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Laboratory Investigation

LABORATORY INVESTIGATION

Role of epigenetic mechanisms in transmitting the effects of neonatal sevoflurane exposure to the next generation of male, but not female, rats

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Abstract

Background: Clinical studies report learning disabilities and attention-deficit/hyperactivity disorders in those exposed to general anaesthesia early in life. Rats, primarily males, exposed to GABAergic anaesthetics as neonates exhibit behavioural abnormalities, exacerbated responses to stress, and reduced expression of hypothalamic K^+-$2Cl^-$ Cl^- exporter (*Kcc2*). The latter is implicated in development of psychiatric disorders, including male predominant autism spectrum disorders. We tested whether parental early life exposure to sevoflurane, the most frequently used anaesthetic in paediatrics, affects the next generation of unexposed rats.

Methods: Offspring (F1) of unexposed or exposed to sevoflurane on postnatal day 5 Sprague-Dawley rats (F0) were subjected to behavioural and brain gene expression evaluations.

Results: Male, but not female, progeny of sevoflurane-exposed parents exhibited abnormalities in behavioural testing and *Kcc2* expression. Male F1 rats of both exposed parents exhibited impaired spatial memory and expression of hippocampal and hypothalamic *Kcc2*. Offspring of only exposed sires had abnormalities in elevated plus maze and prepulse inhibition of startle, but normal spatial memory and impaired expression of hypothalamic, but not hippocampal, *Kcc2*. In contrast to exposed F0, their progeny exhibited normal corticosterone responses to stress. Bisulphite sequencing revealed increased CpG site methylation in the *Kcc2* promoter in F0 sperm and F1 male hippocampus and hypothalamus that was in concordance with the changes in *Kcc2* expression in specific F1 groups.

Conclusions: Neonatal exposure to sevoflurane can affect the next generation of males through epigenetic modification of *Kcc2* expression, while F1 females are at diminished risk.

Keyword: anesthesia; DNA methylation; heredity; neurodevelopmental disorders; pediatrics

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Editor's key points

- Early exposure to general anaesthetics can result in persistent cognitive dysfunction in adult animals, but effects on their offspring are unknown.
- Offspring of rats exposed to sevoflurane as neonates were investigated for behavioural abnormalities, changes in brain gene expression and deoxyribonucleic acid methylation in the genes' promoters.
- Adult male, but not female, progeny of rats neonatally exposed to sevoflurane exhibited abnormalities in epigenetic regulation, gene expression and behaviour.

Most retrospective epidemiological studies of neurocognitive function in older children who had general anaesthesia early in life have found significant deficiencies.[1] Considering the compelling animal data, the US Food and Drug Administration recommended avoiding, when possible, anaesthesia in children <3 yr old, and emphasised the pressing need for further research.[2] The full range of neonatal anaesthesia-induced abnormalities, the mechanisms involved, and the role of sex remain poorly understood even in exposed animals.[3]

We have found that rats exposed as neonates to sevoflurane, propofol, or etomidate, anaesthetics with clinically important effects on GABA type A receptors ($GABA_AR$), exhibit behavioural deficiencies and exacerbated hypothalamic-pituitary adrenal (HPA) axis responses to stress.[4–8] These anaesthetic-induced abnormalities are greater in male rats and reminiscent of those induced by excessive postnatal stress.[9–11] Anaesthetic-enhanced $GABA_AR$ signalling, which is depolarising/stimulatory during early life because of a high Na^+-K^+-$2Cl^-$ (NKCC1)/K^+-$2Cl^-$ (KCC2) Cl^- co-transporter ratio,[12–14] could play an important role in initiating and mediating these abnormalities. Thus, NKCC1 inhibition before anaesthesia was protective, whereas anaesthetised neonatal rats had hypothalamic upregulated *Nkcc1* and downregulated *Kcc2* messenger ribonucleic acid (mRNA) concentrations even in adulthood.[7,8]

During the second postnatal week, $GABA_AR$-mediated neuronal signalling undergoes a fundamental transition from predominantly depolarising/stimulatory to inhibitory caused by concomitant developmental downregulation of NKCC1 and, most importantly, upregulation of neuron-specific KCC2. This shift is brain region- and sex-dependent, occurring earlier in females.[12–14] Anaesthetic-induced delay in the developmental NKCC1/KCC2 ratio maturation could have serious consequences for brain functioning as delay/impairment in NKCC1/KCC2 ratio maturation has been linked to neuropsychiatric disorders, including autism spectrum disorders (ASD) and schizophrenia, which predominate in males.[15–17] A growing number of studies point to co-occurrence of ASD and attention-deficit/hyperactivity disorder (ADHD). Thus, 50–70% of those with ASD exhibit ADHD symptoms, whereas 15–25% of children with ADHD have symptoms of ASD.[18] Importantly, clinical studies report significant increases in ADHD in those who had medical procedures early in life that required exposure to general anaesthesia, with repeated exposures being a prognostic factor for more severe outcome.[2]

Recent studies in rodents demonstrate that the developmental effects of excessive stress early in life can be carried to the next generation or beyond, presumably by epigenetic mechanisms such as non-coding RNAs and deoxyribonucleic acid (DNA) methylation.[19–21] We have found that rats exposed as neonates to sevoflurane exhibited increased expression of hippocampal DNA methyltransferases, in addition to abnormalities at the synaptic and behavioural levels.[22] These enzymes catalyse DNA methylation at the 5′ position of cytosine residues adjacent to guanines (CpG sites), typically leading to long-term transcriptional repression. To investigate whether neonatal exposure to sevoflurane affects exposed parents and their unexposed progeny, neonatal male and female rats were exposed to 6 h of anaesthesia with sevoflurane, and their progeny were tested for inherited behavioural and molecular alterations.

Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Sprague-Dawley rats were housed under controlled illumination (12-h light/dark, lights on at 7:00AM) and temperature (23–24°C) with free access to food and water. Within 24 h of delivery, litters were culled to 12 pups. At 21 postnatal days (P21), pups were weaned and housed in sex-matched groups of two for the rest of the study.

Treatment groups

The P5 male and female rat pups were kept in a temperature-controlled chamber (37°C) with a continuous supply of 30% oxygen in air (1.5 L min^{-1}) during anaesthesia with 6 vol% sevoflurane for 3 min for induction and 2.1 vol% sevoflurane for 357 min as maintenance (sevoflurane group). Previously, we have shown that blood glucose and gas levels after 2.1% sevoflurane for 6 h were in the normal range.[4] Control F0 animals were subjected to animal facility rearing only (control group).

The F0 male and female rats were sequentially evaluated on the elevated plus maze (EPM) starting on P60, for prepulse inhibition (PPI) of the acoustic startle response on P70, and for corticosterone responses to physical restraint for 30 min on ≥P160 followed by isolation of brain and gamete tissue samples for further analyses (Fig. 1). Twenty-four F0 males and 24 females were mated on ~P90 to produce the F1 generation. F0 breeders were randomised into one of the following four groups for mating: 1) control males+control females (con-M*con-F); 2) exposed males+control females (sevo-M*con-F); 3) control males+exposed females (con-M*sevo-F); and 4) exposed males+exposed females (sevo-M*sevo-F). The female was kept alone throughout the entire gestation and postpartum rearing periods. The F1 rats, 144 in total [n=18 per sex (two) per group (four)], which were subjected to facility rearing only, were evaluated in the EPM starting on P60, PPI of startle on P70, Morris water maze (MWM) testing starting on P79, and for the corticosterone responses to restraint for 30 min on ≥P90, followed by isolation of brain tissue samples for further analyses. A separate cohort of F1 rats was sacrificed on P5 to collect brain tissue for bisulphite sequencing.

Basal and stress-induced activity of the HPA axis

Blood samples (~300 μL) were collected at rest and 10, 60, and 120 min after the restraint, as previously described.[7] Serum corticosterone was measured using commercial ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions.[7,8]