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MINIREVIEWS

Neuroendocrine, epigenetic, and intergenerational effects of general anesthetics

Anatoly E Martynyuk, Ling-Sha Ju, Timothy E Morey, Jia-Qiang Zhang

ORCID number: Anatoly E Martynyuk (0000-0002-9915-5584); Ling-Sha Ju (0000-0001-7266-0794); Timothy E Morey (0000-0003-2922-8984); Jia-Qiang Zhang (0000-0002-1367-9644).

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Anatoly E Martynyuk, Department of Anesthesiology and the McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, United States

Ling-Sha Ju, Timothy E Morey, Department of Anesthesiology, University of Florida College of Medicine, Gainesville, FL 32610, United States

Jia-Qiang Zhang, Department of Anesthesiology and Perioperative Medicine, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Zhengzhou 450003, Henan Province, China

Corresponding author: Anatoly E Martynyuk, DSc, PhD, Professor, Department of Anesthesiology and the McKnight Brain Institute, University of Florida College of Medicine, 1600 SW Archer Road, PO Box 100254, Gainesville, FL 32610, United States.
amartynyuk@anest.ufl.edu

Abstract

The progress of modern medicine would be impossible without the use of general anesthetics (GAs). Despite advancements in refining anesthesia approaches, the effects of GAs are not fully reversible upon GA withdrawal. Neurocognitive deficiencies attributed to GA exposure may persist in neonates or endure for weeks to years in the elderly. Human studies on the mechanisms of the long-term adverse effects of GAs are needed to improve the safety of general anesthesia but they are hampered not only by ethical limitations specific to human research, but also by a lack of specific biological markers that can be used in human studies to safely and objectively study such effects. The latter can primarily be attributed to an insufficient understanding of the full range of the biological effects induced by GAs and the molecular mechanisms mediating such effects even in rodents, which are far more extensively studied than any other species. Our most recent experimental findings in rodents suggest that GAs may adversely affect many more people than is currently anticipated. Specifically, we have shown that anesthesia with the commonly used GA sevoflurane induces in exposed animals not only neuroendocrine abnormalities (somatic effects), but also epigenetic reprogramming of germ cells (germ cell effects). The latter may pass the neurobehavioral effects of parental sevoflurane exposure to the offspring, who may be affected even at levels of anesthesia that are not harmful to the exposed parents. The large number of patients who require general anesthesia, the even larger number of their future unexposed offspring whose health may be affected, and a growing number of neurodevelopmental disorders of unknown etiology underscore the translational importance of investigating the intergenerational effects of GAs. In this mini review, we discuss emerging

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experimental findings on neuroendocrine, epigenetic, and intergenerational effects of GAs.

Key words: Brain; General anesthetic; Sevoflurane; Corticosterone; Cortisol; Histone acetylation; Deoxyribonucleic acid methylation; Intergenerational effects; Gamma aminobutyric acid

Core tip: The GABAergic general anesthetics may act as stressors and endocrine disruptors in neonates and young adults. They may induce two distinct types of long-term adverse effects: Neuroendocrine effects (the somatic effects) and epigenetic reprogramming of germ cells (the germ cell effects). The latter may pass neurobehavioral abnormalities to male offspring. Compared to the somatic cells, the germ cells may be more sensitive to the deleterious effects of general anesthetics, raising the possibility that the offspring may be affected even when levels of anesthesia are not harmful to the exposed parents. Further rigorous experimental testing of all these possibilities is required.

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INTRODUCTION

The number of surgeries performed globally has rapidly increased from 226.4 million in 2004 to 312.9 million in 2012, according to 2016 World Health Organization estimates[1]. Most of these surgeries and many non-surgical procedures require general anesthesia, which can be viewed as a state of pharmacologically induced "reversible brain coma"[2]. Despite complete reversibility of the primary effect of general anesthetics (GAs), *i.e.*, induction of the general anesthesia state, many studies in humans and almost all studies in laboratory animals provide evidence that GAs may leave persistent footprints of their brief presence in the body, *i.e.*, anesthetic exposure may lead to long-lasting functional abnormalities[3-7]. Investigations of anesthesia-induced abnormalities are currently restricted primarily to evaluating neurocognitive function in the exposed subjects[8-12], although it is biologically plausible that GAs may affect other functions/systems because their actions are not limited to neuronal effects involved in the mediation of a general anesthesia state. Still, as a result of these studies, the long-term adverse effects of GAs are an increasingly recognized health concern, especially in the very young and elderly[3-7]. The adverse effects of GAs during the perinatal period are of special concern[13-17] because mounting evidence indicates that at this stage of life the central nervous system and other body systems are highly susceptible to reprogramming by environmental factors/stressors[18-21]. Such environmental factors may include GAs, given the multiple molecular targets known to mediate their actions in the brain and throughout the body[22-25]. In support of this contention are reports of learning disabilities, long-term memory impairment, and attention-deficit hyperactivity disorders in patients who had anesthesia early in life[3,5,11,26,27]. Although several recent studies have not found negative neurocognitive consequences of relatively short (≤ 1 h) anesthesia exposures in children[6,7,28], both clinical and laboratory studies, including the most recent clinical assessments[29], agree that prolonged or repeated exposures to GAs that are frequently required for very sick children may result in significant neurocognitive abnormalities later in life[30]. Furthermore, a recent report of the effects on brain development of relatively short anesthesia exposure for cesarean delivery[31] further confirms that the current understanding of this phenomenon remains in an early stage.

Because of a widely accepted dogma that the brain is most susceptible to the deleterious effects of environmental stressors at the extreme of ages, investigations of the long-term adverse effects of GAs in young adults are scarce even in animal models. Several studies have assessed the effects of GAs in young adult rats,

primarily using rats of this age as comparisons to other age groups[4,32-34]. Aside from the fact that these studies found long-term effects of isoflurane in young adult rats, different isoflurane concentrations and exposure regimens make it difficult to compare the effects across these studies. Clearly, further research is needed to elucidate the full range of long-term effects of GAs in young adults. Importantly, the germ cells, which pass the genetic and epigenetic information from parents to offspring, can be susceptible to epigenetic reprogramming by environmental factors throughout the lifespan[35-39].

Laboratory and clinical studies provide evidence that alcohol, stress, endocrine disruptors, obesity, and even physical exercise may affect embryonic development and the phenotype of the offspring[40-44]. GAs share many molecular mechanisms of action with alcohol[45-49] and may act as endocrine disruptors and environmental stressors in animal models and humans. The spectrum of molecular actions of GAs and susceptibility of germ cells to epigenetic reprogramming by environmental factors across the lifespan support the possibility that the offspring may be affected by parental exposure to GAs regardless of the parental age at the time of exposure to GAs. In this mini review, we discuss emerging experimental findings on the neuroendocrine, epigenetic, and intergenerational effects of GAs.

NEUROENDOCRINE, EPIGENETIC, AND SECOND-GENERATION EFFECTS OF GENERAL ANESTHETICS

Research studies on the epigenetic multigenerational effects of environmental factors, such as alcohol, stress, endocrine disruptors, and others, have changed our thinking about the susceptibility of somatic and germ cells to alterations by environmental factors and the persistence of such alterations not only across the lifespan, but also through generations[36-38,40,42]. The potential of GAs to induce similar epigenetic effects, including reprogramming of the germ cell epigenome, and by extension intergenerational effects is supported by the notion that GAs share many molecular mechanisms of action with alcohol and may act as endocrine disruptors and environmental stressors in animal models and humans[45-49].

Endocrine disruptors can broadly be defined as agents that interfere with the functioning of the endocrine system. In support of the neuroendocrine effects of GAs are reports of significant rises in cortisol levels in pediatric patients after surgery or after anesthesia without surgery in healthy children[50,51]. Also, measurements of salivary cortisol levels in response to different levels of sedation in healthy children found a more than threefold increase in cortisol values, with the highest cortisol levels during the recovery phase[52]. Adult patients who received isoflurane-based tracheal general anesthesia compared to those who received bupivacaine-based epidural anesthesia had more than two times higher plasma levels of cortisol at the end of surgery, which was also more than four times higher compared to baseline levels in the same patients[53]. For a recent comprehensive review on cortisol levels associated with anesthesia/surgery see[54]. Importantly, our findings in rodents support the notion that GABAergic anesthetics act *via* specific molecular mechanisms to induce stress-like responses, rather than that GA-caused increases in glucocorticoid levels are the result of the systemic stress response because of uncontrolled physiological parameters during anesthesia. Thus, in neonatal rats, the gamma aminobutyric acid (GABA) type A receptor ($GABA_AR$) antagonist bicuculline at a low dose (0.01 mg/kg) or aromatase inhibitor formestane, administered prior to anesthesia with sevoflurane, prevented the sevoflurane-increased corticosterone secretion without an obvious effect on the sedation depth induced by the anesthetic (unpublished observations).

The inhibitory control of the corticotropin-releasing hormone-secreting hypothalamic paraventricular neurons by $GABA_AR$-mediated signaling and the positive modulation of this signaling by neuroactive steroids is one of the fundamental mechanisms of downregulating the stress response[55,56]. Due to relatively high and low expressions of the Cl^- transporters Na^+-K^+-$2Cl^-$ (NKCC1) and K^+-$2Cl^-$ (KCC2), respectively, immature neurons have elevated intracellular concentrations of Cl^-, the main charge carriers through $GABA_AR$ channels[57-61], a major substrate for the otherwise inhibitory effects of GABAergic anesthetics[22-25]. Activation of $GABA_ARs$ in immature neurons causes Cl^- efflux, membrane depolarization, activation of the voltage-gated Ca^{++} channels, and relief of the Mg^{++}-block of Ca^{++} permeable N-methyl-D-aspartate receptors[58-62]. The GABA-initiated Ca^{++} influxes regulate a wide spectrum of developmental processes from gene expression to synapse formation[61,62]. During the second postnatal week, $GABA_AR$-mediated signaling in the brain undergoes a fundamental transition from predominantly stimulating/excitatory to inhibitory, which is caused by a concomitant developmental downregulation of NKCC1 and,

most importantly, upregulation of neuronal-specific KCC2[61,62]. It is plausible that in the neonatal brain such $GABA_AR$ inhibitory signaling-mediated control of the stress response system is weakened or GABAergic anesthetics may even stimulate the stress response through positive modulation of depolarizing/excitatory $GABA_AR$ signaling at this age.

Consistent with stressor-like effects of GABAergic anesthetics, we found that a single exposure of neonatal rats to the GABAergic GAs sevoflurane or propofol was sufficient to cause multifold increases in corticosterone secretion and electroencephalography-detectable seizures at the time of anesthesia[63-67]. The anesthetic-caused increases in excitatory $GABA_AR$ signaling and corticosterone levels may be required for neonatal GABAergic anesthetic-induced seizures to occur[68,69]. Importantly, a single exposure to sevoflurane or propofol early in life induced neuroendocrine abnormalities[64,66,70-73] similar to those induced by repeated, but not single, maternal separations, a widely used rodent model of developmental effects of early-life stress in humans[74-78]. The GABAergic anesthetic-induced long-term neuroendocrine abnormalities, which were more robust in males, included increased anxiety-like behavior and exacerbated corticosterone responses to stress[64-67]. In addition, the rats, neonatally exposed to anesthesia, had elevated *Crh* mRNA levels in the hypothalamus, as well as up- and downregulated hypothalamic and hippocampal mRNA levels of *Nkcc1* and *Kcc2*, respectively[66,67,73]. Notably, delays in the $GABA_AR$ signaling transition to inhibitory have been linked in animal models and humans to a number of cognitive neuropsychiatric disorders, such as schizophrenia, autism spectrum disorder (ASD), and Rett syndrome[79-85]. For example, in Rett syndrome, a severe form of ASD, the methyl CpG binding protein 2 deficiency-induced KCC2 downregulation may play an important causal role[84,85].

When used at a low dose, bumetanide, a loop diuretic, is the most selective of the available inhibitors of NKCC1 activity[86,87]. Our lab and others have demonstrated that pretreatment of neonatal rats with bumetanide prior to anesthetic exposure ameliorated many of the acute and lasting developmental effects of GABAergic anesthetics, including: (1) Seizures; (2) Downregulated *Kcc2* levels; (3) Elevated levels of *Crh* mRNA; (4) Exacerbated corticosterone responses to acute stress; and (5) Behavioral abnormalities[63-66,88]. Bumetanide's ameliorating effects suggest that anesthetic-exacerbated $GABA_AR$-mediated stimulation/excitation in the neonatal rodent brain is an initial step in anesthetic-induced developmental abnormalities. Importantly, bumetanide exhibits promising therapeutic effects against ASD, schizophrenia, and Fragile X syndrome in animal models and humans[89-93], suggesting that these diseases and the GABAergic anesthetic-induced abnormalities may share similar mediating mechanisms. Our current understanding of rodent and human ontogeny supports the possibility that similar GA-sensitive mechanisms operate in rodents and humans early in life. Based on the intensity of synaptogenesis, a 1-wk-old rat can be compared to a 2- to 3-year-old human[94-98]. In animals, neonatal anesthetic exposure[63,65,72,99,100] and early-life stress[101-103] have profound long-term effects on synaptic morphology and function, suggesting that humans are vulnerable well into the postnatal period. Similar to the rodent brain during the first 2 postnatal weeks, the human brain is more excitable during the first year of life than at any other time, with seizures occurring in 3.5 per 1000 live births[104-106]. Human neonatal seizures are resistant to GABAergic antiepileptic drugs (AEDs) because of depolarizing/excitatory $GABA_AR$ signaling at this age[107,108]. Many human neonatal seizures can be detected only through electroencephalographies because they are not accompanied by clinical signs such as convulsions[109], which helps explain why epileptic seizures are not routinely reported in anesthetized human infants. In neonatal seizures with clinical manifestations, $GABA_AR$-enhancing AEDs depress convulsions but may exacerbate electrographic cortical seizure activity (electroclinical uncoupling)[110]. The NKCC1 inhibitor bumetanide, administered alone or in combination with GABAergic AEDs, may alleviate neonatal seizures in rodents and humans[111,112]. In humans, the KCC2 protein levels at birth are only about 20% of adult levels and significantly increase during the first postnatal year[86]. This late KCC2 increase during brain development may make KCC2 a highly susceptible molecular target for modulation by environmental factors, including GAs.

The heightened corticosterone responses to acute stress months after exposure to the GABAergic anesthetics early in life suggest that stressors in post-anesthesia life can further exacerbate developmental abnormalities, initially programmed by GABAergic anesthetics. This concept is supported by our findings that adult rats exposed neonatally for a relatively short time to anesthesia with etomidate or sevoflurane followed by a subsequent single episode of maternal separation exhibited developmental abnormalities significantly greater than those exposed to only one of the two interventions[66,73,113]. In further support of the idea that the long-term adverse outcomes of early-life anesthesia may result from a combination of the effects of GAs

at the time of anesthesia and the effects of "post-anesthesia" environmental factors, several laboratories have demonstrated that the adverse developmental effects of neonatal anesthesia in rodents may be alleviated, not only by pharmacological interventions before exposure to anesthesia, but also by post-weaning housing of the exposed animals in an enriched environment[114-116]. Environmental factors may alleviate or exacerbate the effects of neonatal anesthesia. In other words, two subjects exposed to the same anesthesia regimen may have different long-term outcomes based on post-anesthesia life experiences. In support of this possibility, Zhang *et al*[117] reported that rats neonatally exposed to sevoflurane and then housed from the time of weaning in isolation in enrichment-deprived environments exhibited reduced levels of brain-derived neurotrophic factor (BDNF), synaptic protein markers, and survival of new granule cells in the hippocampus, as well as behavioral abnormalities. On the other hand, rats that were exposed to the same regimen of anesthesia with sevoflurane and housed in groups in enriched environments had the same outcomes as their counterparts who were not exposed to sevoflurane. These findings further support the possibility that GABAergic anesthetics administered during early life can be considered environmental stressors that predispose the exposed subjects to stress vulnerability later in life. Identifying environmental factors that predispose humans to abnormal stress reactivity later in life and the mechanisms underlying their effects is of important clinical and basic neuroscience concern because dysregulated stress response systems have been linked to the pathophysiology of several neuropsychiatric disorders[118].

Environmental enrichment, which may alleviate the neurodevelopmental effects of early-life exposure to GAs in rodents, is not an issue for the majority of human patients because this is typical for most children. If similar mechanisms operate in humans, then brain development in healthy human patients who experience normal stress levels may be minimally affected, if at all, after exposure to general anesthesia early in life. However, most children who require general anesthesia during the early postnatal period inevitably experience a variety of stressors during life post-anesthesia exposure (*e.g*., diseases, pain, hunger, psychological stress). Such patients may be at risk of developing early-life, anesthetic-programmed neuroendocrine and neurocognitive abnormalities. The exacerbating effects of environmental stressors on long-term adverse outcomes of early-life exposure to GAs may be why several recent studies have not found negative neurocognitive consequences of relatively short (≤ 1 h) anesthesia exposures in healthy children[6,7,28], while studies in very sick children have[29]. It will be important to take this factor into consideration when planning new clinical studies. Further investigation of the interaction of adverse effects of early-life exposure to GAs and post-anesthesia stressors is important because it may identify not only the most vulnerable patients, but also those who are at diminished risk and would not benefit from delaying needed anesthesia-required interventions.

Histone acetylation and DNA methylation are important epigenetic mechanisms whereby environmental factors, in particular stress, affect brain development and function[119-121]. Histone acetylation facilitates gene transcription by enabling chromatin relaxation, while histone deacetylation results in stronger histone interaction with DNA, more compact chromatin structure, and repression of gene transcription. Histone acetylation is regulated by adding and removing acetyl groups to the N-terminal of histone tails by acetyltransferases and histone deacetylases, respectively[122-126]. Jia *et al*[99] found that repeated exposure of neonatal rats to sevoflurane led to increased levels of histone deacetylases 3 and 8 and reduced levels of acetylated histones H3 and H4 in the hippocampus. The sevoflurane-exposed rats had lower hippocampal density of dendritic spines and synaptic protein markers and exhibited impaired hippocampus function-based behavior. These effects of sevoflurane were alleviated by treatments with the histone deacetylase inhibitor sodium butyrate, suggesting a potential role of histone acetylation as one of the mediators of the developmental effects of GAs. The role of histone acetylation in GA-induced abnormalities in neonatal rodents has also been reported by other laboratories[127,128]. Furthermore, histone acetylation may be involved in the mediation of learning and memory dysfunction in offspring after pregnant rats exposed to sevoflurane, isoflurane, or propofol[129-131].

Ju *et al*[100] have found that repeated exposure of neonatal rats to sevoflurane resulted in increased expression of hippocampal DNA methyltransferases 3A/B (DNMT3A/B), but not DNA methyltransferase 1 (DNMT1). These enzymes catalyze DNA methylation at the 5′ position of cytosine residues adjacent to guanines (CpG sites), typically leading to long-term transcriptional repression. The activity of DNMT1 is responsible for maintenance of the remaining 5mC marks during cell divisions because of DNMT1 selectivity to hemi-methylated DNA, while DNMT3A/B are *de novo* DNMTs, which are induced by internal and external (environmental) stimuli[132,133]. In addition to increased expression of DNMT3A/B, the rats neonatally

exposed to sevoflurane had downregulated methyl CpG binding protein 2, hypermethylated the *Bdnf* gene, downregulated BDNF levels, and exhibited behavioral deficiencies[100]. The ability of sevoflurane to induce these abnormalities was significantly diminished in rats that were pretreated with a non-selective DNMT inhibitor, 5-aza-2'-deoxycytidin, prior to sevoflurane exposure[100]. On the other hand, Wu *et al*[134] found that exposure of neonatal rats to isoflurane, another inhaled anesthetic that has positive modulation of $GABA_AR$ activity like sevoflurane, induced a significant increase in the expression of hippocampal DNMT1. More experimental studies are needed before we can draw conclusions on the specific epigenetic mechanisms involved in the mediation of the adverse effects of a given GA or a class of GAs.

It has been established in a wide range of species, including humans, that environmental factors, in particular stress, acting *via* epigenetic mechanisms may affect not only the exposed subjects, but also future generations[135-139]. In contrast to studies of famine or war survivors, in which large groups of people in relatively compact living areas within a specific time period were affected[135,137,140], it is not trivial to link someone's neurodevelopmental abnormalities to his/her parent's relatively short exposure to GAs. Still, the emerging laboratory and clinical data demonstrate that the adverse effects of GAs may include epigenetic modifications, not only in the somatic cells of the exposed subjects, but also in their germ cell epigenome. Of most direct support of the possibility of epigenetic germ cell effects of general anesthesia in humans, Donkin *et al*[141] found that the DNA methylation status of 1509 genes in the sperm of male patients was changed 1 wk after bariatric surgery. The findings of epigenetic changes in spermatozoa just 1 wk after anesthesia/surgery suggest that the anesthesia/surgery may be an important cause of such changes and that even the mature human sperm is susceptible to epigenetic reprogramming by environmental factors. The latter is supported by the presence of DNMTs in mature human sperm[142]. Importantly, of the 1509 genes altered at 1 wk after the anesthesia/surgery, 1004 genes remained altered 1 year later[141]. Alarmingly, several small pilot clinical assessments found that anesthesia care providers may have altered female/male offspring ratios, also suggesting that persistent exposure to traces of the escaped GAs from scavenging in operating rooms might affect germ cells, and, hence, the next generation(s)[143-147].

To test whether neonatal exposure to anesthesia with sevoflurane can affect not only the exposed animals, but also their future offspring, we exposed male and female postnatal day (P) 5 rats (generation F0) to 6 h anesthesia with 2.1% sevoflurane[67]. On P90, the exposed and control rats were used as breeders to produce the second generation of rats (generation F1). We have found that adult offspring of parents who were neonatally exposed to sevoflurane exhibited neurobehavioral abnormalities. Irrespective of whether sires, dams, or both parents were exposed to neonatal sevoflurane, only F1 males, but not F1 females, were affected. F1 males exhibited reduced *Kcc2* Cl^- exporter expression and behavioral abnormalities. Bisulfate sequencing revealed CpG dinucleotide hypermethylation in the *Kcc2* promoter in the F0 sperm and ovary and in the hypothalamus and hippocampus of F1 males[67]. The correlation of impaired hippocampal *Kcc2* expression and hippocampus-dependent behavior in F1 males points to the involvement of epigenetic *Kcc2* modulation in the mediation of the intergenerational effects of sevoflurane[67]. Because DNA methylation is often associated with transcriptional repression, these data suggest that methylation of the *Kcc2* promoter in F0 gametes may contribute to sevoflurane-induced intergenerational impairment in *Kcc2* expression. The role of DNA methylation in the intergenerational effects of neonatal sevoflurane exposure in rats was also reported by Chastain-Potts *et al*[148]. Surprisingly, despite similar, but not identical, neurobehavioral abnormalities in the exposed parents and their male offspring, including downregulation of the *Kcc2* expression, the male offspring, in contrast to their exposed parents, exhibited corticosterone responses to stress that were the same as the corticosterone responses to stress in the male offspring of control parents[67]. These findings, taken together with the effects of GABAergic anesthetics in the exposed animals, suggest that GA-induced modulation of $GABA_AR$ signaling through reduced expression of *Kcc2* may be required but not sufficient to induce exacerbated responses to stress in adulthood.

Importantly, stress-like effects of GABAergic anesthetics may not be limited to the early postnatal period. Thus, exposure of young adult rats to sevoflurane on 3 alternating days starting on P56 resulted in similar increases in serum levels of corticosterone in male and female rats 1 h after the last exposure to the anesthetic[149]. However, long term, the exposed female rats were the same as the controls; they were not affected. On the other hand, contrary to the currently generally accepted view and our initial hypothesis that adult rats are resilient to the long-term adverse effects of GAs, the exposed young adult male rats developed neuroendocrine and behavioral

abnormalities. More than 3 months after exposure to sevoflurane, they exhibited not only an exacerbated hypothalamic pituitary adrenal axis response to stress, but their serum levels of luteinizing and testosterone hormones were also significantly increased, as was their expression of the hypothalamic gonadotropin-releasing hormone gene. The hypothalamic pituitary testicular axis functioning in the exposed adult male rats was also altered at the level of expressions of the hypothalamic aromatase and estrogen receptor α and β genes. The expressions of the aromatase and estrogen receptor α genes were significantly increased, while the expression of the estrogen receptor β gene was slightly, but significantly, decreased[149]. These findings demonstrate that anesthesia with sevoflurane alters (disrupts) not only the functioning of the hypothalamic pituitary adrenal axis, but also the hypothalamic pituitary testicular axis functioning[53]. We have previously demonstrated the role of estradiol in the acute adverse effects of sevoflurane in neonatal rats[69].

Similar to the effects of neonatal exposure to sevoflurane, sevoflurane administered to young adult male rats induced significant impairment in expressions of the hypothalamic and hippocampal *Kcc2* genes. Similar to adult rats that were neonatally exposed to sevoflurane, male and female rats exposed to sevoflurane in young adulthood had hypermethylated *Kcc2* gene in spermatozoa and ovarian tissue, respectively[149]. Interestingly, we have analyzed the same CpG sites in the *Kcc2* promoter region in the germ cells of rats that were exposed to sevoflurane neonatally or in young adulthood and found similar changes in their methylation regardless of the age of sevoflurane exposure[67,149]. These findings suggest that sevoflurane may induce germ cell effects *via* similar mediating mechanisms when administered to rats over a wide range of ages, from neonates to young adults. The similarities between the effects of neonatal and young adult exposure to sevoflurane were also evident in the offspring of the exposed parents. Thus, the *Kcc2* gene was hypermethylated and exhibited reduced expression in the hypothalamus and hippocampus of the F1 male, but not female, offspring of the exposed parents. These changes in the *Kcc2* gene in the hypothalamus and hippocampus of adult F1 male offspring were accompanied by behavioral deficiencies in the elevated plus maze and prepulse inhibition (PPI) of acoustic startle response tests, but their corticosterone responses to stress were not different from the controls[67,149]. The sevoflurane-exposed young adult male and female rats had similar acute increases in serum corticosterone levels and changes in DNA methylation status of the *Kcc2* gene in spermatozoa and ovarian tissue and passed neurobehavioral abnormalities to their male offspring, despite the finding that the exposed dams lacked the somatic effects[149]. These findings allow us to hypothesize that sevoflurane-induced corticosterone secretion at the time of anesthesia is involved in the anesthetic-induced germ cell effects and by extension the intergenerational effects. Also, the findings that the exposed but long-term physiologically unaffected dams, similar to the exposed and affected sires, pass deleterious effects of sevoflurane to their unexposed male offspring suggest that compared to the somatic cells, the germ cells are more sensitive to the deleterious effects of sevoflurane. This raises the possibility that male offspring may be affected even when the anesthesia level/duration is insufficient to induce significant abnormalities in their exposed parents. Future studies will be needed to test these hypotheses that may have important translational applicability.

We have tested the role of the neuron-specific *Kcc2* gene as a mediator of the germ cell and intergenerational effects of sevoflurane only because we have extensive background data supporting its involvement in the mediation of the somatic effects of neonatal and young adult exposure to sevoflurane in the exposed animals[64-67,149]. The findings that the *Kcc2* gene was affected in the parental somatic (brain) cells and germ cells, two effects that may not necessarily have similar mediating mechanisms, was surprising, on the one hand, but on the other hand suggested that the *Kcc2* gene could be one of many genes involved in passing on the intergenerational effects of the anesthetic. Indeed, using genome-wide reduced representation bisulfite sequencing, we found more than 2000 differentially methylated DNA regions in the sperm of adult rats neonatally exposed to sevoflurane compared to unexposed controls (unpublished observations). Future detailed investigation of various genes that are involved in the mediation of the intergenerational effects of sevoflurane and other GAs may help to identify a full spectrum of the biological intergenerational effects of GAs and may lead to new, unexpected parental GA-induced phenotypes in offspring. It is likely that the intergenerational effects of exposure to GAs in young adulthood are not limited to sevoflurane, as Tang *et al*[150] have shown behavioral abnormalities in the offspring of mice that were exposed to enflurane at 11 wk of age.

The weakness of most rodent behavioral paradigms is that they are difficult to directly replicate in humans. The results of our studies demonstrate that the PPI of startle was impaired in F0 rats exposed to sevoflurane as neonates or in young adulthood, as well as in their F1 male offspring[64-67,149]. Also, male and female rats,

exposed to sevoflurane neonatally, and male rats exposed to sevoflurane in young adulthood, exhibited heightened corticosterone responses to stress long term[64-67,149]. In humans, PPI can be measured safely *via* changes in the eyeblink reflex using nearly identical parameters as in rodents[151-155], while heightened stress-related cortisol levels can be readily measured in saliva[156,157]. If GAs induce similar effects in humans, the PPI of startle and saliva levels of cortisol may be used as objective, translatable, and easily and safely measurable biological markers of the adverse effects of GAs in humans. Identification of such a biomarker(s) will facilitate investigation of the underlying mechanisms of the adverse effects of GAs in humans to guide development of safer anesthetic approaches. Of particular relevance, one of the effective, tested therapeutic agents to alleviate the developmental effects of GABAergic anesthetics in rodent models, the NKCC1 inhibitor bumetanide[63-66,88], is approved for the treatment of various pediatric conditions and exhibits promising therapeutic effects in human studies of neurodevelopmental disorders[89-93].

Considering relatively well-studied molecular targets for GA actions and emerging evidence of similarities between the adverse outcomes of exposure to GABAergic anesthetics and psychiatric disorders, understanding the molecular mechanisms of the adverse effects of GAs may help to elucidate the mechanistic basis and etiology of complex neurodevelopmental disorders.

CONCLUSION

The results of recent studies raise many intriguing questions related to the types of adverse effects of GABAergic GAs and their underlying mechanisms, the answers to which may have important translational applicability for establishing safer general anesthesia, in particular, and for better understanding of the nature of neuropsychiatric disorders, in general. They suggest that GABAergic GAs, in particular sevoflurane, may act as stressors and endocrine disruptors in neonates and young adults. These stress-like effects of GABAergic GAs may be involved in the mediation of two distinct types of long-term adverse effects of GAs in the exposed rodents: neuroendocrine effects (the somatic effects) and epigenetic reprogramming of their germ cells (the germ cell effects). The latter may pass neurobehavioral abnormalities to male offspring. The intergenerational effects of sevoflurane are similar, but not identical, when administered to neonatal and young adult rats, suggesting that similar mediating mechanisms are involved over a wide range of ages at the time of anesthesia. The initial data suggest that compared to the somatic cells, the germ cells are more sensitive to the deleterious effects of sevoflurane, raising the possibility that the offspring may be affected even when levels of anesthesia are not harmful to the exposed parents. The long-term adverse effects of GAs in the exposed young adult male rats suggest that current views on the window of vulnerability to the adverse effects of GAs in rodents (up to the first 2 postnatal weeks)[158,159], and, hence, the United States Food and Drug Administration recommendations to avoid GAs in children younger than 3[160], may need to be reconsidered to include more advanced ages. All of these possibilities may have important translational applicability if confirmed; further rigorous experimental testing is required.

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