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MicroRNAs: New Players in Anesthetic-Induced Developmental Neurotoxicity

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Abstract

Growing evidence demonstrates that prolonged exposure to general anesthetics during brain development induces widespread neuronal cell death followed by long-term memory and learning disabilities in animal models. These studies have raised serious concerns about the safety of anesthetic use in pregnant women and young children. However, the underlying mechanisms of anesthetic-induced neurotoxicity are complex and are not well understood. MicroRNAs are endogenous, small, non-coding RNAs that have been implicated to play important roles in many different disease processes by negatively regulating target gene expression. A possible role for microRNAs in anesthetic-induced developmental neurotoxicity has recently been identified, suggesting that microRNA-based signaling might be a novel target for preventing the neurotoxicity. Here we provide an overview of anesthetic-induced developmental neurotoxicity and focus on the role of microRNAs in the neurotoxicity observed in both human stem cell-derived neuron and animal models. Aberrant expression of some microRNAs has been shown to be involved in anesthetic-induced developmental neurotoxicity, revealing the potential of microRNAs as therapeutic or preventive targets against the toxicity.

Keywords

microRNAs; Anesthetics; Developmental Neurotoxicity; Stem Cells

Introduction

In 1979, Steen and Michenfelder compiled several clinical case studies into a review article and highlighted the possible issue of anesthetic-induced neurotoxicity [1]. The case reports in this article detailed children having adverse effects to anesthetic administration. However, it was around this time that the terms “apoptosis” and “excitotoxicity” were just being coined and as such, much of the research in the field relied solely on observations in the clinic rather than on experimental findings. It wasn’t until 1999 that Olney and colleagues first reported, using an experimental animal model, that anesthetics could induce toxicity in

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the developing brain and that this toxicity was specific to the period of rapid synaptogenesis in the brain [2, 3]. They observed that blockade of the N-methyl-D-aspartate receptor (NMDA) receptor could induce significant cell death in the brains of 7-day old rat pups and suggested that their findings "may have relevance to human neurodevelopmental disorders involving prenatal (drug-abusing mothers) or postnatal (pediatric anesthesia) exposure to drugs that block NMDA receptors". Since the majority of anesthetic agents act either as type-A γ-aminobutyric acid receptor ($GABA_A$ receptor) agonists or NMDA receptor antagonists, this seminal study raised important questions about the toxic effects of anesthetics on the developing brain. Understanding whether all anesthetics or only a subset of anesthetics could induce this toxicity and the mechanisms by which this toxicity occurs became the focus of additional studies.

Evidence of developmental neurotoxicity from different models

Animal Studies

It has been reported in several studies that the developing brain is most vulnerable to anesthetics during the period of rapid synaptogenesis [4-6]. This is a time in the developing brain in which many synapses are being formed between neurons. This period ranges differently among species. For example, in rodents it lasts about the first 2 weeks of life, while in humans it ranges from about the 3rd trimester of pregnancy through the 2nd or 3rd year of life [7, 8]. Several studies done in developing rodent models found that nearly all anesthetics including isoflurane, sevoflurane, ketamine, propofol and anesthetic combinations could induce cell death in the brains of these animals and lead to learning and memory impairments later in life [9-13]. For example, Shen and colleagues found that postnatal day (PD) 3 Sprague-Dawley rats displayed significant impairments in spatial learning and memory, as assessed by the Morris Water Maze test following a single exposure to 1% sevoflurane [11]. They found that these effects were dose and exposure number dependent. They also found that 7-week-old rats were insensitive to sevoflurane exposure and displayed equivalent performance in the Water Maze test with or without sevoflurane exposure, confirming that vulnerability to anesthetics is confined to an early period in brain development. In addition, Pesic and colleagues have shown that propofol administration induced neuroapoptosis in the cortex and thalamus of PD7 rat pups through an extrinsic pathway and activation of caspase-3 [14]. Liu and colleagues also observed increases in neuroapoptosis in the frontal cortex of PD7 rat pups exposed multiple times to ketamine. They also found that rat pups exposed to ketamine had altered expression of apoptotic related genes as assessed by microarray [15].

Although these initial rodent studies were extremely important, questions were raised about the translatability of these findings to humans. Brambrink, Creeley and others moved to using Rhesus Macaques to study the effects of anesthetics on the developing brain. This allowed for studies on a model more closely related to humans. In addition, the use of larger animals made it easier to monitor hemodynamic properties of the animals to control for confounding variables of anesthetic administration such as cardiovascular, respiratory or metabolic distress. They found that isoflurane, propofol, and ketamine could all induce increases in cell death in both fetal and neonatal rhesus monkey brains with careful control

of hemodynamic properties [16-21]. They also went on to show that the toxic effects of isoflurane and propofol affected both the neuronal and oligodendrocyte populations, but did not appear to affect the astrocytes in the brain [18, 22]. This was an extremely important finding since oligodendrocytes are key supporting cells in the brain and are critical in neuronal myelination [23, 24]. Although astrocytes are important neuronal supporting cells, they are very low in number in the developing brain and increase rapidly throughout development [25]. The findings from these studies suggested that anesthetics could both directly and indirectly induce neuronal cell death in the developing brain. The extent of anesthetic-induced neurotoxicity may depend on the following variables:

- Anesthetic dose, exposure duration, and number of exposures [26, 27]
- The receptor type being activated or inactivated [9, 16]
- Single anesthetic or combination of different anesthetic agents [9]
- The stage of brain development [12, 16, 28]

Clinical significance and human studies

These and other studies conducted in animals led to a push for human epidemiologic studies and prompted the International Anesthesia Research Society (IARS) to partner with the US Food and Drug Administration to form SmartTots (www.smarttots.org), a research initiative aimed at evaluating the safety of anesthetic use in the pediatric population. In 2009, DiMaggio et al. found that children exposed to general anesthesia prior to the age of 3 were twice as likely to be diagnosed with behavioral or developmental disorders as unexposed children [29]. This was a retrospective study of more than 5,000 children enrolled in the New York Medicaid program and the results of this study raised significant safety concerns about the use of anesthetics in children. In 2010, Wilder and colleagues went on to evaluate the medical and educational records of over 5,000 children in a retrospective study aimed at identifying whether anesthetic administration early in life was linked to learning disabilities later on in humans. In this study, 593 of the children had received general anesthesia prior to the age of 4. They found that there was not a significant increase in learning disability diagnoses in children that had received a single administration of anesthesia, but that there was a significant increase in children that had received multiple administrations of anesthesia [30].

Several additional human retrospective studies found that there was a link between early exposure to anesthetics and later learning and behavioral abnormalities [31-33]. However, additional studies found no connection between early exposure to anesthetics and learning and behavioral abnormalities later in life. For example, in 2009 Bartels and colleagues assessed educational achievement and cognitive performance in over 1,100 monozygotic twin pairs from the Netherlands twin registry for which anesthetic exposure data was available. They found that there was a significant increase in learning disabilities in twins exposed to anesthesia when compared to unexposed twins. However, they found no difference between the unexposed and the exposed twin in discordant pairs suggesting that other factors may be responsible for the increases in learning disabilities rather than the anesthetic exposure [34]. In addition, in 2011 Hansen et al. assessed the academic

performance of 2,689 Danish children that had undergone inguinal hernia repair surgery in infancy. An age-matched control group consisting of 14,575 children was also studied. They found that exposed children performed worse academically than their unexposed counterparts. However, they also found that once the data had been controlled for confounding variables, there was not a statistically significant difference between the groups [35].

Despite the large scale efforts of the SmartTots organization, the effect of anesthetics on the developing human brain remains uncertain. However, the number of laboratory studies over the last 7 years aimed at addressing this issue has increased nearly 5-fold [36]. Many human epidemiological studies are still ongoing as well in this field today including the Pediatric Anesthesia NeuroDevelopment Assessment (PANDA) study out of Columbia University, the General Anesthesia Safety (GAS) study from Children's Hospital Boston and the Mayo Safety in Kids (MASK) study being conducted at Mayo Clinic. Although the results of these human epidemiologic studies will be extremely useful, it will be difficult to properly dissect out the effects of anesthetic exposure from the effects of surgery, underlying medical conditions, socioeconomic status and other potentially confounding variables. At this point, there is not sufficient evidence in humans regarding the toxic effects of anesthetics on the developing brain, and as such, clinicians cannot be properly advised on the matter.

The human epidemiological studies have been unable to determine the safety of anesthetic use in the pediatric population and millions of children are exposed to anesthetics every year in the United States alone [37]. It is critical to develop a better human model by which to study anesthetic-induced developmental neurotoxicity and the mechanisms responsible for this toxicity in order to better guide clinicians and to develop possible preventative strategies. The emerging model of human embryonic stem cell-derived neurons has allowed us to directly assess the effects of anesthetics on developing human neurons and dissect out the mechanisms by which these anesthetics induce toxicity.

Human embryonic stem cell-derived neuron studies

Embryonic stem cells (ESCs) are cells derived from the inner cell mass of a blastocyst. The human embryo reaches the blastocyst stage (a pre-implantation stage) at day 4-5 post fertilization [38]. ESCs are inherently pluripotent, meaning that they can differentiate into cells from all three germ layers (ectoderm, endoderm, and mesoderm) and can replicate indefinitely. This, along with their high differentiation efficiency makes them more advantageous than other stem cell types such as adult stem cells. In 1981, Evans and Kaufman from the University of Cambridge and Martin from the University of California San Francisco discovered mouse ESCs and reported new techniques for culturing these cells *in vitro* [39, 40]. The establishment of these critical *in vitro* techniques opened the doors to many studies aimed at understanding development and disease. It wasn't until 1998 that James Thompson and colleagues at the University of Wisconsin-Madison developed a technique to isolate and culture human ESCs (hESCs) *in vitro* [38]. The seminal work of this group allowed for mechanistic based studies using a human cell line. This eliminated potential concerns regarding the relevancy of animal models to humans.

The generation of neurons from hESCs was first reported in 2001 by several groups. Carpenter and colleagues reported a method for deriving neurons from hESCs using embryoid body (EB) formation and immunoselection [41]. They found that the derived cells stained positive for neuron-specific markers, responded to neurotransmitter application, and displayed voltage-dependent channels on their cell surface. However, these initial protocols involved multi-step approaches and the immunoselection required to purify the populations could result in a mixed population of cells. In 2003, Schulz and colleagues reported a novel technique for the directed differentiation of neurons from hESCs [42]. This protocol involved EB and rosette formation.

Our group followed a similar approach and improved the efficiency of the differentiation by manual selection of rosettes. Neuronal differentiation was observed by morphological assessment in the culture after six days of culturing the hESC-derived neural stem cells (NSCs) in neuronal differentiation medium. Differentiated neurons exhibited round cell bodies with small projections. Two-week-old neurons expressed the neuron-specific marker β-tubulin III, the synaptic marker synapsin-1, the postsynaptic protein Homer 1 [43, 44], and immature neuron marker doublecortin [45]. In addition, differentiated neurons exhibited functional synapses [46]. Development of an *in vitro* neurogenesis system using human stem cells has opened up avenues of research for advancing our understanding of human brain development and the issues relevant to anesthetic-induced developmental toxicity in human neuronal lineages under controlled conditions. Recent studies from our and other groups, showed that isoflurane influenced human NSC proliferation and neurogenesis [47] and ketamine dose- and time-dependently induced hESC-derived neuron death [43]. Additionally, when cells underwent different lengths of exposure to propofol and were subjected to single and multiple exposures, propofol induced cell death in the hESC-derived neurons in a time, dose, and exposure number-dependent manner [45]. These findings in stem cell-derived human neurons have recapitulated the results of the animal and human epidemiologic studies [26, 27] [30].

Current mechanisms of anesthetic-induced developmental neurotoxicity

Despite many findings in animal models that anesthetics induce neurotoxicity in the developing brain, the mechanisms by which this toxicity occurs remain largely unknown. A possible role for calcium signaling, reactive oxygen species (ROS) production, mitochondrial abnormalities, neuroinflammation, and epigenetic changes in the mechanism of anesthetic-induced neurotoxicity have all been reported [43, 48-50]. Sinner et al. found that exposure of cultured rat hippocampal neurons to ketamine resulted in increases in intracellular calcium and neuronal apoptosis [51]. Intracellular calcium levels are tightly regulated under normal conditions. Persistent elevation of intracellular calcium, beyond normal levels, can induce apoptosis [52]. In response to ketamine, there was a significant increase in ROS production in the cytosol and superoxide generation within mitochondria in ketamine-treated hESC-derived neurons, indicating a mitochondrial origin of ROS. Trolox, a ROS scavenger, prevented ketamine-induced ROS production and apoptosis in differentiated human neurons [43]. Several animal studies have suggested that accumulation of ROS was associated with anesthetic-induced mitochondrial damage [53, 54]. Application

of the antioxidant (7-nitroindazole, a nitric oxide synthase inhibitor) attenuated ketamine-induced rat forebrain-derived cultured neuronal cell death [55].

Although many different mechanisms and pathways have been implicated to play a role in anesthetic-induced neurotoxicity, the mitochondria appear to play key roles in this process through their crucial involvement in cellular processes and apoptosis [56]. Ketamine-induced apoptosis in stem cell-derived human neurons was accompanied by a significant decrease in mitochondrial membrane potential and an increase in cytochrome c release from mitochondria into the cytosol. In addition, most control neurons showed elongated and inter-connected, tubular mitochondria while much shorter and smaller mitochondria were prevalent in the ketamine-treated culture [43]. Dynamin-related protein 1 (Drp1) is a key regulator of mitochondrial fission and is primarily distributed in the cytoplasm of a healthy cell, but shuttles between the cytoplasm and the mitochondrial surface. It has also been shown that exposure of neonatal rat pups to general anesthetics induced significant decreases in the level of Drp1 in the cytosol and increases in mitochondrial Drp1 levels [57], leading to increases in mitochondrial fission. Inhibition of mitochondrial fission was shown to prevent mitochondrial cytochrome c release and apoptotic cell death [58]. Loss of mitochondrial membrane potential and release of cytochrome c from mitochondria are key events in initiating mitochondria-related apoptosis [59], indicating that the increased mitochondrial fission possibly plays an important role in the toxic effects of general anesthetics.

It was also reported that neuroinflammation was involved in anesthetic-induced neurotoxicity and cognitive impairment in the developing mouse brain. Exposure to 3% sevoflurane for 2 hours daily for 3 days induced cognitive impairment and neuroinflammation (e.g., increased interleukin-6 levels) in the developing mouse brain but not in adult mice. Anti-inflammatory treatment (ketorolac) attenuated the cognitive impairment, implicating neuroinflammation as a key mediator of anesthetic-induced neurotoxicity [49]. Additionally, alterations in the levels of a variety of neurotrophins have been implicated in anesthetic-induced developmental neurotoxicity. It was observed that exposure of rat pups to propofol induced a significant decrease in the level of nerve growth factor in the thalamus , a protein that is critical for the survival and growth of neurons. Propofol exposure was also shown to alter the expression levels of a variety of key neurotrophic factor receptors and downstream targets such as Akt and Erk [60]. It was recently reported by Han and colleagues that exposure of 7-day old mouse pups to 2 hours of 1.5% sevoflurane increased the phosphorylation of methyl-CpG island binding protein 2 in the hippocampus. The sevoflurane-induced increases of neuronal cell death and phosphorylation of methyl-CpG were reversed by pre-treatment with memantine, a partial antagonist of the NMDAR [48], suggesting that sevoflurane-induced epigenetic alterations might also play important roles in the neurotoxicity. Table 1 depicts example anesthetic-induced developmental neurotoxicity studies and the key findings from these studies.

Despite these findings, the current neurotoxicity mechanisms are incomplete and work remains to be done to fully elucidate these pathways. A potential functional role for microRNAs in anesthetic-induced developmental neurotoxicity has recently emerged.

microRNAs and anesthetic-induced developmental neurotoxicity

MicroRNAs

Mature microRNAs are small non-coding RNA molecules that are approximately 22 nucleotides in length [61]. microRNAs are highly conserved and are believed to be critical components in evolution [62]. MicroRNAs can bind with perfect or imperfect complementary binding to target messager RNA (mRNA), leading to downregulation of protein expression through mRNA cleavage or translational repression [63, 64]. A single microRNA can have multiple mRNA targets and one mRNA can be regulated by one or multiple miRNAs.

As depicted in Figure 1, microRNAs are transcribed in multiple hairpin structures in the nucleus by RNA polymerase II as large primary transcripts (pri-miRNAs). The pri-miRNAs are cleaved by the RNase III enzyme Drosha in the nucleus into hairpin loops called precursor microRNAs (pre-miRNAs). The resulting pre-miRNAs are approximately 70-nucleotides in length. The pre-miRNAs are exported out into the cytoplasm by Exportin 5. Once in the cytoplasm, the pre-miRNAs are further processed by the RNase III enzyme Dicer which removes the hairpin loop forming the mature miRNA strands. The 2 strands unwind and the more thermodynamically unstable strand typically degrades. The mature microRNA strand then incorporates into the RNA-Induced silencing complex (RISC) where it can act to induce silencing of its target mRNA [61, 64].

The first microRNA was discovered in 1993 by Lee and colleagues in C. Elegans [65]. However, it wasn't until 2000 that they were recognized as a distinctive group of RNA molecules responsible for mRNA regulation [66, 67]. In the decade following their initial discovery, microRNA research flourished and their importance in cancer and the heart was quickly discovered along with suitable approaches to manipulate their expression. Thousands of microRNAs have since been identified in various organisms through random cloning and sequencing or computational prediction and shown to be involved in the regulation of almost every cellular event in developmental and physiological processes [68]. Dysregulation of microRNAs has been reported to play a fundamental role in the onset, progression, and dissemination of many human diseases including neurodegeneration, and as such, they have become attractive therapeutic targets.

For example, microRNAs are highly enriched in the brain and have been implicated to play important roles in memory, neurogenesis, synaptic plasticity, and neuronal degeneration [69]. Several studies have cited dysregulation of microRNA expression in the postmortem brain tissue of neurodegenerative disease patients. One study found that there was a downregulation of the brain-specific microRNAs: miRs-9, -29b, and -181 in Alzheimer's disease patients [70] while another study revealed that similar and additional brain-specific microRNAs including miRs-9, -29b, -124a, and -132 were down-regulated in the brains of Huntington's disease patients [71]. However, the potential functions of microRNAs in anesthetic-induced neurotoxicity are just starting to be investigated. More recently, five studies from our and other laboratories pointed to important roles of several microRNAs (e.g., miR-21, miR-34a, miR-34c, miR-124, and miR-137) in anesthetic-induced

developmental neurotoxicity using various experimental models [45, 72-75]. These studies have been summarized and are shown in Table 2.

miR-21

MicroRNA-21 (miR-21) was one of the first microRNAs discovered in humans and its sequence was found to be highly conserved across species [76]. The human miR-21 gene is located within a coding gene known as vacuole membrane protein-1 on chromosome 17q23.2. Despite being located within a coding gene, the human miR-21 gene contains its own promoter and can be transcribed independently [77]. miR-21 has been identified to be involved in many cancers and is a well-established anti-apoptotic factor. Dysregulation of miR-21 has been shown to mediate hypoxia-induced neuroapoptosis [78] while overexpression of miR-21 decreased apoptosis in a rat model of traumatic brain injury [79]. In addition, exposure of fetal cerebral cortical-derived neuroepithelial cells to ethanol, an NMDA receptor antagonist and $GABA_A$ receptor agonist, was shown to suppress miR-21 [80].

Recently, Twaroski et al used hESC-derived neurons for the first time to study microRNA mechanisms governing anesthetic-induced neurotoxicity by exposing 2-week old neurons to 6 hours of 20 μg/mL propofol or the vehicle control, dimethyl sulfoxide. To examine whether microRNAs were playing a role in the observed propofol-induced toxicity, 84 of the most abundantly expressed microRNAs were screened using commercially available qRT-PCR arrays. They found that 20 microRNAs were significantly downregulated following exposure to propofol when compared to vehicle-treated cells [45]. Of these 20 microRNAs, several were of interest based upon their established roles in either physiological or pathological processes. For example, the let-7 family has been shown to be highly expressed in the brain and is important in stem cell differentiation and apoptosis [81]. In addition, miRs 9 and 124 have been shown to play a role in neuronal differentiation [82]. The target of greatest interest was miR-21 which is a well-established anti-apoptotic factor [83, 84].

To confirm that miR-21 was playing a role in the propofol-induced neurotoxicity, miR-21 was artificially up-regulated and knocked down in the stem cell-derived neurons using lipofectamine and a miR-21 mimic and antagomir, respectively. The results showed that miR-21 overexpression attenuated the propofol-induced cell death while miR-21 knockdown exacerbated the effects. There are many established upstream regulators of miR-21 including signal transducer and activator of transcription 3 (STAT3) [85-87]. All members of the STAT family translocate to the cell nucleus once activated by phosphorylation where they act as transcriptional activators [88, 89]. STAT3 was first discovered in 1994 and is activated when phosphorylated at the Tyrosine 705 position [90, 91]. Following propofol exposure, pSTAT3 expression in the neurons was significantly reduced but was not altered following manipulation of miR-21 expression, suggesting that STAT3 may be an important upstream regulator of miR-21 in propofol-induced neurotoxicity [45].

miR-21 also has thousands of established and predicted targets. Of these targets, programmed cell death protein 4 (PDCD4), Sprouty 1 and 2 and phosphatase and tensin homolog (PTEN) are, arguably, the most well studied [92]. There was no change in the expression of PTEN in hESC-derived neurons following propofol exposure, indicating that

PTEN is not involved in this pathway. However, Sprouty 2 expression was significantly increased while the level of activated Akt, a serine/threonine kinase that is involved in many cell survival pathways through inhibition of apoptotic processes [93, 94], was reduced in the propofol-treated hESC-derived neurons. Sprouty 2 knockdown in the hESC-derived neurons using a small interfering RNA (siRNA)-mediated approach significantly attenuated the propofol-induced neuron death and the decrease in activated Akt expression. The authors concluded that Sprouty 2 is the direct target of miR-21 in the neurotoxicity and propofol induced toxicity in human stem cell-derived developing neurons possibly through a STAT3/miR-21/Sprouty 2/Akt dependent mechanism [45].

miR-34a

microRNA-34a (miR-34a) belongs to the miR-34 family of microRNAs comprising three processed microRNAs (miR-34 a/b/c) that are encoded by two different genes. miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript [95]. The hippocampus is an area of the brain involved in learning and memory and has been shown to be a key site of neurotoxicity following exposure to anesthetics in developing animals. In the hippocampus, p53 targets the miR-34 family and this family of microRNAs is essential for cortical brain development [96]. One recent publication indicates the important role of miR-34a in ketamine-induced hippocampal apoptosis and memory impairment through fibroblast growth factor receptor 1 (FGFR1). In this study, one-month old C57/BL6 mice received daily intraperitoneal injections of anesthesia (ketamine, 50 mg/kg) for 7 days. Ketamine induced apoptosis of neurons in region I of the hippocampus [cornus ammonis (CA)1] and upregulated hippocampal miR-34a expression. Lentivirus-mediated inhibition of miR-34a protected against ketamine-induced neuroapoptosis and memory impairment. Luciferase assay demonstrated that FGFR1 was directly regulated by miR-34a in hippocampus and siRNA-induced FGFR1 down-regulation further exaggerated ketamine-induced neuroapoptosis in hippocampus [74]. These findings suggest an important role for miR-34a and FGFR1 in ketamine-induced neurotoxicity.

miR-34c

microRNA-34c (miR-34c) also belongs to the miR-34 family of microRNAs. miR-34c has been implicated to play a role in Alzheimer's disease (AD) [97, 98]. The expression level of miR-34c was increased in both cellular and plasma components of AD patients' circulating blood samples compared to normal age-matched controls. Overexpression of miR-34c in cultures of human embryonic kidney cells (HEK 293) repressed the expression of targets such as Bcl2, SIRT1, Psen1, and Onecut2 that are involved in cell survival and oxidative defense pathways [98], suggesting that increased miR-34c may be one of many factors contributing to an overall systemic weakening of stress defense mechanisms and cell survival in AD patients.

Zhang and colleagues found that miR-34c was upregulated in the hippocampus of neonatal mice exposed to ketamine. They also showed that downregulation of miR-34c could attenuate the ketamine-induced neuronal cell death and cognitive impairment observed in the animals. Knocking down miR-34c activated the protein kinase C (PKC)/extracellular-signal regulated kinase (ERK) pathway, upregulated antiapoptotic protein BCL2, and

ameliorated ketamine-induced apoptosis in the hippocampus. Cognitive examination with the Morris water maze test showed that ketamine-induced memory impairment was significantly improved in the animals by miR-34c downregulation [72]. Thus, miR-34c is appears to be important in regulating ketamine-induced developmental neurotoxicity in the hippocampus.

miR-124

MicroRNA-124 (miR-124) is the most abundant microRNA expressed in the vertebrate central nervous system (CNS). miR-124 is expressed in neurons but not astrocytes and the levels of miR-124 increase over time in the developing CNS. miR-124 is involved in neuronal maturation and differentiation and downregulation of miR-124 has been linked to apoptosis [99]. miR-124 was also required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression [100, 101] and has been shown to induce neurite elongation by indirectly targeting Akt [102]. Xu and colleagues reported that miR-124 was upregulated in the hippocampus of neonatal mice exposed to high doses of ketamine. They found that lentivirus-mediated knockdown of miR-124 reduced ketamine-induced apoptosis in hippocampal CA1 neurons *in vitro* and upregulated the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor phosphorylation and activated PKC-ERK pathway [103]. Morris water maze test demonstrated that miR-124 knockdown improved memory performance of mice treated with ketamine, indicating that inhibiting miR-124 may provide a molecular target to attenuate the neurotoxicity.

miR-137

MicroRNA-137 (miR-137) has been shown to regulate neuronal maturation and dendritic morphogenesis during development [104]. Dysregulation of miR-137 expression may be associated with the pathogenesis and development of Alzheimer's disease [105]. Huang et al showed that upregulation of miR-137 protected against ketamine-induced hippocampal neurodegeneration in young rats [75]. In this study, 1 month old-Sprague-Dawley rats were systemically administrated ketamine (75 mg/kg) once per day for 3 days. Ketamine treatment resulted in neuroapoptosis in the hippocampal CA1 region, down-regulation of miR-137 in the hippocampus, and long-term memory dysfunction. Conversely, overexpression of miR-137 protected the brain against ketamine-induced neuroapoptosis and memory loss [75].

Conclusions and future directions

Mounting evidence from animal, epidemiology, and human stem cell-derived neuron models have shown that anesthetics can induce developmental neurotoxicity. The mechanisms governing the neurotoxicity are likely extremely complex and involve many converging or diverging pathways. Three recent studies from different groups began to clarify the role of microRNAs in anesthetic-induced developmental neurotoxicity using different models. These studies depicted altered profiles of microRNAs in developing human neurons and neonatal rodent brains in response to administration of the intravenous anesthetic drugs propofol or ketamine. Specifically, propofol downregulated miR-21 in stem cell-derived human neurons while ketamine upregulated miR-34a, miR-34c, and miR-124, and

downregulated miR-137 in the mouse and rat hippocampus, respectively. Upregulation of miR-21 and miR-137 or downregulation of miR-34a, miR-34c, and miR-124 attenuated the neurotoxicity conferred by the anesthetics [45, 72-75], suggesting a functional role of these microRNAs in the neurotoxicity. However, the roles of microRNAs in anesthetic-induced developmental neurotoxicity are just beginning to be understood. Despite these findings, there are several important questions left unanswered and these are described below.

Role of other microRNAs in the neurotoxicity conferred by different anesthetic drugs

Since there are over one thousand microRNAs that have been found or predicted to be important in physiology and pathophysiology, it is very likely that other microRNAs could participate in the multiple facets of anesthetic-induced developmental neurotoxicity including neuroapoptosis and other brain developmental events (e.g., neurogenesis, NSC proliferation, and synaptogenesis). Future studies in this area may include expanding the focus of the current studies to identify additional microRNAs that may play a role in the observed anesthetic-induced toxicity. For example, Twaroski et al showed that 20 microRNAs were significantly down-regulated in stem cell-derived human neurons following exposure to propofol, many of which had been implicated in neurological development and disease. For instance, downregulation of miR-9 has been associated with several neurodegenerative diseases and it is known to be involved in neuronal differentiation [71, 106]. Interestingly, a recent study found that miR-9 is involved in neural lineage differentiation of mouse ESCs and this appears to be mediated by STAT3 [107]. Thus, miR-9 may be potential target to pursue in the future.

Role of microRNAs in the neurotoxicity conferred by different anesthetic drugs

As described above, the three published microRNA-based neurotoxicity studies were only focused on the effects of propofol and ketamine [45, 72-75]. However, many other anesthetic agents are used clinically such as sevoflurane and isofluranethat have been shown to induce developmental neurotoxicity either alone or in combination with other anesthetics in animal models. As such, it will also be critical to understand how microRNAs are altered when hESC-derived neurons or young animals are exposed to different anesthetic agents. Future studies focusing on these effects will be very important in understanding whether the mechanisms identified in the previous microRNA studies are relevant to all anesthetic agents or combinations of anesthetics or only to propofol or ketamine and will be critical for the future development of neuroprotective strategies.

Additional microRNA Targets

One microRNA can target many mRNAs. For instance, there are currently thousands of established and predicted targets of miR-21. The study by Twaroski et al focused on the role of Sprouty 2, a direct target of miR-21 in the propofol-induced neurotoxicity. Although Sprouty 2 did appear to play a key role in the propofol-induced neuronal cell death, the role of additional miR-21 targets cannot be ruled out since the knockdown of Sprouty 2 in this study only partially attenuated the propofol-induced cell death [45]. This could be due to the incomplete knockdown of Sprouty 2 or it could suggest a role for additional miR-21 targets. The expression of programmed cell death protein 4 (PDCD4), another direct target of miR-21 was upregulated following exposure to propofol as assessed by Western blot [108].

Nevertheless, future studies will need to include examination of the functional contribution to the observed toxicity of changes in PDCD4 expression and may focus on understanding the intricate balance between miR-21 expression and the expression of its many targets, and how shifts in that balance might be involved in anesthetic-induced neurotoxicity. The use of microRNA target arrays would allow for the assessment of changes in the expression of many predicted and validated targets of individual microRNAs of interest following exposure to anesthetics.

Taken together, the most recent studies suggest a novel microRNA-related mechanism by which propofol and ketamine, widely used anesthetic agents, induce cell death in developing human neurons and animal models, implicating an important role for microRNAs in anesthetic-induced neurotoxicity and further expanding the understanding of how anesthetic agents induce neuronal toxicity. Nevertheless, detailed mechanisms are still poorly understood. Millions of children are exposed to anesthetic agents every year and many of those procedures are unavoidable. Understanding the mechanisms by which anesthetics induce neurotoxicity is critical in order to prevent adverse neurological outcomes following anesthetic exposure in the developing brain. The microRNA findings might lead to the development of novel protective approaches aimed at mitigating the neurotoxic effects of anesthetics in young children.

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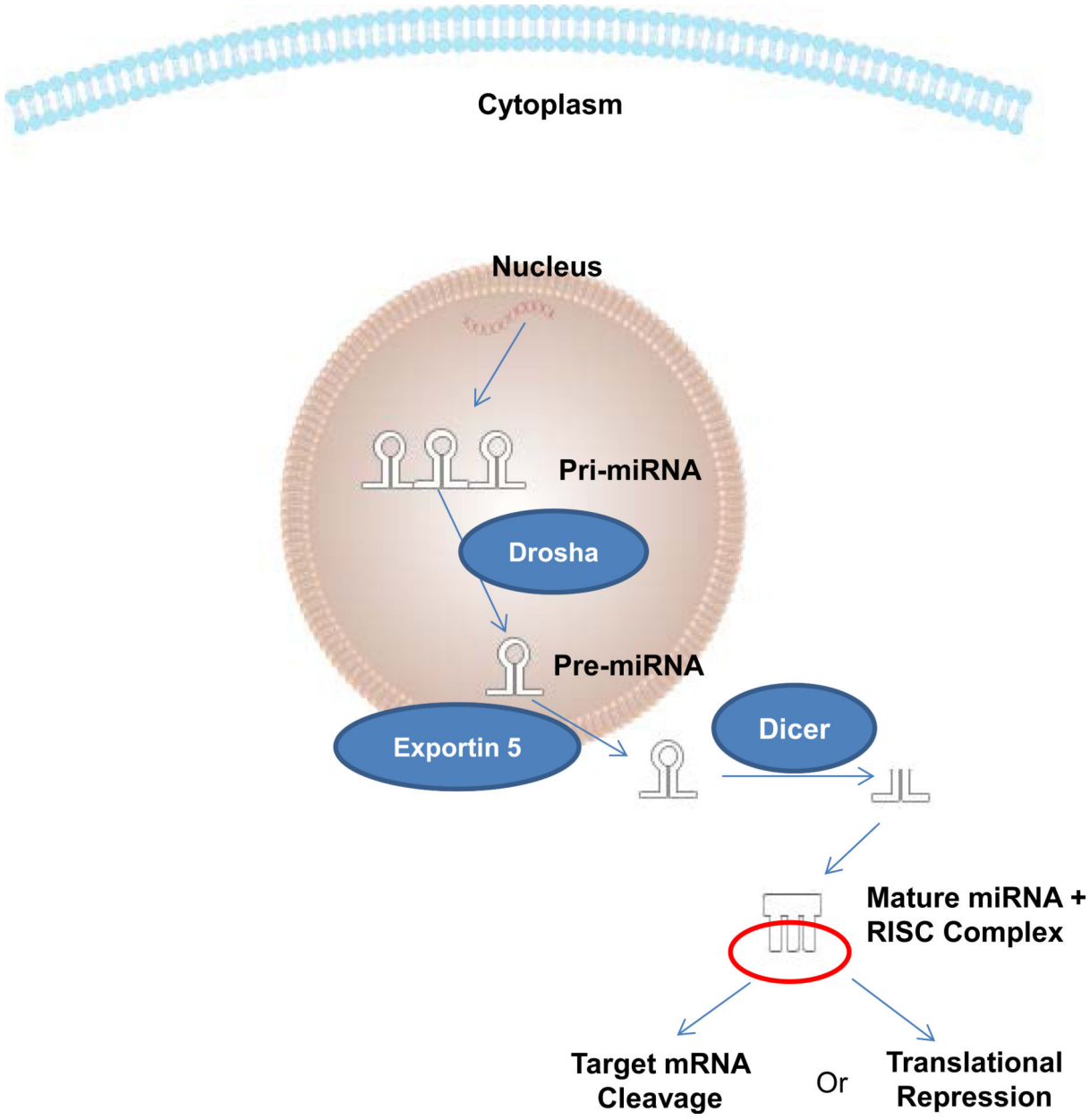

Figure 1. MicroRNA (miRNA) biogenesis and mechanisms of action

miRNAs are transcribed in one or multiple hairpin structures in the nucleus by RNA polymerase II as large primary transcripts (pri-miRNAs). The pri-miRNAs are cleaved in the nucleus into shorter single hairpin loop called precursor miRNAs (pre-miRNAs) by the enzyme Drosha. The pre-miRNAs are exported out into the cytoplasm by Exportin 5. Once in the cytoplasm, the pre-miRNAs are further processed by the enzyme Dicer which removes the hairpin loop and forms the mature double-stranded miRNA. The two strands then unwind and incorporate into the RNA-Induced Silencing Complex (RISC), resulting in the mRNA cleavage or translational repression followed by downregulation of protein expression.

Table 1

The representative animal studies regarding anesthetic-induced developmental neurotoxicity

Anesthetic	Dose/Duration	Model	Main findings	Reference
Sevoflurane	3%, 6 hours	PD7 Sprague-Dawley rats	Sevoflurane elevated caspase-3 activation and ROS levels, decreased mitochondrial cardiolipin contents, altered cellular ultrastructure in the cerebral cortex and metabolic pathways of glucose and intracellular antioxidants.	Liu, et al.[109]
Ketamine	25 μM, 24 hours	Hippocampal neuron cultures from 19-day-old Wistar rat embryos	Ketamine exposure significantly increased the number of apoptotic neurons and the cytosolic calcium concentration. Ketamine also led to a down-regulation of the CaMKII and a decrease in synapsin.	Sinner, et al.[51]
Sevoflurane	1.5%, 2 hours	PD7 mouse pups	Sevoflurane increased the phosphorylation of methyl-CpG island binding protein 2 in the hippocampus and sevoflurane-induced increases of neuronal cell death and phosphorylation of methyl-CpG were reversed by pre-treatment with memantine, a partial antagonist of the NMDAR	Han, et al.[48]
Propofol	25 mg/kg, 1 dose	PD14 Wistar rats	Propofol induced a significant decrease in the level of nerve growth factor in the thalamus and altered the expression levels of a variety of key neurotrophic factor receptors and downstream targets such as Akt and Erk.	Popic, et al.[60]
Midazolam/Nitrous Oxide/Isoflurane	9 mg/kg midazolam, 75% NO, 0.75% iso, 6 hours	PD7 Sprague-Dawley rats	Anesthesia exposure up-regulated reactive oxygen species generation and down-regulated superoxide dismutase. Exposure to the anesthesia was also associated with increased mitochondrial fission.	Boscolo, et al.[57]
Ketamine	100 μM, 24 hours	Human stem cell-derived neurons	Ketamine exposure increased neuronal apoptosis, ROS production, and mitochondrial fission, implicating mitochondrial dysfunction as a key mechanism by which ketamine induces neurotoxicity.	Bai, et al.[43]
Isoflurane	Surgical dose, 5 hours	Fetal rhesus macaques	Isoflurane induced a significant increase in apoptosis of neurons and oligodendrocytes in the fetal monkey brain.	Creeley, et al.[110]

Table 2

Studies depicting a role of microRNAs in anesthetic-induced developmental neurotoxicity

Anesthetic	Model	MicroRNA	Main Findings	Reference
Propofol	Human embryonic stem cell-derived neurons	miR-21	The expression of miR-21 was downregulated following exposure to 6 hours of 20 μg/mL propofol. Overexpression of miR-21 attenuated the propofol-induced cell death. The toxicity occurred through a STAT3/miR-21/Sprouty 2/Akt-dependent mechanism.	Twaroski, et al. [45]
Ketamine	Neonatal mice	miR-34c	miR-34c was upregulated in the hippocampus of neonatal mice exposed to ketamine and downregulation of miR-34c attenuated the ketamine-induced neuronal cell death and cognitive impairment observed in the animals.	Cao, et al.[72]
Ketamine	Neonatal mice	miR-124	miR-124 was upregulated in the hippocampus of neonatal mice exposed to ketamine and knockdown of miR-124 reduced ketamine-induced apoptosis in hippocampal CA1 neurons *in vitro* and activated the PKC-ERK pathway. miR-124 knockdown improved memory performance of mice treated with ketamine.	Xu, et al.[73]
Ketamine	One-month old C57/BL6 mice	miR-34a	Exposure to 50 mg/kg ketamine for 7 days induced apoptosis in hippocampal CA1 neurons and upregulated hippocampal miR-34a. Inhibition of miR-34a protected against anesthesia-induced neuroapoptosis and memory impairment while knockdown of its target, FGFR1 exacerbated the toxicity.	Jiang, et al.[74]
Ketamine	One-month old Sprague-Dawley rats	miR-137	Exposure to 75 mg/kg ketamine for 3 days induced apoptosis in hippocampal CA1 neurons, downregulation of miR-137 in the hippocampus, and long-term memory impairment. Overexpression of miR-137 protected against hippocampal neurodegeneration and memory loss.	Huang, et al. [75]