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Effects of Cohabitation on Neurodevelopmental Outcomes in Rats Discordant for Neonatal Exposure to Sevoflurane

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ABSTRACT

BACKGROUND: Having a sibling with autism spectrum disorder is a risk factor for autism spectrum disorder. We used a rat model in which the general anesthetic sevoflurane (SEVO) induces autism spectrum disorder–like neurodevelopmental abnormalities to test whether they can be transmitted via cohabitation.

METHODS: Male rat pups from several litters were mixed and randomized to 3 new litter types: SEVO-exposed (SEVO), SEVO-unexposed (control), and equal numbers of SEVO-exposed and SEVO-unexposed (MIXED). After weaning, rats in experiment 1 were housed with littermates in SEVO, control, and MIXED (MIXED-exposed and MIXED-unexposed) pairs. In experiment 2, MIXED-exposed and MIXED-unexposed rats were paired with an unfamiliar naïve cagemate. Corticosterone levels, gene expression, central inflammatory markers (experiment 1), and behavior and corticosterone levels (experiment 2) were assessed in adulthood.

RESULTS: In experiment 1, compared with control rats, SEVO rats exhibited abnormalities in the hypothalamic-pituitary-adrenal axis, inflammatory markers, oxytocin, arginine vasopressin, and DNA methylation systems. Almost all these measures in MIXED-exposed and MIXED-unexposed rats were statistically indistinguishable from and similar to those in SEVO or control rats, with most measures in MIXED rats being similar to those in SEVO rats. Experiment 2 showed that pairing with unfamiliar, naïve rats after weaning caused MIXED-unexposed and MIXED-exposed rats' behavior to be no different from that of control and SEVO rats, respectively; however, the 2 groups of MIXED rats also did not differ from each other.

CONCLUSIONS: These findings suggest that neurodevelopmental abnormalities can be transmitted to otherwise healthy individuals through interactions during cohabitation; however, subsequent pairing with unfamiliar, naïve co-habitants may weaken this interaction effect.

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Having a sibling or siblings with autism spectrum disorder (ASD) or attention-deficit/hyperactivity disorder are among the predictors of ASD (1,2). Shared genetic features and common environmental conditions are the most frequently explored reasons for such a link (1–3). However, investigation of the roles of environmental factors in neurocognitive abnormalities in humans is challenging because of the complexity of the environment and the number of variables involved. Environmental factors in general, and social interactions in particular, are never identical—even for individuals within the same family—because each family member has unique social experiences within and outside of their families. Studies with laboratory animals, in which environmental conditions and social interactions can be more strictly controlled, can help to overcome challenges associated with elucidation of the role of social interactions in neurodevelopmental abnormalities.

Investigation of neurobehavioral effects of general anesthetics (GAs) in rodents may represent a promising model for addressing the role of social interactions in neurodevelopmental disorders. Neurodevelopmental consequences of early-life exposure to GAs are the subject of extensive clinical and laboratory research because GAs are administered to hundreds of millions of patients worldwide annually (4–10). Of relevance to developmental neurocognitive disorders in general and ASD in particular, clinical studies have reported significant increases in the prevalence of learning disabilities, attention-deficit/hyperactivity disorder, and communication deficits in individuals who had early-life exposure to GAs, particularly after repeated exposures to GAs (5–7). The relationship between early-life exposure to GAs and the risk of ASD is less certain because some studies have not found such a link (8), while others have (9). Importantly, studies with rats have shown that early-life exposure to GAs induces behavioral and neurobiological abnormalities similar to those found in patients with neurodevelopmental disorders, which supports the validity of this model (10).

Using a rat model of the developmental neurobehavioral effects of one of the most widely used GAs in pediatrics,

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sevoflurane (SEVO), we previously found that neonatal SEVO–induced behavioral abnormalities could be transmitted to SEVO-unexposed cagemates (11). This rat study was driven by findings from human twin studies that evaluated whether early-life medical procedures involving exposure to GAs led to neurodevelopmental/neurocognitive abnormalities (12,13). The twin studies found similarly poor performance on neurocognitive measures in both members of twin pairs who were discordant for early-life GA exposure (12,13). Similarly, we found that in otherwise healthy male rats discordant for neonatal exposure to SEVO, raised in the same litter by a foster dam, and subsequently cohoused with a littermate in a SEVO-exposed/SEVO-unexposed pair, both developed behavioral abnormalities (11). Although cagemates discordant for neonatal SEVO exposure were statistically indistinguishable on all behavioral tests, their behavioral abnormalities tended to be less profound than those exhibited by pairs of SEVO-exposed cagemates (11). The findings that neonatal SEVO exposure–induced neurobehavioral phenotypes can be affected by and transmitted to an unexposed cohabitant are reminiscent of the relatively well-studied phenomenon of social stress buffering and contagion (14–16).

Social stress buffering refers to situations/behaviors in which responses to excessive stress and its negative health outcomes are alleviated by a stressor-unexposed partner. Conversely, stress contagion describes the development of stress-like outcomes in otherwise unstressed individuals who support a stress-exposed partner (14–16). Studies in animal models have provided evidence that stress response systems, specifically the hypothalamic-pituitary-adrenal (HPA) axis, central inflammatory processes, and oxytocin (OT) and arginine vasopressin (AVP) signaling systems, may mediate interactions between stressor-exposed and stressor-unexposed partners (14–17). Notably, stress-like HPA axis effects of SEVO, increased inflammation, changes in OT signaling, and social interaction with cagemates have all been reported to be factors in neurodevelopmental outcomes in rats neonatally exposed to GAs (10,18–21). Therefore, in the current study, we tested whether the effects of cohabitation—i.e., when cohabitating rats can affect each other's brain development, ameliorating some SEVO-induced deficits in exposed rats and inducing some of these deficits in unexposed rats—share molecular mechanisms with stress contagion/buffering phenomena. Specifically, we evaluated changes in HPA axis activity, central inflammatory markers, OT and AVP signaling, and genes involved in epigenomic regulation in male rat cagemates discordant for neonatal exposure to SEVO, using the same rats whose behavioral phenotypes were reported on previously (11). In addition, because familiarity between stressor-exposed and stressor-unexposed partners may be an important moderator of stress buffering/contagion effects (22,23), we tested in a second experiment whether postweaning housing of rats discordant for neonatal exposure to SEVO with an unfamiliar and exposure-naïve cagemate affects the former's behavioral phenotypes. The latter experimental design also addresses an important translational question because many people leave their families in late adolescence to live with unfamiliar peers (e.g., to attend college), such that social interactions with these peers may overshadow interactions with families.

METHODS AND MATERIALS

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee. The study was conducted in accordance with the ARRIVE (Animal Research Reporting of In Vivo Experiments) guidelines (24). Sprague Dawley rats were bred at the University of Florida animal care facility. The rats were housed under controlled illumination (12-hour light/dark cycle, lights on at 7:00 AM) and temperature (23–24 °C) with free access to food and water.

Treatment Groups

Figure 1 shows an overview of the study design (also see the Supplement for a detailed description of the experimental design and all methods). This study consisted of experiments 1 and 2, which used separate sets of male rats. The design was exactly the same for both experiments until the pups were weaned on postnatal day (P) 21. Specifically, P5 male rats from different litters (typically from 6–8 litters born on a given day) were mixed together and randomized to 3 new litter types consisting of pups: 1) subsequently exposed to 2.1% SEVO for 6 hours (SEVO), 2) SEVO-unexposed (control), and 3) equal numbers of SEVO-exposed and SEVO-unexposed pups (MIXED). Each of these litters, with 8 pups/litter, had newly assigned foster dams. SEVO was administered on P5 in a temperature-controlled chamber (to maintain body temperature at approximately +37 °C) with a continuous supply of 30% oxygen in air (1.5 L/min) during anesthesia—6% SEVO for 3 minutes for anesthesia induction and 2.1% SEVO for 357 minutes for anesthesia maintenance (25). After termination of anesthesia with SEVO, rat pups remained in the same cage for 15 minutes on average and were supplied with 30% oxygen in air (1.5 L/minute) until they regained the righting reflex. After regaining the righting reflex upon termination of anesthesia, the pups were returned to their home cage to their foster dam and, in the case of MIXED litters, to their unexposed littermates. Similar anesthesia regimens that involve exposure of neonatal rats to SEVO for 6 hours have been extensively studied (26,27), including by our laboratory (10,25,28), which makes valid comparisons between studies of neurodevelopmental phenotypes induced by GA easier to make. The control animals and MIXED-unexposed rats were subjected to animal facility rearing only.

Pups in experiment 1 were weaned on P21 and subsequently housed 2 per cage with their littermates for the rest of the study (Figure 1). Thus, rats from control litters were housed 2 per cage (the control group, n = 16), as were rats from SEVO litters (the SEVO group, n = 16). The experiment 1 rats from MIXED litters were also housed 2 per cage after weaning so that in each cage there was 1 unexposed and 1 exposed rat. Based on exposure status, rats from MIXED litters formed 2 experimental groups: the MIXED-unexposed group (n = 16) and the MIXED-exposed group (n = 16). The experiment 1 rats were evaluated in the elevated plus maze (EPM) test on P60, for the prepulse inhibition (PPI) of acoustic startle response on P70, and in the Morris water maze (MWM) starting on P80. The results of behavioral evaluations of experiment 1 rats were reported previously (11). As part of the current study, the

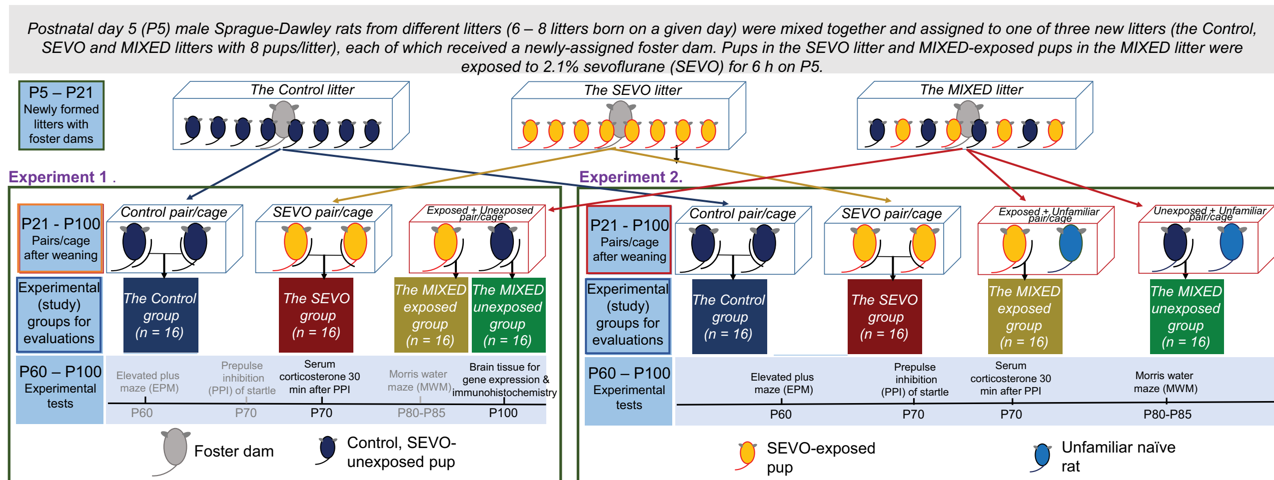

Figure 1. Illustration of the study design. See Methods and Materials for details.

experiment 1 rats were used to assess the function of the HPA axis. This was done by measuring serum levels of corticosterone (CORT) in blood samples collected 30 minutes after completion of the PPI test on P70, as previously described (29). Further mechanistic studies were conducted with tissue samples collected from the experiment 1 rats on P100.

Experiment 2 was designed to test how housing of MIXED littermates with an unfamiliar naïve male cagemate starting after weaning on P21 affected the behavior of rats from the MIXED litters (Figure 1). After weaning on P21, the experiment 2 rats from control litters were housed with a littermate (the control group, n = 16), as were rats from SEVO litters (the SEVO group, n = 16) for the rest of the study, as described above for the experiment 1 rats. In contrast, the experiment 2 rats from MIXED litters were housed in pairs for the rest of the study with a new, unfamiliar, and exposure-naïve age-matched rat so that in each cage there was 1 MIXED-unexposed and 1 unfamiliar naïve rat or 1 MIXED-exposed and 1 unfamiliar naïve rat. The MIXED-exposed and MIXED-unexposed rats housed in pairs with an unfamiliar naïve cagemate formed 2 experimental groups: MIXED-unexposed (n = 16) and MIXED-exposed (n = 16) (Figure 1). Experiment 2 rats were sequentially evaluated in the EPM (29) on P60, for PPI of the acoustic startle response (28,29) on P70, and in the MWM (29–32) starting on P80. Blood samples were collected 30 minutes after completion of the PPI test to measure serum levels of CORT. Detailed descriptions of all experimental techniques and statistical analyses are provided in the Supplement.

Statistical Analyses

The effects of treatment (SEVO-exposed vs. control [SEVO-unexposed]) and cohabitation (littermates with the same treatment condition housed together vs. littermates with different treatment conditions housed together) and treatment × cohabitation interactions were evaluated using the data from both experiments. Two-way analysis of variance with treatment and cohabitation condition as the independent variables was used to assess differences in systemic CORT responses to stress caused by the PPI test, inflammatory markers in the hippocampus, hippocampal and hypothalamic gene transcripts, time spent in the open arms, number of entries to the open arms, total distance traveled during the EPM test, and number of crossings over the former platform location during the MWM probe test. For PPI, MWM escape latency, and time spent in each quadrant, linear mixed models for repeated measures were used, with PPI intensity, day, and quadrant modeled as repeated measures, respectively. These models also included treatment and cohabitation as independent variables. Multiple pairwise comparisons were done using the Holm-Sidak method. p Value $<$ .05 was considered significant. Values are reported as mean ± SEM. The full results of the statistical analyses are reported in Tables 1–4. To focus on effects of cohabitation on SEVO-induced abnormalities in rats discordant for neonatal exposure to SEVO, only results of post hoc comparisons are reported in the text and figures.

RESULTS

Effects of Cohabitation on HPA Axis Abnormalities Induced by Neonatal SEVO Exposure (Experiment 1)

To test whether cohabitation of cagemates discordant for neonatal exposure to SEVO affected their HPA axis responsivity to stress, CORT levels were measured in blood samples collected 30 minutes after completion of the PPI test on P70 (Figure 2A and Table 1). Serum levels of CORT in the SEVO and MIXED-exposed groups were not statistically different, but, surprisingly, there was also no statistical difference between the MIXED-exposed and MIXED-unexposed groups (p = .209). On the other hand, SEVO-unexposed rats housed together with SEVO-exposed cagemates (MIXED-unexposed) had higher serum levels of CORT than rats in the control group.

Next, we assessed how cohabitation of cagemates discordant for neonatal exposure to SEVO affected transcription of genes involved in HPA axis regulation. Consistent with the CORT responses to stress described above, there were similarly increased messenger RNA (mRNA) levels of hypothalamic *Crh* in the MIXED-exposed and MIXED-unexposed groups (p = .718) and significantly elevated levels of *Crh* mRNA in the

Table 1. Results of Statistical Analyses of the Long-Term Effects of Neonatal Exposure to SEVO and Cohabitation on Hypothalamic-Pituitary-Adrenal Axis Activity

Variable	Cohabitation	Treatment: Control and SEVO	Cohabitation × Treatment Interaction
Corticosterone 30 Minutes After PPI	$F_{1,32}$ = 3.491, p = .071	$F_{1,32}$ = 2.896, p = .098	$F_{1,32}$ = 13.550, p < .001[a]
Hypothalamic *Crh* mRNA	$F_{1,19}$ = 0.138, p = .715	$F_{1,19}$ = 7.445, p = .013[a]	$F_{1,19}$ = 10.467, p = .004[a]
Hippocampal *Crh* mRNA	$F_{1,20}$ = 0.410, p = .529	$F_{1,20}$ = 2.700, p = .116	$F_{1,20}$ = 0.884, p = .358
Hypothalamic *Nkcc1* mRNA	$F_{1,20}$ = 1.560, p = .226	$F_{1,20}$ = 26.429, p < .001[a]	$F_{1,20}$ = 7.598, p = .012[a]
Hypothalamic *Kcc2* mRNA	$F_{1,20}$ = 1.718, p = .205	$F_{1,20}$ = 0.176, p = .680	$F_{1,20}$ = 1.868, p = .187
Hypothalamic *Nkcc1/Kcc2* mRNA Ratio	$F_{1,20}$ = 3.494, p = .076	$F_{1,20}$ = 8.576, p = .008[a]	$F_{1,20}$ = 5.758, p = .026[a]
Hippocampal *Nkcc1* mRNA	$F_{1,20}$ = 0.021, p = .887	$F_{1,20}$ = 1.402, p = .250	$F_{1,20}$ = 0.046, p = .832
Hippocampal *Kcc2* mRNA	$F_{1,20}$ = 7.712, p = .012[a]	$F_{1,20}$ = 2.846, p = .107	$F_{1,20}$ = 0.904, p = .353
Hippocampal *Nkcc1/Kcc2* mRNA Ratio	$F_{1,20}$ = 1.573, p = .224	$F_{1,20}$ = 3.476, p = .077	$F_{1,20}$ = 0.114, p = .740
Hypothalamic *Gr* mRNA	$F_{1,19}$ = 0.663, p = .426	$F_{1,19}$ = 2.299, p = .146	$F_{1,19}$ = 1.041, p = .320
Hippocampal *Gr* mRNA	$F_{1,20}$ = 0.382, p = .544	$F_{1,20}$ = 4.100, p = .056	$F_{1,20}$ = 5.271, p = .033[a]
Hypothalamic *Mr* mRNA	$F_{1,20}$ = 0.455, p = .508	$F_{1,20}$ = 0.094, p = .762	$F_{1,20}$ = 0.520, p = .479
Hippocampal *Mr* mRNA	$F_{1,20}$ = 1.957, p = .177	$F_{1,20}$ = 9.454, p = .006[a]	$F_{1,20}$ = 0.142, p = .710

Results obtained from 2-way analyses of variance. Cohabitation includes 1) rats from the same treatment group housed together and 2) rats from different treatment groups housed together.

mRNA, messenger RNA; PPI, prepulse inhibition (of startle test); SEVO, sevoflurane.

[a]Values indicate statistical significance.

MIXED-unexposed group compared with the control group (Figure 2B and Table 1). Neither neonatal exposure to SEVO nor cohabitation had significant effects on hippocampal *Crh* (Figure 2C and Table 1).

SEVO may cause stress-like responses in neonatal rats at least in part by impairing $GABA_A$ (gamma-aminobutyric acid A) receptor–mediated inhibitory control of corticotropin-releasing hormone–secreting neuron in the paraventricular nucleus of the hypothalamus via an increase in the *Nkcc1* Cl^- importer/*Kcc2* Cl^- exporter ratio (30,32–34). Therefore, next we tested whether the effects of cohabitation in cagemates discordant for neonatal exposure to SEVO could be detected at the Cl^- transporter gene transcription level. Importantly, rats in the MIXED-unexposed group had *Nkcc1* mRNA levels similar to those in the MIXED-exposed group (p = .107) but significantly higher than those in the control group (p = .010) (Figure 2D and Table 1). Interestingly, hypothalamic levels of *Kcc2* mRNA in the SEVO, MIXED-exposed, and MIXED-unexposed groups were all lower than in the control group, but these differences did not reach statistical significance (Figure 2E and Table 1). As expected based on the results of previous studies of stress-like effects of SEVO in neonatal rats (18), the resulting hypothalamic *Nkcc1/Kcc2* mRNA ratio was increased in the SEVO group (Figure 2F and Table 1). Consistent with the effect of cohabitation on HPA axis activity, rats in the MIXED-unexposed group had a similar hypothalamic *Nkcc1/Kcc2* mRNA ratio to that of the MIXED-exposed group (p = .712) (Figure 2F).

In contrast to hypothalamic levels, hippocampal levels of transcripts of *Nkcc1*, *Kcc2*, and their ratio were not different between groups except for the reduced level of *Kcc2* mRNA in the MIXED-unexposed group (Figure 2G–I and Table 1).

The HPA axis effects of SEVO may also include reductions in expression of glucocorticoid receptors (encoded by *Nr3c1*, referred to here as *Gr*) and mineralocorticoid receptors (encoded by *Nr3c2*, referred to here as *Mr*) in the brain, which play key roles in the negative feedback of CORT on HPA axis activity (35,36). Therefore, next we examined whether the effects of cohabitation on CORT responses to stress were accompanied by respective changes in transcripts of hypothalamic and hippocampal *Gr* and *Mr*. Neither SEVO nor cohabitation affected mRNA levels of *Gr* or *Mr* in the hypothalamus (Figure 2J, L and Table 1); however, neonatal exposure to SEVO and cohabitation did affect *Gr* and *Mr* transcripts in the hippocampus (Figure 2K, M and Table 1). In support of the effect of cohabitation, the MIXED-exposed and MIXED-unexposed groups had similar mRNA levels of hippocampal *Gr* (p = .850) and *Mr* (p = .071).

Table 2. Results of Statistical Analyses of the Long-Term Effects of Neonatal Exposure to SEVO and Cohabitation on Levels of GFAP and Iba1 in the Hippocampus

Variable	Cohabitation	Treatment: Control and SEVO	Cohabitation × Treatment Interaction
Hippocampal GFAP	$F_{1,12}$ = 21.413, p < .001[a]	$F_{1,12}$ = 49.532, p < .001[a]	$F_{1,12}$ = 19.813, p < .001[a]
Hippocampal Iba1	$F_{1,12}$ = 31.341, p < .001[a]	$F_{1,12}$ = 51.952, p < .001[a]	$F_{1,12}$ = 21.413, p < .001[a]

Results obtained from 2-way analyses of variance. Cohabitation includes 1) rats from the same treatment group housed together and 2) rats from different treatment groups housed together.

GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor 1; SEVO, sevoflurane.

[a]Values indicate statistical significance.

Table 3. Results of Statistical Analyses of the Long-Term Effects of Neonatal Exposure to SEVO and Cohabitation on mRNA Levels in the Hippocampus and Hypothalamus

Variable	Cohabitation	Treatment: Control and SEVO	Cohabitation × Treatment Interaction
Hypothalamic *Ot* mRNA	$F_{1,19}$ = 8.398, p = .009[a]	$F_{1,19}$ = 28.526, p < .001[a]	$F_{1,19}$ = 0.0286, p = .868
Hypothalamic *Oxtr* mRNA	$F_{1,20}$ = 24.409, p < .009[a]	$F_{1,20}$ = 18.362, p < .001[a]	$F_{1,20}$ = 10.504, p = .004[a]
Hippocampal *Oxtr* mRNA	$F_{1,20}$ = 0.041, p = .841	$F_{1,20}$ = 9.277, p = .006[a]	$F_{1,20}$ = 4.834, p = .040[a]
Hypothalamic *Avp* mRNA	$F_{1,19}$ = 1.745, p = .202	$F_{1,19}$ = 13.339, p = .002[a]	$F_{1,20}$ = 0.033, p = .859
Hypothalamic *V1br* mRNA	$F_{1,20}$ = 1.468, p = .240	$F_{1,20}$ = 1.223, p = .282	$F_{1,20}$ = 6.481, p = .019[a]
Hypothalamic *V1ar* mRNA	$F_{1,20}$ = 1.595, p = .221	$F_{1,20}$ = 0.798, p = .382	$F_{1,20}$ = 1.440, p = .224
Hippocampal *V1br* mRNA	$F_{1,19}$ = 0.051, p = .824	$F_{1,19}$ = 2.261, p = .149	$F_{1,19}$ = 2.479, p = .132
Hippocampal *V1ar* mRNA	$F_{1,19}$ = 0.166, p = .688	$F_{1,19}$ = 0.064, p = .802	$F_{1,19}$ = 1.571, p = .225
Hippocampal *Dnmt1* mRNA	$F_{1,20}$ = 12.176, p = .002[a]	$F_{1,20}$ = 2.708, p = .115	$F_{1,20}$ = 2.082, p = .165
Hippocampal *Dnmt3a* mRNA	$F_{1,20}$ = 17.874, p < .001[a]	$F_{1,20}$ = 1.041, p = .320	$F_{1,20}$ = 31.869, p < .001[a]
Hippocampal *Dnmt3b* mRNA	$F_{1,20}$ = 15.404, p < .001[a]	$F_{1,20}$ = 4.933, p = .038[a]	$F_{1,20}$ = 8.051, p = .010[a]
Hippocampal *Mecp2* mRNA	$F_{1,20}$ = 3.356, p = .082	$F_{1,20}$ = 7.614, p = .012	$F_{1,20}$ = 3.294, p = .085
Hippocampal *Tet3* mRNA	$F_{1,20}$ = 1.287, p = .270	$F_{1,20}$ = 3.810, p = .065	$F_{1,20}$ = 0.766, p = .392
Hypothalamic *Dnmt1* mRNA	$F_{1,19}$ = 20.585, p < .001[a]	$F_{1,19}$ = 2.004, p = .169	$F_{1,19}$ = 0.122, p = .731
Hypothalamic *Dnmt3a* mRNA	$F_{1,19}$ = 56.980, p < .001[a]	$F_{1,19}$ = 17.317, p < .001[a]	$F_{1,19}$ = 3.268, p = .087
Hypothalamic *Dnmt3b* mRNA	$F_{1,19}$ = 36.999, p < .001[a]	$F_{1,19}$ = 7.053, p = .016[a]	$F_{1,19}$ = 7.036, p = .016[a]
Hypothalamic *Mecp2* mRNA	$F_{1,19}$ = 2.051, p = .168	$F_{1,19}$ = 6.877, p = .017[a]	$F_{1,19}$ = 0.483, p = .495
Hypothalamic *Tet3* mRNA	$F_{1,19}$ = 1.000, p = .330	$F_{1,19}$ = 4.134, p = .056	$F_{1,19}$ = 0.0996, p = .756

Results obtained from 2-way analyses of variance. Cohabitation includes 1) rats from the same treatment group housed together and 2) rats from different treatment groups housed together.

mRNA, messenger RNA; SEVO, sevoflurane.

[a]Values indicate statistical significance.

Abnormalities in the Hippocampus and Hypothalamus of Adult Male Rat Cagemates Discordant for Neonatal Exposure to SEVO (Experiment 1)

Interactions among stress, inflammation, OT and AVP signaling systems, and epigenomic regulation of gene expression in mediating social behavior are supported by both animal and human studies (37,38). Therefore, next we examined whether these systems, together with the HPA axis, were regulated by cohabitation of cagemates discordant for neonatal exposure to SEVO. We found that changes in biomarkers of activated microglia (Iba1) and astrocytes (GFAP) in the hippocampus exhibited trends consistent with effects of cohabitation (Figure 3A–C and Table 2). Although the MIXED-exposed group had levels of these biomarkers that were comparable to those of the SEVO group (GFAP: p = .903, Iba1: p = .322), the levels of GFAP and Iba1 were also similar in the MIXED-exposed and MIXED-unexposed groups (GFAP: p = .092, Iba1: p = .277).

To gain insight into whether OT and AVP systems are also involved in the transmission of effects of SEVO between cagemates discordant for neonatal exposure to SEVO, we measured transcripts for hypothalamic *Ot* (i.e., the gene *Oxt*), *Avp*, and hippocampal and hypothalamic OT receptor (*Oxtr*) and AVP receptors 1A (*V1ar*) and 1B (*V1br*). Neonatal exposure to SEVO and cohabitation had complex effects on mRNA levels of *Ot* and *Oxtr*, while for the AVP system, hypothalamic *Avp* and hypothalamic *V1br* were affected (Figure 4A, a–h and Table 3). The MIXED-exposed and MIXED-unexposed groups were statistically similar on all these measures (Figure 4A, a–h and Table 3), which is of direct relevance to the topic of this study. The MIXED-unexposed group had a lower average level of hypothalamic *Ot* mRNA than the MIXED-exposed group (p = .001) (Figure 4A, a).

The complex effects of neonatal exposure to SEVO and cohabitation were also evident in transcription of genes that regulate gene expression through DNA methylation at CpG dinucleotide sites (39). DNMT3a and DNMT3b catalyze methylation of new CpG patterns, whereas DNMT1 copies the original DNA methylation pattern onto the newly synthesized daughter strand. TET3 is involved in epigenetic regulation through DNA demethylation (40). MeCP2 is a transcription factor that recognizes methylated CpG sites (41). The effects of neonatal exposure to SEVO and cohabitation on hippocampal and hypothalamic mRNA levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Mecp2*, and *Tet3* are summarized in Figure 4B, a–j and Table 3. With the exception of *Tet3*, which was not significantly affected by neonatal exposure to SEVO and cohabitation, hypothalamic and hippocampal *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Mecp2* expression exhibited profound changes depending on neonatal exposure to SEVO and housing arrangements. Compared with the control group, neonatal exposure to SEVO significantly increased mRNA levels of hippocampal *Dnmt1*, *Dnmt3a*, and *Dnmt3b* and hypothalamic mRNA levels of *Dnmt3a* and *Dnmt3b* in the SEVO group but not in the MIXED-exposed group (Figure 4B, a–j and Table 3). In fact, the transcripts of these genes in the hypothalamus and hippocampus of the MIXED-exposed group were significantly decreased compared with those in the SEVO group but were similar to the mRNA levels of these genes in the MIXED-unexposed group

Table 4. Results of Statistical Analyses of the Long-Term Neurobehavioral Effects of Neonatal Exposure to SEVO and Cohabitation With Unfamiliar Naïve Rats After Weaning

Variable	Cohabitation	Prepulse Intensity/Day of Training/Quadrant	Treatment: Control and SEVO	Cohabitation × Treatment Interaction
Time Spent in Open Arms of EPM	$F_{1,59} = 0.267$, $p = .607$	Not applicable	$F_{1,59} = 16.664$, $p < .001$[a]	$F_{1,59} = 2.891$, $p = .094$
Entries to Open Arms of EPM	$F_{1,59} = 0.256$, $p = .615$	Not applicable	$F_{1,59} = 5.515$, $p = .022$[a]	$F_{1,59} = 1.799$, $p = .185$
Total Distance of EPM	$F_{1,59} = 0.004$, $p = .951$	Not applicable	$F_{1,59} = 4.805$, $p = .032$[a]	$F_{1,59} = 5.142$, $p = .027$[a]
PPI of Acoustic Startle	$F_{1,177} = 0.787$, $p = .376$	$F_{1,177} = 29.443$, $p < .001$[a] (prepulse intensity)	$F_{1,177} = 21.017$, $p = .032$[a]	$F_{1,59} = 3.418$, $p = .066$
Serum CORT After PPI	$F_{1,32} = 1.066$, $p = .310$	Not applicable	$F_{1,32} = 30.887$, $p < .001$[a]	$F_{1,32} = 2.937$, $p = .096$
Escape Latency of MWM	$F_{1,295} = 0.193$, $p = .661$	$F_{4,295} = 68.365$, $p < .001$[a] (day of training)	$F_{1,295} = 0.047$, $p = .828$	$F_{1,295} = 1.515$, $p = .219$
Crossing Times Over the Platform of MWM	$F_{1,59} = 0.660$, $p = .420$	Not applicable	$F_{1,59} = 9.165$, $p = .004$[a]	$F_{1,59} = 0.520$, $p = .474$
Time Spent in Each Quadrant of MWM	$F_{1,236} < 0.001$, $p = .995$	$F_{1,236} = 46.015$, $p < .001$[a]	$F_{1,236} < 0.001$, $p = .998$	$F_{1,236} = 0$, $p = .998$

Results obtained from 2-way analyses of variance. Cohabitation includes 1) rats from the same treatment group housed together and 2) rats from different treatment groups housed together.

CORT, corticosterone; EPM, elevated plus maze; MWM, Morris water maze; PPI, prepulse inhibition; SEVO, sevoflurane.

[a]Values indicate statistical significance.

(Figure 4B, a–j and Table 3). The MIXED-exposed and MIXED-unexposed groups had similarly reduced mRNA levels of hypothalamic *Dnmt1*, even though the transcript for this gene was significantly increased in the SEVO group (Figure 4B, a and Table 3). Interestingly, cohabitation of cagemates discordant for neonatal SEVO exposure resulted in similarly altered transcription of hypothalamic *Dnmt1*, *Dnmt3a*, *and Dnmt3b* genes, which were significantly different from transcripts of

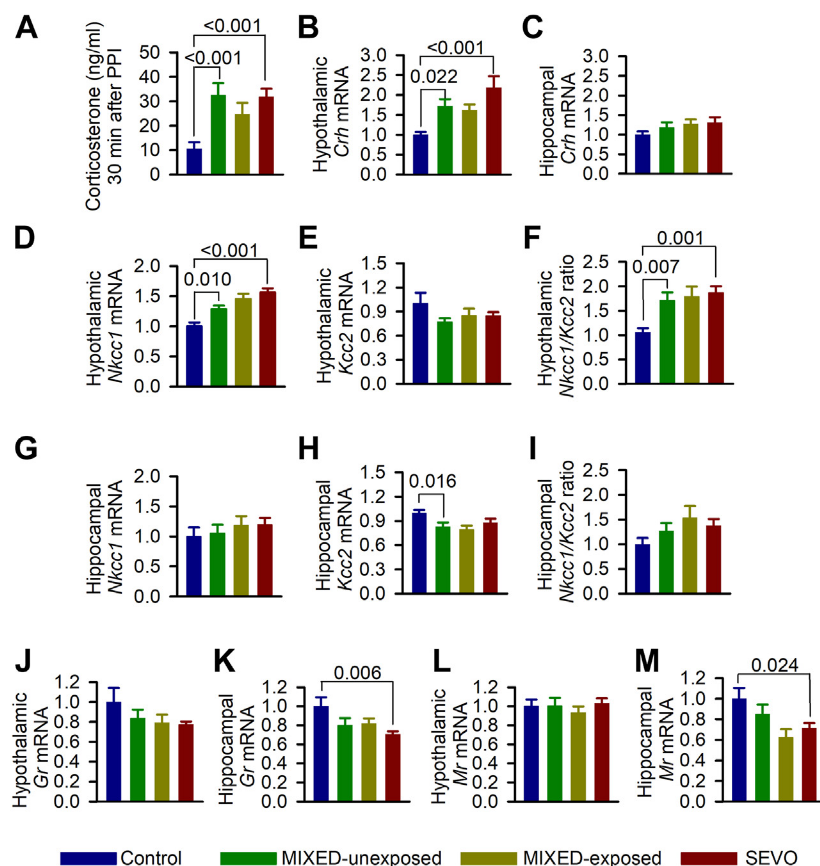

Figure 2. Long-term effects of neonatal exposure to SEVO and housing on hypothalamic-pituitary-adrenal axis activity. **(A)** Levels of serum corticosterone in tail blood samples collected 30 minutes after the PPI test on postnatal day 70. Data are mean ± SEM from 9 rats/group. **(B, C)** The respective levels of hypothalamic and hippocampal *Crh* mRNA. Data normalized against the control group are mean ± SEM from 6 rats per treatment group (n = 5, the control group, hypothalamus). **(D–I)** The respective levels of *Nkcc1* Cl^- importer mRNA, *Kcc2* Cl^- exporter mRNA, and *Nkcc1/Kcc2* mRNA ratio in the hypothalamus and hippocampus. Data are means ± SEM from 6 rats/group. **(J–M)** The respective levels of glucocorticoid receptors (*Gr*) and mineralocorticoid receptors (*Mr*) in the hypothalamus and hippocampus. Data normalized against the control group are mean ± SEM from 6 rats/treatment group (n = 5, the control group, hypothalamus). 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. The color coding of the experimental groups is shown in the figure insets. mRNA, messenger RNA; PPI, prepulse inhibition; SEVO, sevoflurane.

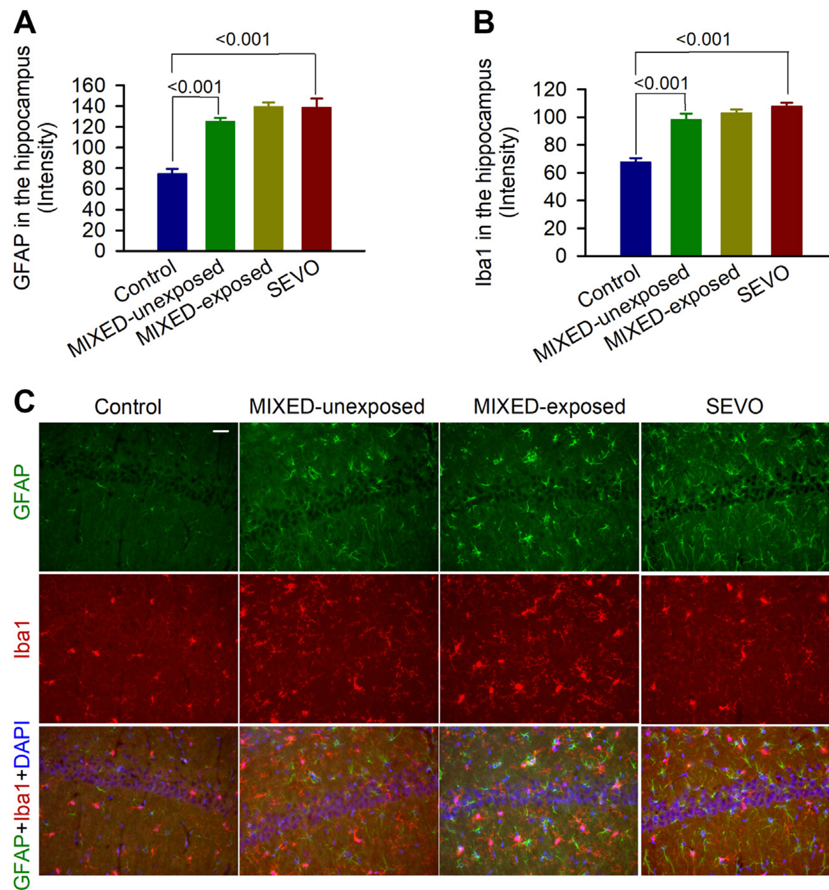

Figure 3. Inflammatory markers in the hippocampus of postnatal day 100 male rat cagemates discordant for neonatal exposure to SEVO. **(A–C)** Quantification of GFAP (astrocyte-specific protein marker) **(A)** and Iba1 (microglia/macrophage-specific protein marker) **(B)** fluorescence and representative images of GFAP and Iba1 in the hippocampus **(C)**. Values presented are means ± SEM from 4 rats/group. **(C)** Scale bar = 50 μm. 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. The color coding of the experimental groups is shown in the figure insets. GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor 1; SEVO, sevoflurane.

these genes in both the control and SEVO groups (Figure 4B, a–c and Table 3).

Abnormalities in Littermates Discordant for Neonatal SEVO Exposure After Post-Weaning Pairing With Unfamiliar Naïve Cagemates (Experiment 2)

Next, we compared behavior and CORT responses with stress in male rat littermates discordant for P5 exposure to SEVO, which after weaning on P21 were paired with a naïve, unfamiliar rat as cagemate. The findings of experiment 2 are summarized in Figure 5 and Table 4. Although the MIXED-exposed and MIXED-unexposed groups were not different from the SEVO and control groups, respectively, in time spent in the open arms of the EPM, the MIXED-exposed and MIXED-unexposed groups also did not differ from each other (p = .100) (Figure 5A). Similarly, the MIXED-exposed and MIXED-unexposed groups were not different from each other on number of open arm entries during the EPM test (p = .483) (Figure 5A and Table 4). Finally, the MIXED-exposed and MIXED-unexposed groups traveled similar distances during the EPM test that were intermediate to the SEVO and control groups (p = .958) (Figure 5A and Table 4).

During the PPI test on P70, compared with the control group, the SEVO group exhibited reduced PPI of startle at prepulse intensities of 3 dB (PP3) and 12 dB (PP12) above background noise (Figure 5B and Table 4). The PPI of startle responses of the MIXED-exposed and MIXED-exposed groups, on the other hand, were not different at all 3 prepulse intensities (PP3: p = .062, PP6: p = .47, PP12: p = .533).

Half an hour after completion of the PPI test, CORT serum levels were measured (Figure 5C and Table 4). In contrast to findings in experiment 1, rat littermates discordant for P5 exposure to SEVO, which after weaning on P21 were paired with a naïve, unfamiliar rat as cagemate, had significantly different serum levels of CORT (p = .011) (Figure 5C and Table 4).

The results of the MWM test are summarized in Figure 5D and Table 4. Spatial memory tests, as indexed by the number of crossings over the escape platform location, showed that the MIXED-exposed and MIXED-unexposed groups did not differ on this measure (p = .111). In addition, the MIXED-exposed and MIXED-unexposed rats spent a similar amount of time in the target quadrant (p = .134) and the entry quadrant (p = .070) of the maze.

DISCUSSION

The findings of this study demonstrate that continuously cohabiting MIXED (SEVO)-exposed and MIXED (SEVO)-

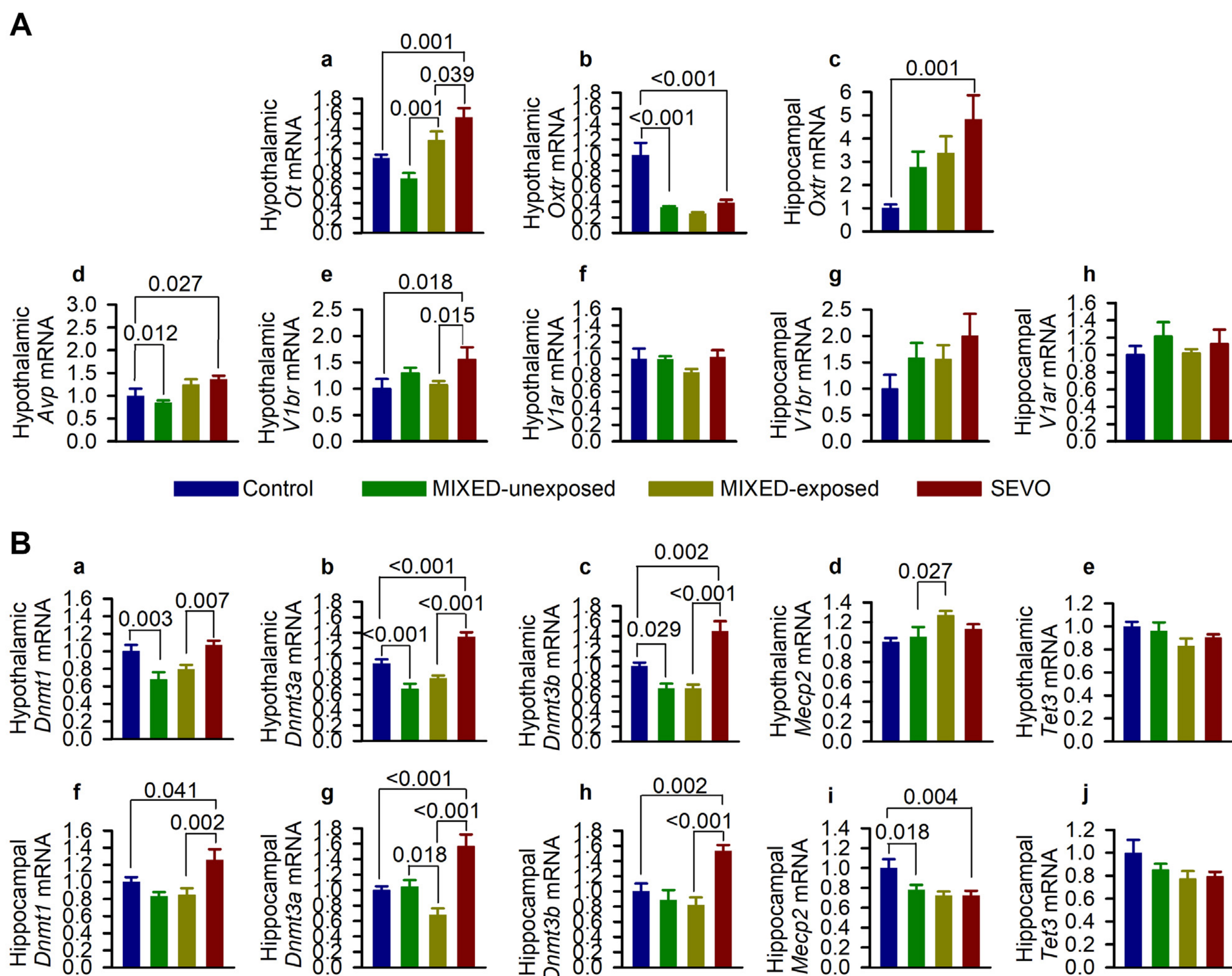

Figure 4. Long-term effects of neonatal exposure to SEVO and cohabitation at the gene expression level. **(A)** The respective mRNA levels of hypothalamic *Ot*, *Avp*, hippocampal and hypothalamic *Oxtr*, *V1ar*, and *V1br*. Data normalized against the control group are mean ± SEM from 6 rats/treatment group (hypothalamic *Ot*: $n = 5$ for the control group). **(B)** The respective levels of *Dnmt1* mRNA, *Dnmt3a* mRNA, *Dnmt3b* mRNA, *Mecp2*, and *Tet3* mRNA in the hippocampus and hypothalamus. Data normalized against the control group are mean ± SEM from 6 rats per treatment group ($n = 5$, the control group, hypothalamus). mRNA, messenger RNA; SEVO, sevoflurane.

unexposed littermates/cagemates developed alterations in HPA axis functioning, central inflammatory markers, neuropeptide signaling, and transcription of genes involved in epigenomic regulation that resemble those found in animal models of social stress buffering and social stress contagion (14–16,24). Such similarities support the idea that effects of cohabitation in rats discordant for neonatal exposure to SEVO and social stress buffering/contagion share common mediating mechanisms. Common mechanisms are also supported by findings of less profound cohabitation effects when the MIXED-exposed and MIXED-unexposed rats were housed with unfamiliar naïve cagemates after weaning because social stress buffering/contagion effects are weaker when stress-exposed animals are placed in a cage with unfamiliar animals unexposed to the stressor (23,24). The findings of this study, in combination with previously reported behavioral changes in the same rats (11), suggest that interactions between familiar rat littermates/cagemates synchronize their biological processes, resulting in indistinguishable neurobehavioral phenotypes. More generally, our findings suggest that not just shared environmental conditions (environmental conditions were the same for all 4 experimental groups) but specifically interactions between cohabitants (presumably social interactions) may attune their neurodevelopmental phenotypes through synchronization of physiological processes in cohabitants.

We speculate that analogous to social stress buffering/contagion phenomena (14–16,23,24,42), SEVO-induced stress-like changes in HPA axis activity, anxiety-like behaviors in the MIXED-exposed cagemate (11), and physiological feedback from the MIXED-unexposed cagemate may be initial triggers that drive the neurobehavioral effects of cohabitation in cagemates discordant for neonatal exposure to SEVO.

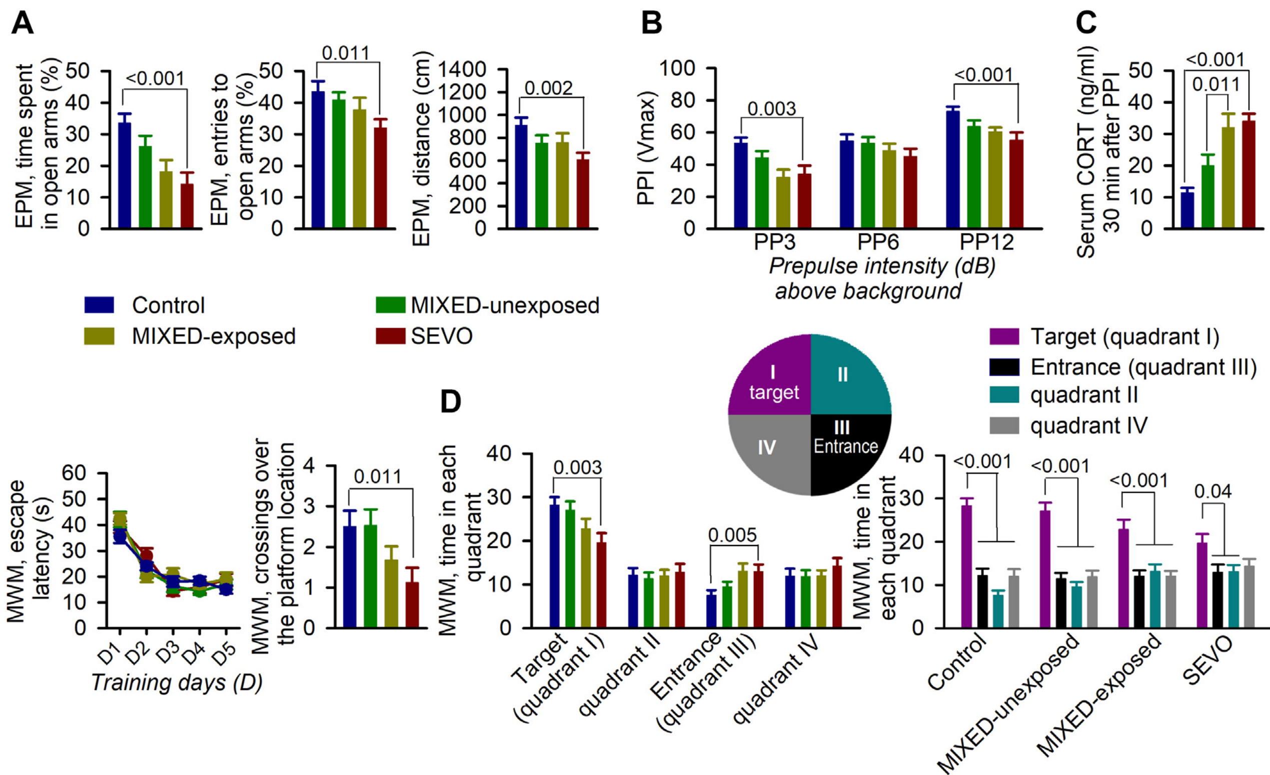

Figure 5. Neurobehavioral effects of SEVO and cohabitation. **(A)** Percentage of time spent in open arms, percentage of entries to the open arms, and total distance traveled during the EPM test. Data are mean ± SEM from 16 rats/group (n = 15, the MIXED-unexposed group). One rat in the MIXED-unexposed group was removed from the study because of a fall. **(B)** Percentage of PPI of the startle responses at a prepulse intensity of 3 (PP3), 6 (PP6), and 12 (PP12) dB. Data are mean ± SEM from 16 rats/group. **(C)** Levels of serum corticosterone in tail blood samples collected 30 minutes after the PPI of the startle test on postnatal day 70. Data are mean ± SEM from 9 rats/group. **(D)** Latencies to reach the escape platform in the water maze across the 5 days of training. Data from the probe trial following the final water maze training day showing the number of times the rat crossed the location of the escape platform and the amount of time spent in each quadrant. Data are mean ± SEM from 16 rats/group. 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. The color coding of the experimental groups is shown in the figure insets. CORT, corticosterone; EPM, elevated plus maze; MWM, Morris water maze; PP, prepulse intensity; PPI, prepulse inhibition; SEVO, sevoflurane.

Notably, specific molecular mechanisms seem to mediate these HPA axis effects of SEVO rather than general stress-like effects resulting from inadequate homeostasis during SEVO exposure. Among such mechanisms is a SEVO-induced shift in $GABA_A$ receptor signaling toward decreased inhibition due to an increase in the *Nkcc1*/*Kcc2* ratio (28,30,33,34). SEVO also reduces transcription of *Gr* and *Mr* in the brain. Both impaired $GABA_A$ receptor–mediated inhibitory control of the HPA axis and a weakened negative feedback effect of CORT on the HPA axis due to reductions in corticoid receptors can contribute to enhanced HPA axis activity (43,44). Remarkably, similar changes in the HPA axis at both molecular and systemic levels were found in SEVO-exposed and SEVO-unexposed cagemates.

The analogy between the effects of cohabitation on neonatal SEVO–induced neurodevelopmental abnormalities in cohabitating rats and social stress buffering/contagion processes is also evident in other signaling systems that are common for both phenomena. For example, published data show that besides abnormal HPA axis activity, changes in inflammatory processes and OT and AVP signaling systems may play key roles in social stress buffering/contagion (15–17,45,46). Similarly, in our study, elevated levels of Iba1 and GFAP in the hippocampus of SEVO, MIXED-exposed, and MIXED-unexposed rats were accompanied by changes in transcripts for hypothalamic *Ot* and *Avp* and hypothalamic and hippocampal OT and AVP receptors. Although the SEVO-induced changes in *Oxtr* transcripts were complex and brain region dependent, with opposite changes in the hippocampus and hypothalamus, they were the same in cagemates discordant for neonatal exposure to SEVO. The same levels of inflammatory markers and *Ot* and *Oxtr* transcripts in cagemates discordant for neonatal exposure to SEVO suggest their involvement in the transmission of neurodevelopmental effects of SEVO between cagemates. The upregulated HPA axis, altered *Oxtr* mRNA levels, and higher levels of hippocampal inflammatory markers in MIXED cagemates discordant for neonatal exposure to SEVO, which were comparable to those found in rats from the SEVO group, suggest that in social interactions between cagemates, stress contagion effects dominate over buffering effects. Conceptually, such a dominant influence of SEVO-exposed rats on their SEVO-

unexposed cagemates is consistent with findings by Bartels *et al.* (12) that twins discordant for early-life exposure to GA exhibited comparable neurocognitive deficiencies.

Involvement of epigenomic processes in the mechanisms initiated by social interactions is supported by findings of similar levels of transcripts for hippocampal and hypothalamic *Dnmt*(s) and *Mecp2* in cagemates discordant for SEVO exposure. The changes in *Dnmt* transcripts in cagemates discordant for neonatal exposure to SEVO approximated those in the control group, in contrast to the upregulated HPA axis, altered transcripts of *Oxtr*, and higher levels of hippocampal inflammatory markers, which approximated those in the SEVO group. Specifically designed future studies will be needed to elucidate whether such opposite changes in HPA axis activity, *Oxtr*, and neuroinflammatory markers on the one hand and *Dnmt* transcripts on the other hand may be a reason that changes in specific behavioral phenotypes in SEVO-discordant cagemates also exhibited shifts in opposite directions (11).

The findings of this study in an animal model suggest that social interaction through cohabitation may be an overlooked environmental factor in interpreting results of human epidemiological studies that have demonstrated higher rates of ASD among siblings (1,2), dementia within couples (47–49), depression in families (50–53), symptoms of posttraumatic stress disorder in posttraumatic stress disorder–unaffected cohabitants (54,55), psychiatric disorders in peer networks (56), and in the interpretation of results of twin studies more broadly (57). A role for continuous social interaction between twins in shaping their phenotypes may also explain increasing differences in epigenomes and resultant phenotypes in monozygotic twins as they become older and live independently (58), an outcome that we also observed in male rats discordant for neonatal exposure to SEVO when they were housed with unfamiliar, naïve cagemates starting after weaning (experiment 2).

Although our study in laboratory rats reared in a strictly controlled environment of same-sex litters and later (after weaning) in same-sex dyads provided initial evidence for involvement of social interaction in transmission of neurodevelopmental abnormalities, this study does not recapitulate the complexity of social interactions experienced by humans or even by rodents in their natural habitats. Future animal and human studies will be needed to model the more complex interactions that typically occur during animal and human development in health and disease. Future mechanistic studies will also place greater focus on the roles of specific brain regions, cell types, and molecular processes in mediating effects of cohabitation on GA-induced neurodevelopmental abnormalities. Finally, given emerging data on the role of the gut microbiota–brain axis in neurodevelopmental disorders, in particular in ASD, and especially experimental evidence that gut microbiota are involved in transmission of parental conditions to neurodevelopmental phenotypes in offspring (59,60), it will be important in future studies to elucidate the role(s) of the gut microbiota–brain axis in interindividual transmission of neurodevelopmental abnormalities.

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AEM, L-SJ, TM, NG, CNS, and BS designed the research; L-SJ and AEM performed the research; L-SJ and AEM analyzed the data; and AEM, L-SJ, TM, NG, CNS, and BS wrote the article.

TM owns equity in Xhale, Inc. In addition, the University of Florida owns equity in Xhale, Inc., a faculty start-up company that produces alar pulse oximeters for clinical use in humans. NG serves as a medical adviser for Teleflex Medical. All other authors report no biomedical financial interests or potential conflicts of interest.

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