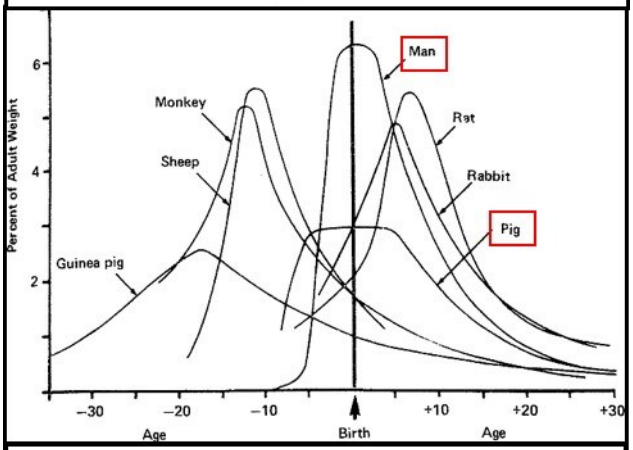
Note: To ensure anesthesia consistency and reduce a Pearson Coefficient effect, anesthesiologists will be recruited to provide anesthesia for the study.

Research Plan

Rationale for the piglet model. The piglet is a well-accepted study alternative to larger animal models used for neuroscience. We pioneered its utilization for pediatric anesthesia neuroinflammation evaluation. Rodent and non-human primate models, used in most prior studies, have not proved to be the ideal animal models to investigate anesthetic neurotoxicity because they are not closely applicable to human neonates. Very few pediatric anesthesia neurotoxicity studies have been done in non-human primates. However, the cost is prohibitive and the animals are extremely sensitive to early rearing conditions, particularly stress and maternal separation.[36-38] The relevance of an animal model must take into consideration the context of human pathology by appreciating the brain maturity and pathobiology involved when compared to the human infant. Very few non-human primate genes for neurotransmitter receptors have been cloned, meaning that crucial receptor-ligand affinities, allosteric modulators, post-translational modifications, alternative splicing variants, and receptor subunit compositions, all relevant to anesthetic-mediated neural injury, are unknown for this species. On the other hand, porcine $GABA_A$ and NMDA receptors have been cloned.[39]

In contrast to newborn rodents, the percentage of adult brain weight at birth in piglets is much closer to that of humans (**Figure 5**).[40] The brain of the pig more closely resembles the human brain and is gyrencephalic, whereas the rodent brain is lissencephalic. Neonatal swine cranial geometries including cortical and basal ganglia topology are similar to human infants. Important similarities between swine and humans have also been demonstrated in hippocampus, entorhinal cortex, basal ganglia, and brainstem, which are the areas of interest in this study.[15] The pig and human brain are similar in growth patterns[36-38] and piglet brain level of development is more analogous to that of full-term gestation human newborns than to ruminants with prenatal brain growth spurts, or rodents with postnatal growth spurts.[36] The multilayer cortical sub-plate of the developing pig brain and the expression pattern of reelin, a glycoprotein that influences neuronal migration, are strongly analogous to the developing human brain.[41,42] Grey and white matter patterns and distributions are similar in human neonates and piglets, and the maturation of the postnatal pig brain is comparable to humans with respect to myelination and electrical activity.[40] **Based on previous work, 10-14 day old piglets correspond to 2 month old human infants with respect to the period of rapid brain growth and maturation.**[40]

Figure 5.



The brain growth spurts of 7 mammalian species expressed as first-order velocity curves of the increase in weight with age. Rates are expressed as weight gain as a percentage of adult weight for each unit of time. [14]

Using this clinically relevant pediatric swine model, we propose to evaluate an anesthetic commonly used in clinical pediatric anesthesia worldwide. Development of a preclinical animal model to study anesthesia-related neuroinflammation is critical to identifying safe pharmacologic regimens for infants and children.

Rationale for anesthetic selection (HC patients). For the conventional anesthesia group, isoflurane is selected for several reasons. Firstly, isoflurane is the most commonly used inhaled anesthetic for neuroanesthesia, likely due to its minimal effects on intracranial pressure and cerebral perfusion pressure.[40] Drugs planned for induction (lidocaine, rocuronium, fentanyl) have not been reported to be neurotoxic or to cause neuroinflammation in regimens used clinically. Isoflurane, however, has been shown to be toxic in multiple animal studies, whether used alone[41] or in combination with other agents.[45] We expect isoflurane to exacerbate neuroinflammation as evidenced by an increase in CSF inflammatory biomarkers after exposure.

For the TIVA group, we selected an anesthetic regimen designed to reduce or eliminate the anesthetic-induced neuroinflammatory response. These patients will receive a total intravenous anesthesia (TIVA) with an infusion of dexmedetomidine, an α_2-agonist and hypnotic agent, and remifentanil, an ultra-short-acting μ opioid agonist medication. Neither dexmedetomidine nor remifentanil have been shown to cause neuroinflammation or neurotoxicity. In fact, dexmedetomidine may attenuate the inflammatory effects caused by other anesthetics.[47,48] This anesthesia regimen will help isolate isoflurane as a strong contributor to neuroinflammation as opposed to surgical insult. The combination of remifentanil and dexmedetomidine is successfully used as a general anesthetic for infants and children undergoing invasive neurological procedures.[49,50]

Rationale for the anesthesia control group (MRI patients). Because direct laryngoscopy, tracheal intubation, and surgery are significant hemodynamic events, these patients will not be subjected to these physiological stresses and consequently represent an excellent, ethical control group. Anesthesia with isoflurane alone is routinely used in children in order to ensure optimal MRI conditions. No other drugs will be given. Anesthesia exposure will be similar in terms of drug, dose and duration in these patients exempted of intracranial pathology or neuroinflammatory disease.

Human Subjects. *Recruitment and Informed Consent.* Participants will not be responsible for the cost of the study. Neither the patients nor the parents will be compensated for their participation. Seventy-five (75) children will be recruited for the study. Parents/guardians will be approached by study personnel during their

preoperative visit in the surgery unit (for hydrocephalus patients) or in the MRI suite (for MRI patients). Parents/guardians will be informed about this study in which their child could be enrolled and which may help improve the anesthesia care of hydrocephalus patients. If they indicate that they are not interested, they will be thanked for their time and no further discussion will occur. If they are interested, study information will be provided. Written informed consent will be signed if they agree to have their child participate. The option of withdrawing their child from the study at any time will be indicated if they become uncomfortable with the participation.

Inclusion/Exclusion Criteria for Human Subjects.

Inclusion Criteria (HC study patients): Pediatric patients, aged 1 month to 16 years, diagnosed with hydrocephalus undergoing a primary or revision VPS procedure under general anesthesia. Participants must have a parent/guardian who is entitled to provide written informed consent in accordance with human investigation committee guidelines.

Exclusion Criteria (HC study patients): Infection of any kind within the last 14 days, treatment in the last 48 hours with corticosteroid medications, anticoagulant administration in the last 48 hours, clinically unstable patients, patients with an American Society of Anesthesiologists physical status ≥4 (severe, life-threatening disease), infants born more than 4 weeks premature if less than 2 years of age, infant born earlier than 28 weeks gestation if greater than 2 years of age but less than 3 years of age.

Inclusion Criteria (MRI with general anesthesia: peripheral blood patients; anesthesia but no surgery): Otherwise healthy pediatric patients, aged 6 months to 10 years, undergoing MRI with general anesthesia for evaluation of non-neurologic, non-inflammatory disease. Participants must have a parent/guardian who is entitled to provide written informed consent in accordance with HIC guidelines.

Exclusion Criteria (MRI with general anesthesia: peripheral blood patients; anesthesia but no surgery): Infection of any kind within the last 14 days, known central nervous system disease, treatment in the last 48 hours with corticosteroid medications, treatment with any drug known to induce or suppress inflammation, patients that have an American Society of Anesthesiologists physical status ≥4 (severe, life-threatening disease), infants born more than 4 weeks premature.

Early Withdrawal Criteria (All Patients): Hypoxemia (arterial oxygen saturation <90% for more than 2 minutes or <80% for greater than 30 seconds); hypotension (systolic or diastolic blood pressure reduced ≥20% from preoperative values for greater than 3 minutes).

Sample Procurement and Analysis.

VPS Patients: 500μL, the minimum volume of CSF required for testing, will be drawn by the neurosurgery team after induction of anesthesia. 5mL of peripheral blood will be obtained before surgery by the anesthesia team via the patient's existing peripheral intravenous (PIV) line. If removal of blood from the PIV line is not possible, a peripheral venipuncture will be performed. At the conclusion of surgery, similar samples will be obtained. An additional 5mL sample of peripheral blood will be drawn in the post-anesthesia care unit (PACU) as described above. All samples will be transported on ice and stored frozen at -80^{o}C within 30 minutes of collection pending further analysis. Cytokines (blood and CSF) will be analyzed using ELISA for TNF-α, IL-1β, MCP-1, PGE2, and NF-κB p65. miR-155, miR-146a, miR-146b, and miR-142-3p will be quantified using qPCR.

MRI Patients. Patients without neurologic or systemic inflammatory disease scheduled for diagnostic MRI under general anesthesia will be recruited for the study. A standardized anesthetic will be given. Peripheral blood will be drawn as above (5mL), before anesthesia, once the peripheral intravenous line is placed as part of routine clinical care. At the conclusion of the scan, another sample of peripheral blood (5mL) will be drawn via the peripheral intravenous line before the child is awakened. Serum handling and analysis will be identical to the above mentioned.

The MRI group will have the same adverse event monitoring as the surgical group:

- Continuously during MRI
- 12 hours, 24 hours, and 1 week after MRI

Additional Clinical Data to be Collected and Reviewed. The following data will also be collected and reviewed to minimize confounding variables: patient weight, sex, preoperative hematology and chemistry values, intraoperative vital signs, and postoperative vital signs per routine care in anesthesia.

Statistical Analysis. We have consulted with three biostatisticians in the development of this study. The sample size (n=25/group) was determined to avoid a Type I (>5%) and Type II (>80%) errors using power analysis in order to show a twofold effect size increase in neuroinflammation in the VPS group receiving isoflurane when compared to the TIVA group. As a pilot study, the analysis is not adjusted for the use of multiple markers or comparisons. Parametric data will be analyzed using a two-tailed Student t-test and expressed as $P<0.05$. Comparison with the control (MRI) group will be performed under the same conditions. This applies to both the human and animal portions of the study, as the same markers will be measured in both groups. Additionally, we will perform a post-hoc analysis using either a Tukey-Kramer test or the Student-Newman-Keuls method for multiple comparisons.

Feasibility: We estimate that at NCH, 2-3 primary VPS surgeries and >30 MRI scans are performed under general anesthesia per week, making the recruitment of the necessary sample size within 1 year very feasible.

Adverse Events.

- Common adverse reactions seen with dexmedetomidine include hypotension, bradycardia, transient hypertension during loading dose, nausea, and dry mouth. These events are usually self-limited and resolve without treatment.
- Adverse reactions associated with infusions >24h include acute respiratory distress syndrome (ARDS), respiratory failure, and agitation.
- Rare serious adverse events (SAEs) of dexmedetomidine include cardiac arrest.
- Common adverse reactions seen with fentanyl include respiratory depression, apnea, rigidity and bradycardia; if these remain untreated, respiratory arrest, circulatory depression or cardiac arrest could occur.
- Other less common adverse reactions include hypertension, hypotension, dizziness, blurred vision, nausea, emesis, laryngospasm and diaphoresis.

The study will be terminated early if >20% of patients experience hypotension or bradycardia (defined as blood pressure or heart rate diminished 20% from baseline for more than one minute). The study will also be terminated early if >20% of patients have an adverse reaction at a dexmedetomidine dose of ≥ 1 mcg/kg/hr. Study termination will occur if adverse reactions occur that have a “reasonable possibility” of being attributable to the investigational drug(s). “Reasonable possibility” is defined according to 21 CFR 312.32(a) under Safety Reporting Requirements for INDs and BA/BE Studies. The investigational drug in this case will be unblinded and standard treatment initiated.

Early withdrawal criteria related to heart rate will be age-dependent due to variability in the normal heart rate for children of different ages.

6-12 mos	HR < 80
12-24 mos	HR < 60
24 mos - <11 years:	HR < 50

Adverse event capture will begin as soon as the patient enters the operating room. Adverse event capture will end once the patient leaves the recovery room. Depending on the type of adverse event, the follow

up period will be 48 hours-4 weeks. All adverse events and the intensity of these events, whether related to study drug or not, will be recorded for the duration of this study. Adverse events that occur will be assessed for causality.

The cause of adverse events in relation to the study medications and procedures will be termed as:
Certain - event or laboratory test abnormality, with plausible time relationship to drug intake; cannot be explained by disease or other drugs; response to withdrawal plausible.
Likely - event or laboratory test abnormality, with reasonable time relationship to drug intake; unlikely to be attributed to disease or other drugs; response to withdrawal clinically reasonable.
Possible - event or laboratory test abnormality, with reasonable time relationship to drug intake; could also be explained by disease or other drugs; information on drug withdrawal may be lacking or unclear.
Unlikely - event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible); disease or other drugs provide plausible explanations.
Unassessible - event or laboratory test abnormality; cannot be judged because information is insufficient or Contradictory; data cannot be supplemented or verified.

The severity of adverse events will be graded ordinally with the following designations: mild (1), moderate (2), severe (3), life-threatening (4).

Dexmedetomidine

Adverse Event	Mild (1)	Moderate (2)	Severe (3)	Life-threatening (4)
Hemodynamic Disturbance	Bradycardia or hypotension (HR or BP decreased >20% from patient's baseline) for 0-30 sec	Bradycardia or hypotension (HR or BP decreased >30% from patient's baseline) for 0-30 sec	Bradycardia or hypotension (HR or BP decreased >30% from patient's baseline) for >30 sec or requiring treatment	Cardiac Arrest
Allergic Reaction	Rash, not including hives	Hives	Bronchoconstriction, non-airway tissue swelling	Leads to anaphylaxis

Fentanyl

Adverse Event	Mild (1)	Moderate (2)	Severe (3)	Life-threatening (4)
Respiratory Depression	Respiratory rate <10	Respiratory rate <8	Respiratory rate <6 or requiring intervention	Respiratory arrest
Allergic Reaction	Rash, not including hives	Hives	Bronchoconstriction, non-airway tissue swelling	Leads to anaphylaxis
Hemodynamic Disturbance	Bradycardia or hypotension (HR or BP decreased >20% from patient's baseline) for 0-30 sec	Bradycardia or hypotension (HR or BP decreased >30% from patient's baseline) for 0-30 sec	Bradycardia or hypotension (HR or BP decreased >30% from patient's baseline) for >30 sec or requiring treatment	Cardiac Arrest
Nausea	Temporary, no vomiting, requires	Vomiting, requires one	Vomiting, requires two therapeutic	Vomiting, requires more than 2

	no treatment	therapeutic medication	medications	therapeutic medications

If 3 or more adverse reactions occur, the study will be temporarily suspended and methodology will be reviewed by the study team and safety officer. If 6 or more adverse reactions occur, the study will be stopped.

Routine preoperative evaluation will be performed on all patients by an attending pediatric anesthesiologist prior to surgery. This includes review of the medical record, review of relevant laboratory studies, history and physician examination including airway examination, and discussion of the anesthetic plan with the parents and/or patient. Unless clinically indicated, patients will not receive preoperative monitoring. Intraoperative monitoring will be in accordance with the American Society of Anesthesiologist standard and will include 5- lead electrocardiogram, pulse oximetry, non-invasive blood pressure, core temperature, end-tidal carbon dioxide, and end-tidal anesthetic concentration (when appropriate.) Monitoring in the operating room is continuous, except for non-invasive blood pressure, which is measured at least every 3 minutes.

In the post-anesthesia care unit, the patient will receive the same monitoring as in the operating room except end tidal carbon dioxide. Again, monitoring will be continuous except for non-invasive blood pressure, which will be monitored at least every 15 minutes.

Pitfalls and Alternatives. We expect to confirm neuroinflammation in the piglet model and in human children based on our preliminary work and existing literature despite not having seen the typical "footprint" of neuroinflammation in the small number of animals studied thus far. Although we expect to detect elevated cytokines in brain homogenates from animals after only 48 hours, we have revised our brain tissue harvest protocol to 72 hours to match reported time after which inflammation is most likely to be evident. Nonetheless, we have considered alternatives in the eventuality that inflammatory injury is not seen. Alternative methods to detect neuroinflammation can be used. Positron emission tomography (PET) and magnetic resonance imaging (MRI) scanning have been used to quantify neuroinflammation in children and in adults.[51,52] These modalities could easily be adapted for use in our protocol.

Future Directions. This translational, pilot study is designed to spawn multiple future studies and to provide the framework for Dr. Whitaker's career in translational investigation. An option for follow-up on our study consent will be included, as children with HC frequently return for surgical revisions. For instance, if we demonstrate less inflammation in our TIVA cohort, a future randomized controlled trial evaluating the neurocognitive outcomes in HC patients based on anesthetic technique will be warranted. In addition, we plan to collaborate with the Department of Behavioral Health here at Nationwide, as it will be important not only to demonstrate neurobiological but also neurocognitive changes.

IRB/IACUC: The animal portion of this study will be performed at The Ohio State University, and IACUC approval has already been obtained. We have been working with this model for nearly 2 years and have received IACUC approval. For the human arm of our study, an IRB application has been submitted and has been approved.

Timeline/Milestones:

1. November - December 2014 (complete):
    a. Complete and submit the IRB application including informed consent form
    b. Establish protocols with investigational pharmacy; ensure study drugs will be available
    c. Educate practitioners (investigators, anesthesia, PACU and neurosurgery) about the study
    d. Order necessary supplies and materials (animal protocol and study are approved and underway)
2. **May 1, 2015: Begin study, pending IRB and IND approval**
3. May – July 2015
    a. Begin to recruit patients during the preoperative visit (Surgery Unit prior to neurosurgery or MRI)
    b. Collect samples (CSF and peripheral blood for VPS patients; peripheral blood for MRI patients)
    c. Assess study procedures and adjust where necessary to ensure safety and efficiency
    d. February 2, 2015: Submit the Ohio State University Davis-Bremer Pre-K Award application ($50,000 for one year; renewable)

4. August 2015:
    a. Evaluate recruitment performance and adjust accordingly
    b. Ad-hoc analysis of preliminary data
    c. Begin ELISA and qPCR analysis of collected samples
5. July 2015– February 2016:
    a. Maintain recruitement and perform data collection
    b. Continue ELISA and qPCR analysis
    c. Completion of patient recruitment and sample collection at the end of year 2015
6. February – May 2016:
    a. Analyze all collected data
    b. Prepare initial manuscripts
    c. Possible manuscripts to which we will submit our manuscripts:
        i. Goal manuscript submission deadline: May 30, 2016
        ii. Potential journals for manuscript submission: *Anesthesiology, Anesthesia and Analgesia, Neuroscience*
    d. Determine the national or international meetings at which we will present our data
    e. Potential annual meetings for data presentation:
        i. American Society of Anesthesiologists (ASA); Society for Pediatric Anesthesia (SPA); International Anesthesia Research Society (IARS); Society for Neuroscience and Critical Care (SNACC)
    f. Dates for annual meetings:
        i. ASA: October 2016
        ii. SPA: October 2016; March 2017
        iii. IARS: March 2017
        iv. SNACC: October 2016
    g. Principal investigator to present data at departmental Grand Rounds
7. April 2016 – May 2016
    a. Complete data analysis
    b. Prepare abstracts and oral presentations for annual meetings
    c. Consider post-hoc analyses
    d. Determine future directions:
        i. Sex differences in anesthetic-induced neuroinflammation
        ii. Positron-emission tomography quantification of anesthetic-induced neuroinflammation
        iii. Central nervous system - enteric nervous system interactions and their influence on anesthetic-induced neuroinflammation
8. May 30, 2016: Complete study

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