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HHS Public Access
Author manuscript
Anesthesiology. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:
Anesthesiology. 2019 November ; 131(5): 1092–1109. doi:10.1097/ALN.0000000000002920.

Intergenerational effects of sevoflurane in young adult rats

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Abstract

Background: Sevoflurane administered to neonatal rats induces neurobehavioral abnormalities and epigenetic reprogramming of their germ cells; the latter can pass adverse effects of sevoflurane to future offspring. As germ cells are susceptible to reprogramming by environmental factors across the lifespan, we hypothesized that sevoflurane administered to adult rats could induce neurobehavioral abnormalities in future offspring, but not in the exposed rats themselves.

Methods: Sprague-Dawley rats were anesthetized with 2.1% sevoflurane for 3 h every other day between postnatal days 56 and 60. Twenty five days later, exposed rats and non-exposed controls were mated to produce offspring.

Results: Adult male but not female offspring of exposed parents of either sex exhibited deficiencies in elevated plus maze (mean ± SD, offspring of control parents *vs.* offspring of both exposed parents, 35 ± 12 *vs.* 15 ± 15 seconds, P < 0.001) and prepulse inhibition of acoustic startle (offspring of control parents *vs.* offspring of both exposed parents, 46.504 ± 13.448 *vs.* 25.838 ± 22.866 %, P = 0.009), and increased methylation and reduced expression of the K^+-Cl^- co-transporter KCC2 gene (*Kcc2*) in the hypothalamus. *Kcc2* was also hyper-methylated in sperm and ovary of the exposed rats. Surprisingly, exposed male rats also exhibited long-term abnormalities in functioning of the hypothalamic-pituitary-gonadal and -adrenal axes, reduced expression of hypothalamic and hippocampal *Kcc2*, and deficiencies in elevated plus maze (control vs. sevoflurane, 40 ± 24 *vs.* 25 ± 12 seconds, P = 0.038) and prepulse inhibition of startle (control *vs.* sevoflurane, 39.905 ± 21.507 *vs.* 29.193 ± 24.263 %, P < 0.050).

Conclusions: Adult sevoflurane exposure affects brain development in male offspring by epigenetically reprograming both parental germ cells, while induces neuroendocrine and behavioral abnormalities only in exposed males. Sex steroids may be required for mediation of the adverse effects of adult sevoflurane in exposed males.

TOC Statement

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Conflicts of Interest:
The authors declare no competing interests.

The neurobehavioral abnormalities in male offspring are accompanied by increased methylation and decreased expression of the K-Cl co-transporter KCC2 gene that regulates neuronal chloride homeostasis, and, thereby, the functional modalities of GABAergic neurotransmission. Sevoflurane exposure also induces hypermethylation of the KCC2 gene in both male and female parental germ cells. These observations suggest that epigenetic reprograming of parental germ cells is involved in transmitting the adverse effects of sevoflurane exposure of adult rats to their male progeny.

Introduction

The United States Food and Drug Administration recommends avoiding anesthesia in children under age 3 whenever possible, as it may negatively affect their brain development.[1] This recommendation is based largely on an earlier body of work in animals, particularly rodents, showing that neonates are especially susceptible to lasting adverse effects of anesthesia during the first two postnatal weeks.[2] In rodents the first two weeks of life are characterized by unique physiological properties, such as excitatory signaling through γ-aminobutyric acid type A receptors ($GABA_ARs$),[3,4] a major substrate for the inhibitory (sedative) effects of GABAergic anesthetics in the more mature brain.[5–7] $GABA_AR$ signaling is excitatory during early life due to elevated levels of intraneuronal Cl^-, maintained by relatively low and high levels of the K^+-Cl^- co-transporter KCC2 and Na^+-K^+-Cl^- cotransporter NKCC1, respectively.[3,4] During the second postnatal week in rats, $GABA_AR$ signaling gradually becomes inhibitory, primarily due to age-dependent increases in KCC2.[3,4] The magnitude of $GABA_AR$ excitatory signaling and the proper timing of its transition from excitatory to inhibitory are key for normal brain development, and impairments in KCC2 and resulting shifts in $GABA_AR$ signaling toward excitation have been linked to neuropsychiatric disorders in both humans and animal models.[8,9] It appears that exposure to GABAergic anesthetics early in life may induce developmental abnormalities at least in part by enhancing the magnitude of $GABA_AR$ excitatory signaling at the time of anesthesia, and by disrupting normal developmental shifts in *Kcc2* expression.[10–12] In support of this contention, exposure of neonatal rats to GABAergic anesthetics, such as sevoflurane, causes electroencephalography-detectable epileptic seizures and multifold increases in corticosterone secretion at the time of anesthesia.[12] After maturing to adulthood, these anesthetic-exposed rats have down-regulated *Kcc2* levels, exacerbated hypothalamic-pituitary-adrenal (HPA) axis responses to stress, and behavioral abnormalities.[10,11] Inhibition of NKCC1 at the time of anesthesia ameliorates acute seizure activities, as well as many of the lasting developmental effects of GABAergic anesthetics,[11,12] suggesting that anesthetic-exacerbated $GABA_AR$-mediated excitation in neonatal brain is an important initiating step in anesthetic-induced neurobehavioral abnormalities in the exposed animals.

Surprisingly, neonatal parental sevoflurane also impairs *Kcc2* expression in the brain of future male offspring by initiating DNA methylation at the 5′ position of cytosine residues adjacent to guanines (CpG sites) in the *Kcc2* gene in parental gamete cells.[10] These findings suggest that neonatal sevoflurane can induce two distinct types of adverse effects in the exposed animals – excitatory $GABA_AR$ signaling-dependent effects in the exposed neonatal brain and epigenetic reprogramming of their germ cells, which can pass adverse effects of

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neonatal parental sevoflurane to future unexposed offspring.[10] Epidemiological studies and research in animal models suggest that parental germ cells can be susceptible to reprogramming by environmental factors across the lifespan.[13–15] Therefore, here we tested the hypothesis that sevoflurane can affect future offspring even when administered to adult rats. We further hypothesized that the primarily inhibitory $GABA_A$R signaling in the brain of young adult rats[3,16] renders them resistant to the adverse neuroendocrine and behavioral effects of sevoflurane.

Materials and Methods

Animals

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

Treatment groups

Male and female rats (generation F0) were randomized for treatment and breeding pairs using a randomization plan generator (http://www.randomization.com/) and the investigators were blind to group assignments. The F0 rats underwent anesthesia on postnatal days 56, 58 and 60. During this period, rats were held in a temperature-controlled chamber to maintain body temperature at ~+37 °C with a continuous supply of 30% oxygen in air (1.5 L/min) during anesthesia, which was induced by 6% sevoflurane for 3 min followed by 2.1% sevoflurane for 177 min for anesthesia maintenance (the Sevoflurane group). Gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Helsinki, Finland), which sampled from the animal chamber interior. Rats in the F0 control group (Control) were not subjected to anesthesia. A subset of the rats in the F0 Control and Sevoflurane groups was sacrificed 1 h after the last exposure to sevoflurane (or equivalent timepoint in the Control group) to collect blood and brain tissue samples to measure serum levels of corticosterone and to evaluate expression of KCC2 in the paraventricular nucleus (PVN) of the hypothalamus (See Fig. 1 for schematic of experimental design). The remaining Control and Sevoflurane F0 male and female rats were mated on P85 to produce offspring (generation F1). Only rats from different litters were mated. F1 rats were categorized as the offspring of: 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 females were housed individually throughout the entire gestation and post-partum rearing periods.

Sixty-four F0 rats (32 control and 32 sevoflurane exposed) were used as breeders to produce 122 F1 rats, which were not exposed to sevoflurane anesthesia and were subjected to animal facility rearing only. The F0 rats were sequentially evaluated in the elevated plus maze starting on ~P125 and prepulse inhibition of the acoustic startle response on ~P135 (Fig. 1). A subset of these animals were sacrificed on ~P160 to collect trunk blood, brain and testis

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and ovarian tissues for further analyses. The rest of these animals were physically restrained for 30 min on ~P160 to measure corticosterone responses, followed by collection of tissue samples for further analyses. The F1 rats were evaluated in the elevated plus maze starting on P60 and prepulse inhibition of startle on P70 (Fig. 1). One half of these animals were sacrificed on ~P95 to collect trunk blood and brain tissue samples for further analyses, and the rest of these animals were tested for the corticosterone responses to physical restraint for 30 min on ~P95 before collecting the brain tissue samples.

Behavioral Tests

Assessment of behavior in the elevated plus maze—The elevated plus maze studies were performed using an elevated plus maze apparatus and BIO-EPM 3C video tracking software (EB Instruments, Pinellas Park, FL) during the light phase of the dark–light cycle as previously described by our laboratory.[10,12,17] If a fall occurred, the animal was removed from the study (one F0 male rat was removed from the study for this reason).

Assessment of prepulse inhibition of startle—The prepulse inhibition of startle tests were performed using a SR-Lab startle apparatus (San Diego Instruments, San Diego, CA) as previously described by our laboratory.[10,12,17] The % of prepulse inhibition of startle for each prepulse level was calculated using the following formula: % prepulse inhibition of startle =100x [(pulse alone) – (prepulse + pulse)]/pulse alone. Data were collected as V_{max} amplitude.

Basal and stress-induced activity of the hypothalamic-pituitary-adrenal axis—Blood samples (~300 μl) were collected using the "tail clip" method at rest and 10, 60, and 120 min after the restraint. Physical restraint was administered using rodent holders (Kent Scientific Corporation, Torrington, CT). Serum corticosterone was measured using commercial ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Tissue collection—After decapitation, the trunk blood samples were collected and centrifuged at 4°C, 1000 g for 15 min, and then kept at −80°C for hormone assays. The brain was removed from the skull onto ice pads. The hypothalamus was isolated by making an anterior cut at the level of the optic chiasm, a posterior coronal section anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles and a dorsal horizontal cut at the level of the anterior commissure, as described previously.[18] The hippocampus was isolated from the respective blocks. All brain tissue samples were placed in vials filled with RNAlater solution (Invitrogen, Carlsbad, CA) and then stored at −80°C. Testis tissue was removed and washed with 0.9% saline before weighing. Sperm were isolated from the caudal epididymis of adult males into phosphate-buffered saline with 1% bovine serum albumin solution at 37°C using a swim-up assay. After settling for 30 min, sperm-containing supernatant was centrifuged for 5 min at 4000 rpm. Sperm pellets were stored at −80°C. After separation from the adipose tissues, ovaries were stored at −80°C.

Hormonal measurements—Hormone analyses in serum samples isolated from F0 and F1 rats were performed using commercially available kits according to the manufacturer's

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instructions. Serum concentrations of follicle stimulating hormone (CSB-E06869r) and luteinizing hormone (CSB-E12654r) were quantified using ELISA kits (CUSABIO TECHNOLOGY LLC, Houston, TX, USA). Serum testosterone and estradiol concentrations were measured using ELISA kits (582701, Cayman Chemical Company, Ann Arbor, MI, USA and ES180S-100, Calbiotech, Spring Valley, CA, USA, respectively).

Quantitative mRNA measurements—Levels of mRNA for *Nkcc1* and *Kcc2* in the hypothalamus and hippocampus, for corticotropin-releasing hormone, estrogen receptor α, estrogen receptor β , aromatase, gonadotropin-releasing hormone in the hypothalamus, and for glucocorticoid receptor in the hippocampus were analyzed via reverse transcription-PCR (qRT-PCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) as previously described by our laboratory.[10] RNA was extracted from the samples using an RNeasy Plus Kit (Qiagen, Valencia, CA), reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, Hercules, CA), and then analyzed via qRT-PCR. Taqman probes specific for the above genes were obtained from Applied Biosystems (Carlsbad, CA): corticotropin-releasing hormone (Rn01462137_m1), glucocorticoid receptor (Rn00561369_m1), estrogen receptor α (Rn01430446_m1), estrogen receptor β (Rn00562610_m1), aromatase (Rn00567222_m1), gonadotropin-releasing hormone (Rn00562754_m1), *Nkcc1* (Rn00582505_m1) and *Kcc2* (Rn00592624_m1). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA (Rn01775763_g1). Gene expression was calculated using the CT method and data are presented as relative fold change from that of control animals.

Bisulfite sequencing—Genomic DNA was extracted from the sperm pellet and ovaries of adult F0 rats and from hippocampal and hypothalamic tissues of F1 rats using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The sodium bisulfite conversion was performed with EZ DNA Methylation kits (Zymo Research, Irvine, CA) following the manufacturer's instructions. The primers (*Kcc2*: forward: GATTGTAAGTGTTTTTATTATTGAGTTGTATATT; reverse:AATAAACTTTTCCCCTTTTATACCC) were designed for the bisulfite-converted DNA sequences, using previously published sequences.[10] PCR amplification was performed with HotStar Taq (Qiagen, Hilden, Germany). Amplicons were cloned into pCR4-TOPO vector with the TOPO TA cloning kit for sequencing (Life Technologies, Carlsbad, CA). Miniprep was performed on each positive clone using ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA). Sanger sequencing was done by Genewiz (South Plainfield, NJ, USA) using M13R primers. The DNA methylation status of all CpG sites was analyzed using Benchling Molecular Biology 2.0 Software (Benchling, San Francisco, CA). The bisulfite sequencing analysis of the *Kcc2* DNA was performed only in F1 male progeny of con-M*con-F and sevo-M*sevo-F matings.

Immunohistochemistry—Rats were anesthetized with sevoflurane and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were collected and fixed in the 4% paraformaldehyde overnight and then dehydrated in 30% sucrose solution in phosphate-buffered saline at 4○C for 2 days. The brains were cut into 18-um-thick coronal sections using a cryostat. After blocking with 10% normal goat

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serum for 1 h at room temperature, the slices were incubated with the primary antibody (rabbit anti-KCC2, 1:500; MilliporeSigma, Burlington, MA) in 10% normal goat serum at 4○C overnight. After washing with PBS for 3 × 5 min, the slices were exposed to the secondary antibody, Alexa Fluor 549 goat anti-rabbit (Invitrogen, Carlsbad, CA). Slides were then washed with phosphate-buffered saline for 3 × 5 min and incubated for 10 min at room temperature with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 1:1000; Sigma, St. Louis, MO) in phosphate-buffered saline for 10 min. The sections were mounted and covered with coverslip after washing with phosphate-buffered saline for 3 × 5 min. A confocal microscope (IX2-DSU spinning disk confocal fluorescent microscope system, Olympus, Tokyo, Japan) was used to capture the fluorescent images. The immunofluorescence intensity of the KCC2-immunoreactive cells was measured using ImageJ software [National Institutes of Health]. Three images from the paraventricular nucleus of the hypothalamus of each animal were taken and the average intensity values were calculated to use for statistical analysis.

Statistical Analysis

Statistical analyses were conducted on raw data using SigmaPlot 14.0 software (Systat Software, Inc., San Jose, CA), which automatically checks if data set meets test criteria (Shapiro-Wilk for normality test and Brown-Forsythe for Equal Variance Test). Values are reported as mean ± SD. The primary outcome in this study was the neurobehavioral changes in offspring of rats exposed to sevoflurane in young adulthood. All other outcome measures were the secondary outcomes. One F0 male rat was removed from the elevated plus maze study because of a fall from the maze. Another F0 male rat in the control group was removed from the behavioral studies because of injury while mating with a female rat. Boxplots were used to identify outliers. No outliers were detected that were in not plausible range of values for the outcome (indicating measurement or recording error). Therefore, all values were maintained in analyses. An independent t-test was used to analyze F0 data for serum corticosterone levels and *Kcc2* expression 1 h after the last exposure to sevoflurane, elevated plus maze, testis weight, hormone levels for testosterone, estradiol, luteinizing hormone and follicle stimulating hormone and gene expression for corticotropin-releasing hormone, glucocorticoid receptor, estrogen receptor α, estrogen receptor β, aromatase, gonadotropin-releasing hormone*, Nkcc1* and *Kcc2*. To analyze F1 data for elevated plus maze, testis weight, testosterone level and gene expression for corticotropin-releasing hormone, glucocorticoid receptor*, Nkcc1* and *Kcc2*, one-way ANOVA was used. A two-way repeated measures ANOVA was used to analyze the prepulse inhibition of startle data, with the treatment and prepulse intensity as independent variables. A two-way repeated measures ANOVA was used to analyze changes in serum corticosterone levels at rest and at 3 time points after the restraint, with experimental groups and time as the independent variables. To assess differences in total corticosterone concentrations, area under the curve with respect to baseline (levels of corticosterone at rest), was calculated and compared across experimental groups using one-way ANOVA. Two-way repeated measures ANOVA with treatment as 'between'-subject factor and CpG site as 'within'-subject factor was used to analyze data on the frequency methylation of CpG sites in the *Kcc2* gene promoter in F0 and F1 tissue samples. An independent t-test was used to analyze DNA methylation level at all 6 CpG sites. All multiple pairwise comparisons were done with the Holm-Sidak method. All

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comparisons were run as two-tailed tests. P 0.05 was considered statistically significant. Statistical details are presented in text and in figure legends. The sample sizes in this study were based on previous experience with the same experimental techniques.[10,11,17] This study was not designed to detect an anesthetic x sex interaction.

Results

Systemic abnormalities in male offspring of rats exposed to sevoflurane in young adulthood.

In the elevated plus maze test, there was a statistically significant between-subjects effect of young adult parental exposure to sevoflurane on time spent in open arms ($F_{(3,58)} = 8.514$, $P < 0.001$; Fig. 2A) in F1 males. Specifically, F1 male progeny of sevoflurane exposed fathers and unexposed mothers (P = 0.040), unexposed fathers and exposed mothers (P < 0.001), and exposed fathers and exposed mothers (P < 0.001) spent less time in the open arms compared to F1 male offspring of control fathers and control mothers. Also, there were between-subjects effects of parental exposure to sevoflurane on number of crossings ($F_{(3,58)} = 4.657$, P =0.006; Fig. 2B) and distance traveled ($F_{(3,58)} = 6.288$, P <0.001; Fig. 2C) during the elevated plus maze test. Only F1 male offspring of both exposed parents made fewer crossings (P = 0.003) and traveled shorter distances (P = 0.001). All groups of F1 females were similar in respect to time spent in open arms (Fig. 2D), number of crossings (Fig. 2E) and distance traveled (Fig. 2F) during the elevated plus maze test.

There was a statistically significant effect of young adult parental sevoflurane exposure on prepulse inhibition of startle responses in F1 male rats ($F_{(3,174)} = 7.590$, $P < 0.001$; Fig. 2G). Multiple pairwise comparisons indicated that when compared to offspring of unexposed parents only F1 males of both exposed parents (P = 0.009) or those of control fathers and exposed mothers (P = 0.027) exhibited reduced prepulse inhibition of startle at PP3. In contrast to F1 males, there was no treatment effect on prepulse inhibition of startle in F1 female rats ($F_{(3,168)} = 0.584$, P = 0.627; Fig. 2H). The startle amplitudes were similar among all experimental groups of F1 male ($F_{(3,58)} = 1.991$, P = 0.125) and F1 female ($F_{(3,56)} = 0.514$, P = 0.674) rats.

Measurements of serum levels of corticosterone in the ~P95 F1 male and female rats before and after physical restraint revealed no differences among experimental groups of the same sex ($F_{(3,84)} = 0.335$, P = 0.800, males; Fig. 2I,J, and $F_{(3,84)} = 0.142$, P = 0.934, females; Fig. 2K,L).

Reduction in the K^+-Cl^- co-transporter KCC2 gene (Kcc2) expression in male offspring of rats exposed to sevoflurane in young adulthood.

There were similar levels of *Nkcc1* mRNA in the hypothalamus of all four treatment groups of the ~P95 F1 male rats ($F_{(3,20)} = 0.928$, P = 0.446, Fig. 3A), but there was a between-subjects effect of parental sevoflurane exposure on hypothalamic *Kcc2* mRNA levels ($F_{(3,20)} = 5.636$, P = 0.006, Fig. 3B). Specifically, F1 male progeny of sevoflurane exposed fathers and mothers had lower levels of *Kcc2* mRNA when compared to F1 male offspring of control fathers and control mothers (P = 0.012). In the hypothalamus of F1 females there

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were not between-subjects effects of parental sevoflurane exposure on *Nkcc1* mRNA levels ($F_{(3,19)} = 0.886$, P = 0.466, Fig. 3C) or *Kcc2* mRNA levels ($F_{(3,20)} = 0.738$, P = 0.542, Fig. 3D).

In the hippocampus of F1 males there were no effects of parental sevoflurane exposure on *Nkcc1* mRNA levels ($F_{(3,20)} = 1.357$, P = 0.284, Fig. 3E), but there was a significant effect on *Kcc2* mRNA levels ($F_{(3,20)} = 41.375$, P < 0.001, Fig. 3F). Only male offspring of both exposed parents (P < 0.001) and offspring of exposed fathers and control mothers (P < 0.001) had reduced hippocampal *Kcc2* mRNA levels compared to offspring of two control parents. In the hippocampus of F1 females there were not significant between-subjects effects of parental neonatal sevoflurane exposure on *Nkcc1* mRNA level ($F_{(3,20)} = 0.925$, P = 0.447, Fig. 3G) or *Kcc2* mRNA level ($F_{(3,20)} = 0.524$, P = 0.671, Fig. 3H).

Consistent with the observations of normal levels of corticosterone (Fig. 2 I,J), levels of corticotropin-releasing hormone mRNA in the hypothalamus ($F_{(3,20)} = 0.519$, P = 0.674, Fig. 3I) and glucocorticoid receptor in the hippocampus ($F_{(3,19)} = 3.293$, P = 0.043, Fig. 3J) of ~P95 F1 males were not different among all four treatment groups. In addition, there were no significant between-subjects effects of parental sevoflurane exposure on either hypothalamic corticotropin-releasing hormone mRNA ($F_{(3,20)} = 1.225$, P = 0.327, Fig. 3K) or hippocampal glucocorticoid receptor mRNA levels ($F_{(3,19)} = 0.860$, P = 0.479, Fig. 3L) in F1 female rats.

Elevated DNA methylation of the K^+-Cl^- co-transporter KCC2 gene (Kcc2) expression promoter region in sperm and ovary of rats exposed to sevoflurane in young adulthood and in the hypothalamus and hippocampus of their offspring.

Increased DNA methylation in the *Kcc2* promoter in gamete cells of adult rats exposed to sevoflurane as neonates and in the hypothalamus and hippocampus of their male offspring has recently been described.[10] We therefore sought to determine if similar effects would be observed in response to young adult exposure to sevoflurane. There was a significant effect of treatment ($F_{(1,36)} = 30.8$, P < 0.001) and within-subjects effect of CpG site ($F_{(5,36)} = 20.066$, P < 0.001) on the frequency of CpG methylation in the *Kcc2* promoter region in sperm of the ~P160 F0 rats (Fig. 4A). There was also a statistically significant interaction between CpG site and treatment ($F_{(5,36)} = 10.036$, P < 0.001). Pairwise multiple comparison analysis showed that young adult exposure to sevoflurane led to increased methylation in F0 male rats at CpG site 1 (P < 0.001 vs control), and CpG site 2 (P = 0.006 vs control). Similarly, there was a significant effect of treatment ($F_{(1,36)} = 34.714$, P < 0.001) and within-subjects effect of CpG site ($F_{(5,36)} = 42.686$, P < 0.001) on the frequency of CpG site methylation in the *Kcc2* gene promoter in ovarian tissue of F0 rats (Fig. 4B). There was also a statistically significant interaction between CpG site and treatment ($F_{(5,36)} = 12.343$, P < 0.001). Pairwise multiple comparison analysis showed that young adult exposure to sevoflurane led to increased methylation in F0 female rats at CpG site 1 (P < 0.001 vs control), and CpG site 2 (P < 0.001 vs control).

There was a between-subjects effect of parental treatment on CpG site methylation in the promoter of *Kcc2* gene in the hypothalamus of the ~P95 male offspring of both exposed parents ($F_{(1,36)} = 48.286$, P < 0.001), and a within-subject effect of CpG site ($F_{(5,36)} =$

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42.629, $P < 0.001$) (Fig. 4C). There was also a statistically significant interaction between CpG site and treatment ($F_{(5,36)} = 15.886$, $P < 0.001$). Pairwise multiple comparison analyses showed that young adult parental exposure to sevoflurane led to increased DNA methylation in the *Kcc2* gene promoter in the hypothalamus of F1 male progeny of both exposed parents at CpG site 1 ($P < 0.001$ vs F1 males from the con-M*con-F group), CpG site 2 ($P = 0.002$ vs F1 males from the con-M*con-F group) and CpG site 3 ($P = 0.013$ vs F1 males from the con-M*con-F group).

There was also a between-subjects effect of parental treatment on CpG site methylation in the promoter of *Kcc2* gene in the hippocampus of male offspring of both exposed parents ($F_{(1,36)} = 21.740$, $P < 0.001$, and within subject effect of CpG site ($F_{(5,36)} = 20.852$, $P < 0.001$) (Fig. 4D). There was also a statistically significant interaction between CpG site and treatment ($F_{(5,36)} = 5.268$, $P < 0.001$). Pairwise multiple comparison analyses showed that young adult parental exposure to sevoflurane led to increased DNA methylation in the *Kcc2* gene promoter in the hippocampus of F1 male progeny of both exposed parents at CpG site 1 ($P < 0.001$ vs con-M*con-F), CpG site 2 ($P = 0.018$ vs con-M*con-F) and CpG site 3 ($P = 0.018$ vs con-M*con-F).

Systemic abnormalities in male rats exposed to sevoflurane in young adulthood.

Unexpectedly, we found that more than two months after exposure to sevoflurane anesthesia, F0 male rats exhibited behavioral deficiencies. They spent less time in the open arms of the elevated plus maze ($t_{(28)} = 2.18$; $P = 0.038$; Fig. 5A), but did not differ from their control counterparts in number of crossings ($t_{(28)} = 1.456$; $P = 0.157$; Fig. 5B) or distance traveled ($t_{(28)} = -1.351$; $P = 0.188$; Fig. 5C). In F0 females, there was no significant effect of sevoflurane on any of these elevated plus maze parameters (Fig. 5D-F).

As in the elevated plus maze task, there was a significant effect of sevoflurane exposure on prepulse inhibition of startle in adult F0 male rats ($F_{(1,123)} = 6.765$; $P = 0.010$; Fig. 5G), but not in F0 female rats ($F_{(1,60)} = 0.049$; $P = 0.827$; Fig. 5H). Multiple pairwise comparisons indicated that exposure to sevoflurane led to impaired prepulse inhibition of startle responses in F0 male rats at prepulse intensity of 3 dB ($P = 0.042$ vs control). Startle stimuli by themselves caused similar responses in the control and sevoflurane groups of F0 male ($t_{(41)} = -0.969$; $P = 0.338$) and F0 female ($t_{(30)} = 1.465$; $P = 0.153$) rats.

Male F0 rats had higher total corticosterone responses to physical restraint on ~P160 when compared to their control counterparts ($t_{(14)} = -6.209$; $P < 0.001$; Fig. 5I,J). This increase was due to higher levels of corticosterone at 10 min ($P < 0.001$ vs control) and 60 min ($P = 0.036$ vs control) after restraint, as serum levels of corticosterone before the restraint ($P = 0.736$ vs control) and 120 min ($P = 0.787$ vs control) post restraint were not different in control and sevoflurane-exposed rats (Fig. 5I). There was no difference in serum corticosterone levels between control and sevoflurane-exposed F0 female rats (Fig. 5K,L).

During collection of sperm for the DNA methylation studies we incidentally found that sevoflurane-exposed F0 males had reduced testis weight ($t_{(27)} = 4.494$; $P < 0.001$; Fig. 5O) more than 3 months after sevoflurane exposure. Counterintuitively, their serum levels of testosterone were increased ($t_{(14)} = -2.839$; $P = 0.013$; Fig. 5M), although their serum levels

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of estradiol were normal ($t_{(14)} = 0.703$; $P = 0.494$; Fig. 5N). F0 female rats in the sevoflurane and control groups were not different with respect to serum levels of testosterone ($P = 0.743$; Fig. 5P) or estradiol ($P = 0.600$; Fig. 5Q).

Reduction in the K^+-Cl^- co-transporter KCC2 expression in male rats 1 h after the last exposure to sevoflurane.

Our current and recently-published findings[10] indicate that rats exposed to sevoflurane in young adulthood and in the early postnatal period develop comparable systemic abnormalities. Given that sevoflurane acts as a stressor in neonatal rats and initiates developmental abnormalities, at least in part, by potentiating excitatory $GABA_AR$ signaling,[10–12,17] here we tested whether sevoflurane can induce a stress-like[19–22] reduction in KCC2 expression in young adult rats. To measure acute effects of sevoflurane, rats were exposed to 2.1% sevoflurane anesthesia for 3 h on P56, P58 and P60. Brain tissue and trunk blood samples were collected 1 h after sevoflurane anesthesia on P60. Consistent with the stress-like effects of sevoflurane reported in previous work,[10–12,17] the exposed male and female rats had increased serum levels of corticosterone compared to controls ($t_{(6)}= -3.313$; $P=0.016$, males, Fig. 6A; and $t_{(8)}= -3.949$; $P= 0.004$, females, Fig. 6B). Despite similar increases in corticosterone levels in sevoflurane-exposed males and females, immunofluorescence evaluations of KCC2 expression in the PVN of the hypothalamus region found reductions in KCC2 level in male ($t_{(6)}= 3.343$; $P= 0.016$), but not female ($t_{(6)}= 0.773$; $P= 0.469$), F0 rats (Fig. 6C-F).

Abnormalities in the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes in male rats exposed to sevoflurane in young adulthood.

Acute KCC2 and long-term systemic effects of young adult sevoflurane in the exposed males only suggest an involvement of sex steroids. To test whether sevoflurane-induced increases in serum testosterone levels involve changes in functioning of the entire hypothalamic-pituitary-gonadal axis, we measured the expression of gonadotropin-releasing hormone in the hypothalamus and serum levels of follicle stimulating hormone and luteinizing hormone. Consistent with the finding that sevoflurane increased systemic levels of testosterone in ~P160 F0 male rats, these same rats also had increased hypothalamic levels of gonadotropin-releasing hormone mRNA ($t_{(10)} = -2.519$; $P = 0.030$; Fig. 7A) and serum levels of luteinizing hormone ($t_{(14)} = -4.932$; $P < 0.001$; Fig. 7B), while serum levels of follicle stimulating hormone ($t_{(10)} = 1.026$; $P = 0.329$; Fig. 7C) were not different from those in F0 control male rats. There were no treatment effects on the hypothalamic levels of gonadotropin-releasing hormone mRNA (Fig. 7D) or serum levels of luteinizing hormone (Fig. 7E) and follicle stimulating hormone (Fig. 7F) in F0 female rats.

The elevated testosterone may modulate the hypothalamic-pituitary-adrenal axis responses to stress through estrogen receptors after testosterone aromatization to estradiol in the brain. One such mechanism includes modulation by estradiol of the glucocorticoid receptor-mediated negative feedback effect of corticosterone on the hypothalamic-pituitary-adrenal axis activity. Estradiol can both inhibit and enhance the negative feedback effects of glucocorticoids by activating estrogen receptor α and estrogen receptor β, respectively.[23,24] In further support of involvement of testosterone/estradiol in exacerbated hypothalamic-

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pituitary-adrenal axis responses to stress, we found that the hypothalamic levels of aromatase mRNA were increased in F0 sevoflurane-exposed males ($t_{(10)} = -4.333$; $P = 0.002$; Fig. 7G). In addition, the F0 male rats exposed to sevoflurane had increased and decreased hypothalamic levels of estrogen receptor α mRNA ($t_{(10)} = -5.144$; $P < 0.001$; Fig. 7H) and estrogen receptor β mRNA ($t_{(10)} = 3.156$; $P = 0.010$; Fig. 7I), respectively. Again, consistent with the normal hypothalamic-pituitary-adrenal axis responses to stress in the exposed F0 females, the increase in the hypothalamic aromatase mRNA in sevoflurane-exposed F0 female rats did not achieve statistical significance ($t_{(10)} = -1.878$; $P = 0.090$; Fig. 7J). Moreover, in the F0 females the hypothalamic levels of estrogen receptor α mRNA (Fig. 7K) were not different between Sevoflurane and Control groups, while those of estrogen receptor β mRNA were slightly, though significantly increased in the Sevoflurane group ($t_{(10)} = -2.521$; $P = 0.030$; Fig. 7L) when compared to the Control group.

In agreement with the exacerbated corticosterone responses to stress in sevoflurane-exposed F0 male rats at ~P160, these rats had increased hypothalamic corticotropin-releasing hormone mRNA levels 2 hours after the restraint ($t_{(14)} = -3.181$; $P = 0.007$; Fig. 7M), as well as reduced levels of glucocorticoid receptor mRNA in the hippocampus ($t_{(10)} = 2.493$; $P = 0.032$; Fig. 7N). Neither hypothalamic corticotropin-releasing hormone mRNA levels (Fig. 7O) nor hippocampal glucocorticoid receptor mRNA levels (Fig. 7P) were different in sevoflurane-exposed and control F0 female rats.

Finally, considering the controlling roles of $GABA_AR$ signaling in hypothalamic gonadotropin-releasing hormone and corticotropin-releasing hormone neuronal activity, it is plausible that sevoflurane initiates enhancements of gonadotropin-releasing hormone and corticotropin-releasing hormone neuronal activity and, hence, the entire reproductive and stress axes, by downregulating *Kcc2* expression and rendering $GABA_AR$ signaling less inhibitory or even excitatory. To assess whether exposure of young adult rats to sevoflurane led to persistent alterations in expression of Cl^- transporters, brain hypothalamic and hippocampal tissue samples were collected more than 3 months after exposure (~P160). The F0 male rats from the Sevoflurane group had normal *Nkcc1* mRNA levels ($t_{(10)} = 1.065$; $P = 0.312$; Fig 8A), but decreased *Kcc2* mRNA levels ($t_{(10)} = 2.273$; $P = 0.046$; Fig. 8B) in the hypothalamus. In contrast, the F0 female rats from the Sevoflurane group had unaltered levels of both *Nkcc1* mRNA ($t_{(10)} = 0.155$; $P = 0.880$; Fig. 8C) and *Kcc2* mRNA ($t_{(10)} = 1.346$; $P = 0.208$; Fig. 8D) in the hypothalamus. In the hippocampus of F0 male rats from the Sevoflurane group, *Kcc2* mRNA levels, but not *Nkcc1* mRNA levels, were significantly reduced ($t_{(10)} = 2.387$, $P = 0.038$, Fig. 8E,F). The hippocampal mRNA levels for *Nkcc1 and Kcc2* were similar in control and sevoflurane-exposed F0 female rats (Fig. 8G,H).

Discussion

The results of these experiments show that sevoflurane administered to young adult rats of either sex can induce abnormalities in their male offspring, as well as in the exposed male rats themselves. The reduction in *Kcc2* expression in the hypothalamus and hippocampus of the F1 male progeny of the exposed parents, as well as hypermethylation of the *Kcc2* promoter in F0 male and female gamete cells and F1 male hypothalamus and hippocampus, support the involvement of epigenetic mechanisms in transmitting adverse effects of

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sevoflurane exposure in young adult rats to the next generation. The findings that both the physiology and behavior of sevoflurane-exposed F0 females were normal, while their ovarian *Kcc2* promoter was hyper-methylated and male progeny of exposed F0 females exhibited neurobehavioral abnormalities, suggest that sevoflurane affects somatic and germ cells via different mechanisms.

Intergenerational effects of young adult sevoflurane

Human and animal studies provide evidence that exposure to alcohol, endocrine disruptors, and stress can affect future generations.[10,12,13–15,25–27] Surprisingly, the heritable effects of general anesthetics are poorly studied, despite the fact that anesthetic agents share molecular mechanisms of action with alcohol and may act as both environmental stressors and endocrine disruptors.[10–12,17,27–29] Furthermore, anecdotal observations point to the possibility of heritable effects of anesthesia/surgery in humans.[30] Finally, indirect evidence of heritable effects of general anesthetics comes from clinical surveys indicating that anesthesia care providers may have altered female/male offspring ratios.[31–35] Importantly, such sex ratio effects have been linked to alcohol, stress and endocrine disruptors as well.[36–38] Our recently published[10] and present findings support the possibility that sevoflurane can induce intergenerational effects. Obviously, sevoflurane-induced epigenetic modulation of *Kcc2* in F0 gamete cells and F1 male brain may contribute to the intergenerational effects of young adult sevoflurane exposure, but it is unlikely that sevoflurane-induced changes in *Kcc2* are the only mediating mechanism. Indeed, this study found that male progeny of exposed dams and control sires had normal *Kcc2* mRNA levels in the hypothalamus and hippocampus, but exhibited deficiencies in the elevated plus maze and prepulse inhibition of startle behavioral tests. It is possible that *Kcc2* DNA methylation in gamete cells affects other epigenetic mechanisms that program offspring brain development, and/or that multiple genes are independently epigenetically modified by sevoflurane in F0 gamete cells and F1 brain. Furthermore, the epigenetic effects of GABAergic anesthetics may not be limited to DNA methylation, as we and others recently reported experimental evidence for involvement of not only DNA methylation,[10,39] but also histone acetylation[40,41] in adverse effects of neonatal sevoflurane exposure in rats.

Despite different anesthesia regimens in our recent study of intergenerational effects of neonatal anesthesia[10] and the young adult anesthesia in the present study, similarities in the intergenerational effects of sevoflurane outweighed differences between the two models. These findings are also consistent with those reported by Rodgers et al. that 42-day exposure of male rats to stress either throughout puberty or in adulthood leads to similar blunted hypothalamic-pituitary-adrenal axis responses in their progeny.[14] Moreover, repeated exposure of 11 week old male mice to the anesthetic agent enflurane negatively affected their offspring's behavior.[42] The susceptibility of germ cell maturation to sevoflurane over an extended period of the lifespan, from the early postnatal period[10] through young adulthood (this study), suggests that supporting cells, such as granulosa cells in ovaries and Sertoli cells in testis, may be the primary target for epigenetic modifications initiated by sevoflurane. It will be important in future studies to analyze epigenetic effects of parental exposure to anesthetics in F0 oocytes, which represent only a minor part of total ovarian tissue that was tested in this study.

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Irrespective of whether fathers, mothers, or both parents were exposed to sevoflurane as young adults, only male F1 progeny exhibited epigenetic, gene expression, and systemic abnormalities. Given that *Kcc2* was hyper-methylated in both male and female gamete cells in F0 rats, it is plausible that parental sevoflurane initiates mechanisms leading to disruption of embryonic DNA methylation reprograming in F1 males, but not in F1 females, resulting in affected F1 males but normal F1 females. Our findings that the exposed but physiologically unaffected dams, similar to exposed and affected sires, pass deleterious effects of sevoflurane to unexposed male offspring raise the possibility that male offspring may be affected even when anesthesia level/duration is not sufficient to induce significant abnormalities in the exposed parents.

Adverse effects of young adult sevoflurane in the exposed rats

The long-term adverse effects of general anesthesia in early childhood and at advanced ages are a widely recognized health-related concern and the subject of extensive clinical and laboratory research.[43] Investigations of such effects in young adults in their prime reproductive age, however, are relatively scarce. Several studies have assessed effects of isoflurane in young adult rats, primarily using young adult rats as comparisons to other age groups. Isoflurane administered to P60 rats affected progenitor proliferation and improved spatial memory in one study and had no effect in other one.[44,45] Crosby and colleagues also observed improvement in spatial memory in rats anesthetized with 1.2% isoflurane-70% nitrous oxide at 6 month of age.[46] Aside from the fact that two 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. Such comparison is even more problematic with our current findings in F0 males, given that we tested not only a distinct anesthesia regimen, but also a different anesthetic agent. Clearly, further research is needed to elucidate the full range of long-term effects of young adult sevoflurane anesthesia and mechanisms that mediate such effects.

The $GABA_A$R/testosterone/aromatase/estradiol/KCC2 pathway may be a key mediator of sevoflurane's long-term neuroendocrine effects in F0 male rats. Because many gonadotropin-releasing hormone neurons are excited by $GABA_A$R signaling even under basal conditions,[47,48] sevoflurane may initially stimulate gonadotropin-releasing hormone neuronal activity and, hence, the entire reproductive axis. Male-specific factors may be required for sevoflurane's actions to acutely reduce KCC2 expression, as similar increases in corticosterone levels in sevoflurane-exposed female rats were not accompanied by a reduction in KCC2 expression. The $GABA_A$R/testosterone/aromatase/estradiol/KCC2 pathway in F0 male rats may function as a system with a positive feedback effect leading to a persistently up-regulated hypothalamic-pituitary-gonadal axis. Sevoflurane-induced increases in systemic testosterone and in brain aromatase expression may lead to reductions in *Kcc2* expression, and in turn to diminished inhibitory or increased stimulatory control of gonadotropin-releasing hormone neurons by KCC2/$GABA_A$R signaling. The plausibility of this scenario is supported by literature demonstrating that estradiol increases expression of aromatase and decreases expression of KCC2 in the brain.[49,50] Interestingly, consistent with our findings, Galanopoulou and Moshé found that estradiol reduced KCC2 expression in males only.[50]

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The $GABA_AR$/testosterone/aromatase/estradiol/KCC2 pathway is also likely to be involved in dysregulated (exacerbated) hypothalamic-pituitary-adrenal axis responses to stress and stress-dependent behavioral abnormalities. One such mechanism includes modulation by estradiol of the glucocorticoid receptor-mediated negative feedback effect of corticosterone on hypothalamic-pituitary-adrenal axis activity. Estradiol can both inhibit and enhance the negative feedback effects of glucocorticoids by activating estrogen receptor α and estrogen receptor β, respectively.[23,24] In support of this mechanism are our findings that hypothalamic levels of aromatase and estrogen receptor α mRNA were elevated, whereas hypothalamic levels of estrogen receptor β mRNA and hippocampal levels of glucocorticoid receptor mRNA were down-regulated. Higher estradiol concentrations are thought to contribute to higher corticosterone at rest or after stress exposure in adult female rats when compared to their male counterparts.[51–53] Hence, our findings of exacerbated corticosterone responses to stress in F0 males, but not F0 females, suggest that exposure to sevoflurane in young adulthood induces a persistent transformation of the male stress response to a more "female-like" form. Upregulated expression of hypothalamic aromatase and resulting elevated levels of estradiol may also explain why in sevoflurane-exposed males, elevated levels of testosterone were accompanied by exacerbated hypothalamic-pituitary-adrenal axis responses to stress, because it was previously shown that gonadectomy of male rats elevated, while androgen replacement blunted the corticosterone response to stress.[54] The persistent down-regulation of *Kcc2* expression and resulting $GABA_AR$ depolarizing/stimulatory signaling in the hypothalamic PVN is likely to further contribute to dysregulated stress responses and behavioral abnormalities by impairing fundamental mechanisms of HPA axis functioning; e.g., the neuroactive steroid/$GABA_AR$ negative feedback-based mechanism of desensitization to stress.[55] Finally, it is plausible that the negative feedback effect of elevated testosterone contributed to the reduced testis weight in exposed F0 males.

In conclusion, our results demonstrate that parental exposure to sevoflurane in young adulthood epigenetically reprograms germ cells, leading to neurobehavioral abnormalities in adult male progeny. These findings also provide evidence that sevoflurane administered even in young adulthood induces neurobehavioral deficits, profound alterations in the hypothalamic-pituitary-gonadal axis and dysregulated hypothalamic-pituitary-adrenal axis responses to stress in exposed male rats. Exposed young adult female rats exhibited no long-term physiological or behavioral abnormalities, but together with the exposed males, passed the adverse effects of sevoflurane exposure to their unexposed male offspring. These differential effects of sevoflurane exposure in males and females could suggest that distinct mechanisms mediate the somatic and germ cell effects of young adult sevoflurane exposure. It is important to note, however, that the current study was not powered to detect such sex differences, and hence it will be important in future work to conduct direct comparisons between males and females.

Acknowledgement:

The authors would like to thank Terrie Vasilopoulos, Ph.D., Department of Anesthesiology, University of Florida College of Medicine, Gainesville, Florida, for her advice and help with the statistical analyses.

Funding Statement:

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Supported by the National Institutes of Health (R01NS091542 and R01NS091542-S to A.E.M.), the Escher Autism Fund (A.E.M.), the I. Heermann Anesthesia Foundation (L.S.J), and the Jerome H. Modell, M.D., F.A.H.A. Endowed Professorship, Gainesville, Florida (N.G.).

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Boxed Summary

What we already know about this topic:

- Exposure to environmental stressors and endocrine disruptors can inducemultigenerational effects resulting in neurobehavioral or other abnormalities in the offspring.
- Early life anesthesia exposure in rodents alters neurocognitive function in their offspring but whether exposure of adult animals affects offspring has not been previously reported.What this article tells us that is new:
- Repeated exposures of adult rats to sevoflurane (2.1%, 3 times, 3 hours on everysecond day) induce neurobehavioral abnormalities in the exposed males and in male but not female progeny.

What this article tells us that is new:

- The neurobehavioral abnormalities in male offspring are accompanied by increased methylation and decreased expression of the K-Cl co-transporter KCC2 gene that regulates neuronal chloride homeostasis, and, thereby, the functional modalities of GABAergic neurotransmission.
- Sevoflurane exposure also induces hypermethylation of the KCC2 gene in both male and female parental germ cells.
- These observations suggest that epigenetic reprograming of parental germ cells is involved in transmitting the adverse effects of sevoflurane exposure of adult rats to their male progeny.

Summary Statement:

Sevoflurane administered to young adult rats can induce two types of adverse effects – the germ cell effects and the somatic cell effects, which lead to abnormalities in future offspring and the exposed animals, respectively.

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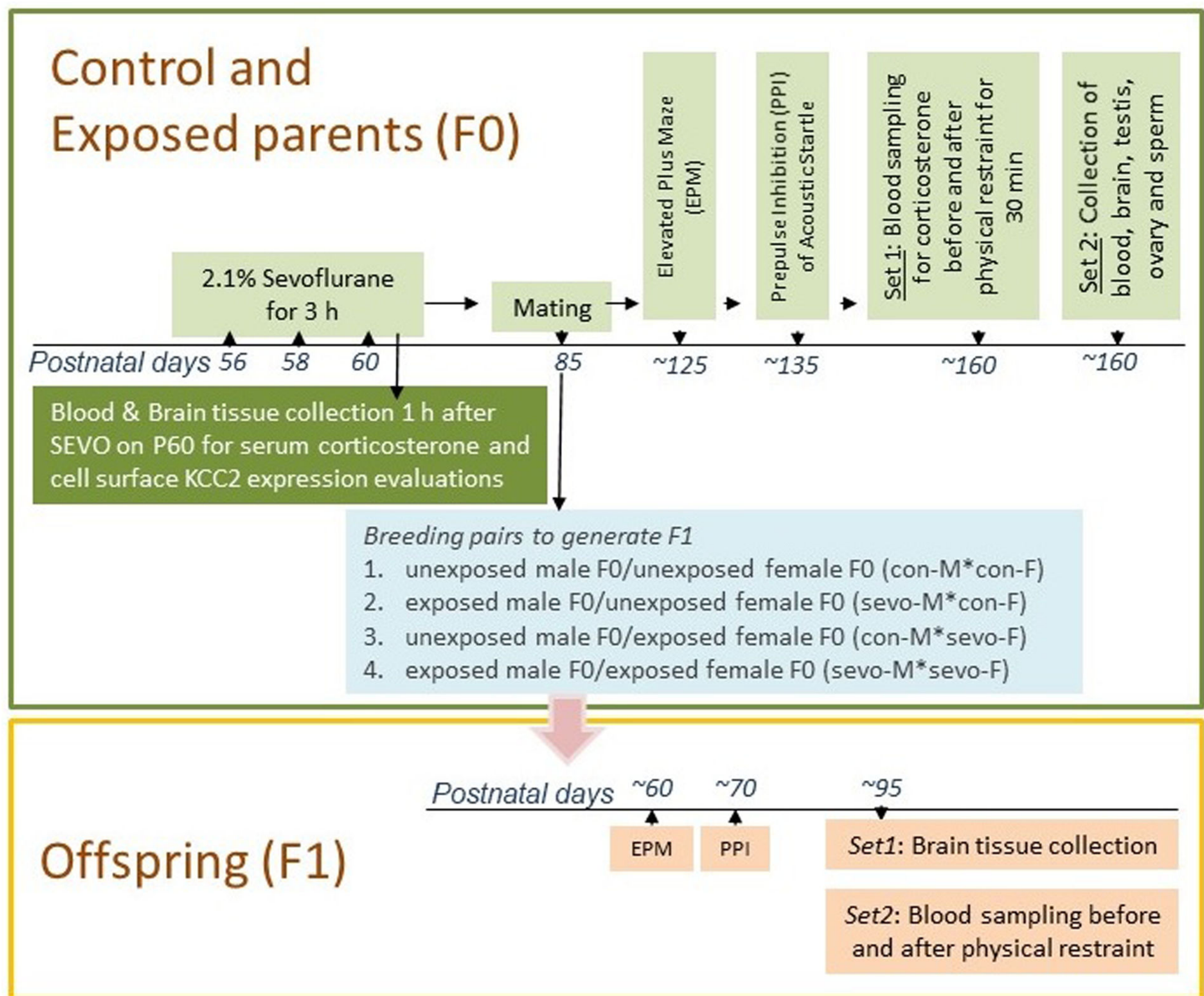

Figure 1.
Study design.

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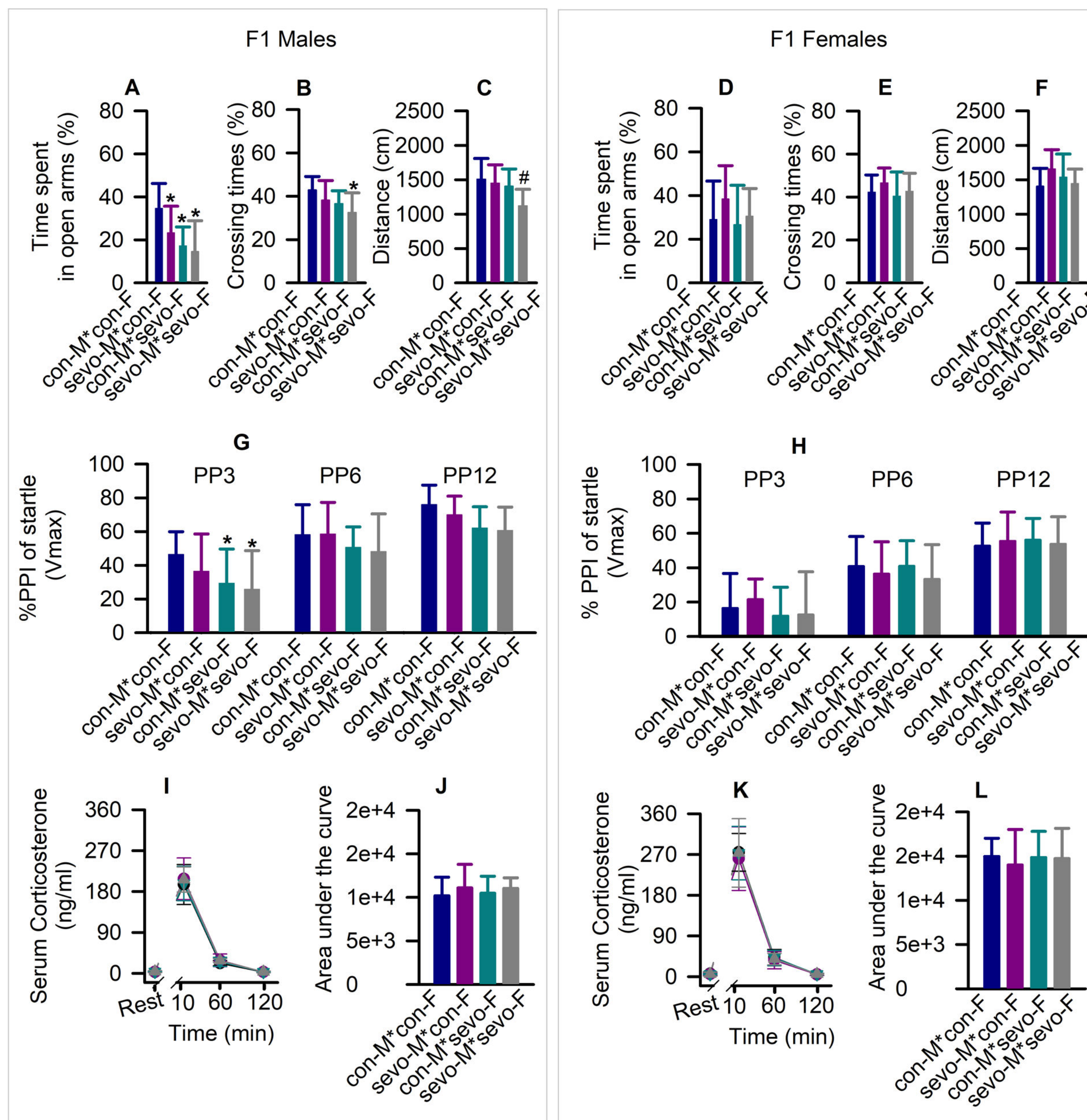

Figure 2.

Systemic second-generation (F1) effects of young adult parental exposure to sevoflurane. (A-F) Time spent in open arms of the elevated plus maze, number of crossing the open arms, and distance traveled by male (A-C) and female (D-F) offspring. F1 rats were categorized as the offspring of: 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). Data are means ± SD from 15–16 rats/group. *P < 0.05 vs. con-M*con-F. (G,H) % of prepulse inhibition of startle responses at prepulse (PP) intensity of 3 dB, 6 dB and 12 dB in male (G) and female (H) offspring. Data are means ± SD from 14–16 rats/group. *P < 0.05 vs. con-M*con-F. (I-L) Plots showing the respective levels of serum corticosterone across each collection point, as well as the total

corticosterone responses in male (I,J) and female (K,L) offspring. Serum levels of corticosterone at rest were taken as baselines for calculations of the total corticosterone responses. Data are means ± SD from 8 rats/group. Multiple pairwise comparisons were done with the Holm-Sidak method. Color coding of experimental groups in J and L is also applicable to I and K.

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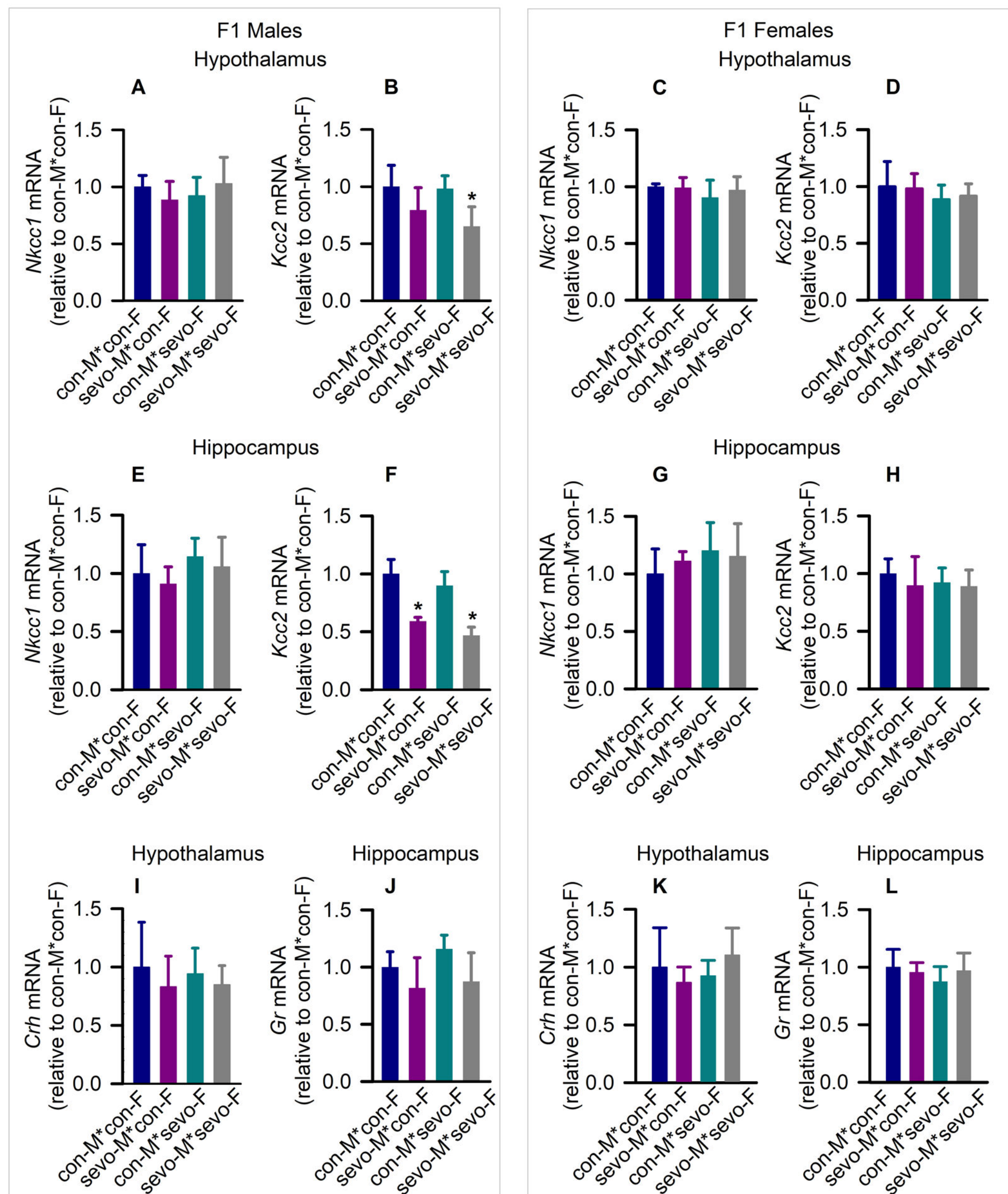

Figure 3.

Molecular second-generation (F1) effects of young adult parental exposure to sevoflurane. (A-H) The respective levels of Na^{+}-K^{+}-Cl^{-} cotransporter NKCC1 gene (*Nkcc1*) mRNA and K^{+}-Cl^{-} co-transporter KCC2 gene (*Kcc2*) mRNA in the hypothalamus of F1 males (A,B) and F1 females (C,D) and in the hippocampus of F1 males (E,F) and F1 females (G,H). F1 rats were categorized as the offspring of: 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). Data normalized against control are means ± SD from 6 rats/group (n = 5, female hypothalamic *Nkcc1* in con-M*con-F). *$P < 0.05$ vs. con-M*con-F. (I-L) The levels of corticotropin-releasing hormone mRNA in the hypothalamus and glucocorticoid receptor mRNA in the

hippocampus in F1 males (I,J) and F1 females (K,L). Data normalized against control are means ± SD from 5–6 rats/group. Multiple pairwise comparisons were done with the Holm-Sidak method.

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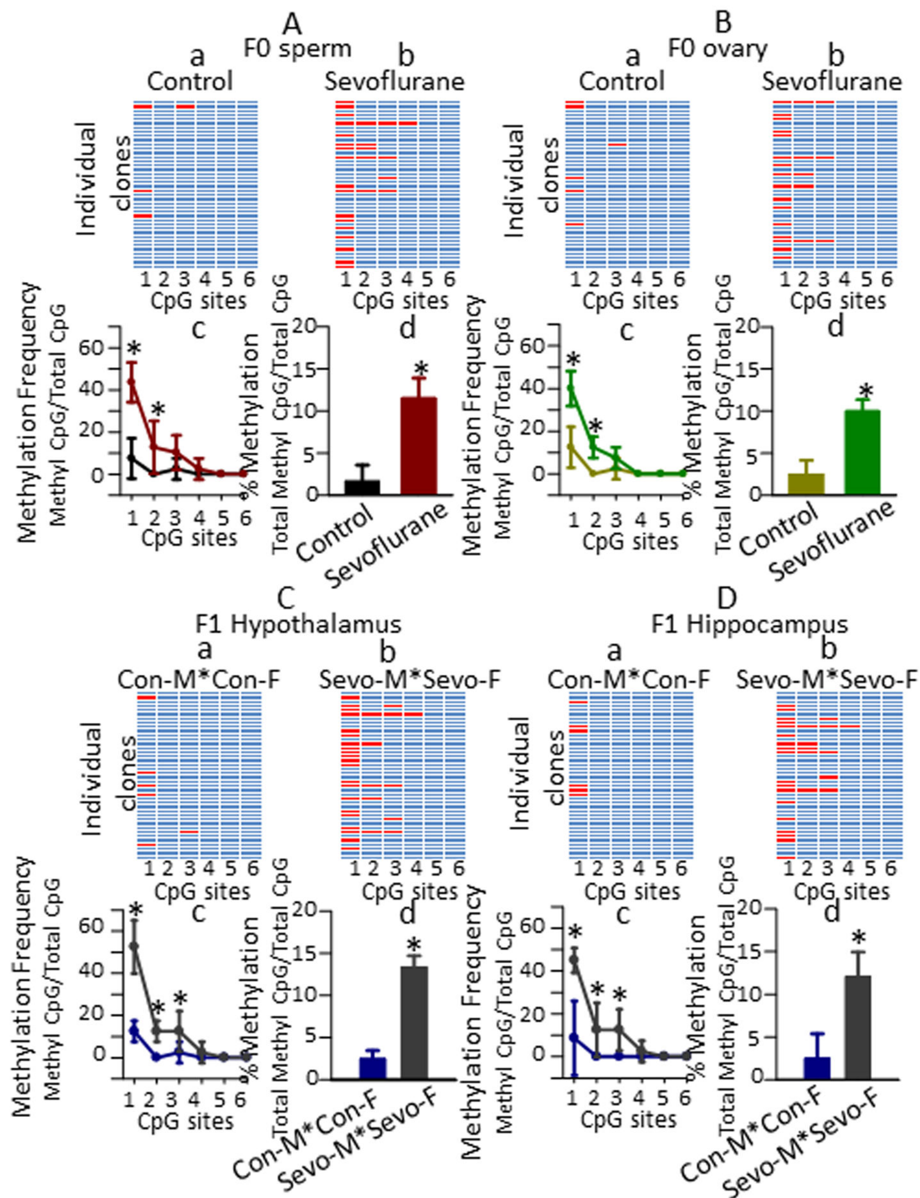

Figure 4.

DNA methylation in the promotor region of K^{+}-Cl^{-} co-transporter KCC2 gene (*Kcc2*) in parental (F0) sperm and ovary and in hypothalamus and hippocampus of their offspring (F1). (A,a-d) Bisulfite sequencing of the 5′ position of cytosine residues adjacent to guanines di-nucleotides (CpG sites) in the *Kcc2* gene of 10 clones from four individual sperm DNA samples isolated from control and sevoflurane-exposed F0 male rats. Heat maps show distribution of unmethylated (blue cells) and methylated (red cells) CpG sites in sperm DNA samples isolated from control (A,a) and sevoflurane-exposed (A,b) F0 male rats. Histograms showing methylation frequency at each CpG site (A,c) and DNA methylation level at all 6 CpG sites (A,d). The results of similar analysis of bisulfite sequencing of CpG di-nucleotides in the *Kcc2* gene of 10 clones from four individual ovary DNA samples

isolated from sevoflurane-exposed and control F0 female rats shown in (B,a-d). Color coding of experimental groups in A,d and B,d is also applicable to A,c and B,c. (C,D) Shown are the respective methylation frequency at each CpG site and DNA methylation level at all 6 CpG sites in the *Kcc2* gene of 10 clones in the hypothalamus (C,a-d) and hippocampus (D,a-d) of F1 male offspring of control sires and control dams (con-M*con-F) and of sevoflurane-exposed sires and dams (sevo-M*sevo-F). Data are means ± SD from 4 rats/group. *$P < 0.05$ vs. Control (A,B) and vs. con-M*con-F (C,D). Color coding of experimental groups in C,d and D,d is also applicable to C,c and D,c. Multiple pairwise comparisons were done with the Holm-Sidak method.

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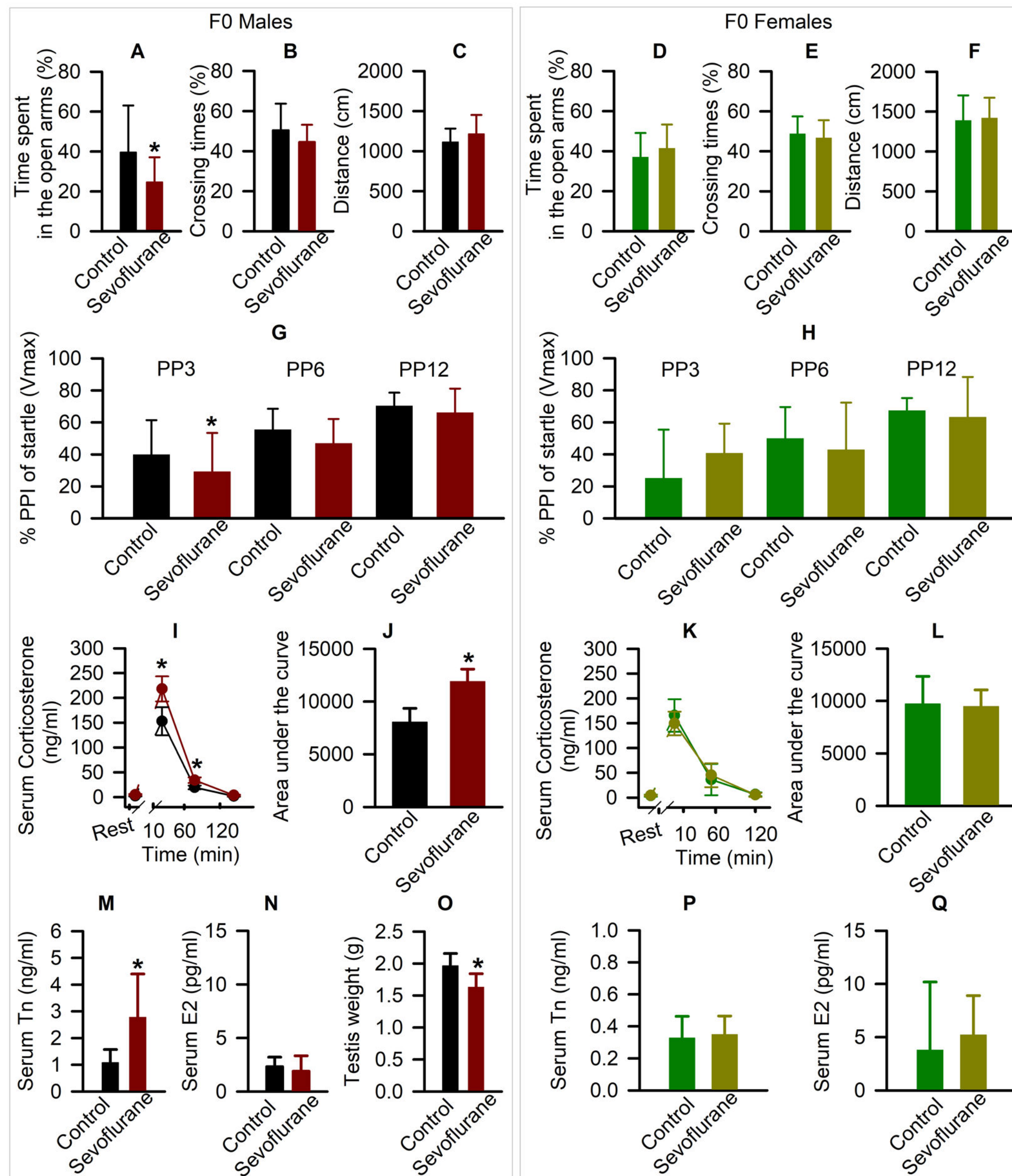

Figure 5.

Systemic first generation (F0) effects of young adult exposure to sevoflurane. The % of time spent in open arms of the elevated plus maze, number of crossing the open arms, and distance traveled by male (A-C) and female (D-F) rats. Data are means ± SD from 15 male and 16 female rats/group. (G,H) The % of prepulse inhibition of startle responses at prepulse intensity (PP) of 3 dB, 6 dB and 12 dB in male (G) and female (H) rats. Data are means ± SD from 21 males in the Control group, 22 males in the Sevoflurane group and 16 females/ group. *P < 0.05 vs. Control. (I-L) The respective levels of serum corticosterone across each collection point, as well as the total corticosterone responses in male (I,J) and female (K,L) rats. Serum levels of corticosterone at rest were taken as baselines for calculations of the total corticosterone responses. Data are means ± SD from 8 rats/group. Color coding of

experimental groups in J and L is also applicable to I and K. (M-O) shown are serum levels of testosterone (M), estradiol (N) and testis weight (O) in male rats. (P,Q) Serum levels of testosterone and estradiol in female rats. Data are means ± SD from 8 rats/group. Data of testis weight are from 16 rats in control group and 13 rats in sevoflurane group. *$P<0.05$ vs. Control.

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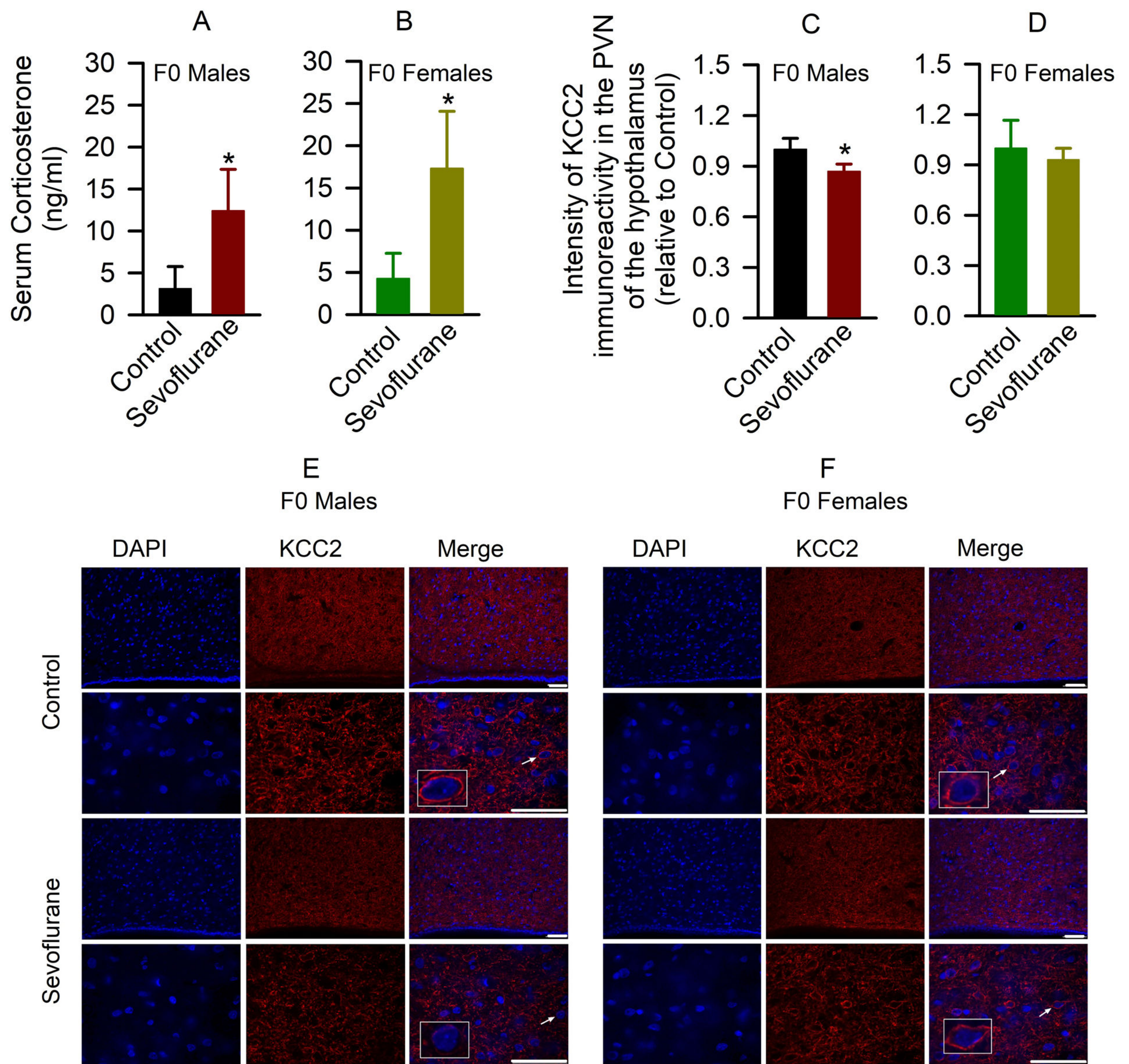

Figure 6.

Acute effects of young adult sevoflurane exposure. (A,B) The respective levels of serum corticosterone in male (A) and female (B) rats. Data are means ± SD (n=4 and n=5 per treatment group in males and females, respectively). *P<0.05 vs. Control. (C-F) Representative confocal images and quantitative analysis of the K^+-Cl^- co-transporter KCC2 immunoreactivity in the paraventricular nucleus (PVN) of the hypothalamus of the control and sevoflurane-exposed male (C,E) and female (D,F) rats. (E) Representative confocal images of the PVN from the control and sevoflurane-exposed male rats, immunostained for 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (left; blue) and KCC2 (middle; red); the merge column shows colocalized images (right; red and blue). The arrowheads indicate the cells shown in the boxed areas at higher magnifications. The KCC2 immunoreactivity, located on the periphery of the neurons (red color), decreased in the PVN neurons from the sevoflurane-exposed male rats. Similar representative confocal images of

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the PVN from the control and sevoflurane-exposed female rats shown in (F). Scale bars, 50 μm. The sevoflurane-exposed males, but not sevoflurane-exposed females, had reduced KCC2 expression (*$P<0.05$ vs. Control; Fig. 6C,E). Data are means ± SD (n=4 rats/group).

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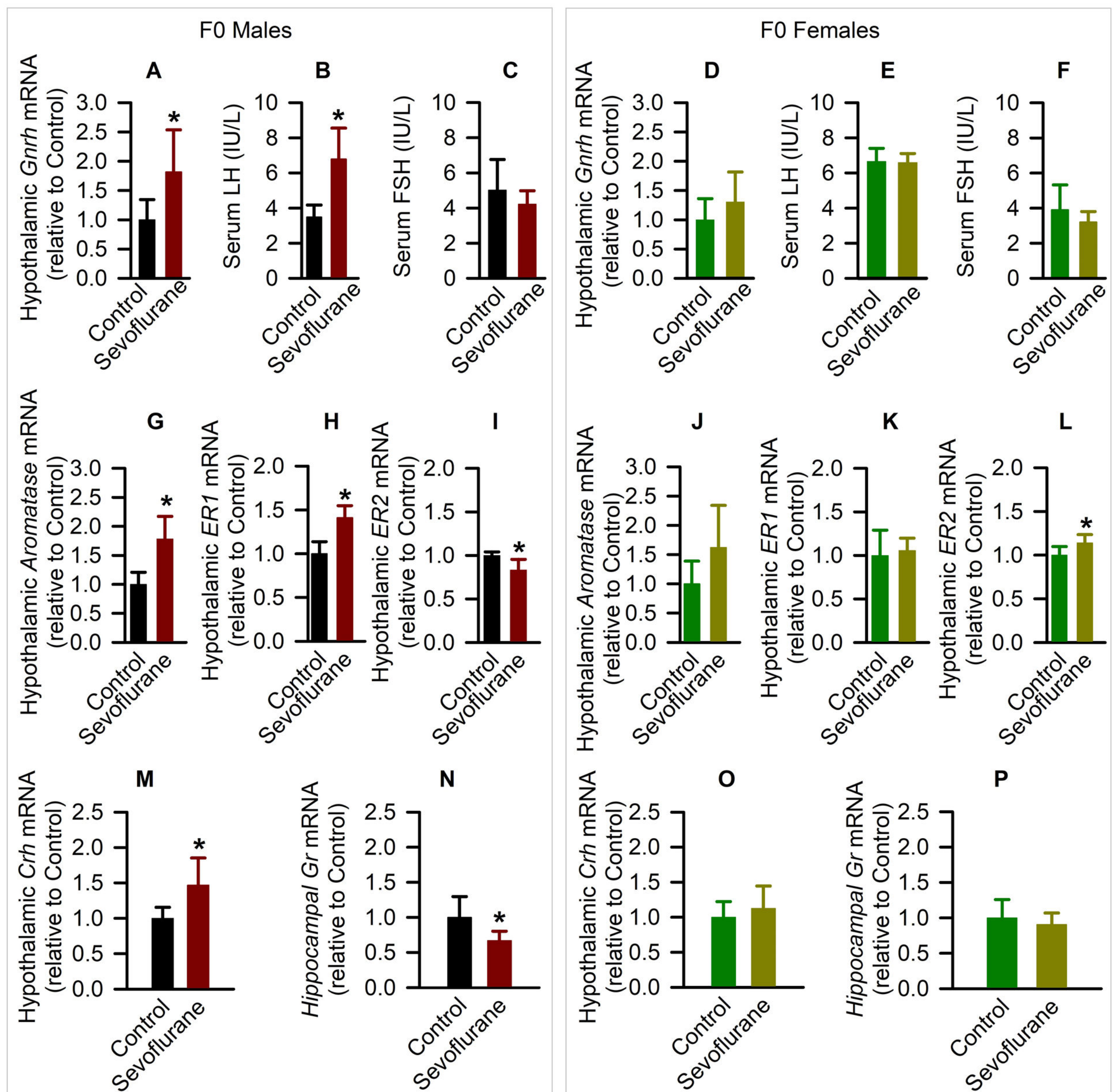

Figure 7.

Molecular first generation (F0) effects of young adult exposure to sevoflurane. (A-F) The levels of hypothalamic gonadotropin-releasing hormone mRNA, serum levels of luteinizing hormone and follicle stimulating hormone of male (A-C) and female (D-F) rats. Data are means ± SD from 8 rats/group (n=6 rats/ group in follicle stimulating hormone). *P < 0.05 vs. Control. (G-L) shown are levels of aromatase mRNA, estrogen receptor α mRNA, and estrogen receptor β mRNA in the hypothalamus of male (G-I) and female (J-L) rats. Data normalized against control are means ± SD from 6 rats/group. *P < 0.05 vs. (M-P) Shown are the respective levels of hypothalamic corticotropin-releasing hormone mRNA and hippocampal glucocorticoid receptor mRNA in males (M,N) and females (O,P). Data normalized against control are means ± SD from 6 rats/group (n=8, male corticotropin-

releasing hormone; n=5 in the Control group and n=4 in the Sevoflurane group in female glucocorticoid receptor). *P < 0.05 vs. Control.

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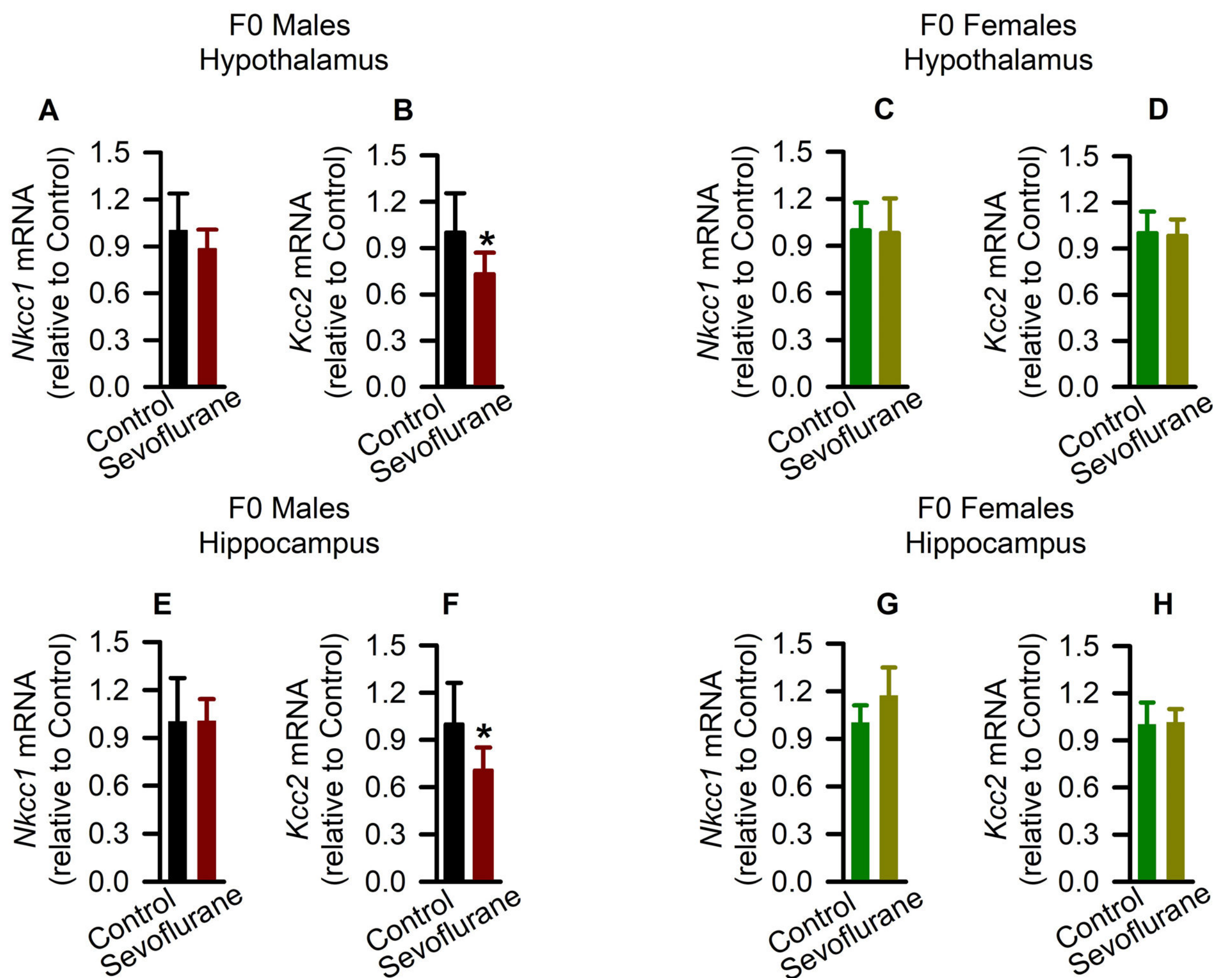

Figure 8.

Effects of sevoflurane exposure on hypothalamic and hippocampal K^+-Cl^- co-transporter KCC2 gene (*Kcc2*) and Na^+-K^+-Cl^- cotransporter NKCC1 gene (*Nkcc1*) mRNA in F0 rats. (A-H) Shown are the respective levels of *Nkcc1* mRNA and *Kcc2* mRNA in the hypothalamus of F0 males (A,B) and F0 females (C,D) and in the hippocampus of F0 males (E,F) and F0 females (G,H). Data normalized against control are means ± SD from 6 rats/ group (n = 5, female hippocampus in sevoflurane group). *P < 0.05 vs. Controls.