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

L.-S. Ju[1], J.-J. Yang[1], T. E. Morey[1], N. Gravenstein[1,2], C. N. Seubert[1], J. L. Resnick[3], J.-Q. Zhang[4] and A. E. Martynyuk[1,2,*]

[1]Department of Anesthesiology, University of Florida College of Medicine, Gainesville, FL, USA, [2]The McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL, USA, [3]Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL, USA and [4]Department of Anesthesiology, Zhengzhou University, Zhengzhou, China

*Corresponding author. E-mail: amartynyuk@anest.ufl.edu

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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]

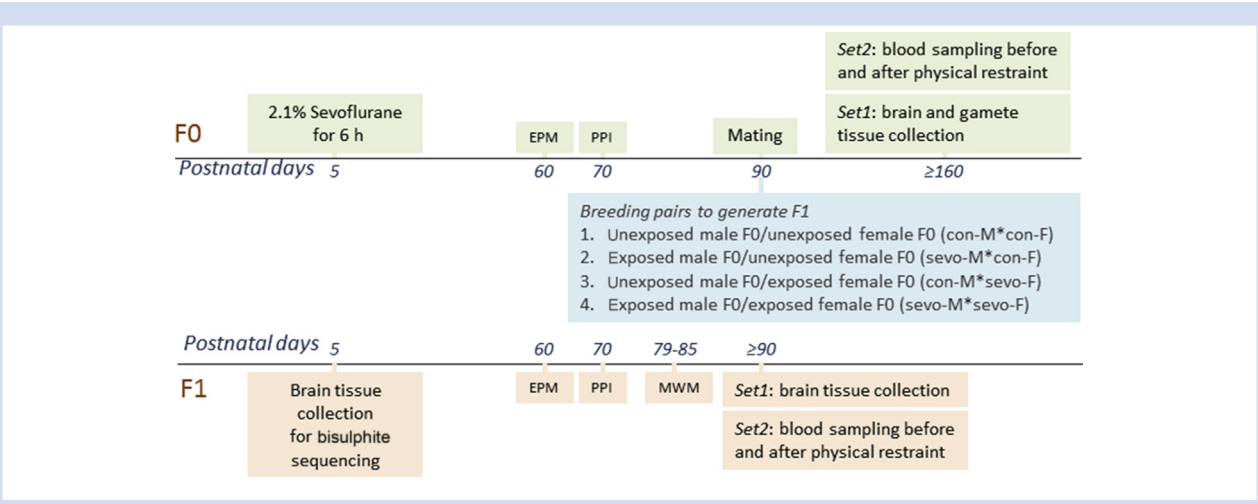

Fig 1. Study design. EPM, elevated plus maze; PPI, prepulse inhibition; MWM, Morris water maze.

Behavioural tests

The EPM, acoustic startle response, PPI of startle, and MWM tests were performed as previously described.[4–8]

Tissue collection

Adult rats were anaesthetised with sevoflurane and decapitated. Whole brains were removed and immediately put in a stainless steel adult rat brain slicer matrix with 0.5 mm coronal section slice intervals (Zivic Instruments, Pittsburgh, PA, USA). Hypothalamic paraventricular nucleus (PVN) tissue was punched out with a 1-mm ID glass capillary tube. The hippocampus was isolated from the respective slices. Tissues were placed in vials filled with RNAlater solution (Invitrogen, Carlsbad, CA, USA) and stored at −80°C. Sperm were isolated from the caudal epididymis of adult males and stored at −80°C. After separation from the adipose tissues, ovaries were stored at −80°C.

Analyses of mRNA levels for *Nkcc1*, *Kcc2*, and glucocorticoid receptors (*Gr*)

The mRNA levels for *Nkcc1*, *Kcc2* in the PVN of the hypothalamus and hippocampus, and for *Gr* in the hippocampus were analysed via qRT–PCR as previously described.[7,8]

Bisulphite sequencing

Genomic DNA was extracted from the sperm pellet and ovaries of adult F0 rats and from hippocampal and hypothalamic tissues of P5 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, USA) following the manufacturer's instructions. The primers (*Nkcc1*: forward: GAGAGGAGTTTATAGGGTT; reverse: AACCCTAC(A/G)CTAACCAACCTC; *Kcc2*: forward: GATTGTAAGTGTTTTTATTATTGAGTTGTATATT; reverse: AATAAACTTTTCCCCTTTTATACCC) were designed for the bisulfite-converted DNA sequences, using previously published sequences.[23,24] PCR amplification was performed with HotStar Taq (Qiagen). Amplicons were cloned into pCR4-TOPO vector with the TOPO TA cloning kit for sequencing (Life Technologies, Carlsbad, CA, USA). Miniprep was performed on each positive clone using ZR Plasmid Miniprep kit (Zymo Research). Sanger sequencing was done by Genewiz (South Plainfield, NJ, USA) using M13R primers. The DNA methylation status of all CpG sites was analysed using Benchling Molecular Biology 2.0 Software (Benchling, San Francisco, CA, USA).

Statistical analysis

Values are reported as mean (standard deviation). Statistical analyses were carried out on raw data using SigmaPlot 13.0 software (Systat Software, Inc., San Jose, CA, USA). To assess differences in total corticosterone concentration, EPM behaviour and gene expression for *Nkcc1*, *Kcc2*, and *Gr*, *t*-test and one way analysis of variance (ANOVA) were used for F0 and F1 generations, respectively. Two way ANOVA with experimental groups and time as the independent variables was run to analyse changes in serum corticosterone concentrations at rest and at three time points after the restraint. Two way ANOVA was used to analyse the PPI data, with the treatment and prepulse intensity as independent variables, and the MWM latencies to escape data, with experimental groups and days of training as the independent variables. One-way ANOVA was used to analyse time spent in the target quadrant and numbers of crossings during the MWM probe test. Two way measures ANOVA with treatment as 'between'-subject factor and CpG site as 'within'-subject factor was used to analyse the frequency methylation of CpG sites. Multiple pairwise comparisons were done with the Holm-Sidak method. All comparisons were run as two-tailed tests. A P value <0.05 was considered significant. The sample sizes in this study were based on previous experience with the same experimental techniques.[6-8]

Results

Neuroendocrine and behavioural abnormalities in F0 rats

Adult F0 rats, exposed to sevoflurane as neonates, had significantly higher total corticosterone responses to restraint

stress compared with F0 controls [males, $t_{(8)}=-8.09$, $P<0.001$; and females, $t_{(8)}=-3.05$, $P=0.015$]. These increases in corticosterone responses were because of higher concentrations of corticosterone 10 min after restraint ($P<0.001$, males, Fig. 2a and b; and $P<0.001$, females, Fig. 2c and d).

The F0 male rats, exposed to sevoflurane as neonates, spent a shorter time in open arms [$t_{(20)}=2.67$, $P=0.015$, Fig. 2e] and travelled shorter distances during the EPM test [$t_{(20)}=2.27$, $P=0.034$, Fig. 2f]. In F0 females, there was no significant between-subjects effect of neonatal sevoflurane exposure on time spent in open arms and distance travelled during the EPM test (Fig. 2g and h).

There were significant effects of neonatal exposure to sevoflurane on PPI of startle in adult F0 rats [$F_{(1,66)}=14.80$, $P<0.001$, males, Fig. 2i; and $F_{(1,66)}=9.13$, $P=0.004$, females, Fig. 2j]. Startle stimuli by themselves caused similar responses in the control and sevoflurane groups of F0 male and female rats.

Hypothalamic and hippocampal *Nkcc1/Kcc2* mRNA ratios in F0 rats

The F0 male rats from the sevoflurane group had increased *Nkcc1* mRNA levels [$t_{(11)}=-3.29$, $P=0.007$, Fig. 3a] and decreased *Kcc2* mRNA levels [$t_{(11)}=2.24$, $P=0.047$, Fig. 3b] in the PVN of the hypothalamus, resulting in significantly increased *Nkcc1/Kcc2* mRNA ratios [$t_{(11)}=-6.97$, $P<0.001$, Fig. 3c]. The F0 female rats from the sevoflurane group had increased *Nkcc1* mRNA levels [$t_{(10)}=-2.91$, $P=0.016$, Fig. 3d], but not significantly altered *Kcc2* mRNA levels (Fig. 3e) in the PVN of the hypothalamus. Still, the resulting *Nkcc1/Kcc2* mRNA ratios in sevoflurane exposed F0 females were increased [$t_{(10)}=-3.17$, $P=0.01$, Fig. 3f]. In the hippocampus of F0 male rats from the sevoflurane group, only *Kcc2* mRNA levels were reduced [$t_{(10)}=4.17$, $P=0.002$, Fig. 3h]. Overall, changes in hippocampal *Kcc2* mRNA and *Nkcc1* mRNA resulted in an increased *Nkcc1/Kcc2* mRNA ratio in F0 males [$t_{(10)}=-3.27$, $P=0.008$, Fig. 3i]. In contrast, hippocampal mRNA

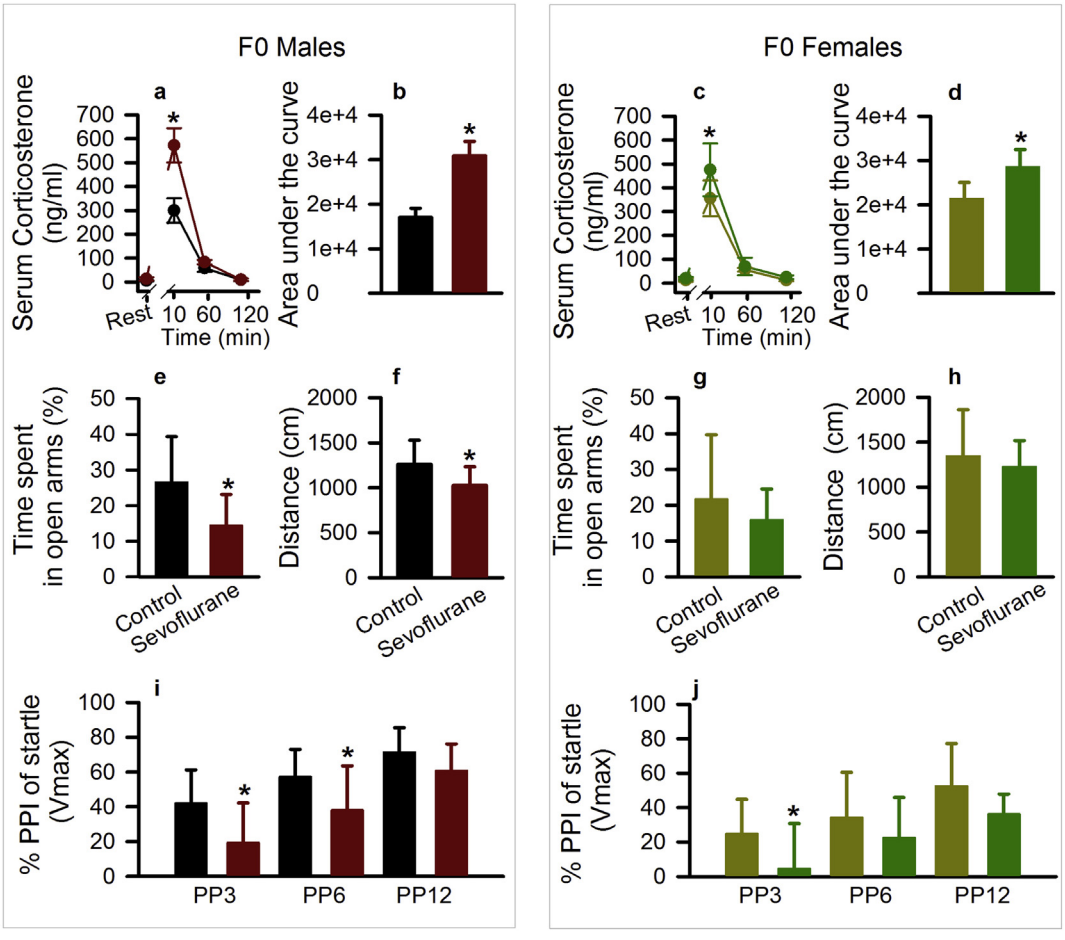

Fig 2. Adult F0 rats, exposed to sevoflurane on postnatal Day 5, exhibited exacerbated corticosterone responses to physical restraint for 30 min, impaired behaviour in the elevated plus maze (EPM) and reduced prepulse inhibition (PPI) of startle. Shown are the respective concentrations of serum corticosterone across each collection point, and the total corticosterone response in male (a, b) and female (c, d) rats. To assess differences in total corticosterone concentrations, area under the curve in respect to ground (concentrations of corticosterone at rest were taken as a ground), was calculated. Data are means [standard deviation (SD)] from five rats per treatment group. (e–h) Shown are time (%) spent in open arms of the EPM and distance travelled by male (e, f) and female (g, h) rats. Data are means (SD) from 11 male and 12 female rats per treatment group. (i, j) Shown are %PPI responses in male (i) and female (j) rats. Data are means (SD) from 12 rats per treatment group. Colour coding in (e–h) is applicable to all figures. *$P<0.05$ *vs* control.

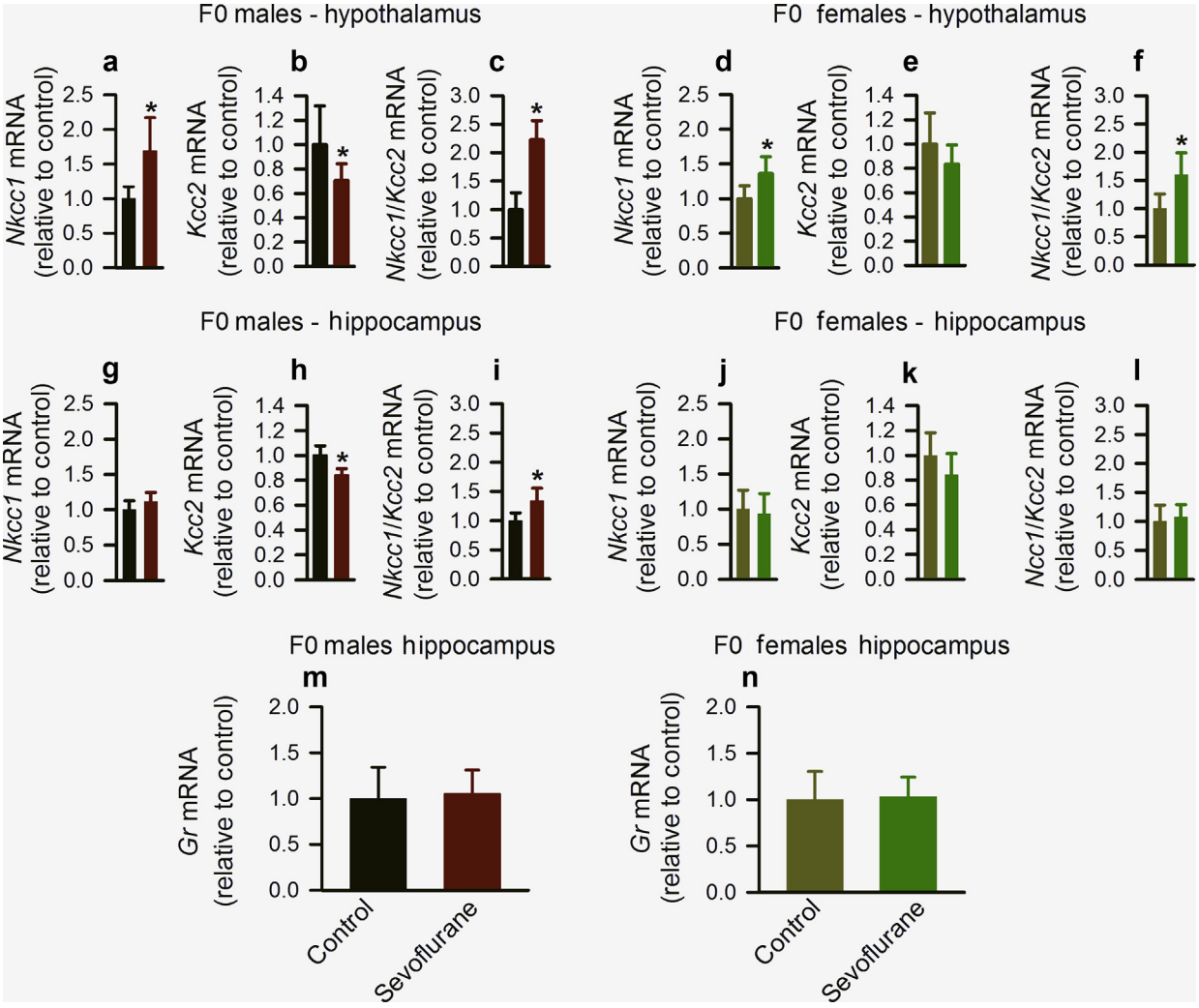

Fig 3. Gene expression of Na^+-K^+-$2Cl^-$ (*Nkcc1*), K^+-$2Cl^-$ (*Kcc2*) and glucocorticoid receptors (*Gr*) in the paraventricular nucleus (PVN) of the hypothalamus and hippocampus of adult F0 rats, exposed to sevoflurane on postnatal Day 5. Shown are the respective levels of *Nkcc1* messenger ribonucleic acid (mRNA), *Kcc2* mRNA and the resulting *Nkcc1/Kcc2* mRNA ratios in the PVN of the hypothalamus of males (a–c) and females (d–f) and in the hippocampus of males (g–i) and females (j–l). Data normalised against control are means [standard deviation (SD)] from a minimum of six rats per treatment group (n=7, male sevoflurane group, hypothalamus). (m, n) Shown are levels of *Gr* mRNA in the hippocampus of male (m) and female (n) rats. Data normalised against control are means (SD) from six rats per treatment group. Colour coding in m and n is applicable to all figures. *P<0.05 *vs* control.

levels for *Nkcc1*, *Kcc2*, and *Nkcc1/Kcc2* were similar in control and sevoflurane exposed F0 female rats (Fig. 3j–l). The hippocampal levels of *Gr* mRNA were similar in control and sevoflurane exposed F0 male (Fig. 3m) and female rats (Fig. 3n).

Behavioural abnormalities and corticosterone responses to stress in F1 rats

Serum concentrations of corticosterone in male and female rats from the F1 generation were not different among all experimental groups within the same sex (Fig. 4a–d).

In F1 males, there was a significant between-subjects effect of parental neonatal exposure to sevoflurane on time spent in open arms [$F_{(3,67)}$=3.51, P=0.02; Fig. 4e], but there was no significant effect on distance travelled (Fig. 4f) during the EPM test. Only F1 male progeny of exposed males and unexposed females spent shorter time in open arms of the EPM. The time spent in open arms and distance travelled during the EPM test were not different amongst all experimental groups of F1 female rats (Fig. 4g and h).

There was a significant effect of parental sevoflurane exposure on PPI of startle responses in F1 male rats [$F_{(3,204)}$=9.19, P<0.001; Fig. 4i]. Only male progeny of exposed sires exhibited reduced PPI of startle at PP3 (P=0.014 *vs* F1 males of con-M*con-F), and PP6 (P=0.007 *vs* F1 males of con-M*con-F). There was no significant treatment effect on PPI of

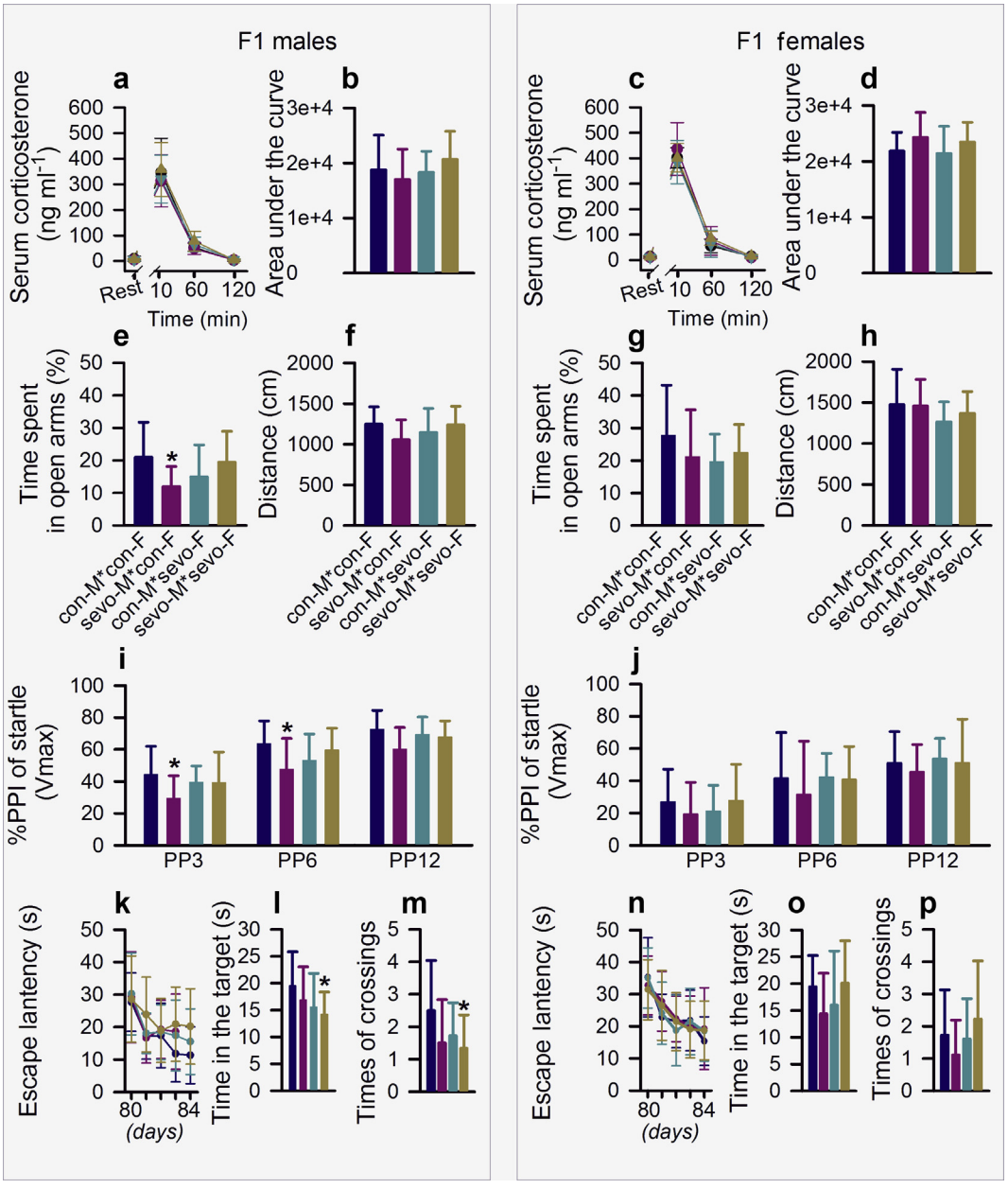

Fig 4. F1 male, but not female, offspring of sires exposed to sevoflurane on postnatal Day 5, exhibit behavioural abnormalities, while both F1 females and F1 males had normal corticosterone responses to stress. Shown are the respective concentrations of serum corticosterone across each collection point, and the total corticosterone responses in male (a, b) and female (c, d) F1 rats. To assess differences in total corticosterone concentrations, area under the curve in respect to ground (concentrations of corticosterone at rest were taken as a ground), was calculated. Data are means [standard deviation (SD)] from six animals per treatment group. Shown are % of time spent in open arms of the elevated plus maze (EPM) and distance travelled by male (e, f) and female (g, h) F1 rats. Data are means (SD) typically from 18 animals per treatment group (n=17, male con-M*con-F group). (i, j) Shown are %PPI responses at prepulse intensity (PP) of 3 dB, 6 dB, and 12 dB in male (i) and female (j) F1 rats. Data are means (SD) from 18 rats per treatment group. (k) Plots showing the values of escape latencies during the 5-day training period from P80 to P84 for F1 male rats. (l, m) Histograms showing the time spent in the target quadrant and the number of times the rat crossed the previous location of the escape platform. (n–p) Shown are respective data for F1 female rats collected during the Morris water maze (MWM) tests. Data are means (SD) from 18 animals per treatment group. Colour coding in (e–h) is applicable to all figures. *$P<0.05$ *vs* F1 males from the con-M*con-F group.

startle in F1 female rats (Fig. 4j). The startle amplitudes were similar among all experimental groups of F1 male and F1 female rats.

In males, the MWM test showed no significant between-subjects effect of parental sevoflurane exposure on the escape latencies across the 5-day training period, but there was a significant within-subjects effect of day of training [$F_{(4,272)}$=30.03, P<0.001; Fig. 4k]. There were significant effects of parental sevoflurane exposure on time in the target quadrant [$F_{(3,68)}$=2.75, P=0.049; Fig. 4l] and times of crossing over the platform [$F_{(3,68)}$=3.06, P=0.034; Fig. 4m]. Only male offspring of both exposed parents spent significantly shorter time in the target quadrant (P=0.04 *vs* F1 males of con-M*con-F) and made less crossings over the former platform (P=0.04 *vs* F1 males of con-M*con-F). There were no significant group effects in the MWM tests of F1 female rats (Fig. 4n–p).

Hypothalamic and hippocampal *Nkcc1/Kcc2* mRNA ratios and hippocampal *Gr* mRNA levels in F1 rats

In F1 males there was a significant between-subjects effect of parental sevoflurane exposure on *Nkcc1* mRNA levels [$F_{(3,22)}$=4.55, P=0.013, Fig. 5a], *Kcc2* mRNA levels [$F_{(3,22)}$=13.53, P<0.001, Fig. 5b], and the *Nkcc1/Kcc2* mRNA ratios [$F_{(3,22)}$=5.68, P=0.005, Fig. 5c] in the PVN of the hypothalamus. In contrast, F1 females showed no such between-subjects effects in the PVN of the hypothalamus (Fig. 5d–f).

In the hippocampus of F1 males, there was no significant between-subject effect of parental neonatal sevoflurane exposure on *Nkcc1* mRNA levels (Fig. 5g), but there was a significant effect on *Kcc2* mRNA levels [$F_{(3,20)}$=3.55, P=0.03, Fig. 5h] and thus the *Nkcc1/Kcc2* mRNA ratio [$F_{(3,22)}$=5.52, P=0.006, Fig. 5i]. In the hippocampus of F1 females, there were no

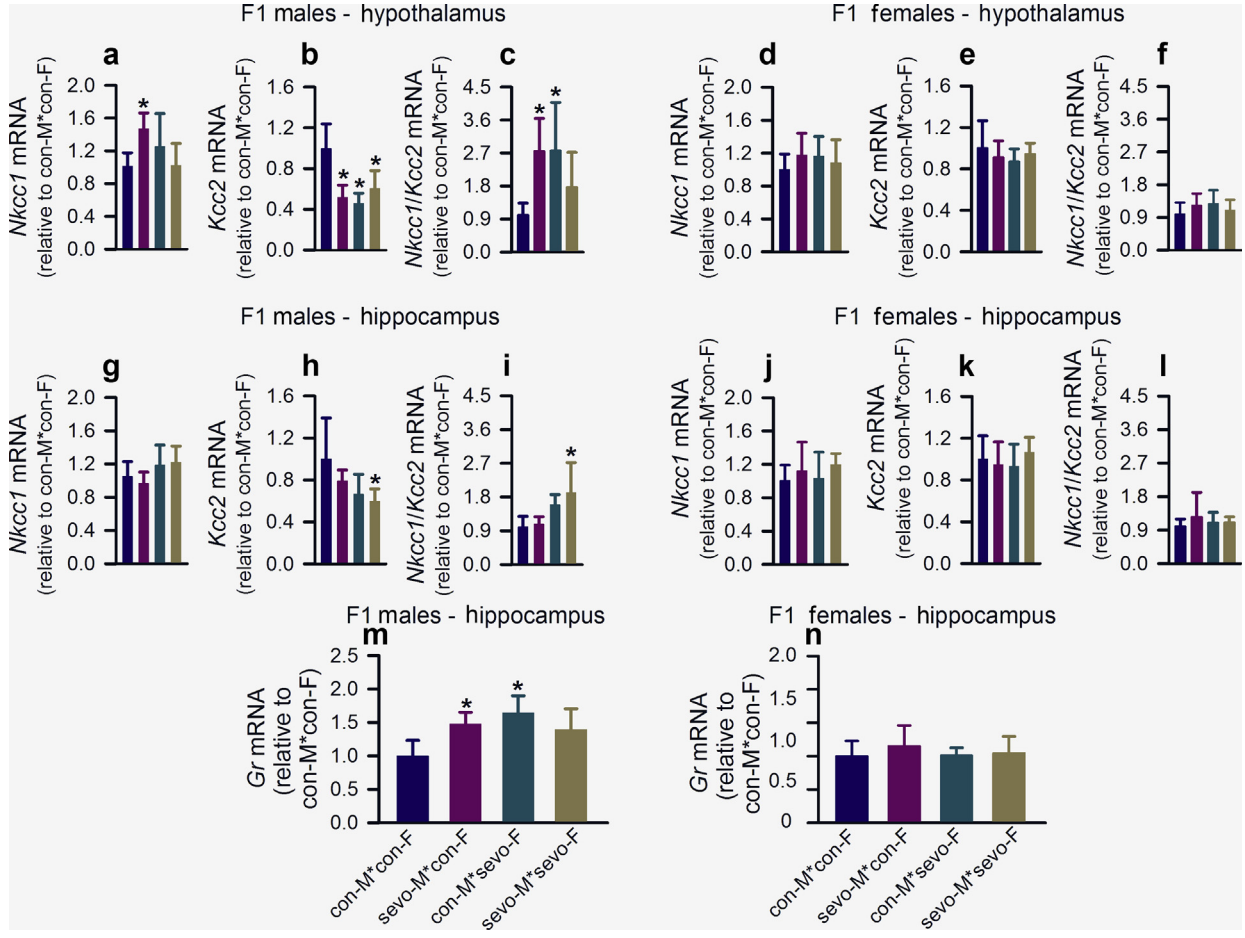

Fig 5. Gene expression for Na^+-K^+-$2Cl^-$ (*Nkcc1*), K^+-$2Cl^-$ (*Kcc2*) and glucocorticoid receptors (*Gr*) in the paraventricular nucleus (PVN) of the hypothalamus and hippocampus of F1 rats. Shown are the respective levels of *Nkcc1* messenger ribonucleic acid (mRNA), *Kcc2* mRNA, and the resulting *Nkcc1/Kcc2* mRNA ratios in the PVN of the hypothalamus of F1 males (a–c) and F1 females (d–f) and in the hippocampus of F1 males (g–i) and F1 females (j–l). Data normalised against control are means [standard deviation (SD)] from at least six rats per treatment group (n=7, male con-M*con-F and sevo-M*con-F groups). (m, n) Shown are levels of *Gr* mRNA in the hippocampus of male (m) and female (n) rats. Data normalised against control are means (SD) from six rats per treatment group. Colour coding in (m) and (n) is applicable to all figures. *P<0.05 *vs* F1 males from the con-M*con-F group.

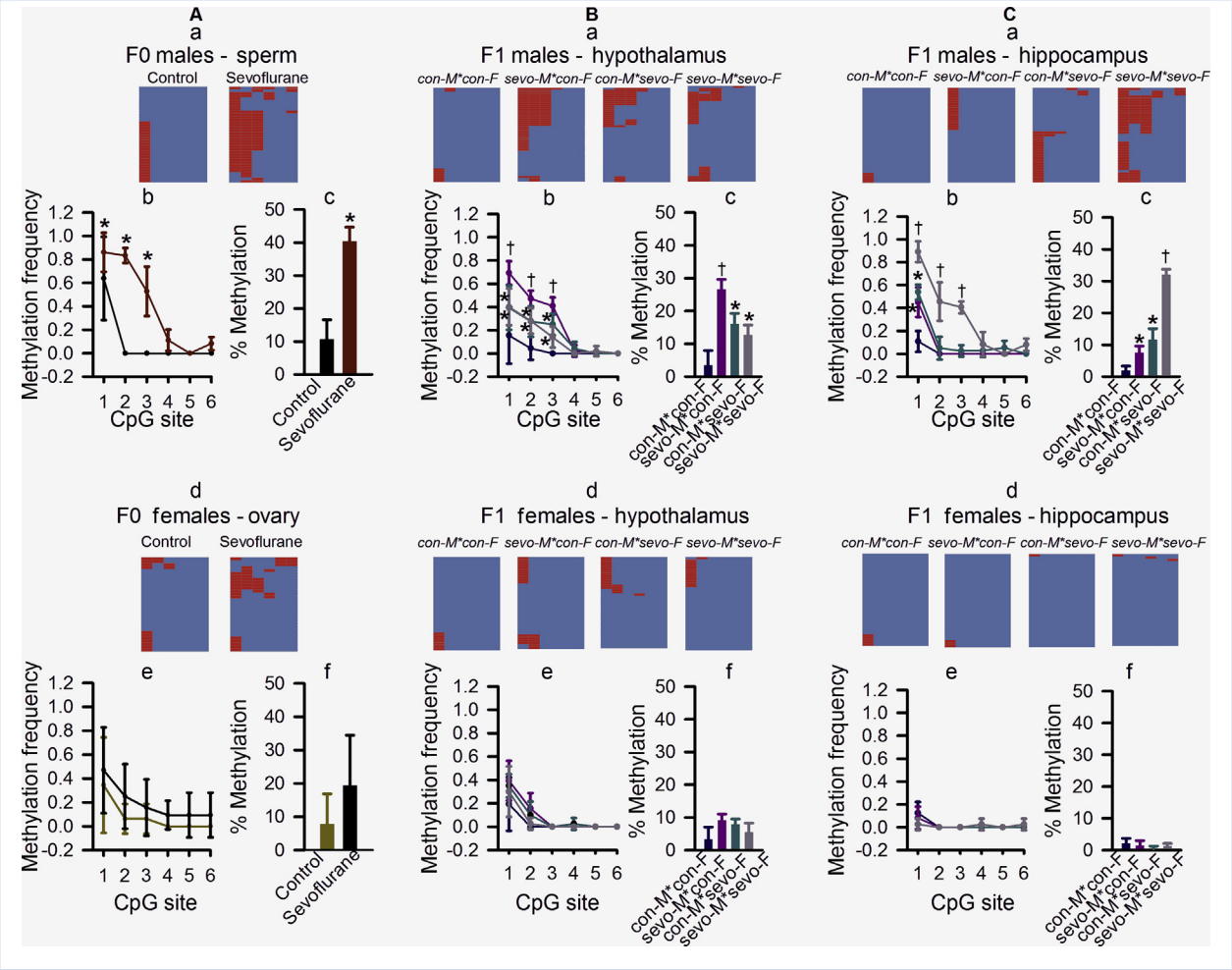

Fig 6. Methylation in promoter region of *Kcc2* gene in sperm and ovary deoxyribonucleic acid (DNA) of F0 rats and in the hypothalamus and hippocampus DNA of F1 rats. (A) Bisulphite sequencing of CpG sites in the *Kcc2* gene of nine clones from four individual sperm (A,a–c) and ovary (A,d–f) DNA samples isolated from sevoflurane-exposed and control F0 rats. Heat maps show DNA methylation status of CpG sites in the promoter region of the Kcc2 gene in sperm (A,a) and ovaries (A,d) of F0 rats. Red cells show methylated sites. X axis—CpG sites; Y axis—clones. Histograms showing methylation frequency at each CpG site (A,b—males; A,d—females) and DNA methylation level at all six CpG sites (A,c—males; A,f—females). (B) Shown are the DNA methylation status of CpG sites, methylation frequencies at each CpG site and DNA methylation level at all six CpG sites in the *Kcc2* gene of 9–10 clones from the hypothalamus of F1 male rats (B,a–c) and F1 female rats (B,d–f). (C) The results of similar analyses as in (B) for hippocampus of F1 rats. Data are means (standard deviation) from four rats per treatment group (n=5, hypothalamus samples isolated from male rats). *P<0.05 *vs* F1 males from the con-M*con-F group. †P<0.05 *vs* all other treatment groups. Colour coding in A,c is applicable to A,b; in A,f to A,e; in B,c,f to B,b,e; in C,c,f to C,b,e.

significant between-subjects effects of parental neonatal sevoflurane exposure on Cl^--transporter mRNA (Fig. 5j–l).

There was significant between-subjects effect of parental neonatal sevoflurane exposure on the hippocampal *Gr* mRNA levels in F1 males [$F_{(3,20)}$=7.44, P=0.002, Fig. 5m], but not in F1 females (Fig. 5n).

DNA methylation in the *Kcc2* gene promoter

In sperm of F0 rats there was significant effect of treatment [$F_{(1,36)}$=59.06, P<0.001, Fig. 6A,a–c] and within-subjects effect of CpG site [$F_{(5,36)}$ = 37.80, P<0.001] on methylation frequency. There was a trend but no significant difference between CpG site methylation frequency in the *Kcc2* gene promoter in ovaries of control and sevoflurane-exposed adult female rats (Fig. 6A,d–f). Greater methylation changes might be present in oocytes, a minor fraction of the cells in the ovary.

There was significant effect of parental treatment on the frequencies of CpG site methylation in the hypothalamus in F1 male [$F_{(3,96)}$=32.09, P<0.001, Fig. 6B,a–c], but not female progeny (Fig. 6B,d–f).

The CpG site methylation frequency in the hippocampus of F1 male rats was largest if both parents were exposed to sevoflurane as neonates [$F_{(3,72)}$=96.83, P<0.001, Fig. 6C,a–c], while F1 females were not significantly affected (Fig. 6C,d–f). We did not detect significant differences in the frequency of CpG site methylation in the promoter in the *Nkcc1* gene in F0 sperm of control and rats exposed to sevoflurane as neonates.

Discussion

A single exposure of neonatal rats to sevoflurane, the most frequently used general anaesthetic in paediatrics, led to significant behavioural abnormalities and changes in DNA methylation not only in exposed rats in adulthood, but also in their adult male offspring that were never exposed to sevoflurane. These effects of sevoflurane were strongly sex-dependent. The findings that male offspring only, but not female littermates, were affected indicate that it is unlikely that sevoflurane-induced abnormalities are transmitted to the next generation through sevoflurane-altered behaviour of the exposed dams. Furthermore, in the EPM and PPI of startle behavioural tests, male offspring of control females and exposed males were the only experimental group that exhibited significant abnormalities, even though F0 males did not have a direct contact with their progeny. These findings, together with increased hypothalamic and hippocampal *Nkcc1/Kcc2* mRNA ratios in exposed parents and their male offspring and similarly increased DNA methylation of the *Kcc2* gene promoter in the sperm of F0 exposed sires and hypothalamic and hippocampal tissues of their male, but not female progenies, strongly support involvement of epigenetic mechanisms in the effects of parental neonatal exposure to sevoflurane to the next generation.

The similarities between the developmental effects of exposure to GABAergic anaesthetics[4–8] and perinatal stress[9–11] early in life suggests similarities in the underlying mechanisms of both phenomena. Recent studies in rodents also report heritable multigenerational effects of perinatal stress.[19–21] Similar to our findings of normal corticosterone responses to physical restraint in progeny of sevoflurane-exposed parents, Morgan and Bale[19] found normal corticosterone responses in offspring of prenatally stressed males and control females. Similar to our findings that only males were affected by neonatal exposure of their parents to sevoflurane, developmental effects of paternal prenatal stress were detected in male offspring only.[19] Among plausible explanations for normal corticosterone responses in male offspring of the exposed rats could be increased expression of *Grs* in the hippocampus, PVN, pituitary, or all three consistent with our finding of increased concentrations of *Grs* mRNA in the hippocampus of F1 male rats where only one parent had been exposed to sevoflurane. The *GRs* mediate the negative feedback of corticosterone on HPA axis activity.[25]

Male offspring of exposed male F0/unexposed female F0 exhibited reductions in PPI of acoustic startle and in time spent in open arms of the EPM. These PPI and EPM abnormalities were accompanied by greater increases in hypothalamic PVN *Nkcc1/Kcc2* mRNA ratios. Also, male progeny of exposed male F0/unexposed female F0 had significantly higher CpG methylation frequencies in the promoter of the *Kcc2* gene in the hypothalamus. In contrast, abnormalities in spatial memory during the MWM test, a standardised and widely used behavioural test that strongly correlates with hippocampal synaptic plasticity,[26] were most prominent in male offspring when both parents were exposed. Again, consistent with behavioural findings, the greatest increase in hippocampal *Nkcc1/Kcc2* mRNA ratio was found in male offspring of this group. Furthermore, this group had significantly higher CpG methylation in the promoter of the *Kcc2* gene in the hippocampus. Together, these findings support an important role of epigenetic mechanisms in the mediation of heritable developmental effects of early life exposure to sevoflurane. Another novel observation is that a delay or postponement in the developmental maturation in the *Nkcc1/Kcc2* ratio in the PVN of the hypothalamus can selectively affect EPM and PPI behaviour with no significant effect on MWM behaviour, while impaired developmental maturation of the *Nkcc1/Kcc2* ratio in the hippocampus can have profound consequences for MWM behaviour, with no significant effects on EPM and PPI behaviour. In future studies it will be important to elucidate how closely the observed changes in gene expression in each specific experimental group translate to changes in respective protein concentrations.

Why male offspring of exposed male F0/unexposed female F0 exhibit significant deficiencies in the EPM and PPI of startle tests, especially when compared with offspring of both exposed parents, remains to be elucidated, as does why only male progeny were affected. A greater HPA axis response to stress in exposed F0 males,[6] as opposed to greater stress responses in naïve females,[27] suggest that anaesthesia alters postnatal brain sex differentiation, at least as it relates to HPA axis function. The primary female sex steroid hormone 17β-oestradiol, synthesised in neonatal brain through aromatisation of testis-derived testosterone, directs brain sexual differentiation by organisational actions during a critical period.[28] Of relevance, 17β-oestradiol is known to down-regulate neuronal *Kcc2* expression.[13,14] It would be important to explore whether sex-dependent developmental effects of sevoflurane include effects on brain sexual differentiation. Even though an increase in the *Nkcc1* mRNA level was detected only in the hypothalamus of one group of second generation male rats, the male progenies of exposed male F0/unexposed female F0, it remains to be elucidated how such an increase in hypothalamic *Nkcc1* mRNA level was passed to the next generation, as we were not able to detect significant changes in the methylation pattern of the *Nkcc1* gene promoter in sperm of exposed F0 males.

In summary, our results demonstrate for the first time that neurobehavioural abnormalities induced by neonatal exposure to the general anaesthetic sevoflurane can be transmitted to the next generation in a complex, sex- and brain region-specific mode through epigenetic mechanisms. This basic science study deals with a complex biological phenomenon of intergenerational heritability of the effects of environmental factors, in general, and with a newly uncovered potentially important translational problem (i.e. intergenerational heritability of the effects of early in life general anaesthesia exposure). Mechanisms of sevoflurane-induced sex- and brain region-specific effects across two generations are exciting and challenging topics for future studies. To further substantiate translational applicability of this phenomenon, additional animal studies using different neonatal anaesthesia paradigms that more broadly model stages of human postnatal brain development at the time of anaesthesia exposure and duration of anaesthesia exposure in young human patients will be needed.

Authors' contributions

Designed research: A.E.M., L.-S.J., T.E.M., N.G., C.N.S., J.L.R., J.-Q.Z.
Performed research: L.-S.J., J.-J.Y.
Analysed data: L.-S.J., J.-J.Y., A.E.M.
Wrote the paper: A.E.M., T.E.M., N.G., C.N.S., J.-Q.Z.
Approved the final manuscript: all authors.

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Declaration of interest

The authors declare that they have no conflicts of interest.

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