4-2015

Certain General Anesthetics Used in Pediatrics Hinder Neurological Development in Infants

Michael R. Hirschi
Southeastern University - Lakeland

Certain General Anesthetics Used in Pediatrics Hinder Neurological Development in Infants

Michael R. Hirschi

Department of Natural and Health Sciences. Southeastern University, Lakeland, Florida

Table of Contents:

List of Figures:

Chapter 1: Overview of General Anesthetics Use in Infants

Abstract: General anesthetics act by either blocking N-methyl-d-aspartate (NMDA) receptors or over stimulating γ-aminobutyric acid (GABA) receptors.[1–3] The actions of these receptors are responsible for the anesthetized state and are also crucial in the neurological development of infants.[2,4–8] Animal studies, although limited, provide vital information about general anesthesia's neurotoxicity its hindrance of neurological development. Exposure to general anesthesia can severely hinder proper neuronal migration, synaptogenesis, and can drastically increase neuronal apoptosis in infant animals.[9–16] General anesthetics are more neurotoxic to infant animals in combination compared to individually.[10,16,17] Additionally, multiple exposures to general anesthesia tend to have compounding deleterious effects on neurological development in infant animals.[12] It is likely that repeated exposure and combinational exposure to general anesthetics are the most detrimental to neurological development. Many retrospective studies on human infants show a correlation between exposure to general anesthesia and an increased risk of neurodevelopment disorders.[18–25] However, these studies are statistically limited due to confounding factors. These confounding factors are the reason direct evidence of the neurotoxic effects of general anesthetics has been so elusive. *In vitro* human stem cell models provide an ethical alternative to clinical studies. However, clinical trials are necessary and are the most promising methods for obtaining direct evidence of the deleterious effects of general anesthesia on the developing human brain. Different methods used in clinical trials on infants help to minimalize ethical dilemmas, increase recruitment rates, and maximize safety and expediency. Specifically, this paper will evaluate the efficacy and safety of different methods used in clinical trials and will propose how clinical trials can be designed for future studies.

How General Anesthesia Works

According to the 2004 National Hospital Discharge Survey, approximately three million children in the United States undergo surgical procedures that require general anesthesia.[26] This does not include children that require general anesthesia for nonsurgical purposes such as dental procedures or imaging studies. A large portion of these children are infants when they are first exposed to general anesthesia. This fact is concerning considering that many general anesthetics exhibit neurotoxicity in infant animals (including non-human primates). Furthermore, retrospective studies demonstrate that children requiring surgery as infants have lower cognitive performance than children who did not require surgery.[20–22,24,25] Unfortunately, many have criticized this data claiming that these studies could be irrelevant due to confounding factors.

The concern for the long-term neurological effects of general anesthesia on infants prompted the FDA in 2007 to look into the data concerning general anesthesia's neurotoxicity.[26] The FDA determined that no changes could be made to which general anesthetics are administered to infants based on current evidence.[26,27] However, they urged that more comprehensive and conclusive clinical studies be conducted promptly to determine which general anesthetics were neurotoxic to infants or children.[26,27] The FDA also recommended that purely elective surgery be deferred to after 6 months of age at a minimum.[26,27] Clinical studies in infants are crucial considering how long the critical period of neurological development lasts. General anesthetics have been shown to have the most severe detrimental effects on neurological development in animals during critical periods known as synaptogenesis.[9,10,28] The length of synaptogenesis in humans lasts from approximately the second trimester of pregnancy to three

years after birth.[21,25,28–30] This is troubling considering many surgical procedures on infants require general anesthesia.

The two broad classes of anesthesia are local and general. Local anesthetics work by blocking neural pain signals in a specific site of the body.[31] General anesthetics alter the function of neurons in the central nervous system (CNS) by directly interacting with multiple ion channels.[31] These ion channels control the excitability of neurons by regulating the flow of ions. An excitatory effect is caused by the neuron depolarizing.[31] An inhibitory effect is caused by the neuron hyperpolarizing.[31] The flow of ions (and therefore the polarization of the neuron) is determined by the anesthetic agent.

The six general anesthetics that are commonly used in pediatric care fall into two categories: inhalational and intravenous. The inhalational general anesthetics are nitrous oxide, isoflurane, sevoflurane, and desflurane.[31] These are called inhalational anesthetics because they are administered through a respirator. The intravenous general anesthetics are propofol and ketamine, which are administered directly into the blood stream.[31] General anesthetics act by either blocking N-methyl-d-aspartate (NMDA) receptors or over-activating γ-aminobutyric acid (GABA) receptors.[1–3] The NMDA receptor is a primary excitatory neurotransmitter receptor in the CNS.[32] The GABA receptor is the most abundant fast inhibitory neurotransmitter receptor in the CNS.[31] The two modes of action for general anesthetics are blocking excitatory signals or enhancing inhibitory signals.[31] Ketamine and nitrous oxide primarily act through blocking NMDA receptors.[2,31] However, they also over-activate GABA receptors but to a lesser degree than other general anesthetics. Isoflurane, sevoflurane, desflurane, and propofol act primarily by over-activating GABA receptors.[31]

The deleterious effects of ethanol on the developing brain are due to ethanol blocking NMDA receptors and over-activating GABA receptors.[33] Ethanol is known to cause severe neurological developmental disorders in fetuses, such as fetal alcohol syndrome, when ingested by the mother during pregnancy.[33–35] Pregnant women are warned to stay away from alcohol throughout their entire pregnancy due to the risk of fetal alcohol syndrome. Unfortunately, many pregnant women or infants cannot be withheld from general anesthesia if surgery is required due to its necessity for most surgical procedures. Since general anesthesia is essential, it is extremely important to understand how different general anesthetics affect the developing brain in order to prevent developmental impairment in infants.

Effects of Blocking NMDA and Over-activating GABA

There are several reasons why blocking NMDA receptors and over-activating GABA receptors hinder neurological development. The developing brain goes through critical periods where GABA receptors are stimulated by NMDA receptors.[2,7,8] GABA receptors need to be stimulated in the developing brain in order for immature neurons and oligodendrocytes to mature properly.[36] Stimulation of GABA receptors also strengthens NMDA receptor stimulation and is vital in the formation of neural networks.[4–7] This period of brain development is called synaptogenesis because new synapses or neuronal connections are being formed.[28,37] Connections that are not stimulated are deemed unnecessary by the body and are removed through a process known as synaptic pruning.[38] This process allows poorly stimulated neurons to go through apoptosis throughout neurodevelopment.[4,5] This is one reason why strengthening neural networks through proper stimulation is so vital to normal neurodevelopment. A study in mice showed that neurons do not migrate properly to form neural networks when NMDA receptors are not expressed.[39] This same study showed that increasing NMDA receptor

expression increased neuronal migration and neural network formation.[39] The NMDA receptor is known to be vital to learning and memory.[32] Therefore, it is likely that hindered synaptogenesis or neuronal migration may impair learning and memory.

The proximity of NMDA and GABA receptors allows them to synergistically initiate key events in neurological developmental. A study in infant mice found that GABA receptor density was 90 times higher around NMDA receptors than in other parts of the brain.[8] These receptors work in combination to achieve synchronous synaptic activity.[8] Synchronous synaptic activity occurs when neurons are rhythmically stimulated with other neurons in the same neural network.[8,40] Synchronous synaptic activity is vital to proper neurological development and neural network formation.[8,41] A study on rat neurons *in vitro* found that synchronous synaptic activity led to the maturation of immature synapses through the addition of AMPA receptors to synapse containing the NMDA receptor.[40] The addition of AMPA receptors allows NMDA receptors to be stimulated.[40] AMPA receptors also prevent afferent connections from forming later on in neurological development.[40]

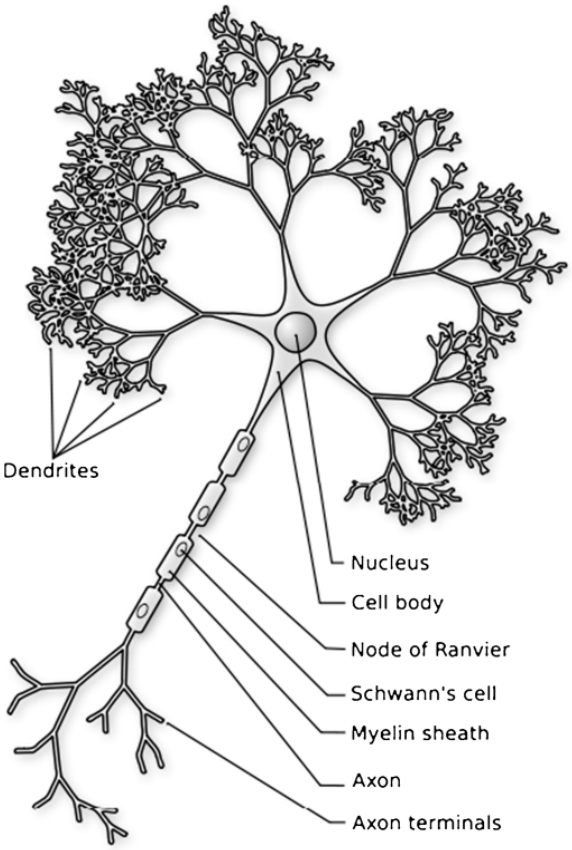

Figure 1: **Diagram of a Neuron and its Structures.** Dendritic spines are located on the shafts of the dendrites. The synapse is formed between the axon of one neuron and the dendrite(s) of another neuron.[48]

There are serious detrimental developmental effects when NMDA receptors are blocked. *In vivo* studies of infant mice and rats have shown that the developing brain goes through excessive neuroapoptosis when NMDA receptors are blocked for hours at a time.[2,42,43] This is in contrast to normal neuroapoptosis that occurs in the developing brain during critical periods in order to strengthen neural circuits and remove unnecessary connections.[28] When the brain undergoes excessive neuroapoptosis during development, there are life-long detrimental effects. Learning impairments and cognitive disability are common side effects of extensive neuroapoptosis during neurodevelopment.[28] This outcome is not surprising considering that an excessive amount of neurons are dying off leading to gaps in neural networks.

Inhalational Anesthesia and Neurological Development

Antiepileptic drugs have been shown to severely impair cognitive development in infant rats.[44] One large clinical study looked at the frequency of physical birth defects in babies from epileptic mothers who were prescribed antiepileptic drugs. The study found that exposure to antiepileptic drugs *in utero* significantly increased a baby's risk of being born with physical abnormalities including major malformations, microcephaly, growth retardation, and hypoplasia

of the midface and fingers.[45] Antiepileptic drugs act on GABA receptors by over-stimulating them in a manner similar to many general anesthetics.[44]

Sevoflurane, isoflurane, and desflurane are all inhalational general anesthetics that act by over-stimulating GABA receptors.[31] Sevoflurane, isoflurane, and desflurane have all been shown to alter dendritic spine density on dendritic shafts *in vivo* in infant rats after two or more hours of exposure.[46,47] Dendritic spine shafts are main sites of synaptic connections in neural networks.[46,47] The dendrites of a healthy neuron (where the dendritic spines are located) can be seen in **figure 1.**[48] A primate study demonstrated a 13-fold increase in neuronal apoptosis in rhesus macaque brain cells *in vitro* after five hours of exposure to clinical concentrations of isoflurane compared to controls.[15] These results are unfortunate considering that isoflurane is capable of passing from mother through the placenta into the fetus in humans.[49] This indicates that mothers who receive isoflurane during cesarean sections are inadvertently exposing their unborn babies to a potentially neurotoxic general anesthetic for the duration of the procedure. The findings of one *in utero* study on rats add evidence to the danger of *in utero* exposure to isoflurane. The rats in this study were in a stage comparable to the second trimester of pregnancy in humans.[9] The rat mothers were exposed to clinical concentrations of isoflurane for four hours.[9] Babies that were exposed to isoflurane *in utero* had impaired spatial memory, impaired learning, and reduced anxiety compared to control rats.[9] These symptoms are most likely signs of improper neurological development.

Studies have found that the use of multiple general anesthetics is actually more detrimental than using only one anesthetic.[10,16,17] Two studies performed on infant rats using clinically relevant concentrations of nitrous oxide and isoflurane showed that these general anesthetics used in combination caused severe neuroapoptosis and prevented proper synapse formation in developmentally vulnerable regions of the brain.[10,17] Specifically, these general anesthetics modified synaptic protein levels. These modifications impaired the function of the proteins and prevented synaptogenesis.[10] This impairment of normal neurological development and synapse formation could lead to long-term learning impairments and memory deficits. A study involving *in utero* guinea pigs showed that isoflurane in combination with nitrous oxide, compared to isoflurane treatment alone, drastically increased neuronal apoptosis in vulnerable brain regions as well.[16] These studies indicate that inhalational general anesthetics need to be studied further individually before they can safely be used in combination.

Intravenous Anesthesia and Neurological Development

Propofol is a general anesthetic that acts by over-activating the GABA receptor. Propofol has been shown to induce neuroapoptosis and cause learning impairments.[11,50] An *in vivo* study on infant mice found that a quarter of the concentration of propofol needed to induce anesthesia was sufficient to induce neuronal apoptosis.[50] An *in vivo* study found on infant rats injected with clinical concentrations of propofol showed signs of neurodegeneration and had learning impairment compared to control rats.[11] These are both expected symptoms from GABA over-activation considering the role of GABA receptors in establishing and strengthening neural networks.[5,7]

Ketamine is a general anesthetic that acts by blocking the NMDA receptor. Ketamine is commonly used in infants during surgical procedures.[3] Ketamine has been shown to cause extensive neuroapoptosis during synaptogenesis.[14,43] An *in vivo* study on infant rats showed that exposure to ketamine significantly increased neuroapoptosis.[43] The infant rats were exposed to seven doses of ketamine leading to a 28-fold increase in neuroapoptosis compared to controls.[43].

An *in vivo* study on infant rhesus monkeys showed that exposure to ketamine significantly increased necrotic and apoptotic neuronal cell death.[14] Additionally, the researchers found that messenger RNA for the NMDA was significantly increased in areas that underwent extensive cell death.[14] Rhesus monkeys that were earlier in development had increased cell death compared to rhesus monkeys three weeks further in development.[14] It is concerning that several animal studies have shown that concentrations of ketamine, propofol or isoflurane that are approximately a quarter of that needed for anesthesia still induce neuronal apoptosis in infant mice.[13,50,51]

The studies done on primitive mammals provide evidence that the negative effects of anesthesia on the developing brain are significant. They provoke the need for further research into the detrimental effects of anesthesia on human infants. However, the clinical significance of the results of these studies is limited. This is due to the fact that the studies were performed on primitive mammals. These studies are indicative of neurotoxicity at best and completely irrelevant at worst. The data from non-human primate studies are more clinically significant. Non-human primate studies are the closest *in vivo* model that can translate to humans besides humans themselves. The agreement in evidence from primitive mammal and non-human primate studies is sufficient to begin large scale clinical trials on the effects of general anesthetics on infants.

Current Clinical Research on Infants

Several clinical studies have analyzed the long-term effects of general anesthetics on infants. They all show that exposure to general anesthetics in infancy increases the risk of neurodevelopmental disorders.[18–25] The studies look at infants that required surgery for various reasons. They compare the long term neurological development (in areas such as learning, memory and motor control) to baseline values determined from infants that did not require surgery. The studies assessed neurological development at varying intervals ranging from two to ten years after surgery. Collectively, these retrospective studies underscore that infantile exposure to general anesthesia significantly increases the risk of learning and memory impairment.[18–25] The critics of these studies often point out that confounding factors cannot be taken into account in these retrospective studies. They claim that this reduces the statistical significance of the studies. Due to ethical concerns, many critics are skeptical about relying on evidence from retrospective studies and animal studies as a basis for clinical trials on infants.

The lack of clinical trials leaves doctors lacking accurate information about the safety of general anesthetics in infants. This ignorance may lead doctors to inadvertently expose babies to general anesthetics that are neurotoxic. This most likely is already happening as was shown in the results of one retrospective study. A retrospective study on pregnant women found that maternal exposure to general anesthesia during the first trimester increased the risk of babies being born with CNS defects.[52] Fortunately, a new *in vitro* system has been developed using human stem cells to create a close resemblance to *in vivo* neurological development.[53] This is not a perfect system because it does not exactly mimic a true *in vivo* system. However, it is a step forward in the ethical dilemma that surrounds clinical trials in infants. This *in vitro* system would be useful in providing more evidence and data so that clinical trials in infants can proceed. This stem cell system would be perfect for testing which general anesthetics are neurotoxic during neurological development.[53] This would also be extremely effective at determining methods of preventing general anesthetics neurotoxic effects.[53]

History of Clinical Research on Infants and Children

In the 1930s, the use of drugs on children was widely unregulated.[54] Children were often administered drugs with questionable safety.[54] This lead to several adverse events that could have been avoided. Due to an ingredient used to manufacture the drug elixir of sulfanilamide, almost 100 children died in the late 1930s.[54] Consequentially, an amendment was made to the Federal Food and Drug Act in 1938 requiring drug ingredients to be labeled honestly and with documentation of the drug's safety.[54] Sadly, many of the ethical guidelines and regulations behind clinical trials and requirements for human drug testing have come about due to the occurrence of severely adverse reactions similar to the example mentioned above.[54]

In the 1960s, the drug thalidomide was found to cause massive birth defects and malformations.[54] This led to the passing of the Harris–Kefauver Amendment to the Food, Drug and Cosmetic Act in 1962 which required drugs to be tested on animals before moving to clinical trials in humans.[54] The ethics of clinical trials have often been difficult to discern, leading to an ever-evolving landscape of ethical guidelines, requirements, and practices.

Informed consent is a perfect example of how greatly ethical guidelines and policies have changed over the years. The Supreme Court ruled in January 31, 1944 that parents may be free to become martyrs themselves. But it does not follow they are free, in identical circumstances, to make martyrs of their children before they have reached the age of full and legal discretion when they can make that choice for themselves.[55] The implications of this ruling were profound. All nontherapeutic clinical research on minors was considered unlawful even if the parents had consented. In accordance with the advice from legal experts of the day, this was the stance that many government agencies took after this 1944 ruling.[55]

This legal stance was largely met with resistance by practicing physicians. In 1967, The Royal College of Physicians emphasized the importance that clinical investigations be conducted with expediency and little regulation.[56] Their main recommendation was that doctors should be free of strict government control so as not to defer clinical trials from occurring.[56] They express their belief that attempting to rigidly control clinical trials would discourage physicians from conducting such research.[56] This would in turn lead to a stagnation in the advancement of medically relevant clinical knowledge. The conflicting stances of the U.S. government and medical organizations caused confusion and an ethically difficult situation. The Supreme Court ruling had little effect on clinical research in humans. Guidelines and regulations were worded in such a way as to indirectly promote the continuance of nontherapeutic clinical research on minors. For example, The Royal College of Physicians stated in 1973,

> Clinical research investigation of children… which is not of direct benefit to the patient should be conducted, but only when the procedures entail negligible risk or discomfort and subject to the provisions of any common and statute law prevailing at the time.[55]

This statement does not directly contradict the Supreme Court ruling. However, it does infer that nontherapeutic clinical research on minors is permissible if it causes negligible harm. The problem with this guideline is that it is rather subjective. A physician must use their personal discretion when designing and implementing clinical trials. The lack of clear regulation and conflicting opinions led to several medical organizations developing their own ethical guidelines and requirements.

The World Medical Association has developed the Declaration of Helsinki over the last fifty years. The Declaration of Helsinki is a document that lays out ethical guidelines for how clinical trials in humans should be conducted.[57,58] The document has become increasingly

inclusive and extensive as ethical issues over clinical trials on humans have arisen.[59] The importance of this document cannot be understated. It has become a standard that is internationally recognized in the field of bioethics. The Declaration of Helsinki states that the advancement of medical knowledge is brought about through research which must occasionally rely on some experimentation involving human patients.[57,58] There are several factors that must be considered when dealing with humans. For example, the Declaration of Helsinki requires that the well-being of the patient take precedence over the advancement of medical knowledge no matter how great the benefit.[57,58] The foundation of the Declaration of Helsinki was brought about, like many other guidelines and regulations, as a result of gruesome events. What some scholars believe to be one of the most important revisions occurred in 1975.[59] This revision required independent committees to review research protocols involving human patients in order to promote ethical accountability.[59]

Recently, a group of physicians found that up to 90% of drugs given to neonates were not used in an authorized manner or were unlicensed.[60] This is a frightening statistic because this would cause neonates to be at a greater risk of drug toxicity. A study conducted in 1998 found that only 36% of drugs given to children were not used in an approved manner.[61] Both these studies reveal that minors, and especially in neonates, need to be better protected by conducting further clinical research. Unfortunately, the lack of knowledge on drugs' effects on children, infants, and neonates is widespread in medicine. *The Physicians' Desk Reference* contains dosing information based on age, weight, and other criteria. Unfortunately, less than 30% of the entries in the Physicians' Desk Reference have statements that the safety and efficacy of the drug in question has not been determined or dosing information for children, infants, or neonates.[54] It is not ethical to continue to allow these age groups to receive substandard care because of a lack of knowledge.

This is the main reason the European Medicines Agency met in 2006. They conducted a workshop that dealt with ways to introduce incentives to researchers in order to promote the study of drugs safety and efficacy in children, infants, and neonates.[62] The Best Pharmaceuticals for Children Act was passed in 2002 to promote a similar type of incentive in the United States. The goal of the Best Pharmaceuticals for Children Act was to promote clinical studies of drugs used off-label in children, infants, and neonates.[63] Since the act's passing in 2002, the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) have been encouraging researchers to conduct studies to evaluate the efficacy and safety of the drugs of drugs used off-label in these vulnerable age groups.[63] It is important to understand how drugs are approved for clinical trials in humans and what kind of guidelines regulate the clinical trials themselves.

The Harris–Kefauver Amendment to the Food, Drug and Cosmetic Act (mentioned earlier) led to the formation of the investigational new drug (IND) process for testing drug safety and efficacy in clinical trials.[54] The IND created the basic requirements of how new drugs are tested for safety and efficacy even to this day.[54] The IND involves three phases of clinical testing after the initial preclinical testing. Phase I of the IND involves testing the drug for safety, methods of metabolism, and its eventual removal from the body of the patient (pharmacokinetics).[54] Phase II involves evaluating the drug for efficacy and proper dosage according to numerous variables in the patient such as weight and age.[54] In phase III, multiple clinical trials are conducted in order to compare their results.[54] One of the most important considerations during a clinical trial is the risk-to-benefit ratio.

The risk-to-benefit ratio is the amount of risk a patient is exposed to in order to obtain any kind of beneficial result.[54] This ratio takes into account how much risk the patient is being

subjected to compared to how much the patient or medical community will benefit.[54] Ethically, physicians should attempt to design trials to achieve low risk and high benefit. Unfortunately, the nature of the risk-to-benefit ratio makes it rather subjective when implemented.[54] Therefore, it is up to ethical committees and physicians to ensure the risk-to-benefit ratio is accurate and reasonable.

Ethics of Clinical Research on Infants

A requirement common to all clinical trials (and an especially sensitive topic when dealing with infants) is informed consent. Problems arise with informed consent because of subjectivity of updating the patient. Informed consent implies that the patient must be educated of what clinical experimentation they will be subjected to.[64] Additionally, informed consent implies that the patient must be educated so that they have a thorough understanding of the risks.[64] The education aspect of informed consent presents many problems to the physicians conducting the clinical research. Education of patients can be time-consuming and counter-productive, leading to poor recruitment rates. This creates an ethical dilemma for the investigating physician. It is often tempting for the investigating physician to bypass the educational aspect of informed consent. However, this is a gross violation of the patient's right to autonomy. The right of autonomy means the patient should have complete control over his or her medical care without the coercion of others. The nature of informed consent makes it especially difficult when conducting clinical research on infants.

The ethical dilemmas of informed consent have caused some critics to suggest that the informed consent requirement be forgone in incompetent patients. Informed consent is often time-consuming and can lead to poor recruitment rates. Therefore, it may often seem that informed consent is counterproductive. Critics of informed consent in the case of incompetent patients have suggested that ethical committees approve or disapprove clinical trials to take the burden off of parents and guardians. However, a study on the parents of neonates involved in clinical trials determined that this would be met with great resistance. Over 80% of the parents in the study stated that they would be unhappy with not giving informed consent and relying on an ethical committee.[65] Other critics have suggested that the attending physician should decide whether or not to enroll an incompetent patient in a clinical trial. Yet, the same study mentioned above found that over 90% of parents said they would be unhappy with the attending physician making the decision.[65] Other studies have also shown that parents prefer to make decisions over their children in regards to clinical trials.[66] However, clinical trials involving infants can achieve high recruitment rates while still leaving decision-making in the hands of the parents.

Written consent appears to be what harms recruitment rates the most for clinical trials involving incompetent patients such as infants. It is believed that the anxiety of parents along with the inability to fully process all the written consent forms leads to poor participation.[65,67,68] Morally, it is unacceptable for informed consent to be bypassed altogether.[69,70] This kind of practice would imply that a benefit to society trumps individual rights. However, other methods of consent can limit anxiety and provide parents with a better understanding of what their infant will be subjected to. It has been suggested that a progressive approach where parents are informed throughout the clinical trial will help reduce anxiety and increase recruitment rates.[65,67,68] Additionally, time constraints can be reduced by delegating responsibilities to different members of ethical committees.[68] This streamlining of clinical trial processing would also assist recruitment rates by giving parents additional time to consider the pros and cons of the clinical trial. Streamlining clinical trial processing could also decrease the duration of clinical

trials, leading to an increased rate of medical advancement. Clinical trials in infants must also be designed in a precautious and safe manner in order to encourage trust and further participation. Streamlining clinical trials in infants can only be taken so far.

There are several factors that must be taken into careful consideration when designing clinical trials involving infants. The smaller dosages that are required for infants make them more susceptible to drug overdose. Even a small percentage of error in dosages can result in seriously adverse reactions due to the limited maturation of organ systems and low body weight of infants.[71] Additionally, the recommended dosages and infants has to be adjusted in accordance with their development. For example, after one to two months the dosage of a particular drug may need to be increased to accommodate organ maturation and weight gain.[71] Based on this understanding, it is important to have extra regulation and precaution when dealing with infants. This extra care has been criticized by activists who argue that clinical trials in infants need to be conducted in haste. Unfortunately, this criticism is largely misplaced and probably results from a lack of understanding concerning the delicate situation of dealing with infants.

What distinguishes neonates from infants is their age. Infants are generally regarded as babies one month to one year old.[71] The term neonate is used to describe a baby just after birth until they are approximately one month old.[71] However, preterm babies are often regarded as neonates for longer periods of time due to the immaturity of their organ systems.[71] Neonates are more vulnerable than infants due to the inability of their organs to metabolize drugs effectively.[71] This is largely because their organs systems are still very immature.[62,71] This is also why infants are considered less vulnerable than neonates. With this information in mind, it is better to recruit infants and neonates at various stages in development in order to obtain the most reliable information in regards to drug safety from clinical trials. This particularly important considering the age of a baby plays an important role in influencing parental consent and emotions.

Interestingly, one study found that parents from a lower socio-demographic background were more likely to consent to their children being involved in clinical trials.[72] Their main motivation behind participation was the advancement of society.[72] This raises an important question. Should physicians seek to further educate parents from a lower socio-demographic background or take advantage of their increased participation? Ethically, it is never acceptable to exploit a parent who is emotionally distressed over his or her child. However, it is possible that parents from a lower socio-demographic background are simply more altruistic and should be praised. Either way, physicians must take special care to inform parents from all backgrounds and to not exploit parental emotions to bolster recruitment.

Discussion

The proper stimulation of NMDA and GABA receptors are vital to neurological development.[2,4–8] These are also the receptors that many general anesthetics act upon.[1–3] The NMDA and GABA receptors play roles in neural network formation, neuronal migration, and SSA.[2,4–8,39,40] These and other neurodevelopmental events are important for learning and memory.[2,4–8] Neurodevelopmental disorders involving learning and memory deficits are expected if these receptors are blocked or dysregulated. General anesthetics act by blocking NMDA receptors and over-stimulating GABA receptors.[1–3] This means that infants exposed to general anesthetics can have hindered neurological development during the entire duration of surgery. Both animal and clinical studies demonstrate that the earlier the exposure to general anesthetics the more detrimental the effect.[14,17,25] General anesthetics most likely impair normal neurological developmental by blocking stimulation for extended periods of time. NMDA and GABA

receptors are important in establishing and strengthening neural networks.[4–8] A lack of proper stimulation can lead to neuroapoptosis and subsequent gaps in neural networks.[28] These gaps could likely lead to improper neurological development and negatively impact learning and memory in the long-term.

Animal models have confirmed that several general anesthetics cause neurodevelopment disorders by obstructing proper neurological development. Exposure to general anesthesia during infancy in animals can cause widespread neuroapoptosis and alterations to various structures in the brain and cause learning and memory impairments.[9–16] Even a single exposure to general anesthesia can drastically increase neuroapoptosis.[15] Sevoflurane, isoflurane, and desflurane altered dendritic spine density after two or more hours of exposure at clinically relevant concentrations.[46,47] This is concerning considering the normal duration of surgical procedures on infants is longer than two hours. A large number of infants are exposed to detrimental amounts of potentially neurotoxic general anesthetics considering the typical duration of surgeries. This follows from the fact that general anesthetics are neurotoxic even after short, single exposures. Infants that require multiple surgical procedures and exposures to general anesthetics have an increased risk of neurological developmental disorders. Animal studies show that multiple exposures to general anesthetics significantly impair neurological development and increase neuroapoptosis by 28-fold compared to a single exposure.[12,43] It is possible that significant cognitive impairments are only noticeable in infants that have been exposed to general anesthesia multiple times. Multiple exposures to isoflurane have been shown to severely impair memory and cause severe learning deficits.[12] This suggests that multiple exposures to GABA receptor agonists can compound the negative effects of general anesthetics on neurological development. Multiple exposures to general anesthesia causes repeated over-activation of GABA receptors and blockade of NMDA receptors. This could have compounding effects leading to more severe neural network degradation by neuroapoptosis.

Unfortunately, many infants that have to go through multiple surgical procedures possess other developmental disorders that are seen as confounding factors. These confounding factors limit the statistical significance of the study. However, it is possible that the confounding factors are distorting the data and leading to an inaccurately low level of reported neurological disorders due to general anesthesia. In infant rats, multiple exposures to isoflurane drastically increased cognitive and developmental impairment compared to a single exposure.[12] It is unfortunate that the circumstances that bring a baby into the operating room multiple times for surgery may be seen as a confounding factor. This could be why it has been difficult to directly link general anesthesia exposure to cognitive and developmental impairment in human infants.

Several studies have found that the use of multiple general anesthetics is actually more detrimental than using only one anesthetic.[10,16,17] This data indicates that general anesthetics need to be studied further individually before they can safely be used in combination in infants. Individual studies would be useful for providing information about why general anesthetics hinder neurological development and cause extensive neuroapoptosis. The *in vitro* human stem cell system is a tool that could prove immensely useful in this regard. While it is not a perfect replica, the *in vitro* system does model early neurological development and can ethically be used in studies.[53] This is a more ethical solution than testing various combinations of general anesthetics on human infants. This system could also be used to determine useful prevention strategies to combat the neurotoxic effects of general anesthetics on infants. The necessity of general anesthetics on infants requires that all possible avenues be pursued. Retrospective studies are also ethically acceptable and useful. The key to retrospective studies is to minimalize

confounding factors in order to interpret data that is statistically strong. Ethical concerns make studying the effects of general anesthesia on infants difficult. However, they are still necessary to prevent further exposure of infants to neurotoxic general anesthetics. Currently, controlled clinical trials on the effects of general anesthetics on infants are underway. Hopefully, when they are complete they will provide clinical insight in preventing general anesthesia induced neurological disorders in infants undergoing surgery. It is likely that these trials will (at the very least) reveal that repeated exposure and combinational exposure to general anesthetics are the most detrimental to neurological development.

Clinical trials on infants are controversial and raise several ethical dilemmas. Concerns over the extent of informed consent and safety of the infants has made extensive ethical guidelines for clinical trials a necessity. Regulations and ethical guidelines have largely been shaped by adverse reactions and gruesome events. There has been increasing inclusiveness in ethical guidelines to ensure that all interest groups are being equally attended to. However, heavily regulating clinical trials on infants can delay findings and lower recruitment rates. This raises ethical issues over how informed parents should be and how expediently these trials should be conducted or approved. There are techniques and methods that can be employed to maximize participant safety while bolstering recruitment rates and expediency. For example, written consent can lead to poor recruitment rates and should not be used. Instead, parents should be informed continually as the clinical trial progresses. Trial design is crucial in this aspect because it allows medical knowledge to advance at a reasonable rate so that treating neonates and infants becomes safer and more effective. Without these types of clinical trials, physicians would not have sufficient evidence to show that they are helping instead of harming an infant. However, it is best for physicians to err on the side of precaution in all human clinical trials, especially when dealing with neonates and infants.

Chapter 2: The Effects of GABA and its Receptor

Introduction

GABA is the predominant inhibitory neurotransmitter in the CNS of mammals.[31,73] GABA is an atypical neurotransmitter in regards to its biosynthesis and its effects on other neurotransmitter concentrations.[74] For example, most neurotransmitters rely heavily on recycling and reuptake after they have produced their effect.[74] However, the recycling of GABA is not as important as synthesis of new GABA.[74] The receptor for GABA has two main subtypes, $GABA_A$ and $GABA_B$. The $GABA_A$ and $GABA_B$ receptors are ubiquitous inhibitory neurotransmitter receptors in the CNS, found on almost all cortical neurons.[31,73] The NMDA and AMPA receptors work in conjunction with GABA receptors and will be briefly discussed in this chapter. However, the main focus of this chapter will be on the $GABA_A$ receptor due to its important role in neurodevelopment.

GABA Synthesis and Catabolism

The precursor to GABA is glutamic acid (also known as glutamate when negatively charged).[75] The new synthesis of GABA requires neurons to have a supply of glutamate which they are not reliant on *de novo* glutamate synthesis.[75] This requires neurons to import glutamate or glutamine, which can be converted by neurons to glutamate.[75] Not surprisingly, neurons that release GABA express transporters for the import of glutamate and glutamine.[76,77] Glutamine synthetase is the enzyme responsible for converting glutamate to glutamine.[75] Glutamine synthetase is expressed in astrocytes which subsequently release this newly converted glutamine to be taken up by GABA releasing neurons.[75] The conversion of glutamate to glutamine helps to detoxify ammonia and recycle neurotransmitters for inhibitory and excitatory neurotransmission.[75] In GABA releasing neurons, glutaminase type I (GLS1) is the enzyme responsible for the conversion of glutamine back to glutamate.[75] The enzyme glutamic acid decarboxylase (GAD) is responsible for the conversion of glutamate to GABA.[75] After GABA has been synthesized, it is moved to synaptic vesicles to be released into the synapse.[74] Expectantly, inhibiting GAD reduces the amount of GABA containing vesicles released into the synapse.[78]

Neurons are capable of undergoing glucose metabolism including the citric acid cycle. Glutamate can be synthesized from the citric acid cycle intermediate α-ketoglutarate.[75] However, this has minimal contribution to glutamate pools in GABA releasing neurons.[75] Blockade of both GABA synthesis and degradation reduces the amount of GABA-containing vesicles that are released into the synapse.[79] Interestingly, blockade of GABA reuptake by GABA transporters does not affect the amount of GABA-containing vesicles that are released.[79] Taken together, this suggests that GABA reuptake is more important for its ability to enter the citric acid cycle than to be reused as a neurotransmitter.

The excitatory amino acid transporter-3 (EAAT3) is expressed at the terminals of neurons that release GABA. The EAAT3 allows these neurons to reuse glutamate by importing it back into the cell for GABA synthesis. Sodium coupled neutral amino acid transporter 1 and 2, termed SNAT1 and SNAT2 respectively, allow neurons to import glutamine. Astrocytes express EAAT2 which has a similar function to EAAT3.

GABA is broken down by the enzyme GABA transaminase (GABA-T).[75] GABA-T removes and amino group converting GABA into succinc semialdehyde (SSA).[75] SAA is further degraded into succinate by the enzyme SSA dehydrogenase.[75] Succinate can then enter the citric

acid cycle. This breakdown of GABA allows neurons to compensate for the citric acid cycle intermediate α-ketoglutarate that is used to synthesize GABA. An excellent image representing the metabolism of GABA can be seen in **figure 2**.[75]

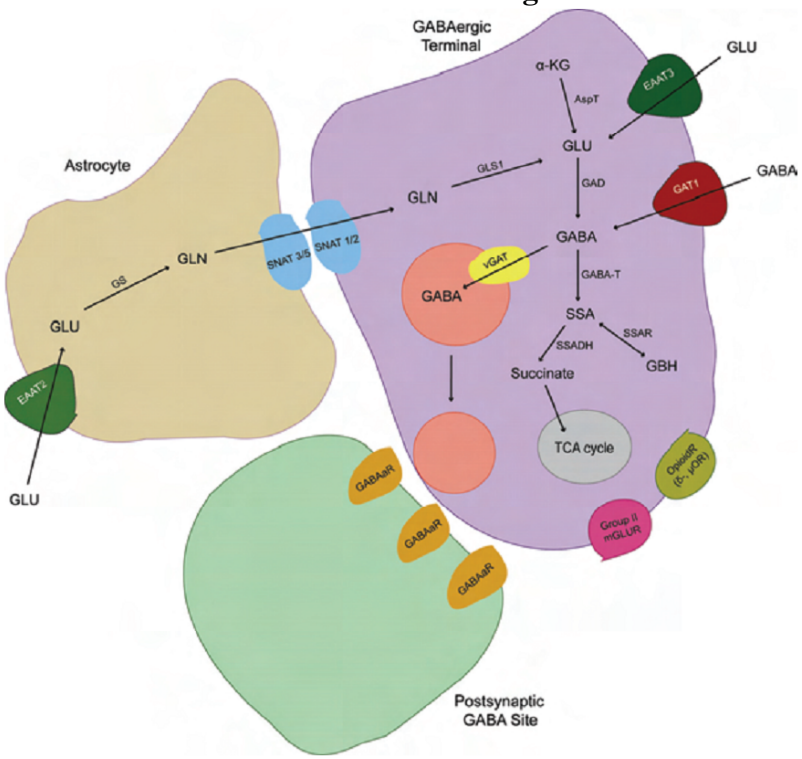

Figure 2: **GABA Metabolism.** The import of substrates needed for GABA synthesis (top left), the transport of GABA to the synapse (bottom middle), and the reuptake of GABA for further use (top right) are all shown in this figure. Relevant abbreviations are mentioned in the text above.[75]

$GABA_A$ and Other Receptors

As one of the earliest synapse systems to form, the importance of the $GABA_A$ receptor and its substrate to neurodevelopment cannot be stressed enough.[80] Over-activating $GABA_A$ receptors results in widespread inhibition throughout the brain that can be detrimental during critical periods such as synaptogenesis. $GABA_A$ receptors must be properly stimulated in order for immature or undifferentiated neurons to mature appropriately.[36] The NMDA receptor is also greatly affected by the activation of the $GABA_A$ receptor. Since the NMDA receptor is one the most widespread excitatory receptors and $GABA_A$ is one of the most widespread inhibitor receptors, they are intrinsically related in the formation of neural networks.[2,7,8] Neural networks cannot properly form without inhibition and stimulation induced by the $GABA_A$ and NMDA receptor respectively.[4–8] Synaptogenesis also relies on the actions of both receptors. Unsurprisingly, there are gaps in neural networks when the NMDA and $GABA_A$ receptors are inhibited or over-activated respectively.[28] This is due to the fact that during synaptogenesis neurons that are not stimulated or "used" get removed by undergoing apoptosis.[4,5] This has concept has been termed synaptic pruning and is most likely the main neurodevelopmental process affected by general anesthetics.[38]

Other receptors are also important in neurodevelopment including the AMPA receptor. The $GABA_A$ and NMDA receptors are located in close proximity to each other. However, AMPA receptors are located essentially right on top of NMDA receptors and affect their signal processing.[40] In order for neurons to fire in coherent patterns, termed synchronous synaptic activity, synapses containing the NMDA receptor must have AMPA receptors added to them.[8,40] The AMPA receptors allow NMDA receptors to become activated by their ligands.[40] After AMPA receptors are activated, GABA and NMDA receptors must work cooperatively to achieve

synchronous synaptic activity.[40] This developmental process helps to strengthen necessary connections within neural networks and remove unnecessary connections.[8,40]

Effects of Receptors

The $GABA_A$ receptor activity is largely controlled by cation-chloride cotransporters (CCC). Binding of GABA to the $GABA_A$ receptor can result in propagation or inhibition of a signal. What determines the fate of the signal is the concentration of chloride ions, which can pass through the receptor upon GABA binding. CCCs are responsible for controlling cellular concentrations of sodium and potassium. A specific type of CCC, the sodium/potassium/chloride cotransporter (NKCC1) increases the chloride concentration inside the cell.[81] NKCC1 imports sodium and potassium with their concentration gradient to force the chloride ions against their concentration gradient.[81] The binding of GABA to the $GABA_A$ receptor causes an efflux of chloride ions resulting in depolarization.[81]

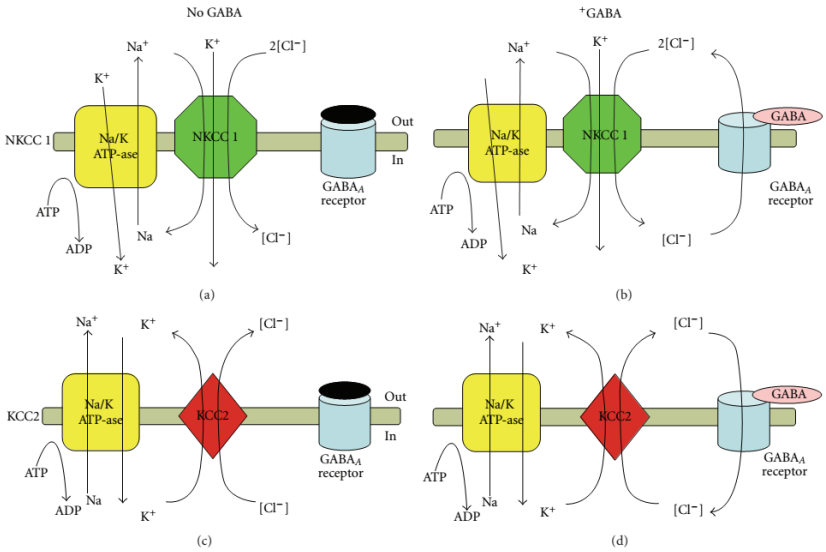

Figure 3: **Cation-Chloride Transporters.** This is a figure depicting the $GABA_A$ receptor and how the flow of ions is affected by its absence (a, c) and upon binding (b, d).[81]

Depolarization results in a propagation of a signal from one neuron onto others. In contrast, the CCC potassium/chloride cotransporter 2 (KCC2) decreases the chloride concentration inside the cell.[81] The KCC2 uses potassium to remove chloride ions from inside the cell.[81] The binding of GABA in this scenario causes an influx of chloride ions resulting in hyperpolarization.[81] Hyperpolarization results in the reduction of a signal and essentially inhibits signal transduction from one neuron to the next. This is how CCCs help to regulate $GABA_A$ receptor mediated inhibition.[81] An excellent figure depicting these interactions can be seen in **figure 3**.[81]

The NMDA receptor is essentially the opposite of the $GABA_A$ receptor, which is an anion channel. The NMDA receptor is a cation channel that controls the flow of sodium and calcium ions into the cell.[82–84] The main ligands for the NMDA receptor are glycine and glutamate. [82–84] Upon binding to glycine and glutamate, the NMDA receptor becomes depolarized resulting in signal propagation.[82–84] The binding of glycine and glutamate causes the NMDA receptor to open allowing the influx of positively charged ions including sodium but especially calcium.[82–84] This high permeability to calcium makes the NMDA receptor rather unique.[82–84]

Another novel feature of the NMDA receptor is that it is blocked by magnesium ions preventing depolarization.[82–84] When NMDA receptors are stimulated for too long of a period, the high amount of calcium influx can affect cellular viability.[82–84] This does not typically happen when general anesthetics are administered. Instead, general anesthetics that are NMDA antagonists, such as isoflurane and nitrous oxide, result in an increase in NMDA receptor expression.[83] The increased density of the NMDA receptor results in greater stimulation upon neurotransmitter release from the pre-synaptic neuron.[83] This can result in an influx of calcium that is virtually identical to excessive stimulation and can ultimately induce apoptosis.[85]

The AMPA receptor (like the GABA and NMDA receptors) is an ionotropic receptor, meaning it forms an ion pore within the membrane.[83] The AMPA receptor allows the influx of sodium, potassium, and in some instances calcium.[83] The AMPA receptor is similar to the NMDA receptor in that it is an excitatory receptor and is one of the most abundant types of receptors in the CNS.[83] The AMPA and NMDA receptor both have a tetrameric structure that is depicted in **figure 4**.[85] The exact roles of AMPA receptors have not been completely elucidated.[83] However, it is known that AMPA receptors are extremely fast excitatory receptors that serve a similar, although quicker, role as NMDA receptors in regards to signal propagation.[83]

The $GABA_A$ receptor is a heteropentameric receptor that spans the membrane four times while the $GABA_B$ receptor is a heterodimeric receptor that spans the membrane seven times.[73] The $GABA_A$ receptor is an ionotropic receptor, meaning it permits ion influx when bound to its ligand, specifically chloride or bicarbonate ions.[73] The $GABA_B$ receptor is a G-protein coupled

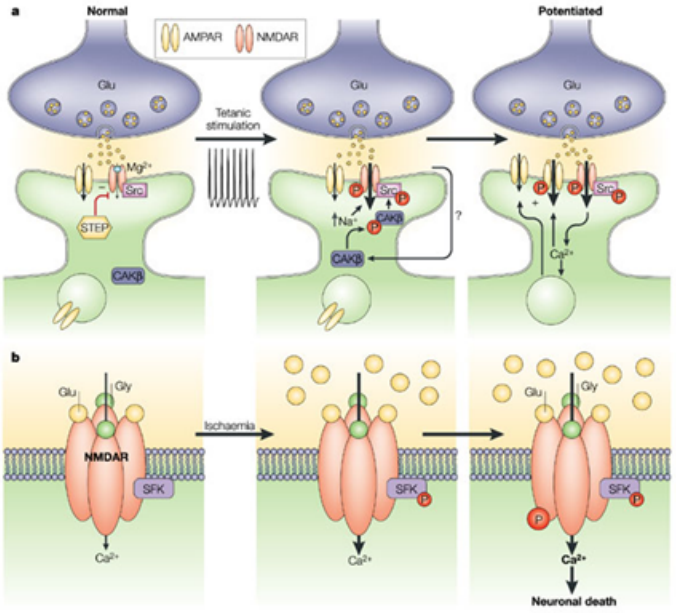

Figure 4: The Effects of Normal and Excessive Activation of the NMDA Receptor. The levels of calcium influx resulting from normal and excessive activation of the NMDA receptor (b). The binding of glutamate and glycine for excessive periods of time can induce calcium-mediated neurotoxicity. The theorized tetrameric structure of the NMDA receptor embedded within the membrane is also accurately depicted in part b. The far left image of part a shows the NMDA under normal conditions. The middle and right images show the NMDA receptor under rapid (tetanic) and excessive stimulation respectively. Rapid and excessive stimulation results in the removal of magnesium ions that block the pore of the NMDA receptor. Lastly, excessive stimulation of the NMDA receptor results in large calcium influx that promotes cell death through the activation of apoptotic cascades. The AMPA receptor has a very similar structure to the NMDA receptor.[85]

receptor.[73] When a ligand binds to the $GABA_B$ receptor it activates a G-protein that goes on to turn on an effector.[73] An excellent figure showing the differences between the $GABA_A$ and $GABA_B$ receptor can be seen in **figure 5**.[73]

As mentioned above, the $GABA_A$ receptor is made up of five protein subunits to form an ion channel that is selectively permeable to chloride and bicarbonate ions.[73] Each subunit of the receptor contains four hydrophobic alpha-helices that span the membrane.[73] The C and N termini are located on the extracellular surface of the cell membrane.[73] However, there is a large intracellular loop that contains phosphorylation sites that are important in regulating the receptor.[73]

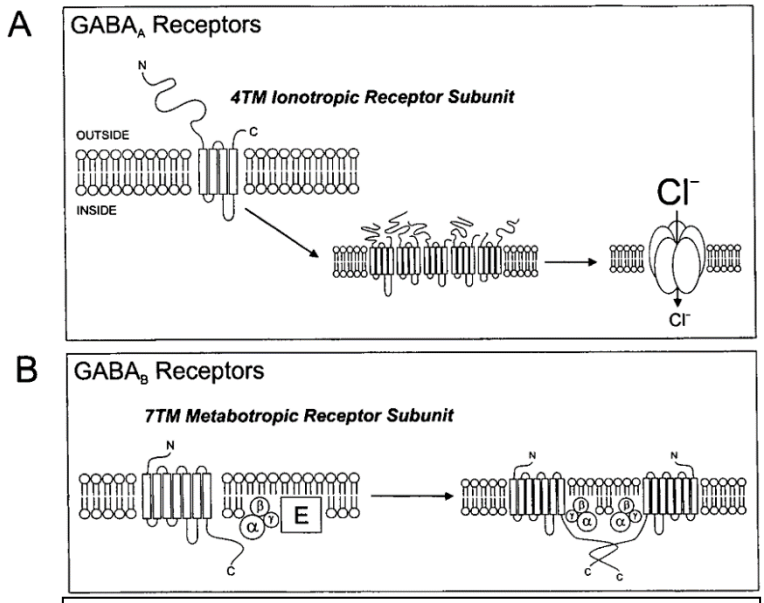

Figure 5: The Structure of the $GABA_A$ and $GABA_B$ receptor. This figure depicts the $GABA_A$ and $GABA_B$ receptor embedded in the plasma membrane along with their relative structures and modes of activation (voltage change in part a and secondary messenger in part b).[73]

Discussion

Commonly used general anesthetics in pediatric practice all affect the $GABA_A$ to some extent. Therefore, it is the arguably the most important receptor to discuss when evaluating how general anesthetics may affect neurodevelopment. However, the importance of the AMPA and NMDA receptors in neurodevelopment is significant as well. Additionally, the AMPA and NMDA receptors are

Table 1
Effects of General Anesthetics on Ligand-gated Ion Channels.

a. Volatile anesthetics	**$GABA_A$**	**AMPA**	**NMDA**
Isoflurane	+++	---	-/0
Nitrous oxide	+++	-/0	---
Sevoflurane	+++	unknown	unknown
b. Intravenous anesthetics	**$GABA_A$**	**AMPA**	**NMDA**
Ketamine	+/0	0	---
Propofol	+++	-/0	-/0

Table 1: +++ represents activation and --- represents inhibition of the receptor at clinically relevant concentrations. +/0 represents a small activation while -/0 represents a small inhibition of the receptor at clinically relevant concentrations. 0 represents no effect at any concentration.[83]

affected by activation of the $GABA_A$ receptors especially during early development. Due to the complex interactions of these receptors, it is important to understand how general anesthetics affect all these receptors to fully elucidate the negative effects of general anesthetics on neurodevelopment. Evidence from animal and *in vitro* studies suggest that general anesthetics affect neural network formation causing potentially life-long negative effects on learning and memory. This is most likely due to the widespread inhibition caused by general anesthetics resulting in increased apoptosis.

A table displaying the current scientific consensus, which is supported by a multitude of studies, on how five commonly used general anesthetics in pediatric care act on important and widely distributed receptors in the CNS can be seen in **table 1**.[83] The AMPA, NMDA, and $GABA_A$ receptors are all affected by general anesthetics although every general anesthetic does not affect each receptor. Isoflurane inhibits AMPA receptors to the greatest extent followed by nitrous oxide and propofol. Isoflurane, nitrous oxide, propofol, and sevoflurane activate $GABA_A$ receptors to the greatest extent while ketamine only slightly activates. Nitrous oxide and ketamine inhibit NMDA receptors to the greatest extent while isoflurane and propofol only slightly inhibit.

Chapter 3: Proposing a Clinical Trial in Infants

Introduction

In 2007, the FDA investigated the long-term neurological effects of general anesthesia on infants due to concerns over neurotoxicity.[26] They concluded that clinical studies should be conducted expediently to determine conclusively which general anesthetics should and should not be administered to infants [26]. Infants are especially vulnerable to neurotoxins due to the extensive amount of neurodevelopment taking place during the first years of life. One critical period of neurological development, known as synaptogenesis, is severely hindered by general anesthetics. The human brain undergoes synaptogenesis from approximately the second trimester of pregnancy to three years after birth.[21,25,28] Experiments on infant animals have shown that general anesthetics negatively influence neurological development.[9,10,28] There is a desperate need for more information about general anesthetic safety considering the vulnerability of infants and their frequency of general anesthesia exposure due to non-elective surgery.

The developing brain must be properly stimulated in order for neurological development to take place. Without proper stimulation, neurons within neural networks will undergo apoptosis in an effort to remove unneeded or unused connections.[28] General anesthetics alter the stimulation of two vital receptors, NMDA and GABA.[2,4–8] NMDA and GABA receptors are crucial in the formation of neural networks, stimulating neuronal migration, establishing firing patterns necessary for coherent thought processes.[2,4–8,39,40] Early formation of neural networks and neuronal migration have long-term effects on memory and learning.[2,4–8] Additionally, while it is not clear why some general anesthetics are less detrimental to neurological development, our study aims to elucidate more information on the relative safety of general anesthetics. Animal studies have demonstrated that early exposure to general anesthesia during neurodevelopment has more pronounced negative effects.[14,17,25]

How General Anesthetics Induces Apoptosis

A strong signal for a cell to undergo apoptosis is caspase-3 activation.[86–88] Caspase-3 is activated through the actions of cytochrome c.[86–88] Cytochrome c is released from mitochondria due to an increase in permeability of the mitochondrial membrane.[86–88] One signal that increases mitochondrial membrane permeability is large influxes of calcium into mitochondria.[86–88] Several *in vitro* and *in vivo* studies have shown that exposure to general anesthetics induce neuroapoptosis through caspase-3 activation.[89–95] Two *in vitro* studies on mice and human neurons showed that exposure to clinically relevant levels of isoflurane increased levels of cytochrome c.[89,96]

There are several proposed mechanisms that could explain why isoflurane causes the release of cytochrome c leading to caspase-3 activation. Some studies have indicated that isoflurane can directly interact with the mitochondrial permeability transition pore (mPTP) causing it to open.[97,98] mPTP opening allows cytochrome c to be released subsequently inducing neuroapoptosis.[86–88] Magnesium and propofol are both mPTP blockers.[97,98] Two studies showed that magnesium and propofol are effective at blocking isoflurane-induced mPTP opening, reducing caspase-3 activation, and inhibiting neuroapoptosis *in vitro* in human neurons and *in vivo* in mice.[97,98] This is one example of how a GABA agonist can increase calcium concentrations inside the cell induce neuroapoptosis. The NMDA antagonist ketamine also affects mitochondrial membrane permeability causing cytochrome c to be released.[99] This has

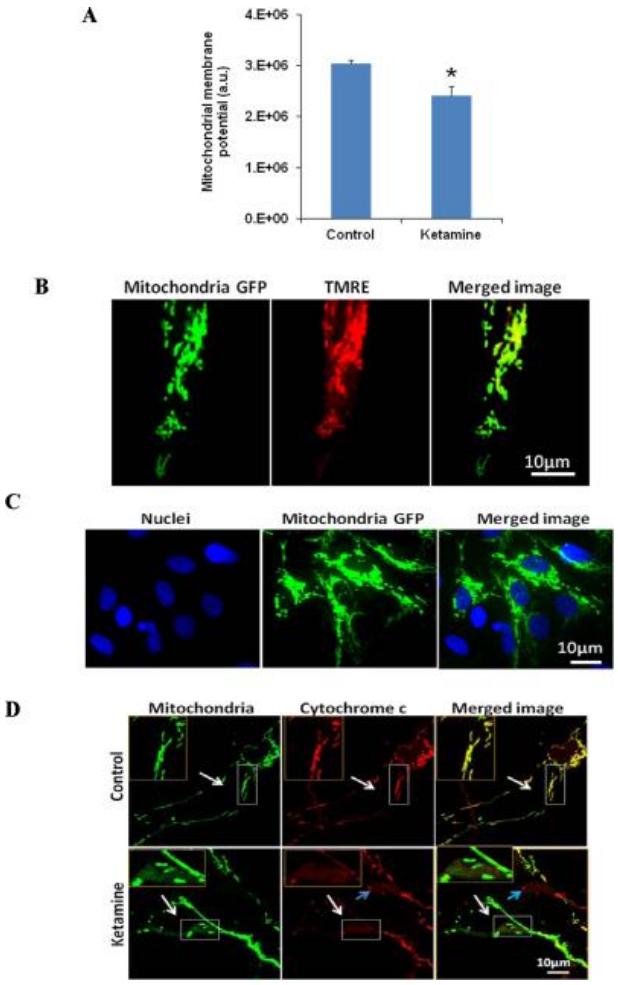

Figure 6: Results of Neuron Exposure to Ketamine. A. Neurons exposed to ketamine had increased mitochondrial membrane permeability. B. Not relevant to the topic being discussed. C. DAPI staining was used to visualize nuclear DNA (shown in blue) while mitochondria were tagged with a green fluorescence protein (shown in green). D. Cytochrome C localization was analyzed using an immunofluorescence staining (shown in red). The merged images clearly depict that ketamine treatment results in a cytochrome C exiting the mitochondria (where they are present in normal cells) and instead localizing in the cytosol where they can induce apoptosis.[99]

led many experts to believe that many general anesthetics induce apoptosis in this fashion.[99] The results of this experiment are shown and explained in **figure 6**.[99] Isoflurane has other potential mechanisms by which it can induce apoptosis such as altering calcium.

Calcium plays a major role in cell signaling and the inositol 1, 4, 5-triphosphate (IP3) receptor is a major route for mobilizing calcium stores in almost all cell types.[100] The IP3 receptor is located on the endoplasmic reticulum (ER) that regulates calcium release.[100] Due to the close proximity of IP3 receptors and mitochondria, over-activation of the IP3 receptor causes calcium depletion in the ER and calcium uptake in mitochondria.[100,101] Therefore, over-activation of the IP3 receptor can induce caspase-3 activation and cellular apoptosis by increasing mitochondrial membrane permeability.[86,87,100,101] *In vitro* studies have shown that isoflurane increases intracellular calcium levels in rat neurons.[102,103] One study on rat neurons showed that isoflurane at clinically relevant concentrations interacts with the IP3 receptor.[103] This interaction causes calcium dysregulation that induces neuroapoptosis.[103] Moreover, isoflurane did not induce apoptosis in neurons that lacked IP3 receptors at any concentration or length of exposure.[103] Two *in vitro* studies have shown that compounds that block IP3 receptor activity inhibit isoflurane-induced apoptosis.[103,104] Isoflurane also induces caspase-3 activation which is capable of triggering additional calcium dysregulation.[89–95] Caspase-3 activation can cleave IP3 receptors causing permanent calcium leakage from the ER.[104] **Figure 7** shows the different pathways that can lead to an imbalance of calcium and induce cellular apoptosis.[51]

Inhibition of the NMDA receptor causes neurons containing this receptor to compensate by increasing NMDA receptor expression.[27] Upon relief of inhibition, these neurons typically have too much calcium influx due to activation of an excessive amount

of NMDA receptors.

This can lead to apoptosis through cytochrome c release from the mitochondria. This appears to be the main mechanism by which general anesthetics that are NMDA antagonists induce apoptosis.[27] However, as demonstrated by isoflurane, there may be other unknown mechanisms by which NMDA receptor antagonists or GABA receptor agonists that contribute to general anesthetic neurotoxicity. For example, ketamine is a NMDA antagonist that also causes cytochrome c release.

Animal Models and Clinical Trials

There is mounting evidence that general anesthetics used in pediatric care are damaging to the developing brain. General anesthetics act through NMDA antagonism and/or GABA stimulation have been show to induce apoptosis in the developing rat brain.[105] Studies on infant mice and rats have shown that NMDA blockade or GABA over-activation for hours at a time increases the rate of neuroapoptosis in the developing brain.[9–16] Several anesthetics have been shown to induce apoptosis in a dose dependent manner including: propofol, isoflurane, and ketamine.[9–16,27,106] Normally, the brain undergoes periods of intense neuroapoptosis during development in order to remove extraneous connections and strengthen neural circuits. [28] The increase in neuroapoptosis during development can lead to life-long learning disabilities.[28] This is most likely due to the loss of connections that are necessary for the proper functioning of important neuronal circuits. Dendritic spines are also drastically altered in structure upon exposure to general anesthetics.[46,47] Dendritic spines are necessary for proper synapse formation and function.[46,47] The loss of dendritic spines can lead to problems with learning and memory dud to gaps in neural networks. [46,47]

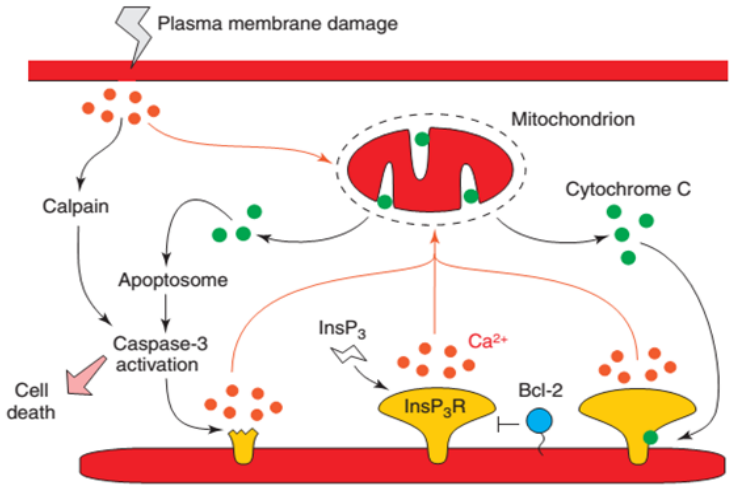

Figure 7: Calcium Dysregulation and Apoptosis. The Bcl-2 protein controls calcium homeostasis by interacting with the IP3 receptor. Excessive release of calcium from ER through the IP3 receptor increases mitochondrial membrane permeability. This allows cytochrome c to be released and activate caspase-3. Caspase-3 induces apoptosis and cleaves the IP3 receptor leading to permanent calcium leak from the ER.[51]

A group of researchers looked at how isoflurane affected dendritic spines. They found that exposure to isoflurane caused a reduction in dendritic spines.[47] However, the addition of the proteases plasmin and tissue plasminogen activator (tPA) were both shown to reduce the isoflurane-induced reduction of dendritic spines.[47] tPA is the protease that converts plasminogen to plasmin.[47] Plasmin and tPA, through their effects on plasmin levels, affect the concentration of a ligand called pro-brain derived neurotrophic factor (pro-BDNF).[47] Pro-BDNF is converted to mature BDNF by plasmin.[47] Pro-BDNF is capable of binding to a receptor that induces apoptosis, the p75NTR receptor.[47] Isoflurane reduces the levels of tPA released by decreasing

neuronal activity through over-activating GABA receptors. tPA is released in response to neuronal activity.[47] Studies using animal models have shown that BDNF is important for promoting neuronal growth, survival, and differentiation.[107] This is one potential mechanism by which isoflurane may promote neuroapoptosis. A figure depicting the results of the study can be seen in **figure 8**.[47]

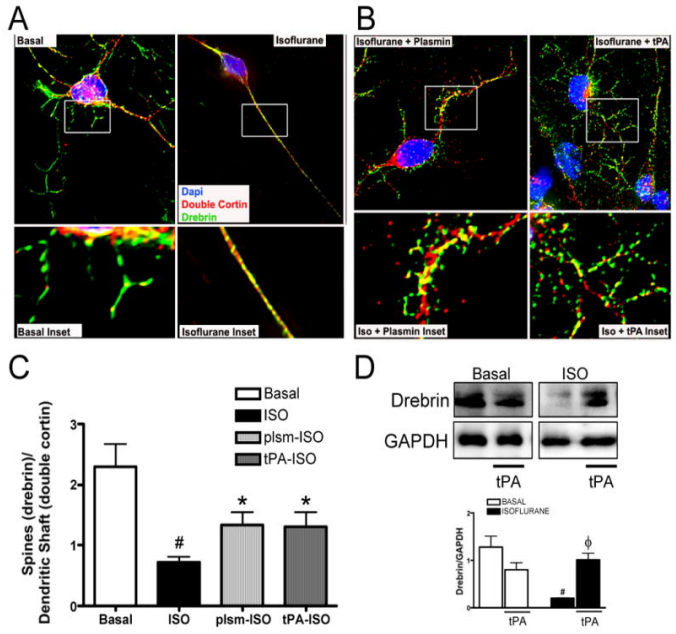

Figure 8: Dendritic Spine Density. Slides of neonatal primary neurons *in vitro* using drebrin immunofluorescence microscopy (part A and B). The primary neurons were exposed to isoflurane for 4 hours at a concentration of 1.4% (clinically relevant) and subsequently stained 2 hours later for debrin and doublecortin. Isoflurane exposure reduced levels of drebrin (shown in green) expression in the primary neurons. Yet, doublecortin (shown in red) expression levels were not affected. Plasmin and tPA were administered to the neurons and significantly reduced the loss of dendritic spines induced by isoflurane. The neurons were also stained with DAPI (shown in blue) to highlight nuclear DNA. Areas of overlap appear as combinations of the colors mentioned. The quantification of the data can be seen in part C.[47]

There is evidence to suggest that a combination of general anesthetics, particularly those that act primarily on different receptors, are more detrimental to neurodevelopment.[10,16,17,105] A study looked at the effects of clinically relevant exposure of rats to midazolam, nitrous oxide, and isoflurane for six hours.[105] Nitrous oxide acts as an NMDA antagonist, causing inhibition. However, isoflurane acts as a GABA agonist, causing inhibition. Nitrous oxide acts as a GABA agonist as well (albeit to a slightly lesser degree). Wide-spread apoptosis in the developing brain of these rats suggests that exposure to a combination of general anesthetics that act primarily on different receptors is more detrimental than exposure to a single anesthetic agent.[105] This is an agreement with studies in other animal models. Furthermore, this study showed that the infant rats that were exposed had spatial learning and memory deficits as adolescents and adults compared to controls.[105] Besides a negative combinatorial effect, there is evidence that multiple exposures to general anesthetics is much more detrimental than a single exposure. One study showed that apoptosis in neurons was elicited in infant rats after repeated exposure to ketamine over a nine hour period.[106] This study is just one of many studies that demonstrates that repeated exposure to general anesthetics is more detrimental than a single exposure.[12,43,106]

Several studies using infant rodents and primates have revealed that the greatest damage to neurodevelopment induced by general anesthetics is during periods of peak synaptogenesis.[9–16,108] The infant brain undergoes periods of synaptogenesis from the second trimester of pregnancy to around three years of

age.[21,25,28–30] Virtually all of the brain undergoes increased neuronal growth throughout the period of synaptogenesis with the growth of various areas, such as the temporal and frontal lobe, peaking at different points.[27] This could contribute to the difficulty in evaluating the detrimental of effects of general anesthesia in a clinical setting on infants and should be taken into consideration.

One of the main concerns many experts have with the current research done in animal models is clinical translation.[37] The timing of development and exact clinically relevant concentrations of general anesthetics are difficult to standardize and extrapolate when working between species.[37] However, this concern does not change the fact that the most current research at a minimum warrants that clinical trials in infants be conducted. It is time for an answer concerning whether general anesthetics are safe for the infant brain.

There are several studies that have tracked and directly assessed infant development following surgical repair. However, none of these studies recorded the concentration or duration of general anesthesia exposure. Additionally, many of the studies did not address confounding factors, making their results irrelevant to the current topic.[107] Yet, there are studies that have utilized the digitization of medical records to track the development of large cohorts of children exposed to general anesthesia in retrospective and epidemiological studies.

Many retrospective studies on human infants show a correlation between exposure to general anesthesia and an increased risk of neurodevelopment disorders especially in learning and memory.[18–25] Learning and memory deficits are consistent with data from animal models.[105] Animal studies on the effects of general anesthetics on neurodevelopment have shown that the hippocampus is the area of the brain that is most severely affected.[27] The hippocampus is one of the most important areas of the brain in regards to learning and memory formation. Also, the NMDA receptor is known to be vital to learning and memory.[32] The agreement of clinical trials, extensive studies using animal models, and *in vitro* experiments is strong evidence that general anesthetics affect learning and memory development in some form or fashion.

Unfortunately, the types of retrospective and epidemiological studies performed thus far are statistically limited due to confounding factors. Unfortunately, a correlation is usually not enough to cause changes in clinical practice. Causation is the gold standard that must be achieved before the scientific community can come to a consensus that eventually leads to changes in clinical practice. Regardless, the results of these studies are still concerning and should be taken very seriously considering their possible implications. For example, one study that evaluated children exposed to general anesthesia before age three found that they had reduced language and cognitive development even after one exposure.[109] Several studies have also found that young children exposed to anesthesia have chronic behavioral irregularities that last up to one month after exposure.[18,25,109–112] This occurs in up to 50% of young children that receive general anesthesia.[18,25,109–112] The most common behavioral abnormalities include irritability, anxiety, and insomnia.[25,112,113] Unsurprisingly, the younger the child is at the time of exposure results in more pronounced behavioral irregularities.[25,112,113]

Interestingly, regional anesthetics are being evaluated for their safety in clinical practice in order to reduce or eliminate the need for general anesthetics during procedures. Regional anesthetics are similar to local anesthetics except that they block pain signals over relatively large portions of the body. While local anesthesia is typically administered to the tissue surrounding the desired site, regional anesthesia is administered to bundles of nerves or even the spinal cord to deliver broad nerve blockade over a relatively large portion of the body. Dexmedetomidine is a regional anesthetic being evaluated due to its minimal side-effects.[114] It

can be used in conjunction with general anesthetics to reduce dosage requirements.[114] Other regional anesthetics are being looked at for similar reasons.[114] One study evaluated the outcomes of over 5,000 births to determine the effects of giving birth under regional or general anesthesia. The study concluded that mother's that received regional anesthesia during childbirth had children with fewer instances of learning disabilities than those who received general anesthesia.[110] However, the process of proving the safety of these regional anesthetics takes time and the results of these studies are far off. Therefore, it is important to evaluate the safety of a variety of drugs used to achieve anesthesia in case the safety of some proves to be questionable and are no longer used in practice. Withholding anesthesia altogether is not an ethical option due to known detrimental effects of severe stress or pain on neurodevelopment.[107] Severe stress or pain drastically increases neuroapoptosis, stress hormones, pain tolerance, and instances of aberrant behavior.[107]

Human Stem Cell Model Design

This trial will employ the use of an *in vitro* human stem cell model made up of neural stem cells (NSCs) to study neurodevelopment. As mentioned above, general anesthetics have been shown to be most detrimental during times of intense synaptogenesis. The developing brain goes through intense synaptogenesis until the age of two.[115] There has been great success in experiments using neural stem cell models to evaluate the effects of various drugs on the developing brain.[53] NSCs can be acquired from infant and adult nervous tissue.[53] The ability to derive these cells from adults makes the acquisition and use of these cells less controversial and more universally accepted. Embryonic mouse fibroblasts will be used as feeder cells to ensure the NSCs remain viable *in vitro*.[53] A transmission electron microscope will be employed to evaluate the level of synaptogenesis and neural network formation throughout the duration of the experiment.[53] The transmission electron microscope will also be used to evaluate the ultrastructure of the NSCs to visualize any abnormalities in organelles or cell structure upon exposure to general anesthetics agents.[53]

The employment of an *in vitro* human stem cell model has several unique advantages. It is an ethical manner to obtain experimental data that has high translation to *in vivo* human neurodevelopment. The use of NSCs ensures that a virtually unlimited supply of cells can be maintained for exhaustive research opportunities. Additionally, the *in vitro* nature of the model allows tight control of experimental parameters, such as concentration of general anesthetic exposure. By avoiding animal sacrifice, NSCs can be evaluated more directly and frequently in a cost-efficient manner. This model has the potential to elucidate the molecular action of individual general anesthetics due to the several advantages mentioned above.

In order to properly set up the neural stem cell model, a chemically defined a media is needed. The protocol described in Bai and Bosnjak will be used to ensure that iPSCs differentiate properly into mature neurons.[113] This process is described in more detail in **figure 9**.[116] The media helps to mimic the *in vivo* conditions of the brain causing neural stem cells to differentiate into several neuronal lineages including neurons, oligodendrocytes, and astrocytes.[113] This model has been effective in producing functional synapses that mimic *in vivo* synapses.[117] This model has proven it to be quite efficient in causing neurons to undergo differentiation. One study achieved 90% differentiation of cells into NSCs using this model.[99]

The NSCs will be exposed to sevoflurane, propofol, ketamine, isoflurane, and nitrous oxide, the most commonly used general anesthetics currently used in infants. The dosage to which NSCs will be exposed to will vary between anesthetic agents. However, all agents will be evaluated at .5, 1, 2, and 4 times the clinically recommended concentration. Experiments will be conducted at a duration of 1, 2, 4, or 8 hours of general anesthetic exposure. The frequency of exposure to each general anesthetic will be 1, 2, or 3 times. Controls will not be exposed to the general anesthetic agent. However, they will be exposed to all the same conditions with only the carrier gas used to deliver the individual anesthetic agents.

A variety of assays will be used in conjunction with the transmission electron microscope to obtain a better idea of how general anesthetics may cause neurotoxicity. Cellular proliferation will be evaluated using bromodeoxyuridine (a thymidine analog that gets incorporated into newly formed DNA) and Ki67 (a non-histone, nuclear protein that is not expressed in dormant cells).[53] Cell viability will be measured using an LDH and MTT assay. A 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye is used to measure mitochondrial function and cell viability.[118] The MTT dye is degraded by mitochondrial dehydrogenase activity, producing a colored

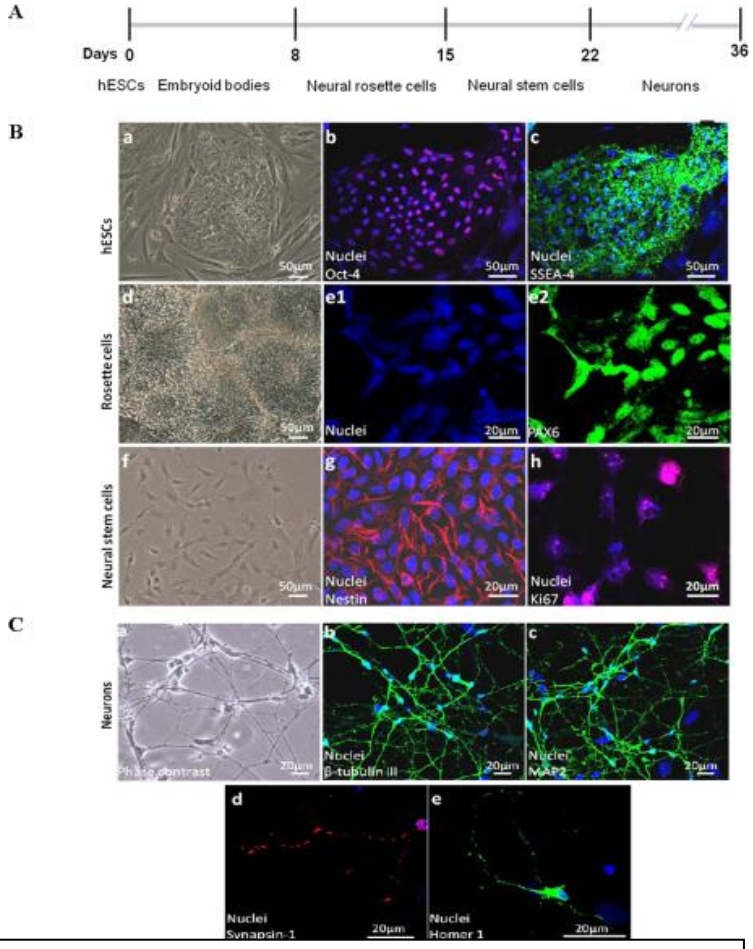

Figure 9: Neural Stem Cell Model. This figure depicts how human embryonic stem cells (hESCs) can be induced to differentiate into various types of neurons in a four step process that is translatable to iPSCs. The fours step process causes hESCs to differentiate into embryoid body formations, neural rosette cells, neural stem cells, and various types of neurons in that order (part A). Stem cell markers are shown in pink and green in part b and c to indicate differentiation is starting to occur. In parts d and e, nuclear DNA is shown in blue and a neuroepithelial marker is shown in green (only part e), indicating the hESCs had developed into embryoid bodies. In part g, a biomarker specific to neural stem cells is shown in red, indicating the embryoid bodies have differentiated into neural stem cells. In part C, the cells have fully differentiated into neurons and are expressing two biomarkers specific to neurons, β-tubulin III shown in green (part b) and MAP2 which is also shown in green (part c). Additionally, the neurons expressed two biomarkers specific to synapses, synapsin-1 shown in red (part d) and Homer 1 shown in green (part e).[116]

product.[118] This concentration of this colored product will then be quantified using a spectrophotometer. Lactate dehydrogenase (LDH) is an enzyme that can pass through the cellular membrane if it is damaged.[118] The concentration of LDH in culture will be quantified using an LDH assay to determine the amount of cells that are damaged or nonviable.[118] Real-time PCR will also be employed to elucidate which genes are over- or under-expressed upon general anesthetic exposure. RNA will be extracted from experimental and control cells. This RNA will be reverse transcribed and amplified using PCR. The resulting cDNA will be run through a database and quantified to determine levels of gene expression.[118]

It is believed that the primary mechanism of neurotoxicity by general anesthetics is increased apoptosis.[118] DNA fragmentation can be visualized using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end labeling (TUNEL) staining. DNA fragmentation is indicative of cellular apoptosis.[118] The level of DNA fragmentation will be recorded using a confocal microscopy to quantify the amount of cells going through apoptosis.[118] A caspase-3 colorimetric assay will also be used to quantify the number of cells going through apoptosis.[118]

This model has been used to effectively evaluate the neurotoxic effects of ketamine and isoflurane individually. Both studies found that prolonged exposure to either general anesthetic increased neuroglia cell proliferation and neuronal apoptosis.[99,116] Isoflurane and ketamine both caused apoptosis by increasing cytochrome c and reactive oxygen species levels.[99,116] While the concentration and duration of general anesthetic exposure were outside of clinical relevance, these studies do show that a neural stem cell model is an ideal method for evaluating how general anesthetics influence cellular processes inducing apoptosis *in vitro*.

Clinical Trial Design

Participants will be selected from a pool of infants that are two years of age or younger that are requiring surgery for issues unrelated to neurodevelopment. The age was decided to correlate with the critical neurodevelopmental period of synaptogenesis. Synaptogenesis occurs in the human brain from the time of birth to approximately three years of age.[37] During surgery, the general anesthetic(s) used will be recorded along with the concentration and duration of exposure. Other drugs used and any complications during surgery will also be noted. Participants will receive free-of-charge anesthesia upon commitment to assessments of cognitive and behavioral development every other year for eight years. Assessments every other year will allow a greater range of development to be assessed for a more cost-effective approach. The assessments will be based on the outcome of the neural stem cell experiment as well as previous clinical trial conclusions.

The assessments employed will be the Wechsler Abbreviated Scale of Intelligence (WASI) and NEuroPSYchological Assessment, second edition (NEPSY II). WASI will be used to evaluate global cognitive function by determining global IQ and verbal skills.[119] NEPSY II will be used to evaluate specific cognitive functions.[119] A behavioral assessment will be conducted by providing the Child Behavior Checklist ages 2-10 to the parents.[119] The sibling of participants will perform identical assessments with the same professional at the same age as their sibling. Age-specific instruments will be used during the assessments to reduce statistical variability and potential error.[26] This will not be an issue since siblings will perform identical assessments at the same age as their sibling that was exposed to general anesthesia. Sibling-pairing has been shown to be an effective method for reducing confounding factors because inter-sibling differences are minimal in comparison to random selection.[26] We hypothesize that

depending on the age of exposure different areas of the brain will be affected, producing a slight variation of symptoms. This is based on the fact that synaptogenesis peaks at different times in different areas of the brain.[27] However, we also hypothesize that learning and memory will be affected the most, which would agree with current data from animal model studies.[27]

Discussion

There is a drastic need for more information about the safety of using general anesthetics in infants. Too many infants are being exposed to potentially neurotoxic agents that could have devastating effects on neurodevelopment. Unfortunately, while the evidence suggests that general anesthetics could be neurotoxic, the lack of consensus by the medical community has prevented any changes in clinical practice from occurring.

The critical periods of brain development are important in the synaptogenesis in the formation of neural networks. However, during this critical time period, neuronal stem cell proliferation, neuronal migration, and the formation of axons and dendrites also occurs within this time.[113,120,121] Therefore, general anesthetics have the potential to affect several neurodevelopmental processes.[113,120,121] These neurodevelopmental processes can be stimulated in neural stem cells, providing an ideal model for evaluating general anesthetic neurotoxicity *in vitro*.[113] An additional benefit of using neural stem cells is that they can replicate the phenotype of a patient with an heritable disease.[113] Assuming the *in vitro* neural stem cell model is effective in the trial proposed, it could capture the interest of researchers and lead the utilization of stem cells in future studies. This stem cell model would be a useful method for carefully and repeatedly evaluating how genetic factors influence pathology. One of the only downsides of this technology is that the results of studies cannot be directly translated to a true *in vivo* system. However, this is a fault that is necessary due to the nature of *in vitro* studies. The current study would greatly benefit if stem cell technologies were capable of culturing many types of neurons to gather to more accurately mimic the conditions of an *in vivo* brain. As new technologies emerge and interest grows, future studies could utilize this type of stem cell model to produce more conclusive and translatable data.

This study is unique and provides valuable contributions to the field in several ways. First, this will be one of a few studies to evaluate the neurotoxicity of general anesthetics using the *in vitro* neural stem cell model. Second, this is the first clinical trial to evaluate general in anesthetics *in vivo* and *in vitro* simultaneously. Third, this is currently one of the first studies directly assessing neurodevelopmental outcomes of children exposed to general anesthetics as infants using sibling-paired controls. Lastly, no other study has evaluated the number of general anesthetics we will be evaluating using the *in vitro* neural stem cell model.

Bibliography

1. Chen H-SV, Lipton SA. The chemical biology of clinically tolerated NMDA receptor antagonists. *Journal of Neurochemistry*. 2006;97(6):1611-1626. doi:10.1111/j.1471-4159.2006.03991.x.

2. Fredriksson A, Pontén E, Gordh T, Eriksson P. Neonatal Exposure to a Combination of N-Methyl-d-aspartate and γ-Aminobutyric Acid Type A Receptor Anesthetic Agents Potentiates Apoptotic Neurodegeneration and Persistent Behavioral Deficits. *Anesthesiology*. 2007;107(3):427-436.

3. Reich DL, Silvay G. Ketamine: an update on the first twenty-five years of clinical experience. *Can J Anaesth*. 1989;36(2):186-197. doi:10.1007/BF03011442.

4. Owens DF, Boyce LH, Davis MBE, Kriegstein AR. Excitatory GABA Responses in Embryonic and Neonatal Cortical Slices Demonstrated by Gramicidin Perforated-Patch Recordings and Calcium Imaging. *J Neurosci*. 1996;16(20):6414-6423.

5. Owens DF, Liu X, Kriegstein AR. Changing Properties of GABAA Receptor–Mediated Signaling During Early Neocortical Development. *Journal of Neurophysiology*. 1999;82(2):570-583.

6. D'Souza SW, McConnell SE, Slater P, Barson AJ. Glycine site of the excitatory amino acid N-methyl-D-aspartate receptor in neonatal and adult brain. *Arch Dis Child*. 1993;69(2):212-215.

7. Wang DD, Kriegstein AR. GABA Regulates Excitatory Synapse Formation in the Neocortex via NMDA Receptor Activation. *J Neurosci*. 2008;28(21):5547-5558. doi:10.1523/JNEUROSCI.5599-07.2008.

8. Cserep C, Szabadits E, Szonyi A, Watanabe M, Freund TF, Nyiri G. NMDA Receptors in GABAergic Synapses during Postnatal Development. *PLoS One*. 2012;7(5). doi:10.1371/journal.pone.0037753.

9. Palanisamy A, Baxter MG, Keel PK, Xie Z, Crosby G, Culley DJ. Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults. *Anesthesiology*. 2011;114(3):521-528. doi:10.1097/ALN.0b013e318209aa71.

10. Lunardi N, Ori C, Erisir A, Jevtovic-Todorovic V. General Anesthesia Causes Long-Lasting Disturbances in the Ultrastructural Properties of Developing Synapses in Young Rats. *Neurotoxicity Research*. 2010;17(2):179-188. doi:10.1007/s12640-009-9088-z.

11. Bercker S, Bert B, Bittigau P, et al. Neurodegeneration in Newborn Rats Following Propofol and Sevoflurane Anesthesia. *Neurotoxicity Research*. 2009;16(2):140-147. doi:10.1007/s12640-009-9063-8.

12. Murphy KL, Baxter MG. Long-Term Effects of Neonatal Single or Multiple Isoflurane Exposures on Spatial Memory in Rats. *Front Neurol*. 2013;4. doi:10.3389/fneur.2013.00087.

13. Loepke AW, McCann JC, Kurth CD, McAuliffe JJ. The Physiologic Effects of Isoflurane Anesthesia in Neonatal Mice: *Anesthesia & Analgesia*. 2006;102(1):75-80. doi:10.1213/01.ANE.0000181102.92729.B8.

14. Slikker W, Zou X, Hotchkiss CE, et al. Ketamine-Induced Neuronal Cell Death in the Perinatal Rhesus Monkey. *Toxicol Sci*. 2007;98(1):145-158. doi:10.1093/toxsci/kfm084.

15. Brambrink AM, Evers AS, Avidan MS, et al. Isoflurane-induced Neuroapoptosis in the Neonatal Rhesus Macaque Brain: *Anesthesiology*. 2010;112(4):834-841. doi:10.1097/ALN.0b013e3181d049cd.

16. Rizzi S, Carter LB, Ori C, Jevtovic-Todorovic V. Clinical Anesthesia Causes Permanent Damage to the Fetal Guinea Pig Brain. *Brain Pathol*. 2008;18(2). doi:10.1111/j.1750-3639.2007.00116.x.

17. Nikizad H, Yon J-H, Carter LB, Jevtovic-Todorovic V. Early Exposure to General Anesthesia Causes Significant Neuronal Deletion in the Developing Rat Brain. *Annals of the New York Academy of Sciences*. 2007;1122:69-82. doi:10.1196/annals.1403.005.

18. DiMaggio C, Sun LS, Kakavouli A, Byrne MW, Li G. A retrospective cohort study of the association of anesthesia and hernia repair surgery with behavioral and developmental disorders in young children. *J Neurosurg Anesthesiol*. 2009;21(4):286-291. doi:10.1097/ANA.0b013e3181a71f11.

19. Garcia Guerra G, Robertson CMT, Alton GY, et al. Neurodevelopmental outcome following exposure to sedative and analgesic drugs for complex cardiac surgery in infancy. *Pediatric Anesthesia*. 2011;21(9):932-941. doi:10.1111/j.1460-9592.2011.03581.x.

20. Hintz SR, Kendrick DE, Stoll BJ, et al. Neurodevelopmental and Growth Outcomes of Extremely Low Birth Weight Infants After Necrotizing Enterocolitis. *Pediatrics*. 2005;115(3):696-703. doi:10.1542/peds.2004-0569.

21. Kabra NS, Schmidt B, Roberts RS, et al. Neurosensory Impairment after Surgical Closure of Patent Ductus Arteriosus in Extremely Low Birth Weight Infants: Results from the Trial of Indomethacin Prophylaxis in Preterms. *Journal of Pediatrics*. 2007;150(3):229-234.e1. doi:10.1016/j.jpeds.2006.11.039.

22. Laing S, Walker K, Ungerer J, Badawi N, Spence K. Early development of children with major birth defects requiring newborn surgery. *Journal of Paediatrics & Child Health*. 2011;47(3):140-147. doi:10.1111/j.1440-1754.2010.01902.x.

23. Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. *Arch Dis Child Fetal Neonatal Ed*. 2007;92(3):F193-F198. doi:10.1136/adc.2006.099929.

24. Schulzke SM, Deshpande GC, Patole SK. Neurodevelopmental outcomes of very low-birth-weight infants with necrotizing enterocolitis: A systematic review of observational studies. *Arch Pediatr Adolesc Med*. 2007;161(6):583-590. doi:10.1001/archpedi.161.6.583.

25. 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. doi:10.1097/01.anes.0000344728.34332.5d.

26. Sun LS, Li G, DiMaggio C, et al. Anesthesia and Neurodevelopment in Children: Time for an Answer? *Anesthesiology*. 2008;109(5). http://journals.lww.com/anesthesiology/Fulltext/2008/11000/Anesthesia_and_Neurodevelopment_in_Children__Time.2.aspx.

27. SPECIAL ARTICLE – Long term neurotoxicity by general anesthetics in infants | Anaesthesia, Pain & Intensive Care. http://www.apicareonline.com/?p=2350. Accessed February 24, 2015.

28. Rice D, Barone S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. 2000;108(Suppl 3):511-533.

29. Korkman M, Kemp SL, Kirk U. Effects of Age on Neurocognitive Measures of Children Ages 5 to 12: A Cross-Sectional Study on 800 Children From the United States. *Developmental Neuropsychology*. 2001;20(1):331-354.

30. Sowell ER, Peterson BS, Thompson PM, Welcome SE, Henkenius AL, Toga AW. Mapping cortical change across the human life span. *Nature Neuroscience*. 2003;6(3):309.

31. Garcia PS, Kolesky SE, Jenkins A. General Anesthetic Actions on GABAA Receptors. *Curr Neuropharmacol*. 2010;8(1):2-9. doi:10.2174/157015910790909502.

32. Li F, Tsien JZ. Memory and the NMDA Receptors. *N Engl J Med*. 2009;361(3):302-303. doi:10.1056/NEJMcibr0902052.

33. Young C, Klocke BJ, Tenkova T, et al. Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. *Cell Death Differ*. 2003;10(10):1148-1155. doi:10.1038/sj.cdd.4401277.

34. Ikonomidou C, Bittigau P, Ishimaru MJ, et al. Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome. *Science*. 2000;287(5455):1056-1060. doi:10.1126/science.287.5455.1056.

35. Young C, Roth KA, Klocke BJ, et al. Role of caspase-3 in ethanol-induced developmental neurodegeneration. *Neurobiology of Disease*. 2005;20(2):608-614. doi:10.1016/j.nbd.2005.04.014.

36. Plotkin MD, Snyder EY, Hebert SC, Delpire E. Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: A possible mechanism underlying

GABA's excitatory role in immature brain. *Journal of Neurobiology*. 1997;33(6):781-795. doi:10.1002/(SICI)1097-4695(19971120)33:6<781::AID-NEU6>3.0.CO;2-5.

37. Huttenlocher PR, Dabholkar AS. Regional differences in synaptogenesis in human cerebral cortex. *J Comp Neurol*. 1997;387(2):167-178. doi:10.1002/(SICI)1096-9861(19971020)387:2<167::AID-CNE1>3.0.CO;2-Z.

38. Kim WR, Sun W. Programmed cell death during postnatal development of the rodent nervous system. *Development, Growth & Differentiation*. 2011;53(2):225-235. doi:10.1111/j.1440-169X.2010.01226.x.

39. Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. *Science*. 1993;260(5104):95.

40. Voigt T, Opitz T, Lima AD de. Activation of Early Silent Synapses by Spontaneous Synchronous Network Activity Limits the Range of Neocortical Connections. *J Neurosci*. 2005;25(18):4605-4615. doi:10.1523/JNEUROSCI.3803-04.2005.

41. Watt AJ, Cuntz H, Mori M, Nusser Z, Sjostrom PJ, Hausser M. Traveling waves in developing cerebellar cortex mediated by asymmetrical Purkinje cell connectivity. *Nat Neurosci*. 2009;12(4):463-473. doi:10.1038/nn.2285.

42. Ikonomidou C, Bosch F, Miksa M, et al. Blockade of NMDA Receptors and Apoptotic Neurodegeneration in the Developing Brain. *Science*. 1999;283(5398):70-74. doi:10.1126/science.283.5398.70.

43. Scallet AC, Schmued LC, Slikker W, et al. Developmental Neurotoxicity of Ketamine: Morphometric Confirmation, Exposure Parameters, and Multiple Fluorescent Labeling of Apoptotic Neurons. *Toxicol Sci*. 2004;81(2):364-370. doi:10.1093/toxsci/kfh224.

44. Manent J-B, Jorquera I, Mazzucchelli I, et al. Fetal Exposure to GABA-Acting Antiepileptic Drugs Generates Hippocampal and Cortical Dysplasias. *Epilepsia*. 2007;48(4):684-693. doi:10.1111/j.1528-1167.2007.01056.x.

45. Holmes LB, Harvey EA, Coull BA, et al. The Teratogenicity of Anticonvulsant Drugs. *New England Journal of Medicine*. 2001;344(15):1132-1138. doi:10.1056/NEJM200104123441504.

46. Briner A, De Roo M, Dayer A, Muller D, Habre W, Vutskits L. Volatile Anesthetics Rapidly Increase Dendritic Spine Density in the Rat Medial Prefrontal Cortex during Synaptogenesis. *Anesthesiology*. 2010;112(3). http://journals.lww.com/anesthesiology/Fulltext/2010/03000/Volatile_Anesthetics_Rapidly_Increase_Dendritic.13.aspx.

47. Head BP, Patel HH, Niesman IR, Drummond JC, Roth DM, Patel PM. Inhibition of p75 Neurotrophin Receptor Attenuates Isoflurane-mediated Neuronal Apoptosis in the Neonatal Central Nervous System. *Anesthesiology*. 2009;110(4):813-825. doi:10.1097/ALN.0b013e31819b602b.

48. Sullivan EV, Pfefferbaum A, Rohlfing T, Baker FC, Padilla ML, Colrain IM. Developmental Change in Regional Brain Structure over 7 Months in Early Adolescence: Comparison of Approaches for Longitudinal Atlas-based Parcellation. *Neuroimage*. 2011;57(1):214-224. doi:10.1016/j.neuroimage.2011.04.003.

49. Dwyer R, Fee JPH, Moore J. Uptake of halothane and isoflurane by mother and baby during Caesarean section. *Br J Anaesth*. 1995;74(4):379-383. doi:10.1093/bja/74.4.379.

50. Cattano D, Young C, Straiko MMW, Olney JW. Subanesthetic Doses of Propofol Induce Neuroapoptosis in the Infant Mouse Brain: *Anesthesia & Analgesia*. 2008;106(6):1712-1714. doi:10.1213/ane.0b013e318172ba0a.

51. Young C, Jevtovic-Todorovic V, Qin Y-Q, et al. Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. *Br J Pharmacol*. 2005;146(2):189-197. doi:10.1038/sj.bjp.0706301.

52. Sylvester GC, Khoury MJ, Lu X, Erickson JD. First-trimester anesthesia exposure and the risk of central nervous system defects: a population-based case-control study. *Am J Public Health*. 1994;84(11):1757-1760.

53. Bai X, Twaroski D, Bosnjak ZJ. Modeling anesthetic developmental neurotoxicity using human stem cells. *Semin Cardiothorac Vasc Anesth*. 2013;17(4). doi:10.1177/1089253213495923.

54. Blumer JL. Off-Label Uses of Drugs in Children. *Pediatrics*. 1999;104(Supplement 3):598-602.

55. Dworkin G. Legality of consent to nontherapeutic medical research on infants and young children. *Arch Dis Child*. 1978;53(6):443-446.

56. Rosenheim ML. Medical Ethics: Supervision of the Ethics of Clinical Investigations in Institutions: Report of R.C.P. Committee. *Br Med J*. 1967;3(5562):429-430.

57. World Medical Association Declaration of Helsinki. *Bulletin of the World Health Organization*. 2001;79(4):373.

58. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. *World Medical Journal*. 2013;59(5):199-202.

59. Carlson RV, Boyd KM, Webb DJ. The revision of the Declaration of Helsinki: past, present and future. *Br J Clin Pharmacol*. 2004;57(6):695-713. doi:10.1111/j.1365-2125.2004.02103.x.

60. Conroy S, McIntyre J, Choonara I, HULL PS. Unlicensed and off label drug use in neonates. *Arch Dis Child Fetal Neonatal Ed*. 1999;80(2):F142-F145.

61. Turner S, Longworth A, Nunn AJ, Choonara I. Unlicensed and off label drug use in paediatric wards: prospective study. *BMJ*. 1998;316(7128):343-345.

62. Development of medicines for neonates. *WHO Drug Information*. 2006;20(4):265-265.

63. Amin SB, McDermott MP, Shamoo AE. Clinical Trials of Drugs Used Off-Label in Neonates: Ethical Issues and Alternative Study Designs. *Accountability in Research: Policies & Quality Assurance*. 2008;15(3):168-187. doi:10.1080/08989620802194392.

64. Campbell AGM. Infants, Children, and Informed Consent. *Br Med J*. 1974;3(5926):334-338.

65. Stenson B, Becher J, McIntosh N. Neonatal research: the parental perspective. *Arch Dis Child Fetal Neonatal Ed*. 2004;89(4):F321-F324. doi:10.1136/adc.2002.021931.

66. Burgess E, Singhal N, Amin H, McMillan D, Devrome H, Fenton A. Consent for clinical research in the neonatal intensive care unit: a retrospective survey and a prospective study. *Arch Dis Child Fetal Neonatal Ed*. 2003;88(4):F280-F286. doi:10.1136/fn.88.4.F280.

67. Tobias JS. BMJ's present policy (sometimes approving research in which patients have not given fully informed consent) is wholly correct. *BMJ*. 1997;314(7087):1111-1114.

68. Knellwolf A-L, Bauzon S, Alberighi ODC, et al. Framework conditions facilitating paediatric clinical research. *Ital J Pediatr*. 2011;37:12. doi:10.1186/1824-7288-37-12.

69. Paediatrics RC, Committee CH, HULL PS. Guidelines for the ethical conduct of medical research involving children. *Arch Dis Child*. 2000;82(2):177-182. doi:10.1136/adc.82.2.177.

70. Mulford RD. Experimentation on Human Beings. *Stanford Law Review*. 1967;20(1):99. doi:10.2307/1227417.

71. Ligi I, Boubred F, Grandvuillemin I, Simeoni U. Clinical research in newborn infants: difficulties and specificity. *European Journal of Clinical Pharmacology*. 2011;67:29-32. doi:10.1007/s00228-010-0921-3.

72. Harth SC, Thong YH. Sociodemographic and motivational characteristics of parents who volunteer their children for clinical research: a controlled study. *BMJ*. 1990;300(6736):1372-1375.

73. Sarnat HB, Simeone TA, Donevan SD, Rho JM. Molecular Biology and Ontogeny of γ-Aminobutyric Acid (GABA) Receptors in the Mammalian Central Nervous System. *Journal of Child Neurology*. 2003;18(1):39-48.

74. Rae C, Hare N, Bubb WA, et al. Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. *Journal of Neurochemistry*. 2003;85(2):503-514. doi:10.1046/j.1471-4159.2003.01713.x.

75. Solis Jr E. The Influence of GABA Metabolism on GABA Neurotransmission: The Role of Metabolic Regulatory Points and a Neuronal Glutamate Transporter. 2010.

76. Mathews GC, Diamond JS. Neuronal glutamate uptake Contributes to GABA synthesis and inhibitory synaptic strength. *J Neurosci*. 2003;23(6):2040-2048.

77. Sepkuty JP, Cohen AS, Eccles C, et al. A Neuronal Glutamate Transporter Contributes to Neurotransmitter GABA Synthesis and Epilepsy. *J Neurosci*. 2002;22(15):6372-6379.

78. Engel D, Pahner I, Schulze K, et al. Plasticity of rat central inhibitory synapses through GABA metabolism. *J Physiol*. 2001;535(Pt 2):473-482. doi:10.1111/j.1469-7793.2001.00473.x.

79. Jensen K, Chiu C-S, Sokolova I, Lester HA, Mody I. GABA Transporter-1 (GAT1)-Deficient Mice: Differential Tonic Activation of GABAA Versus GABAB Receptors in the Hippocampus. *Journal of Neurophysiology*. 2003;90(4):2690-2701. doi:10.1152/jn.00240.2003.

80. Tanaka M, DeLorey TM, Delgado-Escueta A, Olsen RW. GABRB3, Epilepsy, and Neurodevelopment. In: Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, eds. *Jasper's Basic Mechanisms of the Epilepsies*. 4th ed. Bethesda (MD): National Center for Biotechnology Information (US); 2012. http://www.ncbi.nlm.nih.gov/books/NBK98178/. Accessed February 13, 2015.

81. Briggs SW, Galanopoulou AS. Altered GABA Signaling in Early Life Epilepsies. *Neural Plasticity*. 2011;2011:1-16. doi:10.1155/2011/527605.

82. Bartlett TE, Wang YT. The intersections of NMDAR-dependent synaptic plasticity and cell survival. *Neuropharmacology*. 2013;74:59-68. doi:10.1016/j.neuropharm.2013.01.012.

83. Krasowski MD, Harrison NL. General anaesthetic actions on ligand-gated ion channels. *Cell Mol Life Sci*. 1999;55(10):1278-1303.

84. Laube B, Hirai H, Sturgess M, Betz H, Kuhse J. Molecular Determinants of Agonist Discrimination by NMDA Receptor Subunits: Analysis of the Glutamate Binding Site on the NR2B Subunit. *Neuron*. 1997;18(3):493-503. doi:10.1016/S0896-6273(00)81249-0.

85. Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci*. 2004;5(4):317-328. doi:10.1038/nrn1368.

86. Berridge MJ, Lipp P, Bootman MD. The Versatility and Universality of Calcium Signalling. *Nature Reviews Molecular Cell Biology*. 2000;1(1):11-21. doi:10.1038/35036035.

87. Cerella C, Diederich M, Ghibelli L. The Dual Role of Calcium as Messenger and Stressor in Cell Damage, Death, and Survival. *Int J Cell Biol*. 2010;2010. doi:10.1155/2010/546163.

88. Danial NN, Korsmeyer SJ. Cell Death: Critical Control Points. *Cell*. 2004;116(2):205-219. doi:10.1016/S0092-8674(04)00046-7.

89. Zhang Y, Pan C, Wu X, et al. Different effects of anesthetic isoflurane on caspase-3 activation and cytosol cytochrome c levels between mice neural progenitor cells and neurons. *Front Cell Neurosci*. 2014;8(14):1-8. doi:10.3389/fncel.2014.00014.

90. Dong Y, Wu X, Xu Z, Zhang Y, Xie Z. Anesthetic Isoflurane Increases Phosphorylated Tau Levels Mediated by Caspase Activation and Aβ Generation. *PLoS One*. 2012;7(6). doi:10.1371/journal.pone.0039386.

91. Zhang G, Dong Y, Zhang B, et al. Isoflurane-Induced Caspase-3 Activation Is Dependent on Cytosolic Calcium and Can Be Attenuated by Memantine. *J Neurosci*. 2008;28(17):4551-4560. doi:10.1523/JNEUROSCI.5694-07.2008.

92. Yan H, Xu T, Zhao H, Lee K-C, Wang H-Y, Zhang Y. Isoflurane Increases Neuronal Cell Death Vulnerability by Downregulating miR-214. *PLoS ONE*. 2013;8(2):1-10. doi:10.1371/journal.pone.0055276.

93. Pan C, Xu Z, Dong Y, et al. The Potential Dual Effects of Anesthetic Isoflurane on Hypoxia-Induced Caspase-3 Activation and Increases in β-Site Amyloid Precursor Protein-Cleaving Enzyme Levels. *Anesth Analg*. 2011;113(1):145-152. doi:10.1213/ANE.0b013e3182185fee.

94. Xie Z, Culley DJ, Dong Y, et al. The common inhalation anesthetic isoflurane induces caspase activation and increases Aβ level in vivo. *Ann Neurol*. 2008;64(6):618-627. doi:10.1002/ana.21548.

95. Xie Z, Dong Y, Maeda U, et al. The Common Inhalation Anesthetic Isoflurane Induces Apoptosis and Increases Amyloid β Protein Levels. *Anesthesiology*. 2006;104(5):988-994.

96. Zhang Y, Dong Y, Wu X, et al. The Mitochondrial Pathway of Anesthetic Isoflurane-induced Apoptosis. *J Biol Chem*. 2010;285(6):4025-4037. doi:10.1074/jbc.M109.065664.

97. Zhang Y, Dong Y, Xu Z, Xie Z. Propofol and magnesium attenuate isoflurane-induced caspase-3 activation via inhibiting mitochondrial permeability transition pore. *Med Gas Res*. 2012;2:20. doi:10.1186/2045-9912-2-20.

98. Zhang Y, Zhen Y, Dong Y, et al. Anesthetic Propofol Attenuates the Isoflurane-Induced Caspase-3 Activation and Aβ Oligomerization. *PLoS One*. 2011;6(11):1-9 (e27019). doi:10.1371/journal.pone.0027019.

99. Bai X, Yan Y, Canfield S, et al. Ketamine Enhances Human Neural Stem Cell Proliferation and Induces Neuronal Apoptosis Via Reactive Oxygen Species-Mediated Mitochondrial Pathway. *Anesth Analg*. 2013;116(4):869-880. doi:10.1213/ANE.0b013e3182860fc9.

100. Hanson JC, Bootman MD, Roderick LH. Cell Signalling: IP3 Receptors Channel Calcium into Cell Death. *Current Biology*. 2004;14(21):R933-R935. doi:10.1016/j.cub.2004.10.019.

101. Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. *Cell Death & Differentiation*. 2006;13(3):385-392. doi:10.1038/sj.cdd.4401778.

102. Kindler CH, Eilers H, Donohoe P, Ozer S, Bickler PE. Volatile Anesthetics Increase Intracellular Calcium in Cerebrocortical and Hippocampal Neurons. *Anesthesiology*. 1999;90(4):1137-1145.

103. Zhao Y, Liang G, Chen Q, et al. Anesthetic-Induced Neurodegeneration Mediated via Inositol 1,4,5-Trisphosphate Receptors. *J Pharmacol Exp Ther*. 2010;333(1):14-22. doi:10.1124/jpet.109.161562.

104. Wei H, Liang G, Yang H, et al. The Common Inhalational Anesthetic Isoflurane Induces Apoptosis via Activation of Inositol 1,4,5-Trisphosphate Receptors: *Anesthesiology*. 2008;108(2):251-260. doi:10.1097/01.anes.0000299435.59242.0e.

105. Jevtovic-Todorovic V, Hartman RE, Izumi Y, et al. Early Exposure to Common Anesthetic Agents Causes Widespread Neurodegeneration in the Developing Rat Brain and Persistent Learning Deficits. *J Neurosci*. 2003;23(3):876-882.

106. Hayashi H, Dikkes P, Soriano SG. Repeated administration of ketamine may lead to neuronal degeneration in the developing rat brain. *Paediatric Anaesthesia*. 2002;12(9):770.

107. Loepke AW, Soriano SG. An Assessment of the Effects of General Anesthetics on Developing Brain Structure and Neurocognitive Function. *Anesthesia & Analgesia*. 2008;106(6). http://journals.lww.com/anesthesia-analgesia/Fulltext/2008/06000/An_Assessment_of_the_Effects_of_General.14.aspx.

108. Stratmann MD, Ph.D. ,Greg, Sall MD, Ph.D. ,Jeffrey W., May MA, Laura D.V., et al. Isoflurane Differentially Affects Neurogenesis and Long-term Neurocognitive Function in 60-day-old and 7-day-old Rats. *The Journal of the American Society of Anesthesiologists*. 2009;110(4):834-848. doi:10.1097/ALN.0b013e31819c463d.

109. Ing C, DiMaggio C, Whitehouse A, et al. Long-term Differences in Language and Cognitive Function After Childhood Exposure to Anesthesia. *Pediatrics*. 2012;130(3):e476-e485. doi:10.1542/peds.2011-3822.

110. Sprung J, Flick RP, Wilder RT, et al. Anesthesia for Cesarean Delivery and Learning Disabilities in a Population-Based Birth Cohort. *Anesthesiology*. 2009;111(2):302-310. doi:10.1097/ALN.0b013e3181adf481.

111. Flick RP, Katusic SK, Colligan RC, et al. Cognitive and Behavioral Outcomes After Early Exposure to Anesthesia and Surgery. *Pediatrics*. 2011;128(5):e1053-e1061. doi:10.1542/peds.2011-0351.

112. Hansen MD, Ph.D. ,Tom G., Pedersen MS, Jacob K., Henneberg MD, Ph.D. ,Steen W., et al. Academic Performance in Adolescence after Inguinal Hernia Repair in InfancyA Nationwide Cohort Study. *The Journal of the American Society of Anesthesiologists*. 2011;114(5):1076-1085. doi:10.1097/ALN.0b013e31820e77a0.

113. Bai X, Bosnjak ZJ. Emerging model in anesthetic developmental neurotoxicity: human stem cells. *Int J Clin Anesthesiol*. 2013;1:1002.

114. Bajwa S, Kulshrestha A. Dexmedetomidine: An Adjuvant Making Large Inroads into Clinical Practice. *Ann Med Health Sci Res*. 2013;3(4):475-483. doi:10.4103/2141-9248.122044.

115. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Human Development*. 1979;3(1):79-83. doi:10.1016/0378-3782(79)90022-7.

116. Zhao X, Yang Z, Liang G, et al. Dual effects of isoflurane on proliferation, differentiation and survival in human neuroprogenitor cells. *Anesthesiology*. 2013;118(3):537-549. doi:10.1097/ALN.0b013e3182833fae.

117. Johnson MA, Weick JP, Pearce RA, Zhang S-C. Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte coculture. *J Neurosci*. 2007;27(12):3069-3077. doi:10.1523/JNEUROSCI.4562-06.2007.

118. Bosnjak ZJ, Yan Y, Canfield S, et al. Ketamine induces toxicity in human neurons differentiated from embryonic stem cells via mitochondrial apoptosis pathway. *Curr Drug Saf*. 2012;7(2):106-119.

119. Sun LS, Li G, DiMaggio CJ, et al. Feasibility and Pilot Study of the Pediatric Anesthesia NeuroDevelopment Assessment (PANDA) Project. *J Neurosurg Anesthesiol*. 2012;24(4):382-388. doi:10.1097/ANA.0b013e31826a0371.

120. De Roo M, Klauser P, Briner A, et al. Anesthetics Rapidly Promote Synaptogenesis during a Critical Period of Brain Development. *PLoS One*. 2009;4(9). doi:10.1371/journal.pone.0007043.

121. Culley DJ, Boyd JD, Palanisamy A, et al. Isoflurane Decreases Self-Renewal Capacity of Rat Cultured Neural Stem Cells. *Anesthesiology*. 2011;115(4):754-763. doi:10.1097/ALN.0b013e318223b78b.