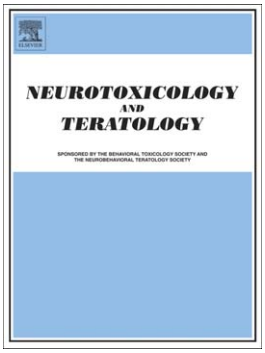

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Neurogenesis and Developmental Anesthetic Neurotoxicity

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Abstract

The mechanism by which anesthetics might act on the developing brain in order to cause long term deficits remains incompletely understood. The hippocampus has been identified as a structure that is likely to be involved, as rodent models show numerous deficits in behavioral tasks of learning that are hippocampal-dependent. The hippocampus is an unusual structure in that it is the site of large amounts of neurogenesis postnatally, particularly in the first year of life in humans, and these newly generated neurons are critical to the function of this structure. Intriguingly, neurogenesis is a major developmental event that occurs during postulated windows of vulnerability to developmental anesthetic neurotoxicity across the different species in which it has been studied. In this review, we examine the evidence for anesthetic effects on neurogenesis in the early postnatal period and ask whether neurogenesis should be studied further as a putative mechanism of injury. Multiple anesthetics are considered, and both in vivo and in vitro work is presented. While there is abundant evidence that anesthetics act to suppress neurogenesis at several different phases, evidence of a causal link between these effects and any change in learning behavior remains elusive.

Introduction

The combination of epidemiologic studies showing an association between anesthesia and surgery with reduced cognitive function in young children (DiMaggio et al., 2012; DiMaggio et al., 2009; Ing et al., 2012; Wilder et al., 2009; Zhu et al., 2010) and a large number of preclinical studies in which early postnatal rodents exposed to anesthesia demonstrate lasting impairments in learning and memory e.g. (Erasso et al., 2012; Huang, L. et al., 2016; Jevtovic-Todorovic et al., 2003; Lee et al., 2014; Levin et al., 1991; Ramage et al., 2013; Satomoto et al., 2009; Shih et al., 2012; Zhu et al., 2010)has given rise to a concern that anesthetic may have harmful effects on the developing brain, which has been much discussed in the anesthetic literature e.g. (Block et al., 2012; Dimaggio et al., 2011; Hudson and Hemmings, 2011; Jevtovic-Todorovic et al., 2013; Kuehn, 2011; Mann and Kahana, 2015; Nemergut et al., 2014; Rappaport et al., 2015; Vutskits, 2011). Studies in nonhuman primates show pathologic changes in the brains of animals exposed to anesthetics during development (Brambrink et al., 2010), and early studies of neurocognitive function have demonstrated an association between anesthesia exposure and changes in behavior related to emotional reactivity (Raper et al., 2015) and cognition (Paule et al., 2011). A small scale human trial showed a correlation between anesthesia plus surgery exposure and a decrease in performance on a recognition memory task suggesting a plausible comparison to similar findings in the animal model (Stratmann et al., 2014). However, more comprehensive clinical studies leave much unanswered. Two recent large scale human trials provide some reassurance that that short, single exposures to general anesthesia in otherwise healthy children do not have detectable harmful effects on neurocognitive function (Davidson et al., 2016; Sun et al., 2016), but the original epidemiologic reports cited above, as well as the predominance of animal model research, all indicate that either multiple exposures or exposures of at least several hours duration are required to cause measurable deficits. The clinical significance of this phenomenon, which we will refer to as developmental anesthetic neurotoxicity (DAN), remains unproven and incompletely understood as limited human data are available.

Establishing the mechanism of injury in DAN is of critical importance. In order for the phenomenon to be considered as potentially clinically relevant, it must be shown to be biologically plausible. Furthermore, this information could guide clinical trials and primate studies towards appropriate outcome measures and potentially allow for the inclusion of treatment arms in such studies. Most studies of mechanism have been conducted in the rodent model, where numerous laboratories have independently confirmed that anesthetic exposure at early postnatal ages causes deficits in spatial learning. Some areas that have been explored include direct cytotoxicity of anesthetics via apoptotic pathways, impairments in the formation of synapses, and disruptions of neuronal growth (Jackson et al., 2016). No definitive conclusions have been reached, and it is quite possible either that multiple mechanisms of injury are at work or that some, or even all, of the mechanisms that have been studied are epiphenomena or are relevant only in the rodent. A relatively under-examined, but promising mechanism of injury is via anesthetic effects on neural stem cells. In this review, we ask whether anesthetics might exert their deleterious effects on brain development by disrupting neurogenesis in early postnatal animals.

Overview of postnatal neurogenesis

Although most neurons are generated prior to birth in mammals, new neurons are continuously added to certain brain areas throughout life (Ming and Song, 2005). These neurons are derived from a population of neural stem cells (NSCs) that reside primarily in two distinct areas of the brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). It has recently been appreciate that postnatal neurogenesis occurs on a very large scale and it is critically important for brain function, particularly in the hippocampus. Postnatal neurogenesis in the DG is critically important for learning (Deng et al., 2010) and disruptions of this process may be associated with psychiatric disease (Kang et al., 2016). While it has been appreciated that postnatal neurogenesis is an important phenomenon for rodent brain function, recent data shows that it is highly relevant in humans as well. By measuring the concentration of nuclear-bomb-test-derived ^{14}C in genomic DNA, a recent study found that

one-third of hippocampal neurons in adult humans are subject to exchange, and 1400 new neurons are added to the brain through hippocampal neurogenesis daily, corresponding to 1.75% of the neurons per year within the DG, with a modest decline during aging (Spalding et al., 2013). In many species including rodents and nonhuman primates, newly born neuroblasts in the SVZ migrate towards the olfactory bulb through the rostral migratory stream and replace GABAergic and dopaminergic interneurons in the granular layer (GRL) and glomerular layer (GCL) of the olfactory bulb (Lledo et al., 2008; Merkle et al., 2007). Although newborn neurons have not been observed in the olfactory bulb of humans, there appears to be substantial neurogenesis in the human striatum. It has been calculated that 2.7% of the striatal neurons are exchanged per year, and a subset of the generated neurons express calretinin (Ernst et al., 2014).

Postnatal neurogenesis is a particularly interesting target to study in anesthetic neurotoxicity, because it is one of few developmental processes that is still ongoing at high levels after birth, when potentially toxic anesthetic exposures occur. In rats peak levels of dentate gyrus neurogenesis occur between the first and fifth postnatal day, with a change to a lower rate process resembling adult neurogenesis at approximately 2 weeks of age (Altman and Bayer, 1990). Most rodent DAN studies have involved anesthetic exposures on or around postnatal day 7. Anatomical studies conducted in nonhuman primates show that approximately 25% of the lifelong population of the dentate gyrus of the hippocampus is generated in the SGZ during a peak period of neurogenesis that occurs between birth and three months of age, and that neurogenesis continues to occur at very high levels from three months to one year. Studies showing evidence of DAN in nonhuman primates have generally involved exposures done within the first postnatal month e.g. (Brambrink et al., 2010; Raper et al., 2015). In humans, histological examination of brain specimens obtained from autopsy and neurosurgical resection demonstrates a large number of NSCs in the SVZ in the first 18 months of life, which declines substantially thereafter (Sanai et al., 2011). Thus, the window of vulnerability to DAN, generally believed to be the first three years of life in humans (Rappaport et al., 2015), coincides with substantial ongoing postnatal neurogenesis. One of the principal

concerns related to the clinical relevance of DAN is that the developmental timeline of brain development of the different species under study does not necessarily suggest any easy translation from rodent models to the human patient. However, what is intriguing about the study of neurogenesis is the strong correlation between the timing of substantial dentate gyrus neurogenesis and known or suspected windows of vulnerability to anesthetic neurotoxicity specific to rodents, nonhuman primates, and humans. This makes altered neurogenesis a very plausible mechanism that would potentially connect the different model systems in DAN investigation.

Another compelling reason to consider toxic effects on neurogenesis as a mechanism for DAN relates to the known function of neurogenesis in the dentate gyrus. In recent years postnatal neurogenesis in the dentate gyrus has attracted a lot of attention in neuroscience research due to its involvement in higher cognitive functions such as spatial learning, memory retention, memory retrieval, forgetting, clearance of memory traces and mood regulation (Christian et al., 2014; Deng et al., 2010; Kang et al., 2016; Kitabatake et al., 2007). In particular, spatial learning is clearly compromised in rodent models of DAN, which suggested a rationale to study neurogenesis as a mechanism for DAN. Perturbation of hippocampal neurogenesis in animal models has been associated with cognitive deficits that are often present in the patients with psychiatric disorders such as major depression, schizophrenia, anxiety disorders, and addictive behaviors (Christian et al., 2014). Additionally, studies have now shown that the level of adult hippocampal neurogenesis is correlated with pathological conditions such as aging, epilepsy, stroke, neurodegenerative disorders, and psychiatric disorders in humans (Bishop et al., 2010; Lupien et al., 2007; Parent, 2003; Raber et al., 2004; Rola et al., 2004; Sahay and Hen, 2007; Winner et al., 2011). Although it remains to be determined whether impaired hippocampal neurogenesis contributes to the pathogenesis of psychiatric disorders or other relevant neurological symptoms, a growing body of evidence strongly suggests that the hippocampus may be an area that is highly susceptible to various physiological, pathological and pharmacological stimuli, including anesthetic toxicity.

Postnatal hippocampal neurogenesis is a multistep process which comprises a series of sequential developmental events, which can be identified through the use of immunohistochemical markers in most cases. The study of neurogenesis has been greatly aided by the use of thymidine analogs such as bromodeoxyuridine (BRDU) which are taken up in newly born cells, allowing them to be identified from that point onwards (Kuhn and Cooper-Kuhn, 2007). Neurogenesis begins with the activation of the self-renewing multipotent radial glia-like cell (RGL). These cells can be identified by markers such as glial fibrillary acid protein (GFAP), nestin, and paired box 6 (Pax-6) (von Bohlen und Halbach, 2011). The RGLs are the NSCs of the DG and reside in the SGZ at the border of inner granule cell layer and hilus. The RGL can undergo both symmetric and asymmetric cell division, and upon the asymmetric divisions the RGL gives rise to intermediate progenitor cells (IPCs), which are a population of transit amplifying cells that undergo limited rounds of division before generating neuroblasts (Berg DA, 2015; Bond et al., 2015). Intermediate cells are best identified by labeling for neurogenic differentiation protein (NeuroD) (von Bohlen und Halbach, 2011). These initial phases of neurogenesis serve to expand the population of NSCs and neuroblasts (Kempermann et al., 2015). The neuroblasts then migrate a short distance along the SGZ to dwell in the inner granular cell layer of the DG where they develop into immature granule neurons (Sun et al., 2015), during which time they express high levels of doublecortin (DCX) (von Bohlen und Halbach, 2011) During the postmitotic maturation phase, neurons go through dendritic and axonal development and start forming synapses. The newly formed granule neurons provide excitatory input to pyramidal neurons of CA3 region of the hippocampus by sending axonal projections along the mossy fiber tract while they receive synaptic input from the entorhinal cortex (Ming and Song, 2005). Interestingly, neurotransmitters have been shown to regulate the different stages of neurogenesis (Berg et al., 2013). For example, γ-aminobutyric acid (GABA), released from local interneurons is a key player in regulating proliferation and fate determination of NSCs to maturation of newly born neurons in the adult DG (Duveau et al., 2011; Ge et al., 2006; Jagasia et al., 2009; Li et al., 2009; Sun et al., 2009; Tozuka et al., 2005). This is of particular interest as many anesthetic agents that have been implicated in DAN,

particularly isoflurane, sevoflurane, and propofol, act on GABA receptors, suggesting a possible means by which anesthetics could act on neurogenesis.

Methodology

In order to arrive at a complete picture of our understanding of neural stem cells as a putative target in DAN, we have conducted a literature search. The following terms were used to identify relevance to anesthesia: Isoflurane, Sevoflurane, Desflurane, Ketamine, and Propofol. The following terms were searched in order to identify relevance to neurogenesis: Neural Stem Cell, Neural Progenitor Cell, and Neurogenesis. The search consisted of all possible combinations of one anesthesia term and one neurogenesis term. Review articles were scrutinized to determine whether any original research was missed in the initial literature search. Original research manuscripts were chosen for inclusion in the review based on what they contributed to the literature and on the authors' evaluation of sound methodology.

Effects of Potent Volatile Anesthetics on Neurogenesis

While there is increasing evidence that many different anesthetic agents may be neurotoxic during development, the potent volatile anesthetics are among the most frequently implicated in studies of DAN. Numerous investigations of isoflurane and several of sevoflurane have been conducted to explore their potentially deleterious effects on neurogenesis, with mixed results. The earliest comprehensive study was conducted by Stratmann and co-workers, who exposed P7 rats to isoflurane for 4 hours at a variable concentration between 3.5% and 1.5% titrated to the response to tail clamp. Injection of BRDU prior to the anesthetic and co-labeling with antibodies for BRDU and NeuroD, an early stage marker for NSCs transitioning to become fate-committed neural progenitors (von Bohlen und Halbach, 2011) was conducted and assessed using quantitative microscopy in the dentate gyrus. This approach is designed to measure differentiating NSCs, which are committed to a neuronal fate and very recently post-mitotic at

the time of anesthesia exposure (Stratmann et al., 2009). No difference was measured between control and isoflurane exposure conditions. Along with this finding, the authors do show an increase in $BRDU^+/NeuroD^+$ cells in P6 rats exposed to the same condition, which can be seen as an important positive control validating the effectiveness of their BRDU injection dose and timing. In the same manuscript, the authors examined the acute effects of isoflurane on proliferation of NSCs by quantifying BRDU+ cells in the dentate gyrus after injection of BRDU during the isoflurane exposure. The authors found a 38% decrease in the isoflurane-exposed P7 group, indicating a likely reduction in proliferation of NSCs. While this result is reasonably convincing, it would have benefited from co-labeling with markers such as Nestin, GFAP, or Pax-6, which are markers for the rapidly proliferating, uncommitted Stage 1 NSCs (von Bohlen und Halbach, 2011), which would have allowed the authors to definitively state that proliferation was decreased in NSCs. Also, a confirmatory measurement with cell division markers such as Ki-67 or proliferating cell nuclear antigen (PCNA) in place of BRDU and in conjunction with a stage 1 NSC marker would have been additionally convincing. Nevertheless, this is strong evidence of an acute suppression of NSC proliferation due to isoflurane.

Several other studies in the literature support and extend the hypothesis that potent volatile anesthetics suppress neurogenesis. Zhu and coworkers found that three consecutive daily 35 minute exposures to 1.7% isoflurane accompanied by acute BRDU injection resulted in a decrease in $BRDU^+NeuN^+$ cells and an increase in $BRDU^+S100^+$ 45 days later (Zhu et al., 2010). This suggests that the anesthetic exposure results in a change in the behavior of NSCs such that there is an increase in glial fate at the expense of neuronal fate. In the same manuscript, the authors show a striking decrease in $Sox\text{-}2^+GFAP^+$ neurons with isoflurane exposure, which is measurable one day after exposure and increased 45 days later (Zhu et al., 2010). GFAP and Sox-2 together identify earliest stage NSCs, which are multipotent and typically quiescent (von Bohlen und Halbach, 2011). They also found a similar pattern of decreased dentate gyrus labeling for P-H3, a marker of mitotic cell division. There is a significant decrease in P-H3 labeling at one day and a greater decrease at 45 days. Taken together these findings suggest an injury to the

population of dentate gyrus NSCs that is propagated over time (Zhu et al., 2010). However, the nature of the exposure used in this study differs fairly substantially from other published work and it would be of great interest to see whether this effect can be achieved with a single, long exposure to determine whether it is the same DAN phenomenon that has been observed in many rodent investigations of DAN. Isoflurane is often studied, but may not be the principal volatile anesthetic used in pediatric practice, and thus it is always of interest to consider studies of sevoflurane when possible. Literature on this topic is limited, but a reduction in $BRDU^+$ cells in the dentate gyrus with sevoflurane exposure in P7 rats between 3% and 5% for four hours has been shown (Fang et al., 2012), which may constitute a comparable result to what has been observed with isoflurane.

Not all volatile anesthetic exposures that have been employed show changes in neurogenesis. Qiu and coworkers conducted a short, single exposure in P7 and P15 rats of 30 minutes of sevoflurane at 2.5% (Qiu et al., 2016). They conducted measures of neurogenesis similar to others that have been published. To assess acute proliferation they measured BRDU+ cells 6 hours after the anesthetic and to assess for chronic changes in proliferation they measured PCNA+ neurons 3 months after exposure. Additionally they tested for changes in differentiation to neuronal versus glial fate of exposed cells by determining percentages of $BRDU^+NeuN^+$ and $BRDU^+S100^+$ cells. None of these assays showed any significant difference between control and sevoflurane exposed conditions (Qiu et al., 2016). It is possible that these findings demonstrate that either longer exposure in the range of several hours or shorter repeated exposures are required in order to have measurable effects on neurogenesis. However, Qiu et al. did one assay at a longer exposure time of 4 hours at their dose of 2.5% sevoflurane. For this condition they measured only the number of $BRDU^+$ cells in the dentate gyrus six hours after anesthesia exposure (Qiu et al., 2016). They found no significant differences between anesthetic and control groups, a finding which differs from the literature already presented. However, no other assays, either to more fully assess short term proliferation or to test long-term effects on proliferation or differentiation, were conducted for this longer duration of exposure so it is difficult to make complete comparisons to other studies. It is possible

that the dose used in Qiu et al. may be too low to appreciate an effect, as the closest comparable study of sevoflurane used a range from 3% to 5% for 4 hours(Fang et al., 2012), and the dose of 2.5% for 4 hours may ultimately result in a considerable difference in delivered amounts of anesthetic. There is some evidence that lower doses of anesthetic have different effects on neurogenesis. In one report a six hour 1.8% sevoflurane exposure in P4 to P6 rats resulted in enhanced neurogenesis both acutely and chronically in the dentate gyrus as measured by BRDU injection and cell counts (Chen et al., 2015). As with so many proposed mechanisms of DAN, it is clear that the effects of potent volatile anesthetics have dose and duration thresholds, and it seems likely that repeated exposure also increases the likelihood of measuring any pathologic consequences. Whether these doses, which are naturally high in young rodent models, are relevant to pediatric clinical practice is uncertain.

There is a reasonable amount of evidence to support the hypothesis that exposure to volatile anesthetics during early postnatal development causes impairments in neurogenesis, but it is challenging to prove that this phenomenon is actually responsible for the observed changes in neurocognitive function in DAN. There is some evidence to this effect, however. Stratmann and coworkers show reduced performance on contextual fear conditioning (Stratmann et al., 2009). This behavioral paradigm does depend on dentate gyrus function (Hernandez-Rabaza et al., 2008), but alternate explanations for reduced performance such as changes in synaptic function in the hippocampal circuit are equally plausible. A similar argument can be made for the correlation between anesthesia-induced deficits in neurogenesis and performance on the Morris water maze, which was reported by Fang and coworkers (Fang et al., 2012). One strong line of evidence of a connection comes from Zhu et al., who showed greater impairments in performance on an object recognition task at 10 weeks than at 4 weeks (Zhu et al., 2010). This progressive decline in function mirrored progressive impairments in neurogenesis, and an escalating pattern of deficits is a plausible consequence of an early loss of neural stem cells that leads to a long-term deficiency in neurogenesis. One explanation for this phenomenon is that the early loss or suppression of the pool of rapidly dividing, multipotent precursors might create an ongoing deficit in neurogenesis that worsened

over time as the gap in NSC numbers between normal controls and exposed animals widened with rapid cell division. Another study that suggests a specific connection between neurogenesis and cognitive dysfunction in DAN showed that environmental enrichment rescued deficits in learning and memory (Shih et al., 2012). Enhanced neurogenesis has been shown to upregulate neurogenesis in rodents (Kempermann et al., 1997), and so a plausible, but not completely substantiated interpretation of these data is that environmental enrichment acted by restoring neurogenesis. Taken together, the evidence supports the possibility that altered neurogenesis could explain some component of cognitive dysfunction in DAN, but conclusive proof is lacking, as it is with most other mechanisms of injury under study in DAN.

Effects of Intravenous Anesthetics on Neurogenesis

Propofol is one of the most commonly used intravenous anesthetic and sedative drugs in pediatric medicine. Recent evidence suggests that early postnatal exposure to propofol in rodents results in apoptotic cell death (Creeley et al., 2013; Milanovic et al., 2016; Milanovic et al., 2010) and deficits in performance of a wide array of behavioral tests of learning e.g (Gao et al., 2014; Gonzales et al., 2015; Man et al., 2015). Thus propofol is increasingly widely accepted as a likely cause of DAN.

The data suggesting that developmental exposure to propofol suppresses neurogenesis are relatively sparse. There is evidence of hippocampal cell death after developmental propofol administration, including a reduction in the number of granule cells in the experimental groups which exhibited impaired memory (Man et al., 2015; Yu et al., 2013). This finding could reflect an alteration in neurogenesis, but it is equally likely to be simply the result of cytotoxicity. Another recent study in showed reduced BRDU incorporation in proliferating dentate gyrus neuronal precursors, reduced levels of the neurogenic transcription factor Sox-2, and reduced immunolabeling for mature dentate gyrus neurons after 60mg/kg intraperitoneal propofol injections performed on three subsequent days (Huang, J. et al., 2016). In

experiments on adult neurogenesis in rats, propofol has been shown to decrease the survival of newborn neurons and to inhibit dendritic arborization and spine formation. It is very uncertain whether these effects, which occurred with exposure of 17 day old but not 11 day old neurons relate to neurogenesis during development (Krzisch et al., 2013)

Ketamine is a N-methyl-D-aspartate (NMDA) receptor antagonist frequently used for sedation, analgesia, and anesthesia in children. It has been appreciated for some time that ketamine has potentially neurotoxic effects in neonatal rodents, including increased levels of neuronal apoptosis (Ikonomidou et al., 1999). Further research has focused mostly on the toxic effects of ketamine to mature neurons and astrocytes. Recently, potential effects of ketamine on the development and migration of neural stem cells has been examined in vivo in rodent models. Huang et al. examined the effect of intraperitoneal ketamine on neural stem cell (NSC) proliferation in the subventricular zone of postnatal day 7 rats compared to controls. While they found decreased NSC proliferation, decreased astrocyte differentiation, and increased neuronal differentiation in the ketamine-treated rats compared to the saline treated rats, the effects were transient (Huang et al., 2015).

Cell Culture Models

The use of cell culture models to study the effects of anesthetics on neurogenesis has several advantages. First and foremost, it is possible to confirm findings from in vivo studies using a model that is free of the possible confounds of hypoxia, hypercarbia, acidosis, and hypothermia that are difficult to completely avoid in rodent studies. Sall and coworkers found that cultured rat neural stem cells exposed to 3.4% isoflurane for 6 hours do not exhibit evidence of direct cytotoxicity, as measured by LDH and MTT assays (Sall et al., 2009). The fold increase in cell number over 48 hours was reduced by 42% in the isoflurane group as compared to control (Sall et al., 2009), strongly suggesting that isoflurane inhibits NSC proliferation. As corroborative evidence the authors found that isoflurane decreased mRNA levels for Ki-67, a cell cycle marker, and Sox-2, an early NSC marker. In a notable difference from published in

vivo work, when cultures were labeled for a neuronal marker, TuJ1, and a glial marker, GFAP, the percentage of neurons increased and the percentage of glia remained unchanged (Sall et al., 2009). At first glance this result seems at odds with that Zhao and co-workers who found a decrease in neuronal fate and an increase in glial fate (Zhao). However the timescales were dramatically different, 4 days in vitro versus a month in vivo, and context plays a critical role in development of NSCs, and thus some differences are to be expected. Similar results were found by Culley and coworkers, who treated rat embryonic neural stem cell cultures for six hours with isoflurane doses ranging from 0.7% to 2.8%. They found a reduction in BRDU incorporation and in Sox-2^{+} cells, suggesting a decrease in proliferation, and there was no evidence of cell death (Culley et al., 2011). In vitro studies also provide further evidence that propofol and ketamine can both can inhibit neurogenesis (Bai et al., 2013; Dong et al., 2012; Sall et al., 2012). Taken together, these in vitro studies support the hypothesis that anesthetics suppress neurogenesis, although they do not give any greater insight into whether effects on neurogenesis are a mechanism of DAN.

New technology has made obtaining stem cells derived from human tissue relatively easy, and thus the opportunity exists to ask whether the effects on neurogenesis observed in rodents cells translate to human cells. Zhao and co-workers tested the effects of isoflurane on ReNcell CX, a human neural progenitor cell line. They found that at high doses and long exposures, isoflurane caused cytotoxicity, impaired proliferation, and promoted glial fate over neuronal fate (Zhao et al., 2013). The cytotoxicity effect, as measured by LDH and MTT assays, required isoflurane doses of 1.2% or greater for exposures of 12 to 24 hours, which is potentially beyond a clinically relevant dose and duration. By contrast, tests of the effects of isoflurane on proliferation as measured by BRDU incorporation, showed widely varying results depending on the dose. One hour of isoflurane exposure at 0.6% enhanced proliferation, whereas the same duration of exposure at 2.4% isoflurane suppressed proliferation and a 1.2% isoflurane dose had no significant effect (Zhao et al., 2013). This finding strongly supports the conclusions of in vivo rodent

studies which demonstrate that the putative effects of anesthetics on neurogenesis are highly dependent on the dose of anesthetic involved.

The molecular mechanisms by which anesthetics inhibit neurogenesis remains unclear, and the few studies to address this are largely those conducted in cell culture where pharmacologic and genetic manipulation of neural stem cells is easier. None of the mechanisms that have been proposed by these studies have been extensively explored, but it is possible to get some sense of what molecular systems may be involved. For instance, Zhao and co-workers found that effects of isoflurane on proliferation in a human neural progenitor cell line could be prevented by xestospongin C or dantrolene, which are, respectively, inhibitors of the inositol 1,4,5-trisphosphate receptor and the ryanodine receptor (Zhao et al., 2013). This finding suggests that release of intracellular calcium stores mediated by isoflurane is required for suppression of neurogenesis. Studies of rodent embryonic neural stem cells have implicated a variety of signaling systems in the effects of isoflurane on neurogenesis, including p53, wnt-catenin, and ERK1/2 (Hou et al., 2015; Nie et al., 2013; Zhang et al., 2013). Interestingly, Sall et al working in cell culture found evidence supporting a role for propofol in suppressing neurogenesis and also showed that this effect did not correlate with caspase activation and it was no blocked by GABA receptors antagonists (Sall et al., 2012). The significance of these findings is difficult to assess. None of these studies attempted to translate their findings to an in vivo model, and also it is difficult to say whether the anesthetics act directly on these systems or whether their involvement is simply an epiphenomenon that reflects the broad importance of these molecules in neuronal growth and differentiation.

Conclusion

In summary, there is considerable evidence to suggest that anesthetics, including isoflurane, sevoflurane, propofol, and ketamine, can and do disrupt fundamental processes in neurogenesis. This includes proliferation, differentiation, and fate selection, and experiments with different anesthetics done in vivo and in vitro are fairly well in agreement on this point. The effects on neurogenesis are dependent on dose

and duration of the anesthetic exposure, which is unsurprising. It is striking that two of the major studies that support a role for neurogenesis in the rodent model involve multiple exposures and relatively low doses (Huang, J. et al., 2016; Zhu et al., 2010), whereas a study done with the more traditional single, long, high dose exposure showed only a short term effect on neurogenesis, which is a considerably less robust result that is less likely to have a long-term impact on cognitive function (Stratmann et al., 2009). On the one hand, this opens up questions about the extent to which alterations of neurogenesis are likely important to the most prevalent rodent model of DAN. On the other hand, it seems increasingly possible that if DAN is clinically important, it may require multiple exposures—something which is a feature of newly emerging primate data and older epidemiologic studies that have reported adverse associations with anesthetic exposure (DiMaggio et al., 2009; Raper et al., 2015; Wilder et al., 2009) . So it is possible that if substantial changes in neurogenesis-dependent DAN require multiple exposures that this reflects a greater clinical relevance of this mechanism of injury than those which can be provoked by single exposure only. Further exploring this issue may help us better understand the relevance of the most prevalent animal models of DAN.

What is principally lacking at this time is evidence of a direct link between altered neurogenesis and functional deficits in learning or any other cognitive domain. This is an elusive goal in the study of DAN, which will require an understanding of exactly how anesthetics act on NSCs to alter their function, in order to prevent or reverse these deficits. The ideal experiment to address this question would be one in which an intervention specific to neurogenesis was given in a model in which both disruptions in neurogenesis and cognitive impairments were known to occur, and a conclusive result would require a convincing rescue of both the neurogenesis and the cognitive function. While the evidence provided by studies of the effects of environmental enrichment is a step in that direction, it falls well short of the mark and this is likely why neurogenesis did not figure prominently into the authors' conclusions (Shih et al., 2012). One plausible approach would be to take advantage of new advances in neural stem cell biology which have allowed for transplantation of exogenous neural stem cells into rodents that go on to develop

and incorporate themselves into neuronal circuitry (Juopperi et al., 2011). Anesthesia exposed animals could undergo a transplantation of exogenous neural progenitor cells and then be assessed for the degree to which this intervention provided a rescue. A further goal that should be addressed is to searh for any evidence of translation to more complex species such as non-human primates. With ongoing primate studies, it should easily be possible to do a comprehensive immunohistochemical screening using a panel of stem cell markers to assess for changes in stem cell populations caused by anesthetic exposure e.g. (Kwak et al., 2015). At this point it is likely that a point of diminishing returns has been reached with further cell culture or rodent in vivo studies that simply ask whether neural stem cells are vulnerable to anesthetics. Further work in these models should be directed towards specific molecular targets of anesthetics in neurogenesis, identifying interventions that are specific to neurogenesis, or testing for differences in the type of exposure paradigm that is required to provoke changes in neurogenesis versus changes in other pathologic manifestations of DAN such as apoptosis or synaptic changes.

While much remains to be done and there is a great deal of uncertainty as to the outcome, investigation into the hypothesis that neurogenesis is a key target in DAN remains a promising one. There is solid evidence that under the right conditions anesthetics can interfere with neurogenesis, and it remains to be determined what the relevance of this to any clinical manifestations of DAN. Furthermore, the fact remains that postnatal neurogenesis is a major developmental event that occurs during the putative window of vulnerability to DAN across species and that could putatively explain the many of the proven or suspected cognitive deficits associated with DAN. Thus, we conclude that further study in this area is warranted, but that it should be focused on the areas outlined above.

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Highlights for Review, Kang et al, Neurogenesis and Developmental Anesthetic Neurotoxicity

- In animal models anesthetic exposure during the early postnatal period can cause alterations in brain development and lasting cognitive dysfunction.
- There are concerns that the same phenomenon could occur in humans.
- Neurogenesis is a critical developmental event that is ongoing during the early postnatal period in humans as well as in primate and rodent animal models of developmental anesthetic neurotoxicity.
- There is evidence that under the right conditions anesthetics can interfere with neurogenesis.
- It is not yet clear whether early postnatal anesthetic effects on neurogenesis could explain lasting toxic effects on cognitive function, and this potential mechanism of injury should be investigated further.