Published in final edited form as:

Exp Neurol. 2012 September ; 237(1): 207–210. doi:10.1016/j.expneurol.2012.06.023.

Developmental neurotoxicity screening using human embryonic stem cells

Zeljko J. Bosnjak, PhD [Professor and Vice Chairman for Research]

Departments of Anesthesiology and Physiology, Medical College of Wisconsin, 8701 W. Watertown Plank Rd. Milwaukee Wisconsin, 53226, Tel: 414-456-5687

Zeljko J. Bosnjak: zbosnjak@mcw.edu

Abstract

Research in the area of stem cell biology and regenerative medicine, along with neuroscience, will further our understanding of drug-induced death of neurons during their development. With development of an *in vitro* model of stem cell-derived human neural cell lines investigators can, under control conditions and during intense neuronal growth, examine molecular mechanisms of various drugs and conditions on early developmental neuroapoptosis in humans. If the use of this model will lead to fewer risks, or identification of drugs and anesthetics that are less likely to cause the death of neurons, this approach will be a major stride toward assuring the safety of drugs during the brain development. The ultimate goal would be not only to find the trigger for the catastrophic chain of events, but also to prevent neuronal cell death itself.

Introduction

The mechanisms of drug action and pathophysiology of various diseases are mostly studied in animals and need to be validated in human models. However, research efforts are hampered by limited access to human tissue, especially those that have not been exposed to disease, drugs, remodeling etc. For those reasons, many investigations are using derived human cells (i.e. neurons, cardiomyocytes, hepatocytes) from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (iPSCs). This innovative, patient-specific approach is relatively new and was made possible due to seminal discovery in 2006 that terminally differentiated mouse somatic cells such as skin fibroblasts can be reprogrammed into a pluripotent state that is highly similar to embryonic stem cells (Takahashi and Yamanaka 2006). The resulting iPSCs are generated typically by transfer of reprogramming transcription factors, such as Oct4, Klf4, Sox2, and cMyc. Because iPSCs can be generated from any patient, including those with heritable diseases, iPSC studies offer the unique possibility of investigating the cellular consequences of various conditions or diseases by being able to investigate the genetic vs. environmental causes of altered phenotypic responses. That the above approach is going to be very useful comes from the results from recent studies showing, for instance, that iPSC-derived cardiomyocytes obtained from patients with long QT syndrome recapitulate the long action potential phenotype in cellular culture (Itzhaki et al. 2011, Moretti et al. 2010). Because neither traditional expression studies nor various animal models are able to faithfully mimic the human disease phenotype, this approach will help the investigators to model and examine, for instance, potential channelopathies associated with specific human mutations in cardiomyocyte lineages derived from patients with specific genetic mutations. Moreover, if the patient is unresponsive to conventional treatments, experiments conducted on patient-specific iPSCs have a great potential to discover mechanisms involved and the chance to formulate new and more effective drug therapies. Clearly, these innovative approaches have the capability to provide the necessary results and contribute to personalized medicine by identifying genetic-

related disease aggravators, environmental confounding factors, and at the end, the beneficial therapies.

Because hESC and iPSC lines are capable to differentiate into various cell types, they represent a powerful experimental model to screen drugs and study normal and pathologic processes. Derived neurons and cardiomyocytes can phenotypically resemble functional human cells and have also been tested for cell replacement therapies. As in any new and emerging technology, there are expected variabilities between cell line to cell line such as pluripotent hESCs, iPSCs, mesoderm, ectoderm, and definitive endoderm that have been observed in microarray profiles. Moreover, there also appears to be significant heterogeneity in mRNA expressed in different hESC lines. In the future, one approach to better understand how changes in messenger RNA levels in differentiating stem cells and individual cell lines relate to cell function is to study changes in signal transduction and global changes in protein expression. (King CC 2010). In this brief review, potential developmental neurotoxicity of alcohol and general anesthetics are discussed with emphasis on the use of *in vitro* models of stem cell-derived human neuronal cell lines.

Alcohol toxicity

It is well-documented that alcohol abuse and dependency may lead to a fetal alcohol spectrum disorder (FASD). One of the most severe forms of FASD is the fetal alcohol syndrome (FAS) which is characterized by mental retardation, structural abnormalities, and microcephaly of the brain. Vast majority of studies using animal models and examining fetal alcohol toxicity indicate that ethanol exposure during the critical periods of intense synaptogenesis will lead to massive neuroapoptosis. These toxic effects of alcohol are due in large part to either block of NMDA glutamate receptors or activation of GABA_A receptors in the developing brain (Ikonomidou et al. 2000). Not only are these neurotoxic changes secondary to acute alcohol exposure seen in rodent's brain, but also in the non-human primates (Farber et al. 2010). Using the primary neural progenitor culture from E14 rat embryos, it was reported that ethanol exposure suppressed the proliferation of neural progenitor cells and induced early differentiation of neurons (Kim et al. 2010). Other studies have shown that murine neural progenitors are relatively resistant to the apoptosis-inducing effects of ethanol (Prock and Miranda 2007). Using the rat model, the same laboratory also reported that ethanol promoted progenitor cell cycle progression and increased neurosphere number, without inducing apoptosis (Santillano et al. 2005).

Only a few studies have attempted to examine the effects of ethanol on hESCs or neuronal progenitors (NPCs) and effects of ethanol on these cells in the developing human brain are not yet clear. hESCs are pluripotent stem cells derived from the inner cell mass of human embryos at preimplantation stage. hESCs are able to replicate indefinitely and virtually differentiate into every cell type found in the adult body. The differentiation ability of hESCs into committed stem cells or specific lineages is potentially valuable for studying cellular and molecular events involved in early human development under physiological and pathological conditions. Recapitulation of neurogenesis from hESCs *in vitro* allows the investigations of drug-induced developmental neurotoxicity which are difficult to perform in humans. Multiple sequential steps are involved in hESC-neurogenesis and include differentiation of hESCs into NSCs with proliferation and differentiation potential, and differentiation of NSCs into neurons. This *in vitro* neurogenesis system mimics basal processes of brain development, such as cellular neuron differentiation. These processes might be disturbed by various drugs and anesthetics. Thus, using this *in vitro* neurogenesis system one is able to dissect the toxic effect of drugs on the hESCs, NSCs and developing neurons. Recent work has provided some unexpected new insights into how alcohol may cause additional damage to hESCs and NPCs (Nash et al. 2012). These investigators found a

dramatic phenotype of proliferation in response to ethanol in hESC and NPC lines. Exposure to ethanol resulted in larger colonies of undifferentiated hESCs, despite an increase in apoptosis, because of increased proliferation of the undifferentiated cells and neuroblasts. These findings suggest more complicated cellular mechanisms of ethanol toxicity and spotlight the place hESC models have in helping to examine these mechanisms and complementing animal models of drug-induced developmental neurotoxicity. Clearly, this study points to a very promising approach by using hESCs to examine molecular mechanisms of various drugs and conditions of early developmental neuroapoptosis in humans.

Other investigators have examined the effects of ethanol on neuronal progenitors cells isolated from normal second-trimester fetal human brains (Vangipuram et al. 2008). The results indicate that ethanol induced a more rapid coalescence of progenitor cells and an increased volume of neurospheres, most likely due to an increase in cell proliferation as suggested in their subsequent publication (Vangipuram and Lyman 2010). Recent study used iPSCs to generate human neural cultures as a model system to examine the molecular actions of alcohol (Lieberman et al. 2012). These iPSCs were obtained from several alcohol-dependent subjects and several non-alcoholic volunteers. Consistent with previous findings acute ethanol exposure attenuated NMDA receptor response. In contrast the authors did not observe acute ethanol enhancement of GABA_A responses which have been observed in some, but not all, reports using rodent neuronal cultures. This preliminary study also found that chronic alcohol exposure produced increases in mRNA expression of the NMDA receptor subunit genes only in cultures derived from alcoholic, but not non-alcoholic subjects. Although the results of this study are limited due to the small number of subjects, it is an intriguing approach of using patient-derived neural cells. It should provide a powerful model to examine cellular responses to acute or chronic alcohol exposure with respect to between-patient genetic variation. Although preliminary, the above study suggests that patterns of NMDA-mRNA expression maybe different in neural cells derived from alcoholic subjects. Indeed, as suggested by the authors, the heritable traits may lead to a more active alcohol-induced gene expression in the NMDA system in subjects predisposed to alcohol dependence and contribute to tolerance of the inhibitory effects of alcohol on the NMDA receptor activity. It is very likely that using the iPSC approach will give us an opportunity to study the functional effects on neuronal tissue of alleles that have been identified with risk for alcohol dependence.

General anesthetic toxicity

Most of the studies on toxic effects of alcohol centering on NMDA receptor blockade and activation of GABA_A receptors during the rapid brain spurt are drawing on parallel studies performed on chemicals and drugs that also block the NMDA receptor for glutamate and/or activate GABA_A receptors. Indeed many groups have shown that most of the general anesthetics used today cause brain neurons to die by apoptosis during the active synaptogenesis (Stratman 2011). Either volatile or intravenous anesthetics suppress neural activity by also inhibiting NMDA receptors and/or activating the receptors for the inhibitory neurotransmitter GABA. It is likely that upregulation of excitatory receptors by anesthetics or alcohol leads to excessive calcium entry exceeding the buffering capacity of mitochondria followed by membrane potential depolarization, reactive oxygen species production, cytochrome c release into cytosol, and caspase activation, resulting in the neuronal apoptosis. Growing evidence in rodents and non-human primates has demonstrated that prolonged exposure of developing animals to general anesthetics (e.g., ketamine and isoflurane) could induce widespread neuronal cell death followed by long-term memory and learning abnormalities. For instance ketamine, an NMDA blocker, causes neurotoxicity in a variety of developing animal models (Braun et al. 2010, Slikker et al. 2007, Soriano et al.

2010, Takadera et al. 2006, Vutskits et al. 2006, Wang et al. 2006, Zou et al. 2009a, b), leading to a serious concern regarding the safety of pediatric anesthesia. *In vitro* experimental evidence from cultured neonatal animal neurons confirmed the *in vivo* findings (Takadera et al. 2006, Vutskits et al. 2006, Wang et al. 2006). Greatest vulnerability of developing brain to anesthetics occurs at the time of rapid synaptogenesis or so-called brain growth spurt period. Likewise, a few anesthetics might not be toxic at all, such as dexmedetomidine, because they do not affect the cellular receptors most commonly influenced by the majority of general anesthetics (Sanders et al. 2009).

While it is well-documented that exposure to general anesthetics during very active brain growth and formation of synapses in animals will result in considerable death of brain neurons and subsequent learning disabilities (Ben-Ari 2002, Chalon et al. 1981, Jevtovic-Todorovic et al. 2003, Olney et al. 2000), similar studies in humans are not feasible. There is a clear possibility that general anesthetics are also causing neuronal cell death in humans, although it is difficult to prove this definitively. Even more important is whether anesthesia impairs cognition in humans. Some epidemiologic studies in humans, although not all, have implicated that children exposed to anesthesia in early life have higher incidence of learning disabilities later in life (Davidson et al. 2008, Hansen et al. 2009, Loepeke and Soriano 2008, Sun et al. 2008.). These and subsequent studies of multiple anesthetic exposures have shown to be detrimental for cognitive development of children, exhibiting significant delays in measured intelligence, adaptive functioning, and academic performance (DiMaggio et al. 2009, Flick et al. 2011, Kalkman et al. 2009, Wilder et al. 2009). In the United States millions of children are exposed annually to anesthetic agents that have been shown to cause neuronal cell death in immature animals, including non-human primates (Brambrink et al. 2010, Creeley and Olney 2010, Slikker et al. 2007, Zou et al. 2009a, b). In addition, up to 2% of pregnant women undergo anesthesia during their pregnancy for surgery unrelated to the delivery.

Nevertheless, if and how general anesthetics induce human neural cell toxicity is unknown. Recapitulation of neurogenesis from hESCs *in vitro* allows investigation of the toxic effects of anesthetics on hESCs, NSCs and developing neurons which is impossible to perform in humans. Using the hESCs my laboratory has recently examined the effects of clinically relevant concentrations of ketamine on proliferation and toxicity of hESC-derived NSCs and neurons (Bai et al. 2012, Bosnjak et al. 2012). These initial data indicate that similar to alcohol (Nash et al. 2012), clinically relevant doses of ketamine also increased human NSC proliferation and induced a time- and dose-dependent neuronal death. Ketamine also enhanced mitochondrial fission as well as reactive oxygen species (ROS) production compared with no-treatment control. Importantly, Trolox, a ROS scavenger, significantly attenuated the decrease of ketamine-induced cell viability.

In our study, following ketamine exposure at clinically relevant concentrations, there was a significant increase in the caspase-3 activity as well as TUNEL-positive cells with condensed and fragmented nuclei. The central components of the programmed cell death are a group of proteolytic enzymes called caspases which can be activated by various types of stimulation. Loss of $\Delta\Psi_m$ and release of cytochrome c from the mitochondria are key events in initiating mitochondria-involved apoptosis (Budd et al. 2000). The released cytochrome c activates caspase-9, which consequently induces caspase-3 activation, resulting in the cleavage of several cellular proteins, and finally leading to the typical alterations related to cell apoptosis such as DNA fragmentation in cell nuclei (Braun et al. 2010, Zhang et al. 2010). Indeed we found that ketamine-induced neuronal apoptosis was accompanied by the significant decrease in $\Delta\Psi_m$ and an increase in cytochrome c release from mitochondria into cytosol, suggesting that ketamine induces human neurons to undergo mitochondria-mediated apoptosis pathway. Mitochondria are highly dynamic and the alterations in mitochondrial

shape (fusion or fission) can affect a variety of biological processes such as apoptosis and mitosis (Ong et al. 2010). Dynamin-related protein 1 (Drp1) is a fission-related protein, and with an overexpression of Drp1_{K38A} that is a dominant negative mutant of Drp1, leads to prevention of mitochondrial cytochrome c release and apoptotic cell death (Frank et al. 2001). Our data showed the fragmented discrete punctiform mitochondria in the ketamine-treated culture, while mitochondrial shape was elongated with interconnected network in the control cells. Our initial data indicated that mitochondrial fission may mediate the ketamine-induced neuronal apoptosis by increasing ROS production.

To our knowledge, nobody has used this *in vitro*-controlled human stem cell-based neurogenesis model to address such important anesthesia issues and study mitochondrial fission/fusion dynamic- and autophagy-involved toxicity mechanisms. This approach has the potential translational application because identification of the cellular mechanisms and signaling pathways that underlie anesthesia-induced neurotoxicity will allow targeting of the molecules that can prevent this toxic effect. In addition, this established *in vitro* experimental model will provide numerous possibilities for future studies as one could use this high throughput approach to rapidly test the effects of various conditions and anesthetics on developing human neurons, which will lead to major advances toward assuring the safety of anesthesia for fetus and infant. As to the clinical practice, clearly more evidence is needed to guide clinical decision-making on the safety of anesthesia during labor and delivery as well as pediatric anesthesia. The National Center for Toxicological Research, an internationally recognized research center at the U.S. Food and Drug Administration, and several universities are conducting research regarding the effects of anesthetics on the nervous systems of animals during developmental periods of rapid brain growth (smarttots.org). Currently, there is not sufficient evidence to determine whether these findings are translatable to the millions of young children who receive anesthesia each year. Despite the unequivocal neurotoxic effect of anesthetics in animal models, there is no direct clinical evidence showing any such effect in fetuses, infants, and children at any dose. Without data from clinical studies, the question of whether anesthetic agents are neurotoxic in children cannot be answered.

Ultimately, if anesthesia is discovered to cause cognitive decline in developing human brains, we need to know how to prevent it by knowing the mechanisms of anesthesia-induced toxicity. Clearly, more research is urgently needed to determine whether anesthesia impairs brain function in human neurons, what the specific mechanisms are, and how it can be prevented. Research in the area of stem cell biology and regenerative medicine, along with neuroscience, will further our understanding of anesthetic-induced death of neurons during their development. With development of an *in vitro* model of stem cell-derived human neural cell lines investigators can, under control conditions and during intense neuronal growth, test the effects of different drugs including anesthetics on a developing human neuronal network. If the use of this model will lead to fewer risks, or identification of drugs and anesthetics that are less likely to cause the death of neurons, this approach will be a major stride toward assuring the safety of drugs during the brain development along with safe anesthesia and sedation for children. The ultimate goal would be not only to find the trigger for this catastrophic chain of events, but also to prevent neuronal cell death itself. It is of interest that some preliminary data showing evidence of lithium-mediated protection against ethanol neurotoxicity (Ishii et al. 2008, Luo 2010) is also seen in protecting the developing brain against the toxic effects of general anesthetics (Creeley & Olney 2010).

Acknowledgments

This work was supported in part by the National Institutes of Health Grants P01GM066730 and R01HL034708 (to Dr. Bosnjak), Bethesda, Maryland.

References

- Bai X, Yan Y, Canfield S, Muravyeva MY, Kikuchi C, et al. Ketamine enhances human neural stem cell proliferation and induces neuronal apoptosis via reactive oxygen species-mediated mitochondria pathway. 2012 Accepted, *Anesth. Analg.*
- Ben-Ari Y. Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci.* 2002; 3:728–739. [PubMed: 12209121]
- Bosnjak ZJ, Yan Y, Canfield S, Muravyeva MY, Kikuchi C, et al. Ketamine induces toxicity in human neurons differentiated from embryonic stem cells via mitochondrial apoptosis pathway. 2012 Accepted, *Curr. Drug Saf.*
- Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, et al. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. *Anesthesiology.* 2010; 112:834–841. [PubMed: 20234312]
- Braun S, Gaza N, Werdehausen R, Hermanns H, Bauer I, et al. Ketamine induces apoptosis via the mitochondrial pathway in human lymphocytes and neuronal cells. *Br J Anaesth.* 2010; 105:347–354. [PubMed: 20659914]
- Budd SL, Tenneti L, Lishnak T, Lipton SA. Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *Proc Natl Acad Sci U S A.* 2000; 97:6161–6166. [PubMed: 10811898]
- Chalon J, Tang CK, Ramanathan S, Eisner M, Katz R, et al. Exposure to halothane and enflurane affects learning function of murine progeny. *Anesth Analg.* 1981; 60:794–797. [PubMed: 7197490]
- Creeley CE, Olney JW. The young: neuroapoptosis induced by anesthetics and what to do about it. *Anesth Analg.* 2010; 110:442–448. [PubMed: 19955510]
- Davidson AJ, McCann ME, Morton NS, Myles PS. Anesthesia and outcome after neonatal surgery: the role for randomized trials. *Anesthesiology.* 2008; 109:941–944. [PubMed: 19034085]
- 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; 21:286–291. [PubMed: 19955889]
- Farber NB, Creeley CE, Olney JW. Alcohol-induced neuroapoptosis in the fetal macaque brain. *Neurobiol Dis.* 2010; 40:200–206. [PubMed: 20580929]
- Flick RP, Katusic SK, Colligan RC, Wilder RT, Voigt RG, et al. Cognitive and behavioral outcomes after early exposure to anesthesia and surgery. *Pediatrics.* 2011; 128:e1053–1061. [PubMed: 21969289]
- Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell.* 2001; 1:515–525. [PubMed: 11703942]
- Hansen TG, Flick R. Danish Registry Study Group; Mayo Clinic Pediatric Anesthesia and Learning Disabilities Study Group. Anesthetic effects on the developing brain: insights from epidemiology. *Anesthesiology.* 2009; 110:1–3. [PubMed: 19104158]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, et al. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
- Ishii T, Hashimoto E, Ukai W, Tateno M, Yoshinaga T, et al. Lithium-induced suppression of transcription repressor NRSF/REST: Effects on the dysfunction of neuronal differentiation by ethanol. *Eur J Pharmacol.* 2008; 593:36–43. [PubMed: 18671961]
- Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, et al. Modeling the long-QT syndrome with induced pluripotent stem cells. *Nature.* 2011; 471:225–229. [PubMed: 21240260]
- Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci.* 2003; 23:876–882. [PubMed: 12574416]
- Kalkman CJ, Peelen L, Moons KG, Veenhuizen M, Bruens M. Behavior and development in children and age at the time of first anesthetic exposure. *Anesthesiology.* 2009; 110:805–812. [PubMed: 19293699]

- Kim KC, Go HS, Bak HR, Choi CS, Choi I, et al. Prenatal exposure of ethanol induces increased glutamatergic neuronal differentiation of neural progenitor cells. *J Biomed Sci.* 2010; 17:85. [PubMed: 21073715]
- King CC. Culture and preparation of human embryonic stem cells for proteomics-based applications. *Methods Mol Biol.* 2010; 584:151–77. [PubMed: 19907977]
- Lieberman R, Levine ES, Kranzler HR, Abreu C, Covault J. Pilot study of iPS-derived neural cells to examine biologic effects of alcohol on human neurons in vitro. *Alcohol Clin Exp Res.* 2012; 1–10
- Loepke AW, Soriano SG. An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. *Anesth Analg.* 2008; 106:1681–1707. [PubMed: 18499597]
- Luo J. Lithium-mediated protection against ethanol neurotoxicity. *Front Neurosci.* 2010; 4:1–4. [PubMed: 20582256]
- Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flugel L, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med.* 2010; 363:1397–1409. [PubMed: 20660394]
- Nash R, Krishnamoorthy M, Jenkins A, Csete M. Human embryonic stem cell model of ethanol-mediated early developmental toxicity. *Exp Neurol.* 2012; 234:127–135. [PubMed: 22227564]
- Olney JW, Ishimaru MJ, Bittigau P, Ikonomidou C. Ethanol-induced apoptotic neurodegeneration in the developing brain. *Apoptosis.* 2000; 5:515–521. [PubMed: 11303910]
- Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, et al. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation.* 2010; 121:2012–2022. [PubMed: 20421521]
- Prock TL, Miranda RC. Embryonic cerebral cortical progenitors are resistant to apoptosis, but increase expression of suicide receptor DISC-complex genes and suppress autophagy following ethanol exposure. *Alcohol Clin Exp Res.* 2007; 31:694–703. [PubMed: 17374049]
- Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, et al. Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. *Anesthesiology.* 2009; 110:1077–1085. [PubMed: 19352168]
- Santillano DR, Kumar LS, Prock TL, Camarillo C, Tingling JD, et al. Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. *BMC Neurosci.* 2005; 6:59. [PubMed: 16159388]
- Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci.* 2007; 98:145–158. [PubMed: 17426105]
- Soriano SG, Liu Q, Li J, Liu JR, Han XH, et al. Ketamine activates cell cycle signaling and apoptosis in the neonatal rat brain. *Anesthesiology.* 2010; 112:1155–1163. [PubMed: 20418696]
- Stratmann G. Review article: neurotoxicity of anesthetic drugs in the developing brain. *Anesth Analg.* 2011; 113:1170–1179. [PubMed: 21965351]
- Sun LS, Li G, Dimaggio C, Byrne M, Rauh V, et al. Anesthesia and neurodevelopment in children: time for an answer? *Anesthesiology.* 2008; 109:757–761. [PubMed: 18946281]
- Takadera T, Ishida A, Ohyashiki T. Ketamine-induced apoptosis in cultured rat cortical neurons. *Toxicol Appl Pharmacol.* 2006; 210:100–107. [PubMed: 16307766]
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126:663–76. [PubMed: 16904174]
- Vangipuram SD, Grever WE, Parker GC, Lyman WD. Ethanol increases fetal human neurosphere size and alters adhesion molecule gene expression. *Alcohol Clin Exp Res.* 2008; 32:339–347. [PubMed: 18162078]
- Vangipuram SD, Lyman WD. Ethanol alters cell fate of fetal human brain-derived stem and progenitor cells. *Alcohol Clin Exp Res.* 2010; 34:1574–1583. [PubMed: 20586756]
- Vutsksits L, Gascon E, Tassonyi E, Kiss JZ. Effect of ketamine on dendritic arbor development and survival of immature GABAergic neurons in vitro. *Toxicol Sci.* 2006; 91:540–549. [PubMed: 16581949]
- Wang C, Sadovova N, Hotchkiss C, Fu X, Scallet AC, et al. Blockade of N-methyl-D-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. *Toxicol Sci.* 2006; 91:192–201. [PubMed: 16500925]

- Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, et al. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology*. 2009; 110:796–804. [PubMed: 19293700]
- Zhang Y, Dong Y, Wu X, Lu Y, Xu Z, et al. The mitochondrial pathway of anesthetic isoflurane-induced apoptosis. *J Biol Chem*. 2010; 285:4025–4037. [PubMed: 20007710]
- Zou X, Patterson TA, Divine RL, Sadovova N, Zhang X. Prolonged exposure to ketamine increases neurodegeneration in the developing monkey brain. *Int J Dev Neurosci*. 2009a; 27:727–731. [PubMed: 19580862]
- Zou X, Patterson TA, Sadovova N, Twaddle NC, Doerge DR, et al. Potential neurotoxicity of ketamine in the developing rat brain. *Toxicol Sci*. 2009b; 108:149–158. [PubMed: 19126600]