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Intergenerational Perioperative Neurocognitive Disorder in Young Adult Male Rats with Traumatic Brain Injury

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Abstract

Background: We tested the hypothesis that the effects of traumatic brain injury, surgery and sevoflurane interact to induce neurobehavioral abnormalities in adult male rats and/or in their offspring (an animal model of intergenerational perioperative neurocognitive disorder).

Methods: Sprague-Dawley male rats (F0 generation) underwent a traumatic brain injury on postnatal day 60 that involved craniectomy (surgery) under 3% sevoflurane for 40 min followed by 2.1% sevoflurane for 3 h on postnatal days 62, 64, and 66 (injury group). The surgery group had craniectomy without traumatic brain injury, whereas the sevoflurane group had sevoflurane only. On postnatal day 90, F0 males and control females were mated to generate offspring (F1 generation).

Results: Acutely, F0 injury rats exhibited the greatest increases in serum corticosterone and interleukins 1β and 6, and activation of the hippocampal microglia. Long term, compared to controls, F0 injury rats had the most exacerbated corticosterone levels at rest (2.21 ± 0.64 vs. 7.28 ± 1.95 ng/ml, n = 8 - 7; $P < 0.001$) and 10 min after restraint (133.12 ± 33.98 vs. 232.83 ± 40.71 ng/ml, n =8 - 7; P < 0.001), increased interleukins 1β and 6, and reduced expression

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of hippocampal glucocorticoid receptor (*Nr3c1*) (0.53 ± 0.08 fold change relative to control, $P < 0.001$, n = 6) and brain-derived neurotrophic factor genes. They also exhibited greater behavioral deficiencies. Similar abnormalities were evident in their male offspring, whereas F1 females were not affected. The reduced *Nr3c1* expression in F1 male, but not female, hippocampus was accompanied by corresponding *Nr3c1* promoter hypermethylated CpG sites in F0 spermatozoa and F1 male, but not female, hippocampus.

Conclusions: These findings in rats suggest that young adult males with traumatic brain injury are at an increased risk of developing perioperative neurocognitive disorder, as are their unexposed male but not female offspring.

Introduction

Accelerated neurocognitive decline after general anesthesia and surgery, termed postoperative cognitive dysfunction or perioperative neurocognitive disorder, is an important public health problem potentially affecting millions of patients every year. Its exact etiology is unknown.[1,2] Clinical and laboratory evidence suggest that perioperative stress, neuroinflammation, and preexisting neurodegenerative diseases play an essential role in perioperative neurocognitive disorder development.[2–4] Because neurodegenerative diseases become more prevalent and worsen with age, perioperative neurocognitive disorder symptoms are most often studied in the aging population, although younger individuals may also be affected.[1–5]

An important question is whether young adults with pathophysiological conditions that involve dysregulated stress response systems, neuroinflammation, and neurological/neurocognitive abnormalities are more vulnerable to perioperative neurocognitive disorder. One such condition is traumatic brain injury.[6–10] Traumatic brain injury, with >50 million cases per year, is a dominant cause of disability in young adults.[11,12] Patients with a history of traumatic brain injury may also require anesthesia/surgery or sedation to treat conditions unrelated or related to traumatic brain injury.[13]

Sevoflurane is a commonly used halogenated general anesthetic whose polyvalent actions include enhancement of γ-aminobutyric acid type A ($GABA_A$) receptor signaling.[14] We previously found that repeated exposure to sevoflurane in young adult rats, similar to prolonged exposure to sevoflurane in neonatal rats, not only affects the exposed rats, but causes intergenerational neurobehavioral abnormalities.[15,16] Other groups have confirmed such heritable effects of sevoflurane in rodents.[17,18] These experiments show that sevoflurane induces a K^+-$2Cl^-$ Cl^- exporter-mediated excitatory shift in $GABA_A$ receptor signaling, upregulation of the hypothalamic-pituitary-adrenal axis, and multifold increases in secretion of the stress hormone corticosterone. These findings also support the possibility that corticosterone release is involved in mediating sevoflurane-induced neurocognitive abnormalities in the exposed animals as well as transmissible epigenomic changes in their germ cells (in part through changes in DNA methylation).[15,16,19] Notably, a K^+-$2Cl^-$ Cl^- exporter-mediated excitatory shift in $GABA_A$ receptor signaling may also play a role in the pathophysiology of traumatic brain injury,[20] suggesting the involvement of similar mediating mechanisms.

Here, we tested the hypothesis that effects of surgery, traumatic brain injury, and sevoflurane interact to cause both acute and persistent dysregulation of the hypothalamic-pituitary-adrenal axis, increased inflammation, and behavioral deficits in young adult male rats (an animal model of perioperative neurocognitive disorder) and/or in their future offspring who have neither trauma or anesthetic exposure (intergenerational perioperative neurocognitive disorder).

Materials and Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. The study was conducted, and data are reported in accordance with the ARRIVE guidelines.[21] Sprague-Dawley rats (generation F0) were purchased from Charles River (Wilmington, MA). The F0 female rats were used as breeders only to generate offspring (generation F1). Rats were housed under controlled illumination (12-h light/12-h dark, lights on at 7:00 AM) and temperature (23–24 °C) with free access to food and water. Within 24 h of delivery, F1 litters were culled to 12 pups. At 21 days, pups were weaned and housed two per cage for the rest of the study. Experimental data in this study are from 192 male and 72 female rats. One rat in the injury group died immediately after traumatic brain injury induction and two other rats in the surgery group were removed from the study because of suture failure during the recovery period. All three rats were excluded from all analyses.

Treatment Groups

Figure 1 shows an overview of the study design. F0 male rats were randomized into four treatment groups (n = 30/group). Investigators were blinded to group assignments. Rats in the injury group were subjected to all interventions: 1) surgery under 3% SEVO anesthesia for 40 min on postnatal day 60 to conduct a craniectomy and implant an injury hub; 2) a midline fluid percussion-inflicted moderate traumatic brain injury (~2.0 atm average pressure wave; ~9 min time from the initial impact until the rat spontaneously rights itself from a supine position)[20] on the same day; and 3) exposure to 2.1% sevoflurane for 3 h on postnatal days 62, 64, and 66, to model anesthesia/sedation needed for treatment of conditions associated with traumatic brain injury or unrelated ones.[13] Rats in the sevoflurane group had only sevoflurane exposure on postnatal days 60, 62, 64, and 66. Rats in the surgery group had a craniectomy and injury hub implantation but not traumatic brain injury on postnatal day 60. They also had sevoflurane exposure on postnatal days 62, 64, and 66. Rats in the control group were placed in a new cage and housed one per cage for an equivalent amount of time on postnatal days 60, 62, 64, and 66.

A subset of F0 male rats from all groups (n = 12/group) was sacrificed 1 h after recovery from sevoflurane anesthesia on postnatal day 66 or at an equivalent timepoint in the control group to study acute effects. The remaining F0 male rats (n = 18/group) were mated on postnatal day 90 with control female rats to produce offspring. A cohort of 18 breeding pairs of F0 male and female rats were used to produce F1 offspring for a given experimental group (18 F1 rats/group/sex). A given F1 offspring experimental group included 1 to 2 rats

from a given F1 litter. Within 24 h of delivery, F1 litters were culled to 12 pups. The F0 females were housed individually throughout the entire gestation and postpartum rearing periods. At the age of 21 days, pups were weaned and housed in sex-matched pairs for the rest of the study. F1 rats were not exposed to any treatment and were subjected to animal facility rearing only.

The F0 sires and F1 male and female offspring were sequentially evaluated in the elevated plus maze[22], for prepulse inhibition of the acoustic startle response[22,23], and in the Morris water maze[24,25] (Figure 1). To measure corticosterone responses to stress, blood samples were collected immediately prior to physical restraint for 30 min and 10, 60, and 120 min after restraint in F0 sires, and 30 min after confinement for the prepulse inhibition of acoustic startle test in F1 offspring. Ten days after completing the *in vivo* studies, F0 and F1 rats were anesthetized and sacrificed through decapitation to collect trunk blood and brain tissue samples for enzyme-linked immunosorbent assays,[15,16] reverse transcription-polymerase chain reaction,[15,16] and targeted next-generation bisulfite sequencing studies.[26] All methods have been described in the referenced studies[15,16,22–26] (see Supplemental Digital Content 1 for detailed descriptions). In response to peer review, additional experiments were conducted on hippocampal *Nr3c1* methylation in F1 females and serum levels of proinflammatory cytokine interleukin 6 in both generations. In addition, the Morris water maze data were re-analyzed to estimate the time spent in each quadrant by a single group.

Statistical Analyses

The primary outcomes in this study were the neuroendocrine and behavioral changes in F0 sires and in their F1 offspring. All other outcome measurements were secondary outcomes. Sample size calculations were done, assuming a range of anticipated differences in mean outcomes and standard deviation based on background data and past experience with similar measurements in Sprague-Dawley rats.[3,8] These analyses indicated that sample sizes of at least n = 16 rats/group for behavioral studies and n = 5 rats/group for measurements in tissue samples were required to detect differences between treatment groups, with effect sizes of $d \geq 0.8$, assuming an α level of 0.05. Values are reported as mean ± SD. Boxplots were used to identify outliers. No outliers were detected that were not in the plausible range of values for the outcomes; therefore, all data were maintained in analyses. One-way ANOVA was used to assess F0 data for acute serum corticosterone, interleukin-1β and interleukin-6 levels, acute ionized calcium binding adaptor 1 (microglia/macrophage-specific protein marker) expression, long-term resting corticosterone, total corticosterone concentrations before and after the restraint, interleukin-1β and interleukin-6 levels, changes in gene expression, time spent in and number of entries to the open arms and total distance traveled during the elevated plus maze test, and number of crossings over the former platform during the Morris water maze probe test. Two-way repeated measures ANOVA with experimental groups and time as the independent variables was run to analyze changes in serum corticosterone levels before and at three time points after the restraint. Two-way repeated measures ANOVA was used to analyze the F0 prepluse inhibition data, with treatment and prepulse intensity as independent variables. Two-way ANOVA with experimental groups and days of training as the independent variables was used to analyze changes in escape latencies to the escape

platform during the Morris water maze test in F0 rats. Two-way ANOVA was used to analyze the time spent in each quadrant during the MWM probe test in F0 rats, with the treatment and quadrant as independent variables. Two-way ANOVA with treatment and sex as the independent variables were used to assess F1 data for changes in serum corticosterone levels at rest and after the prepluse inhibition test, changes in serum interleukin-1β and interleukin-6 levels, changes in gene expression, time spent in and number of entries to the open arms and total distance traveled during the elevated plus maze test, and Morris water maze platform location crossing times. For F1 prepluse inhibition, Morris water maze escape latency and time in each quadrant, linear mixed models for repeated measures were used, with prepluse inhibition intensity, days of training, and quadrant modeled as repeated measures, respectively. These analyses account for within-subject corrections across repeated measurements. The models also included treatment and sex as main effects, as well as interaction terms. An independent t test was used to analyze methylation level at each CpG site and overall CpG sites of *Nr3c1* gene. Analyses were conducted in SigmaPlot 14.0 software and SPSS v27 (IBM Corp., Armonk, NY, USA). Multiple pairwise comparisons were done with the Holm-Sidak method. $P < 0.05$ was considered significant.

Results

Acute Changes in Hypothalamic-Pituitary-Adrenal Axis Activity and Inflammation in F0 Male Rats

Analyses of blood and brain tissue samples collected 1 h after the last exposure to sevoflurane or at equivalent time points in the control group found a main effect of treatment (or intervention), *i.e.*, control, sevoflurane, surgery, and traumatic brain injury, on serum levels of corticosterone and the proinflammatory cytokines interleukin-1β and interleukin-6, as well as hippocampal levels of ionized calcium binding adaptor 1, a microglia-/macrophage-specific calcium-binding protein (fig. 2A–D and suppl. table 1). Rats in the injury group had higher serum levels of corticosterone than the other groups, except the sevoflurane group. Inflammatory markers were greatest in injury rats, both systemically and within the brain.

Long-term Changes in Hypothalamic-Pituitary-Adrenal Axis Activity, Systemic Inflammation, and Brain-Derived Neurotrophic Factor mRNA Levels in the Hypothalamus and Hippocampus in F0 Males

Assessment of resting corticosterone levels >80 days after the interventions (table 1 and fig. 3A) revealed that the injury group had higher corticosterone levels than all the other groups. There was also an interaction between treatment and the time course of corticosterone levels following restraint stress (fig. 3B,C and suppl. table 1), in that the injury group had higher levels 10 min after restraint than all other groups.

In agreement with the changes in corticosterone levels, there was a main effect of treatment on mRNA levels of hypothalamic corticotropin-releasing hormone (*Crh*), glucocorticoid receptor (*Nr3c1*), and mineralocorticoid receptor (*Nr3c2*) (fig. 3D,F,H and suppl. table 1). When compared to controls, only rats in the injury group had lower *Nr3c1* and *Nr3c2* levels, whereas *Crh* mRNA levels were increased in the injury, surgery, and sevoflurane groups.

There were also changes in mRNA levels of hippocampal *Nr3c1* and *Nr3c2* but not *Crh* (fig. 3E,G,I and suppl. table 1). The injury group had lower *Nr3c1* and *Nr3c2* mRNA levels than the control and sevoflurane groups, whereas the surgery group had lower levels of these receptors than the control group.

There were significant changes in serum levels of proinflammatory cytokines (fig. 3J,K and suppl. table 1), which, compared to controls, were increased in the injury and surgery groups (interleukin-1β) and in the sevoflurane, injury and surgery groups (interleukin-6). There were also main effects of treatment on *Bdnf* mRNA in the hypothalamus and hippocampus (fig. 3L,M and suppl. table 1). The *Bdnf* levels were lower in the injury group than the control and sevoflurane groups (hypothalamus) and the control group (hippocampus), whereas rats in the surgery and sevoflurane groups had lower hypothalamic *Bdnf* mRNA levels than the control group.

Long-term Behavioral Effects of Sevoflurane in F0 Males

Analyses of F0 rats' behavior during the elevated plus maze test revealed main effects of treatment on both time spent in the open arms and number of open arm entries (fig. 4A,B and suppl. table 1), with no effects on total distance traveled (fig. 4C and suppl. table 1). The control group spent more time in the open arms *vs.* all the other groups. Only the injury group made fewer open arm entries than the control group.

There were also effects of treatment and prepulse intensity in the prepulse inhibition of the acoustic startle response test (fig. 4D and suppl. table 1). Multiple pairwise comparisons indicated that rats in the injury group exhibited impaired prepulse inhibition of startle at prepulse intensities of 3 dB, 6 dB, and 12 dB *vs.* the control group.

Spatial learning (escape latencies) during the 5 days of Morris water maze training revealed no differences among treatment groups (fig. 4E and suppl. table 1). During recall testing, however (*i.e.,* in the absence of an escape platform), the injury, surgery, and sevoflurane groups made fewer crossings over the escape platform location than the control group (fig. 4F and suppl. table 1). Furthermore, the injury group exhibited greater impairment in spatial memory by spending more time in the entrance quadrant and less time in the target quadrant (fig. G and suppl. table 1). Analysis of the time that F0 rats from each treatment group spent in each quadrant showed that rats from the control, sevoflurane and surgery groups spent more time in the target quadrant than in any other quadrant (fig. 4H and suppl. table 1). Consistent with an interaction of the detrimental effects of surgery, traumatic brain injury and sevoflurane, however, there was no difference between the times that F0 injury rats spent in the entry and target quadrants (fig. 4H and suppl. table 1).

Changes in Hypothalamic-Pituitary-Adrenal Axis Activity, Inflammatory Markers, and *Bdnf* mRNA Levels in F1 Offspring

Resting corticosterone in F1 offspring was unaffected by paternal treatment, though there was a main effect of sex (greater corticosterone levels in females; (fig. 5A and suppl. table 2). In contrast, stress-induced corticosterone release was affected by paternal treatment in a sex-dependent manner (fig. 5B and suppl. table 2). Specifically, 30 min after completion of the prepulse inhibition test, F1 males of sires from the injury and surgery groups had higher

corticosterone levels than both F1 males offspring of control and sevoflurane sires or the respective groups of F1 females. Stress-induced corticosterone levels in F1 females were not affected by paternal treatments.

There was no main effect of paternal treatment, sex, or sex × paternal treatment interaction on *Crh* mRNA in hypothalamic and hippocampal tissue from non-stressed animals (fig. 5C,D and suppl. table 2). In contrast, and consistent with the differential response to stress, assessment of hypothalamic *Nr3c1* mRNA revealed a main effect of sex and paternal treatment × sex interaction, but no effect of paternal treatment (fig. 5E and suppl. table 2), whereas assessment of hippocampal *Nr3c1* mRNA revealed a main effect of paternal treatment, sex, and paternal treatment × sex interaction (fig. 5F and suppl. table 2). Compared to F1 males of control sires, only F1 males of injury sires had reduced *Nr3c1* mRNA transcripts in the hypothalamus and hippocampus. The *Nr3c1* mRNA in F1 males of the injury and surgery sires (hypothalamus) and the injury, surgery, and sevoflurane sires (hippocampus) were lower than in respective groups of F1 females. Effects of paternal treatment on *Nr3c2* mRNA levels in the hypothalamus were not sufficient to achieve significance (fig. 5G and suppl. table 2); however, there was a main effect of paternal treatment, but not sex or treatment × sex interaction on *Nr3c2* mRNA levels in the hippocampus (fig. 5H and suppl. table 2). Only F1 males of injury sires had lower hippocampal *Nr3c2* mRNA levels than F1 males of control sires.

Consistent with the markers of sustained systemic inflammation in F0 sires, there was a significant between-subjects effect of paternal treatment, sex, and treatment × sex interaction on serum levels of interleukin-1β and interleukin-6 in F1 offspring (fig. 5I,J and suppl. table 2). F1 males of injury sires had higher serum interleukin-1β levels than F1 males of sires from all the other groups or F1 females in the respective groups. Also, F1 males of surgery sires had higher interleukin-1β levels than male rats of control sires (fig. 5I and suppl. table 2). Serum levels of interleukin-6 were increased in F1 male offspring of sires from sevoflurane, injury and surgery groups (fig. 5J and suppl. table 2). Levels of interleukin-1β and interleukin-6 in F1 females were not affected by paternal treatment.

There were main effects of paternal treatment, sex, and paternal treatment × sex interaction on the *Bdnf* mRNA transcripts in both hypothalamus and hippocampus of F1 offspring (fig. 5K,L and suppl. table 2). F1 males of injury and surgery sires (hypothalamus) and the injury, surgery, and sevoflurane sires (hippocampus) had lower *Bdnf* mRNA levels than F1 males of control sires. F1 males of injury, surgery, and sevoflurane sires (hypothalamus) and injury and surgery sires (hippocampus) had lower *Bdnf* mRNA levels than the respective groups of F1 females. The latter were not affected by paternal treatments.

Effects of Paternal Treatment on F1 Offspring Behavior

In the elevated plus maze, there were main effects of paternal treatment and sex, but no paternal treatment × sex interaction on percent time in the open arms (fig. 6A and suppl. table 2). F1 males of injury and surgery sires spent less time in the open arms than F1 males of control sires or respective groups of F1 females. The number of open arm entries and the total distance traveled were not affected (fig. 6B,C and suppl. table 2).

There were overall differences for paternal treatment, sex, and prepulse intensity for prepulse inhibition of startle in F1 rats (fig. 6D and suppl. table 2). F1 males from injury sires were significantly impaired compared to male offspring of control sires at prepulse intensities of 3 dB, 6 dB, and 12 dB and to the male offspring of surgery sires at a prepulse intensity of 3 dB. There was no significant treatment effect in F1 females.

Paternal treatment did not affect escape latencies over the 5-day training period (fig. 6E and suppl. table 2) or number of crossings over the former escape platform (fig. 6F and suppl. table 2) during the Morris water maze test. There was a within-subjects effect of quadrant, but not sex or paternal treatment, on the time spent in the four quadrants of the maze (fig. 6G and suppl. table 2). Male offspring of control sires spent less time in the entrance quadrant and longer in the target quadrant than F1 males of sires from all the other groups. The analysis of the time that F1 rats from a specific treatment group spent in each quadrant showed that in contrast to F0 sires, only F1 male offspring of control sires spent more time in the target quadrant than in any other quadrant (fig. 6H and suppl. table 2). F1 males from sevoflurane and surgery sires spent similar time in the entry and target quadrants, while F1 male offspring of injury sires spent similar time in all four quadrants (fig. 6H and suppl. table 2). Although F1 female offspring of control, surgery, and injury sires spent more time in the target quadrant than in any other quadrant, the time that F1 female offspring of sevoflurane sires spent in the target quadrant was not sufficient to become statistically different from the time that they spent in the entry quadrant (fig. 6H and suppl. table 2).

Increased DNA Methylation in the Promoter Region of the *Nr3c1* Gene in F0 Spermatozoa and F0 male and F1 male, but not F1 female, Hippocampi

To gain insight into potential mechanism(s) by which effects of paternal treatment are transmitted to F1 offspring, we used targeted next-generation bisulfite sequencing to evaluate DNA methylation levels in the *Nr3c1* gene in spermatozoa of F0 injury and control sires and the hippocampus of their F1 offspring, as well as in the hippocampus of F0 injury and control sires. We found differentially methylated CpG sites in the chr18: 31,271,681–31,393,375 region of the *Nr3c1* gene in F0 spermatozoa (9 hyper- and 1 hypomethylated), F1 male, but not F1 female hippocampus (5 hyper- and 1 hypomethylated), and F0 hippocampus (5 hyper- and 1 hypomethylated) (fig. 7B,C). Among hypermethylated CpG sites, 3 F1 male hippocampal and 3 F0 spermatozoa had the same genomic coordinates (CpG sites # −1745, −486, and −385; fig. 7B,C). Interestingly, two of these common hypermethylated CpG sites (CpG sites # −486 and −385) were also hypermethylated in the hippocampus of injury sires. There was a significant effect of treatment on overall methylation levels of *Nr3c1* promoter regions in the spermatozoa and hippocampus of F0 sires and the hippocampus of F1 males, but not F1 females (fig. 7D), in that methylation of 29 CpG sites in the *Nr3c1* promoter was increased in injury sires and their male offspring compared to their respective control groups.

Discussion

A strong cognitive reserve among young adults is a likely reason that the possibility of perioperative neurocognitive disorder has received less attention in patients from this age

group. The findings of this study demonstrate that in young adult male rats, the effects of surgery under sevoflurane anesthesia to induce traumatic brain injury, and to an even greater degree the effects of surgery and traumatic brain injury combined, interact with the effects of subsequent repeated sevoflurane exposure to induce abnormalities in hypothalamic-pituitary-adrenal axis functioning, inflammatory markers, and some, but not all, behavioral tests. The findings of this study also demonstrate that male F1 offspring can develop the same types of abnormalities; i.e., an intergenerational perioperative neurocognitive disorder. Importantly, unexposed F1 offspring were in some cases affected even when their exposed sires did not exhibit overt deficiencies, at least in regard to spatial memory in sevoflurane F0 males and their F1 male offspring. Overlapping hypermethylated CpG sites in the *Nr3c1* gene in the spermatozoa of F0 injury rats and in the hippocampus of their male but not female offspring (particularly in the proximal promoter), reduced *Nr3c1* expression in the F1 male but not female hippocampus, and exacerbated glucocorticoid receptor-dependent hypothalamic-pituitary-adrenal axis responses to stress in F1 males but not females support the involvement of epigenetic mechanisms in the intergenerational transmission of adverse effects of paternal surgery, traumatic brain injury, and sevoflurane exposure. The findings that *Nr3c1* was similarly hypermethylated and exhibited similarly-reduced expression in the brains of F0 injury sires and that both generations exhibited similar neuroendocrine, inflammatory, and neurobehavioral abnormalities point to overlapping initiating mechanisms of the F0 somatic and germ cell effects of surgery, traumatic brain injury, and sevoflurane.

Studies in animal models have shown that parental preconception treatments with corticosterone or synthetic glucocorticoids induce epigenetic changes in spermatozoa and phenotypic alterations in offspring similar to those induced by parental preconception stress.[27–30] In addition, treatment with glucocorticoid receptor antagonists during paternal preconception exposure to stress ameliorates heritable effects of stressful experiences,[29] suggesting that exacerbated glucocorticoid responses are involved in initiation of heritable effects of stressful experiences. Dysregulated stress response systems and elevated corticosterone in particular are likely contributing factors in initiating heritable effects of the combination of surgery, traumatic brain injury, and sevoflurane (this study) as well as sevoflurane alone.[15,16] In support of a role for corticosterone in initiating intergenerational effects of sevoflurane, we recently demonstrated that F0 male and female rats exposed to sevoflurane had high corticosterone levels at the time of exposure, but only F0 males exhibited persistent neurobehavioral deficiencies.[15] Notably, F0 sires and dams had similar epigenomic alterations in the K^+-$2Cl^-$ Cl^- exporter gene in sperm and ovarian tissue, respectively, and both passed abnormalities to F1 male offspring.[15] The hypothalamic-pituitary-adrenal axis effects of sevoflurane in young adult rats can be ameliorated by pretreatments with the Na^+-K^+-Cl^- Cl^- importer inhibitor bumetanide (*the authors' unpublished observations*). Studies to investigate the effects of bumetanide and glucocorticoid receptor antagonists on intergenerational perioperative neurocognitive disorder in young adult rats with traumatic brain injury are in preparation.

An important difference between the intergenerational effects of sevoflurane alone[15,16] and the combination of surgery, traumatic brain injury, and sevoflurane was that only F1 male offspring of surgery, and even more so injury, sires exhibited abnormal corticosterone responses to stress. Notable, sires from the surgery and injury groups had greater increases

in levels of inflammatory markers, suggesting that the interaction between acute stress- and inflammation-like effects of sevoflurane, surgery, and traumatic brain injury may lead to greater germ cell, long-term somatic, and neuroendocrine and neurobehavioral abnormalities in F0 sires. By extension, they may also lead to greater neuroendocrine and neurobehavioral abnormalities in F1 offspring. The interaction of the effects of paternal surgery, traumatic brain injury, and sevoflurane to induce dysregulation of hypothalamic-pituitary-adrenal axis functioning in F0 sires and their F1 male offspring was especially evident in the reduced expression of both hypothalamic and hippocampal corticoid receptor genes. Corticoid receptors are involved in feedback regulation of corticosterone levels within the hypothalamic-pituitary-adrenal axis.[31] Dysregulated stress response systems and inflammation play key roles in the pathogenesis of neurodegenerative/neurocognitive disorders and aging.[32–35] Therefore, these findings further support the possibility that young adults with traumatic brain injury are at a greater risk of developing accelerated neurocognitive decline after surgery/anesthesia, and that such a risk can be inherited by their male offspring.

Because of persistent stress-like effects of sevoflurane alone[15,16] and even stronger persistent stress- and inflammation-like effects of surgery, traumatic brain injury and sevoflurane, we speculate that surgery and/or sedation do not need to be concurrent with traumatic brain injury to result in more severe intergenerational perioperative neurocognitive disorder. In other words, patients who experience traumatic brain injury may develop perioperative neurocognitive disorder because of later distant exposure to surgery and/or sedation. It will be important to test this possibility by investigating intergenerational perioperative neurocognitive disorder in patients with traumatic brain injury subjected to surgery and/or prolonged sedation across different time intervals.

Many additional questions still need to be addressed in future studies that more closely model clinical settings; e.g., the roles of number, duration, and depth of paternal anesthesia and the delay between surgery, traumatic brain injury, and anesthesia exposure and mating. The 3-h sevoflurane regimen on three alternating days employed in this study may be long for the most common clinical cases, but it is still applicable to many patients.[36–38] More recent examples of long exposure to general anesthetics include patients with COVID-19 in the intensive care unit who may require general anesthesia levels of sedation for >20 days.[39] It will be translationally important to elucidate effects of traumatic brain injury of different severity, as well as other models of traumatic brain injury including those that do not involve surgery. It will further be important in future work to investigate a broader range of genes (beyond *Nr3c1*) that could be involved in intergenerational transmission of the effects of surgery, traumatic brain injury, and sevoflurane.

In this exploratory study, we have chosen to start testing the interaction hypothesis of intergenerational effects of traumatic brain injury/surgery/sevoflurane, in general, and of the role of epigenomic transmission in such effects, in particular, in F0 males, because the sires' contribution to offspring development in rodents is largely restricted to passing spermatozoa containing genomic and epigenomic information. Testing of this hypothesis in F0 females, which will be done in future studies, will require embryo or ovary transfer to a nonexposed female and fostering of neonates by a nonexposed surrogate mother. This will allow

investigation of the role of epigenetic modifications in the maternal germline in heritable effects of traumatic brain injury/surgery/sevoflurane, while excluding potential effects of altered maternal physiology and behavior on the offspring's development. Investigations of both maternal and paternal intergenerational perioperative neurocognitive disorders are not only equally important, but, additionally, may help to elucidate mechanisms of heritable effects of parental experiences, as intergenerational effects of stress and general anesthetics may be parent-dependent. For example, studies in humans show that distinct, even opposite, changes in methylation of the *NR3C1* gene in offspring occur depending on whether they are initiated by paternal or maternal stressful experiences that lead to posttraumatic stress disorder.[40] On the other hand, we have recently reported that F0 female rats exposed to sevoflurane in young adulthood are less affected neurobehaviorally than F0 males, but F1 male offspring of both sevoflurane-exposed F0 males and females exhibit neurobehavioral deficiencies.[15] Therefore, it is a possibility that the dams with traumatic brain injury exposed to surgery/general anesthesia might have hypermethylated *Nr3c1* genes in ovary and act as asymptomatic "silent carriers" of perioperative neurocognitive disorder to offspring.

In the current study, abnormalities in all measured parameters were found almost exclusively in F1 males. These findings closely resemble sex-specific intergenerational effects induced by young adult or neonatal exposure to sevoflurane alone,[15,16] suggesting similar initiating mechanisms of intergenerational effects of parental exposure to sevoflurane alone and surgery/traumatic brain injury/sevoflurane. Future studies will be needed to elucidate such mediating mechanisms.

The confounding effects of social, cultural, educational, and behavioral factors are among the reasons that the heritability of adverse effects of surgery, traumatic brain injury or general anesthetics has not been evaluated in humans. Nevertheless, the persistent and heritable adverse effects of stress in specific circumstances, for instance, when war or famine affected large groups of people in relatively compact living areas within a defined period of time, have been extensively studied.[40–42] The findings of such human studies along with the findings of stress- and inflammation-like intergenerational effects of surgery/traumatic brain injury/sevoflurane in young adult male rats in this study, which had the advantage of strictly controlled experimental conditions, draw attention to the need for understanding and management of perioperative neurocognitive disorders in millions of young adult patients with traumatic brain injury. Considering that persistent inflammation and excessive stress are associated with accelerated cognitive decline in later life,[32–35] our findings suggest that surgery/traumatic brain injury/sevoflurane-induced perioperative neurocognitive disorder in the F0 sires and their F1 male offspring might worsen as life progresses. These findings also highlight the need to investigate the dynamics of intergenerational perioperative neurocognitive disorder in human patients with traumatic brain injury across the life span of several generations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding Statement:

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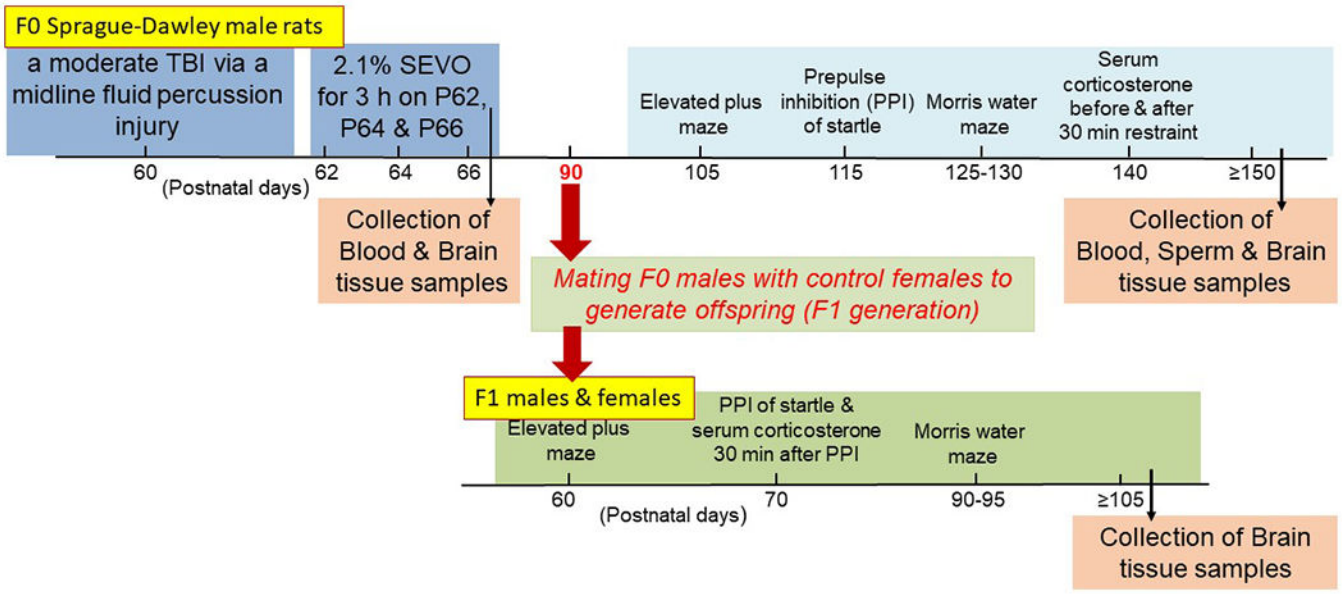

Fig. 1.
Study design.

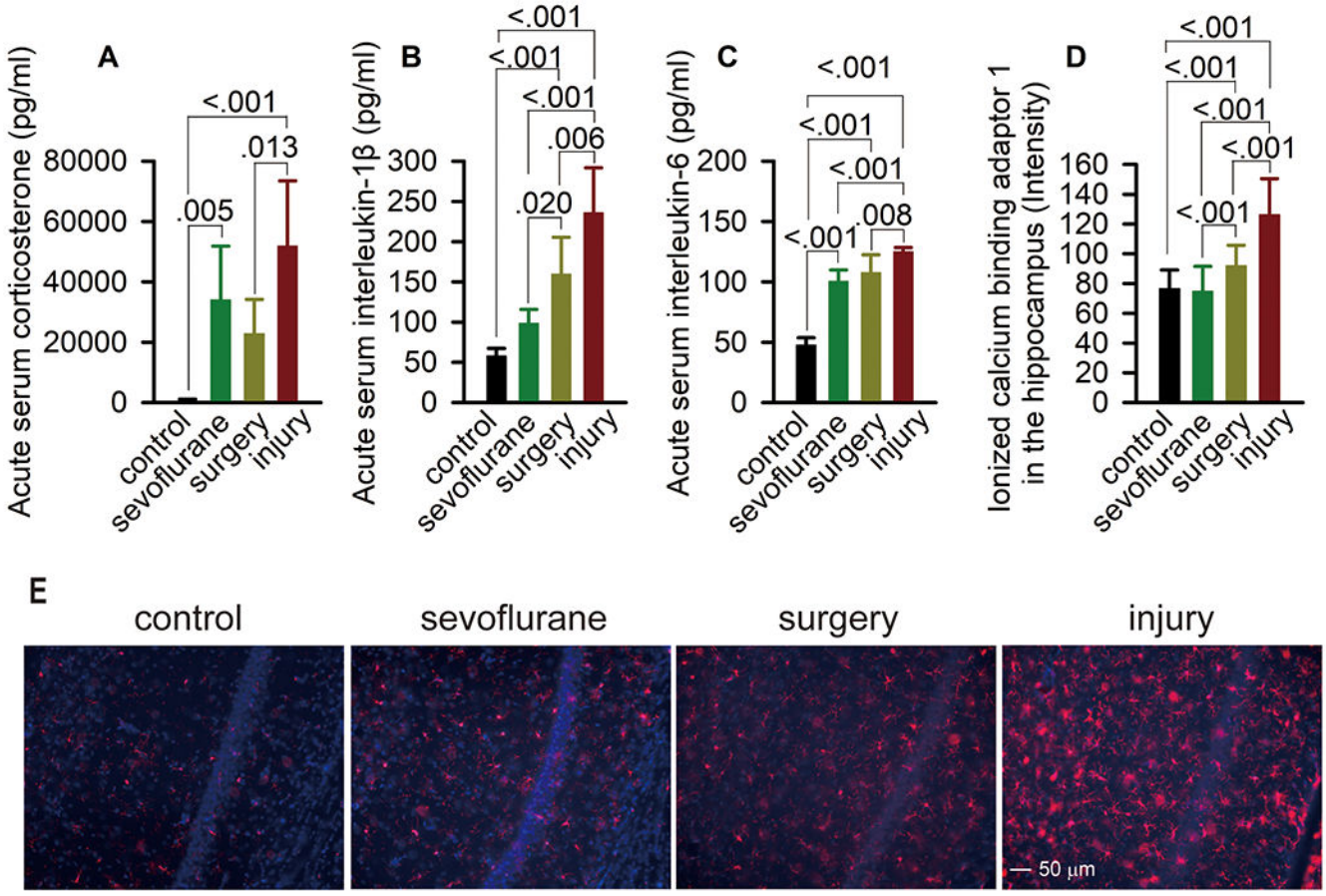

Fig. 2.

Levels of corticosterone and interleukin-1β and interleukin-6 in serum and ionized calcium binding adaptor 1 protein in the hippocampus of F0 sires 1 h after the exposure to sevoflurane on postnatal day 66 (the sevoflurane, surgery, and injury groups) or at an equivalent time point in the control group. (*A-C*) The respective levels of serum corticosterone, interleukin-1β and interleukin-6. (*D,E*) The results of quantification of ionized calcium binding adaptor 1 fluorescence and representative images of ionized calcium binding adaptor 1 in the hippocampus. Data are mean ± SD from 6 rats/group. The *P* values of multiple pairwise comparisons are shown in the respective plots above the horizontal lines. The beginning and end of the horizontal lines correspond to the compared experimental groups.

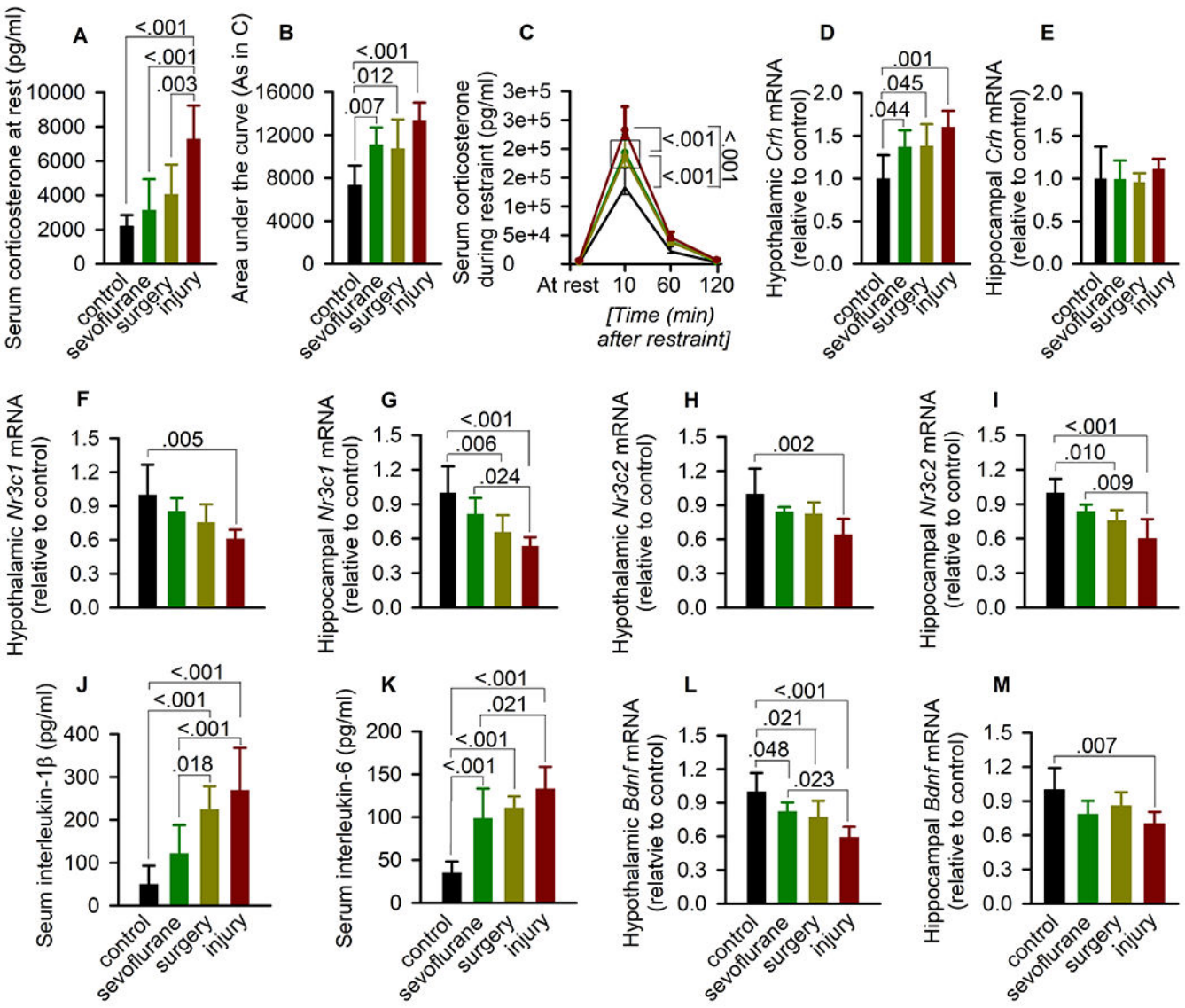

Fig. 3.

Long-term alterations in hypothalamic-pituitary-adrenal axis activity, systemic inflammation, and brain-derived neurotrophic factor (*Bdnf*) messenger RNA (mRNA) levels in F0 sires. (*A*) The resting serum levels of corticosterone in trunk blood samples collected from postnatal day ≥150 rats. Data are mean ± SD from 7 rats/group (n = 8 in control group). (*B,C*) The total serum corticosterone responses (*B*) and the respective levels of corticosterone before physical restraint for 30 min and at 10, 60, and 120 min after the restraint on postnatal day 140 (C). To assess differences in total corticosterone concentrations, area under the curve with respect to ground (levels of corticosterone at rest were taken as ground) was calculated. (*B*) Data are mean ± SD from 7 rats/group (n = 8 in Control group). (*D-I,L,M*) The respective mRNA levels of corticotropin-releasing hormone (*Crh*), glucocorticoid receptor (*Nr3c1*), mineralocorticoid receptor (*Nr3c2*), and *Bdnf* in the hypothalamus and hippocampus tissue samples collected on postnatal day ≥150. Data normalized against control are mean ± SD from 6 rats/group. (*J,K*) Serum levels of

interleukin-1β and interleukin-6 in postnatal day ≥150 F0 sires. Data are mean ± SD from 6 rats per treatment group for interleukin-1β and 8 rats per treatment group for interleukin-6. Color coding of experimental groups in A,B,D,E and J-M is applicable to all figures. The *P* values of the multiple pairwise comparisons are shown in the respective plots above the horizontal lines. The beginning and end of the horizontal lines correspond to the compared experimental groups.

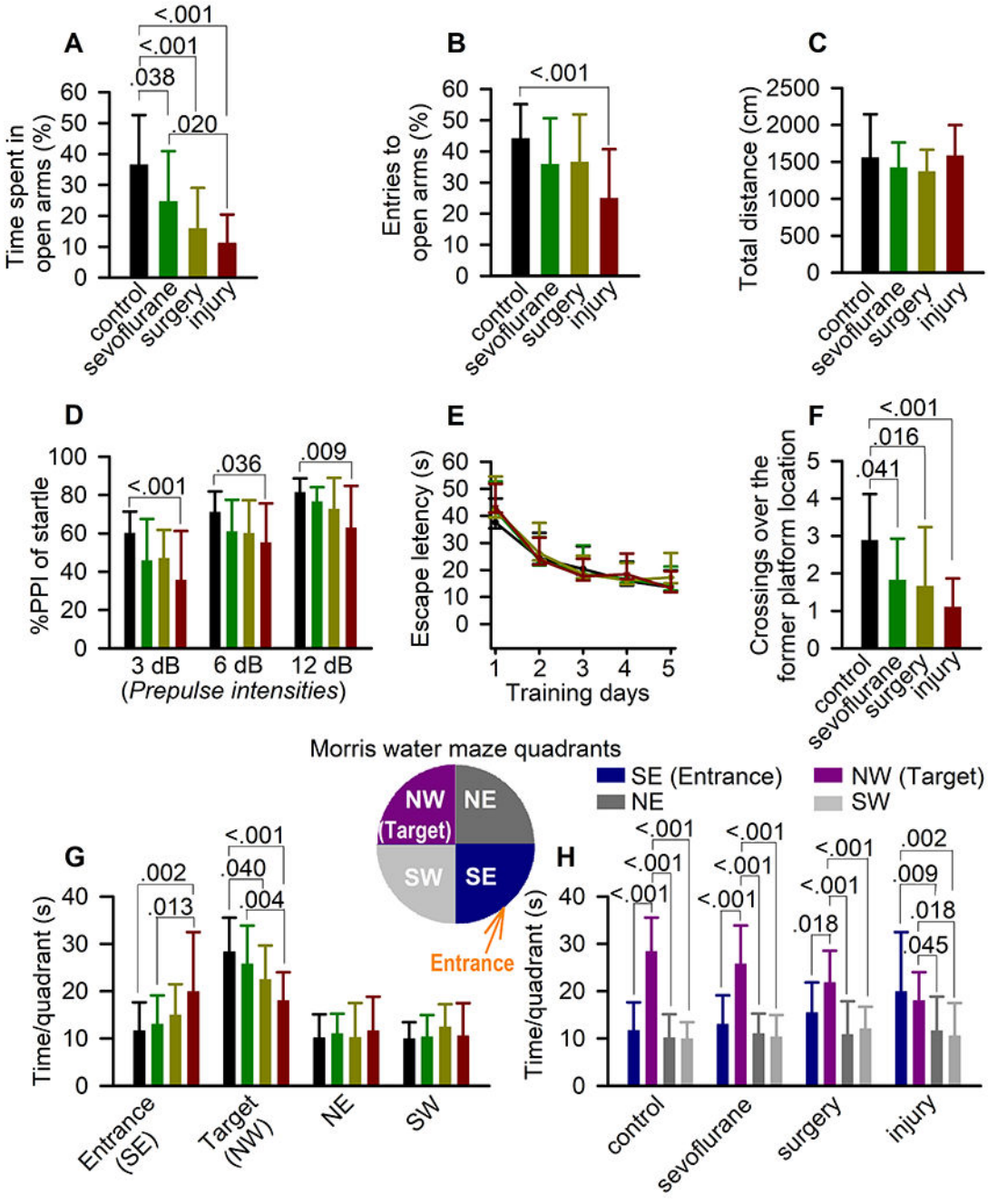

Fig. 4.

The long-term behavioral effects of surgery, traumatic brain injury, and sevoflurane in F0 sires. (*A-C*) The percentage of time spent in the open arms, number of entries to the open arms, and total distance traveled during the elevated plus maze test. Data are mean ± SD from 18 rats/group. (*D*) The percentage of prepulse inhibition of the startle at prepulse intensities of 3 dB, 6 dB, and 12 dB. Data are mean ± SD from 18 rats/group (n = 17 in surgery group). (*E*) Plots showing the values of escape latencies during the 5-day training period of the Morris water maze test. (*F-H*) Histograms showing the number of times that

rats from different treatment groups crossed the previous location of the escape platform (F), the time that rats from different treatment groups spent in each quadrant (G), and the time that rats from a specific treatment group spent in each quadrant (H). Color coding of experimental groups in A-C and F is applicable to all figures except H. (H) Colors of quadrants and quadrant locations (SE – southeast; SW – southwest; NW – northwest; NE – northeast) are shown in the figure insets. The *P* values of the multiple pairwise comparisons are shown in the respective plots above the horizontal lines. The beginning and end of the horizontal lines correspond to the compared experimental groups.

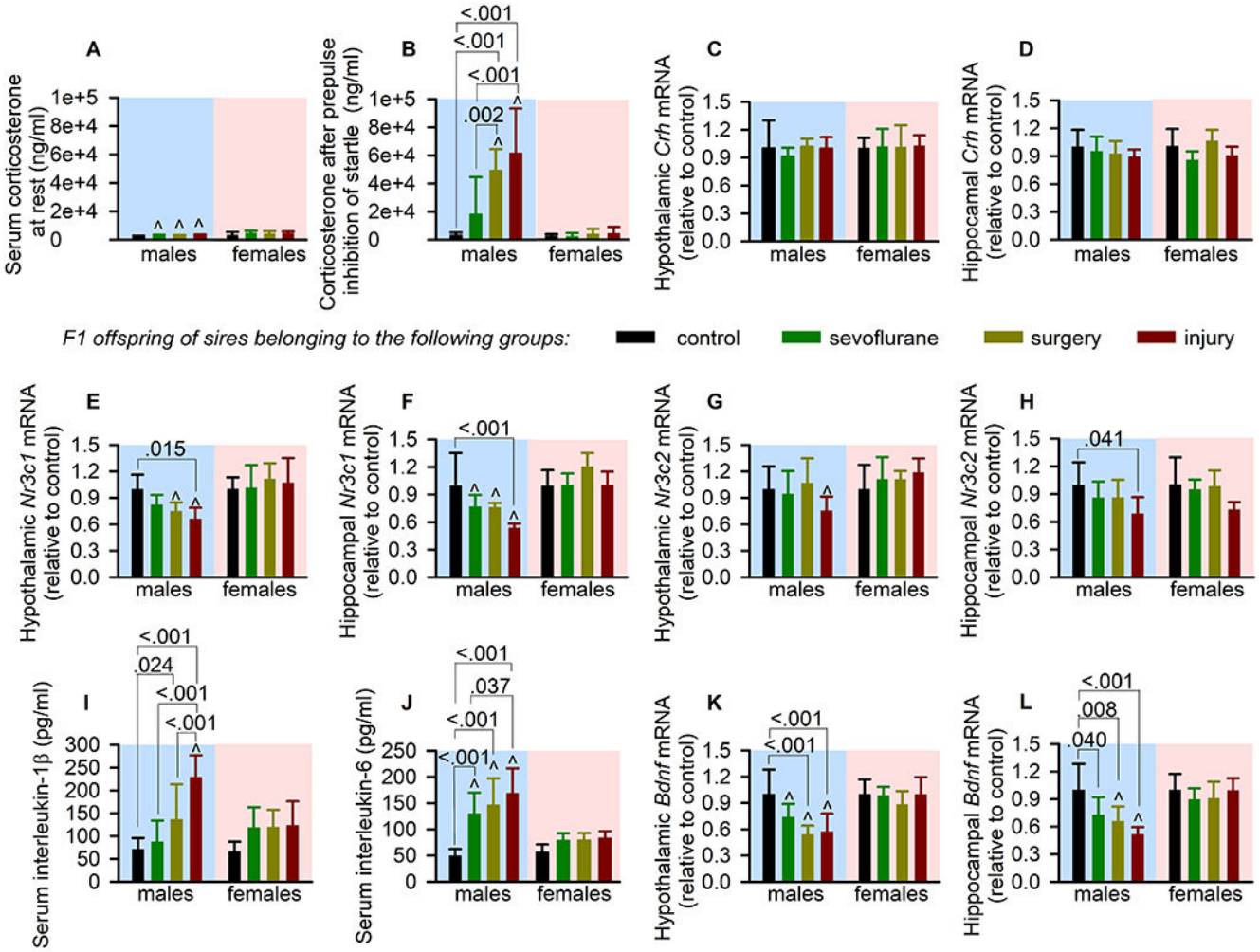

Fig. 5.

The effects of paternal surgery, traumatic brain injury, and sevoflurane on hypothalamic-pituitary-adrenal axis activity, inflammation markers, and the brain-derived neurotrophic factor (*Bdnf*) messenger RNA (mRNA) levels in adult F1 offspring. (*A*) The resting serum levels of corticosterone in F1 trunk blood samples collected on postnatal day ≥105. Data are mean ± SD from 5 to 7 F1 rats/sex/group. (*B*) Levels of serum corticosterone in F1 offspring tail blood samples collected 30 min after the prepulse inhibition of acoustic startle test. Data are mean ± SD from 7 rats/sex/group (n = 6 for F1 females of control sires). (*C-H,K,L*) The respective mRNA levels of corticotropin-releasing hormone (*Crh*), glucocorticoid receptor (*Nr3c1*), mineralocorticoid receptor (*Nr3c2*), and *Bdnf* in the hypothalamus and hippocampus of F1 rats. Data normalized against F1 offspring of control sires are mean ± SD from 6 rats/sex/group (n = 5 for all hypothalamic transcripts in F1 males of sevoflurane sires and n = 4 for hypothalamic *Mr* transcripts in F1 males of injury sires). (*I,J*) Serum levels of interleukin-1β and interleukin-6 in F1 offspring. Data are mean ± SD from 8 rats/sex/group. ^$P < 0.05$ *vs.* respective groups of F1 females. The color coding of the experimental groups is shown in the figure insets. The *P* values of the multiple pairwise

comparisons are shown in the respective plots above the horizontal lines. The beginning and end of the horizontal lines correspond to the compared experimental groups.

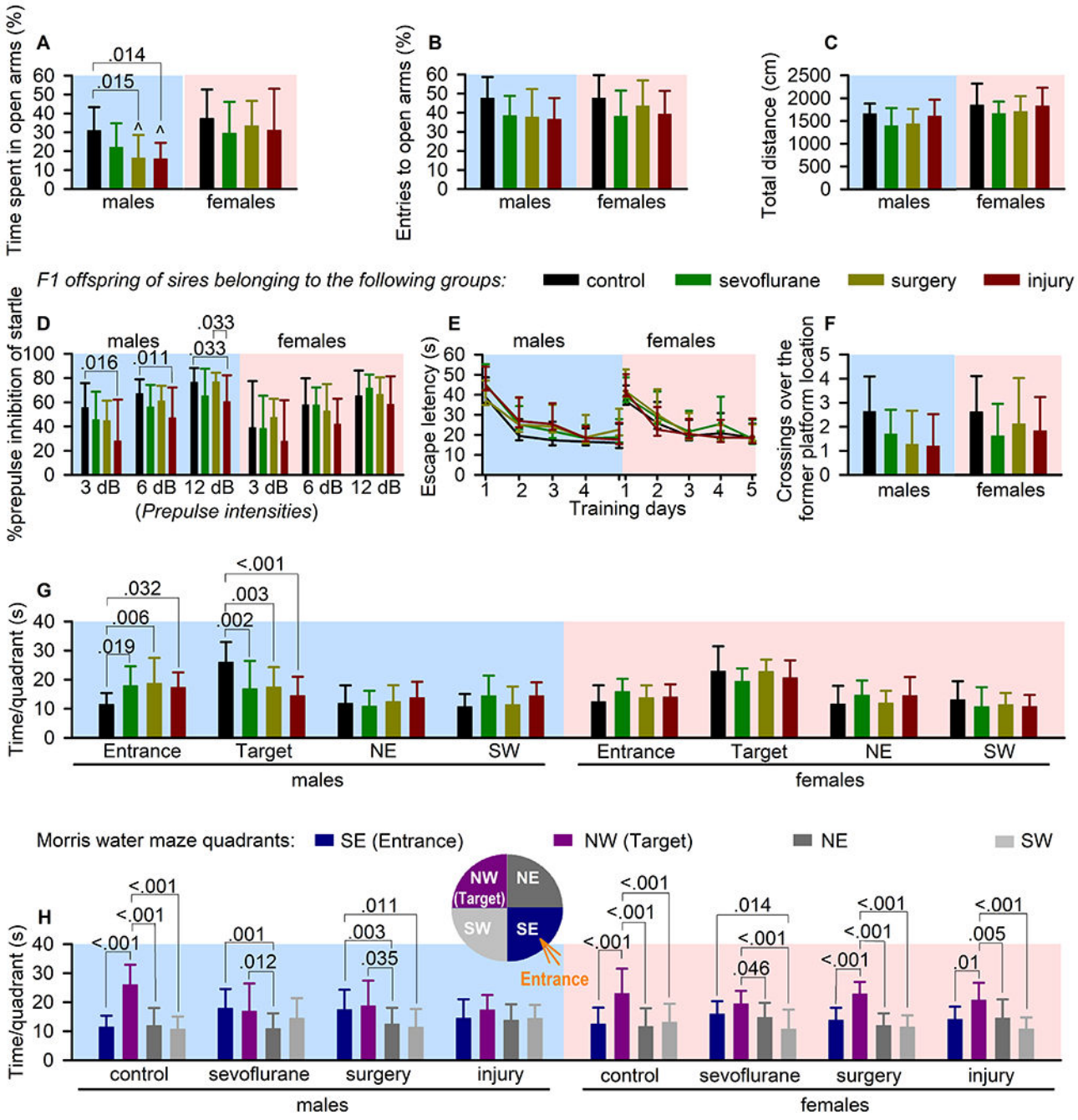

Fig. 6.

The behavioral effects of paternal surgery, traumatic brain injury, and sevoflurane exposure in F1 offspring. (*A-C*) The percentage of time spent in open arms, number of entries to the open arms, and total distance traveled by the F1 offspring during the elevated plus maze test. Data are mean ± SD from 18 rats/sex/group. (*D*) The percentage of prepulse inhibition of acoustic startle at prepulse intensities of 3 dB, 6 dB and 12 dB. Data are mean ± SD from 18 rats/sex/group. (*E*) Plots showing the values of escape latencies during the Morris water maze test in 5-day training period. (*F-H*) Histograms showing the number of times that rats

from different treatment groups crossed the previous location of the escape platform (F), the time that rats from different treatment groups spent in each quadrant (G), and the time that rats from a specific treatment group spent in each quadrant (H). Colors of experimental groups and quadrants, as well as quadrant locations (SE – southeast; SW – southwest; NW – northwest; NE – northeast) are shown in the figure insets. Data are mean ± SD from 14 rats/sex/group. ^$P < 0.05$ *vs.* respective groups of F1 females. The *P* values of the multiple pairwise comparisons are shown in the respective plots above the horizontal lines. The beginning and end of the horizontal lines correspond to the compared experimental groups.

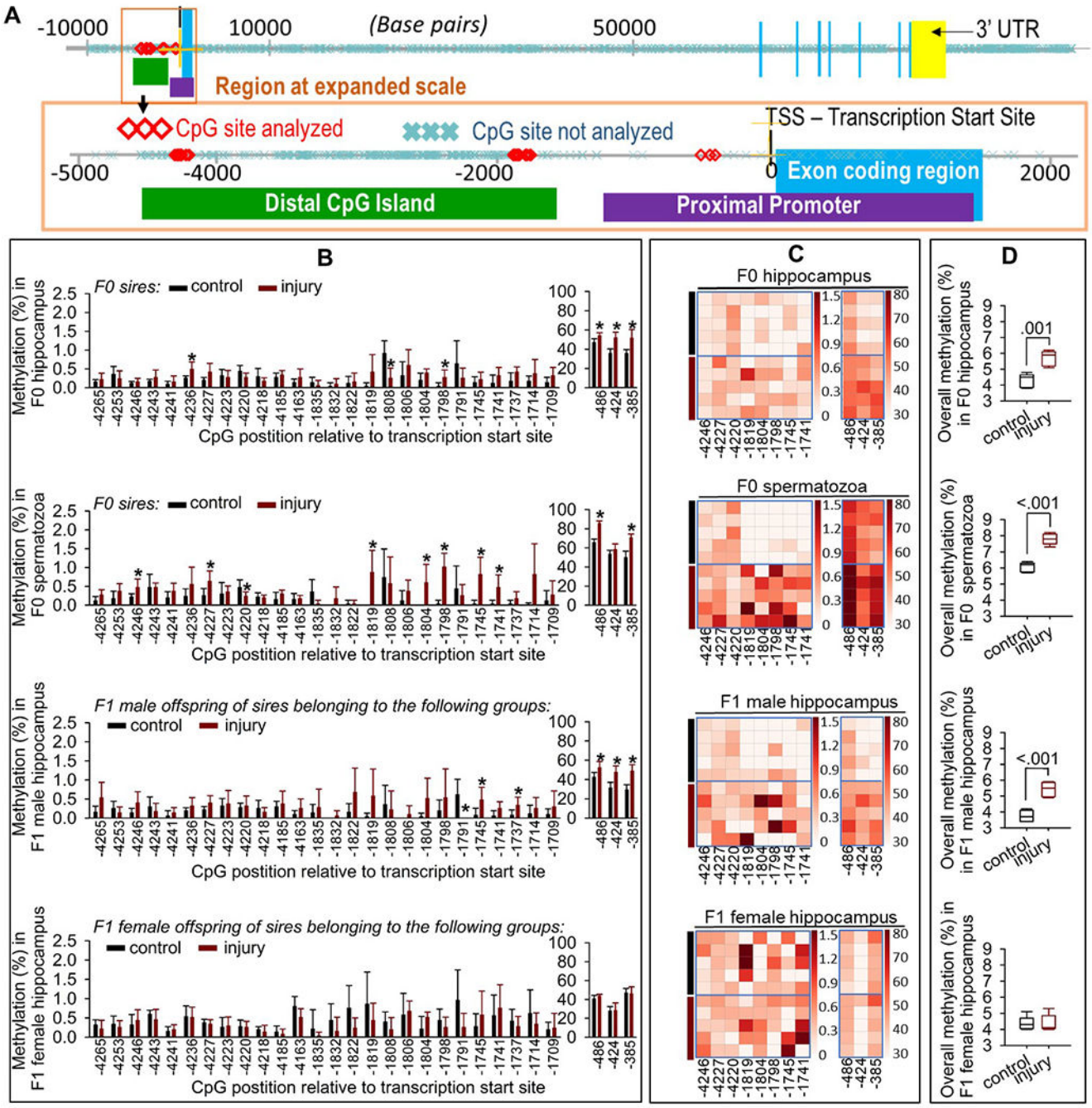

Fig. 7.

Methylation levels for CpG sites of the glucocorticoid receptor (*Nr3c1)* gene in the hippocampus and sperm of F0 injury and control sires and in the hippocampus of their F1 male and female offspring. (*A*) Schematic location of the CpG sites (labeled with a red diamond) within the *Nr3c1* gene that were analyzed by targeted next-generation bisulfite sequencing. The CpG sites located from −4265 to −1709 base pair region are within the distal CpG island; sites located from −486 to −385 base pair region are within the proximal promoter. (*B*) Methylation percentage at each CpG site. Data are mean ± SD from 5 rats/

group. *$P < 0.05$ *vs.* F0 control or F1 offspring of control sires. (*C*) Heatmaps, by depicting the same CpG sites in F0 spermatozoa and F0 male, F1 male and F1 female hippocampi, illustrate overlapping differentially methylated CpG sites in F0 spermatozoa and F1 male hippocampus and F0 male hippocampus. Each column represents a CpG site with percent methylation plotted by using color gradient. Percent methylation axis is from 0 to 1.4 for CpG sites −4246, −4227, −4220, −1819, −1804, −1798, −1745 and-1741; and from 30 to 80 for CpG sites −486, −424 and −385). Each row represents a tissue sample from one out of five animals in total per treatment group (black - control sires and offspring of control sires; red – injury sires and offspring of injury sires). (*D*) Box plots showing the differences between CpG site methylation levels in control and injury sires and offspring of control and injury sires. Box plots indicate the interquartile range and median; whiskers extend to the farthest data point within a maximum of 1.5 × interquartile range. The color coding of the experimental groups is shown in the figure insets.