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The Impact of Chronic Phthalate Exposure on Rodent Anxiety and Cognition

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ABSTRACT

BACKGROUND: There is a growing importance for environmental contributions to psychiatric disorders and understanding the impact of the exposome (i.e., pollutants and toxins). For example, increased biomonitoring and epidemiological studies suggest that daily phthalate chemical exposure contributes to neurological and behavioral abnormalities; however, these mechanisms remain poorly understood. Therefore, the current study was aimed at examining the effects of chronic phthalate exposure on rodent anxiety behaviors and cognition and the impact on hypothalamic-pituitary-adrenal axis function.

METHODS: Adult male mice (C57BL6/J) were administered MEHP via drinking water (1 mg/mL), and anxiety-like behavior and cognition combined with hypothalamic-pituitary-adrenal axis and inflammatory assays were assessed after 3 weeks of MEHP exposure.

RESULTS: MEHP-treated mice exhibited enhanced generalized anxiety-like behaviors, as demonstrated by reduced time spent in the open-arm of the elevated plus maze and center exploration in the open field. Tests of spatial memory and cognition were unchanged. Following MEHP administration, circulating levels of corticosterone and proinflammatory cytokines were significantly increased, while at the tissue level, there were MEHP-dependent reductions in glucocorticoid metabolism genes *Hsd11b1* and *Hsd11b2*.

CONCLUSIONS: These data suggest that chronic MEHP exposure leads to enhanced generalized anxiety behaviors independent of rodent measures of cognition and memory, which may be driven by MEHP-dependent effects on hypothalamic-pituitary-adrenal axis and peripheral glucocorticoid metabolism function.

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Phthalates are synthetic chemicals that are commonly used as plasticizers to impart flexibility to polyvinyl chloride (PVC) for use in plastic consumer and medical products (1). Despite the inherent benefits of plastics, the relative abundance of phthalate chemicals in the environment has raised concerns recently about human health and disease risk (2,3). Human exposure to phthalates can occur through chemical leaching or migration because the phthalate plasticizer is not covalently bound to the PVC matrix. Not surprisingly, given the ubiquity of plastic materials in the environment, human biomonitoring studies have indicated widespread daily exposure to phthalates, with detectable levels in >75% of the general population (4–7). Therefore, additional studies to elucidate the impact of phthalate exposure on biological pathways, specifically related to the impact on brain development and mental health (8), are needed.

Phthalates are classified as endocrine-disrupting chemicals, and growing clinical and preclinical evidence has found that phthalate exposure interferes with neurodevelopment and increases the risk for behavioral disorders (9). For example, multiple epidemiological and clinical-based studies have detected associations between prenatal or early-childhood phthalate exposure and adverse cognitive and neurobehavioral outcomes (10,11), including somatic symptom disorder (12), attention-deficit/hyperactivity disorder-related behaviors (13–16), externalizing behavior including aggression, depression, reduced emotional control (17,18), and anxiety proneness (19). However, the causal and/or consequential mechanisms and neurobiological pathways that underlie the impact of postnatal phthalate exposure on cognitive and behavioral outcomes remain poorly understood (20).

One of the most utilized phthalate esters in plastic production is DEHP, and previous research has indicated that its main metabolite, MEHP, has significant harmful effects on male reproductive health (21–23). Therefore, the current study, using a rodent model, was aimed at investigating the impact of chronic exposure to phthalates during the postnatal period on anxiety, memory, cognitive function, and neuroendocrine function. The findings of this study provide novel evidence suggesting that chronic exposure to MEHP contributes to increased anxiety-like behaviors, possibly due to phthalate-induced changes in neuroendocrine and glucocorticoid metabolism function.

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METHODS AND MATERIALS

Animals

All experimental protocols were approved by the Institutional Care and Use Committee of The George Washington University and were in compliance with National Institutes of Health guidelines. Adult male C57BL/6J mice (10 weeks old, $N = 60$) used in this study were from Jackson Laboratory and were housed in a temperature- and humidity-controlled room on a 12-hour light/dark cycle with water and food available ad libitum.

MEHP Administration

The mice in the study were given drinking water supplemented with MEHP, and previous research has demonstrated that phthalate supplementation does not affect the average daily water intake of the mice, which ranged from 5.0 to 5.3 ± 0.6 mL/day (24). Our previous studies also demonstrated that following chronic phthalate exposure, MEHP serum concentration levels are significantly elevated compared with control animals (24). To optimize the aqueous solubility of MEHP, drinking water was also supplemented with 20 mg/mL captisol cyclodextrin (Cydex Pharmaceuticals). Vehicle control animals were similarly administered 20 mg/mL captisol through drinking water. After 3 weeks of treatment, mice underwent behavioral testing and were subsequently sacrificed at the end of the fifth week. Two cohorts of mice were used for behavioral testing (Figure 1); animals received MEHP or vehicle control drinking water during the entire testing period.

Behavioral Testing

To decrease possible stress caused by room changes, 48 hours before the behavior tests, mice were brought to the behavior room for testing environment habituation. Animal activities were tracked and analyzed via ANY-maze video tracking software (Stoelting Co., https://www.any-maze.com/). Freezing behavior during fear response tests was recorded and quantified using Freezeframe 3.32 (Coulbourn Instruments). The startle reflex was evaluated by the SR-Lab Startle Systems (San Diego Instruments). Additional details are provided in the Supplement.

Tissue and Plasma Collection

After behavioral tests were complete, mice were sacrificed via decapitation, and the brain, liver, and kidneys were dissected and fresh frozen with liquid nitrogen. Whole blood was collected in EDTA-coated tubes (Thermo Fisher Scientific), samples were centrifuged (5000*g*, 10 min), and plasma was collected. Tissues and plasma samples were stored at −80 °C for future study.

Enzyme-Linked Immunosorbent Assay

Plasma corticosterone (AR E-8100, LDN), adrenaline/noradrenaline/dopamine (BA E-5600, LDN), adrenocorticotropic hormone (ACTH) (M046006, MD Bioproducts), and aldosterone (ADI-900-173, LDN) were measured using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Cytokine Analysis

Plasma proinflammatory cytokines were measured using commercially available electrochemiluminescence kits (Meso Scale Discovery). For the V-Plex Plus Proinflammatory Panel1 Mouse Kit (K-15048G1), the plasma samples were run in duplicate at 1:2 dilution, and the assay was performed according to the manufacturer's instructions.

Reverse Transcriptase–Quantitative Polymerase Chain Reaction

The hypothalamus was collected by a 1-mm diameter brain tissue punch. Total RNA was extracted from hypothalamus, liver, and kidney tissue using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Additional details can be found in the Supplement.

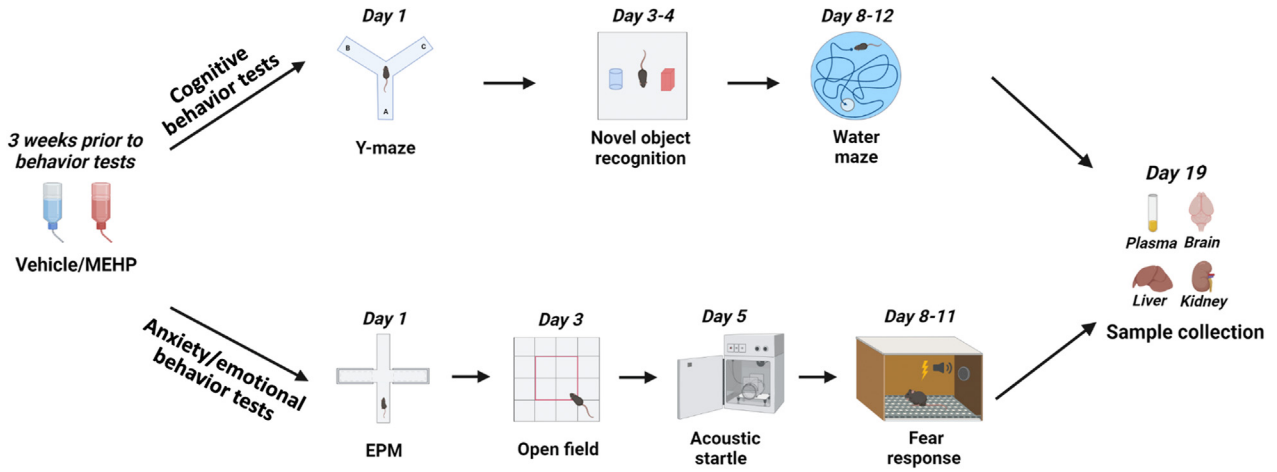

Figure 1. Experimental protocol and timeline. Three weeks following MEHP exposure, mice underwent either cognitive behavioral tests or anxiety/emotional behavior tests followed by blood and tissue sample collection. EPM, elevated plus maze.

Data Presentation and Statistical Analysis

For all data analyses, distributions were checked for nontrivial violation of normality assumptions using graphical methods and were checked for outliers using the Grubbs outlier test (a = 0.05) or Robust Regression and Outlier Removal Technique (Q = 1%) in GraphPad Prism 9.0 software (https://www.graphpad.com/). For normally distributed data, parametric tests were used to compare mean differences between 2 groups, and results were analyzed by unpaired Student's *t* tests. To determine mean differences between 3 or more groups, the two-way analysis of variance with repeated measures was used depending on the number and level of factors (i.e., within subjects, between subjects) to be analyzed. If the main effects between groups or within-subjects reached statistical significance ($p < .05$), an appropriate post hoc analysis (i.e., Bonferroni) was performed.

RESULTS

Chronic MEHP Exposure Does Not Affect Spatial Learning, Recognition, and/or Short-term Memory

To assess whether chronic MEHP administration impacts spatial learning and memory, the Y-maze test was used, and spontaneous alternation behavior was quantified (25). This test is based on an animal's willingness to explore new arms of the maze, and mice typically show a tendency to enter an arm that was not recently visited. If a mouse enters a different arm for 3 consecutive arm entries, a spontaneous alternation occurs (25). MEHP-treated mice and vehicle mice showed a similar percentage of spontaneous alternations (Figure 2A, B) (mean ± SD, vehicle 53.97 ± 5.14 vs. MEHP 52.21 ± 3.91, $p > .05$), suggesting that MEHP exposure did not impair innate exploration and spatial memory.

Rodents have an innate preference for novelty, and the choice to explore a novel object reflects the use of learning and recognition memory (26). Therefore, we next evaluated the effects of MEHP on cognition or recognition memory using the novel object recognition test (Figure 2C–E). Mice were allowed to explore an open field containing 2 objects, and on the second day, one of the objects was replaced with a novel one. As shown in Figure 2C, differences in the exploration time of novel and familiar objects were used to evaluate learning and memory. The 2 groups of mice showed similar novelty performance (novel object exploring time/total exploring time, vehicle 0.55 ± 0.08 vs. MEHP 0.67 ± 0.04, $p > .05$) and discrimination index ([novel object exploring time − familiar object exploring time]/total exploring time, vehicle 0.10 ± 0.15 vs. MEHP 0.35 ± 0.08, $p > .05$) (Figure 2D, E).

Finally, to assess the effects of MEHP on long-term spatial memory, a Morris water maze was conducted. As shown in Figure 2F, H, mice were introduced to the water maze, and the

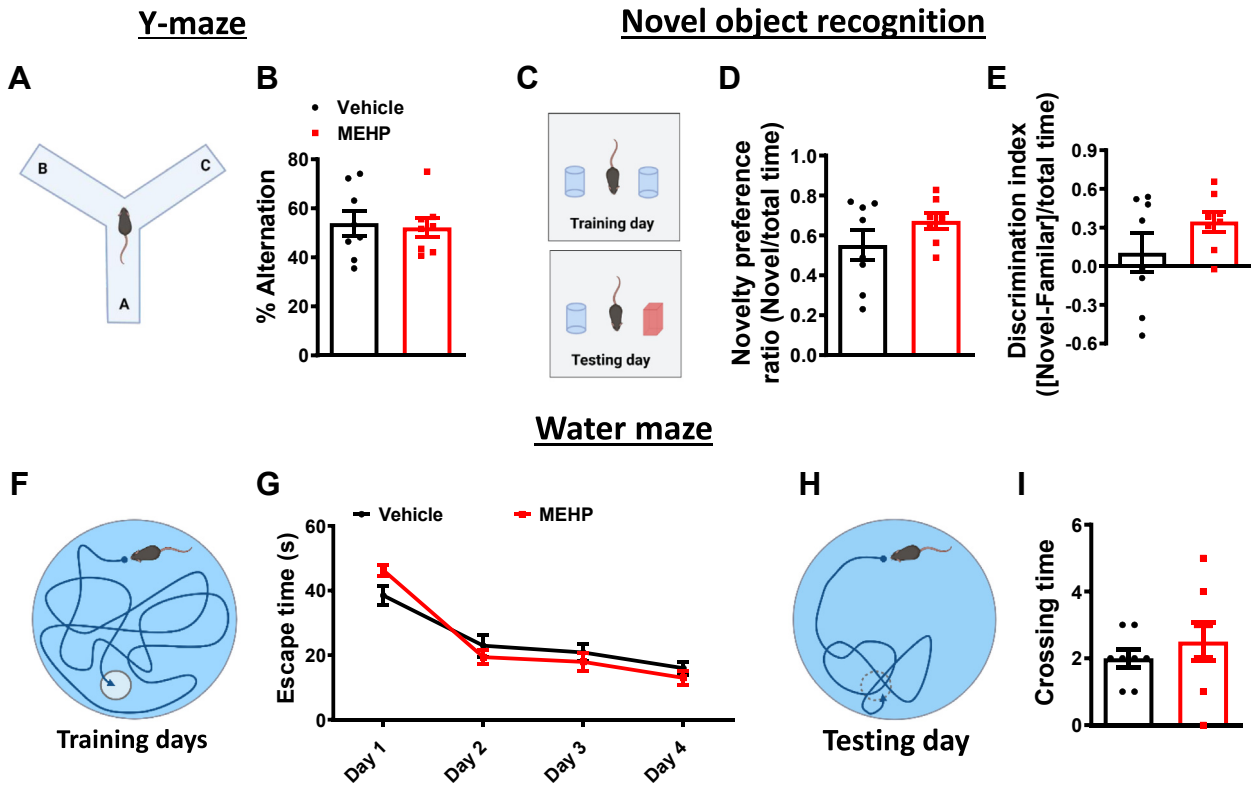

Figure 2. The effects of chronic MEHP exposure on spatial learning, recognition, and/or short-term memory. **(A)** Experimental protocol for Y-maze test. **(B)** Percentage of spontaneous alternations in the Y-maze test. **(C)** Experimental protocol for novel object recognition test. **(D)** Novelty preference ratio in the novel object recognition test. **(E)** Discrimination index in the novel object recognition test. **(F)** Experimental protocol for water maze training days. **(G)** Escape time in the water maze training. **(H)** Experimental protocol for water maze testing. **(I)** Time that the mice crossed the position where the platform used to be during water maze testing. Data are presented as mean ± SEM, $n = 8$.

amount of time they spent searching for a hidden platform (escape time) was recorded. On the last day of the test, the platform was removed, and the number of times that mice crossed the position where the platform had previously been located was measured (crossing time). No significant differences were found in escape time ($F_{1,14}$ = 0.024, $p > .05$) or crossing time (vehicle 2.0 ± 0.27 vs. MEHP 2.5 ± 0.57, $p > .05$) between the MEHP and the vehicle groups (Figure 2G, I), suggesting that long-term spatial memory was not impaired by MEHP exposure.

Chronic MEHP Exposure Increases Anxiety-like Behaviors

Using elevated plus maze and open field testing, next we assessed the effects of chronic phthalate exposure on anxiety-like behaviors. As shown in Figure 3B, the total distance traveled during the 5 minutes of the elevated plus maze testing was comparable between the 2 groups (vehicle 11.16 ± 0.60 vs. MEHP 10.07 ± 0.71, $p > .05$); however, the MEHP-treated mice performed significantly fewer open-arm entries (Figure 3C) (vehicle 15.42 ± 1.29 vs. MEHP 9.25 ± 1.15, $p < .01$), traveled less distance in the open arms (Figure 3D) (vehicle 2.54 ± 0.44 vs. MEHP 1.27 ± 0.31, $p < .05$), and spent significantly less time in the open arms (Figure 3E) (vehicle 30.70 ± 3.93 vs. MEHP 17.24 ± 3.25, $p < .05$) compared with the vehicle group.

To further assess the anxiolytic behavioral effects of MEHP exposure (Figure 3C–E), an open field test was performed with center exploration used as an index of anxiety level. Within the first minute of testing, MEHP-treated animals made significantly fewer center entries and traveled less center distance (Figure 3H, I) (center entries: vehicle 6.9 ± 0.64 vs. MEHP 4.6 ± 0.6, $p < .05$; center distance: vehicle 1.03 ± 0.12 vs. MEHP 0.65 ± 0.12, $p < .05$), but thereafter these parameters became comparable between MEHP-treated and control mice during the 5-minute test (Figure 3H–J) (center entries: vehicle 5.08 ± 0.37 vs. MEHP 5.03 ± 0.57, $p > .05$; center distance: vehicle 0.73 ± 0.07 vs. MEHP 0.69 ± 0.09, $p > .05$). The increased anxiety-like behavior that accompanied MEHP administration was not a result of locomotor impairment because the total distance traveled in the open field remained unchanged between the 2 groups (Figure 3G) (0–1 min: vehicle 4.38 ± 0.24 vs. MEHP 4.24 ± 0.33, $p > .05$; 1–5 min: vehicle 3.62 ± 0.24 vs. MEHP 3.44 ± 0.24, $p > .05$). Locomotor activity was also evaluated for 30 minutes in the open field, and no differences were found between the 2 groups (data not shown).

Chronic MEHP Exposure Increases Fear-Related and Startle Reactivity

To assess the effect of MEHP exposure on learned fear and startle reactivity, an acoustic startle test and a conditioned fear test were performed (Figure 4A, D, G). Compared with the vehicle control, MEHP-treated mice exhibited a higher startle magnitude to the 120 dB white noise bursts, but only during the initial stage of the test (Figure 4B) (interaction of group × burst, $F_{15,330}$ = 2.197, $p < .01$). Furthermore, fear responses as measured by percentage freezing to the conditioned tones (i.e., the conditioned stimulus) were also increased in the MEHP-exposed mice. As shown in Figure 4E, F, H, I, increased

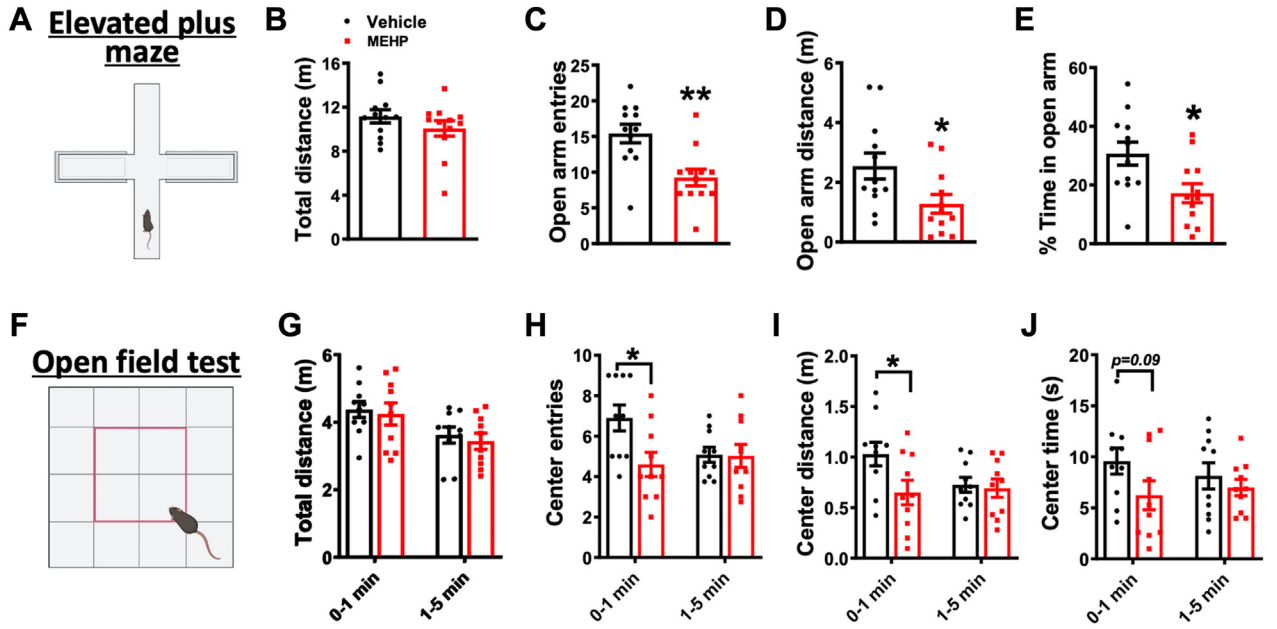

Figure 3. The effects of chronic MEHP exposure on anxiety-related measurements. **(A)** Experimental protocol for EPM test. **(B)** Total distance traveled in EPM. **(C)** Open-arm entries in EPM. **(D)** Distance traveled in the open arm of the EPM. **(E)** Percentage of time spent in the open arm during the EPM test. **(F)** Experimental protocol for open field test. **(G)** Total distance traveled in the open field. **(H)** Time of entries into the center zone of the open field test. **(I)** Distance traveled in the center zone of the open field test. **(J)** Time spent in the center zone of the open field test. Data are presented as mean ± SEM. *$p < .05$, **$p < .01$, two-tailed Student's t test, n = 10–12. EPM, elevated plus maze.

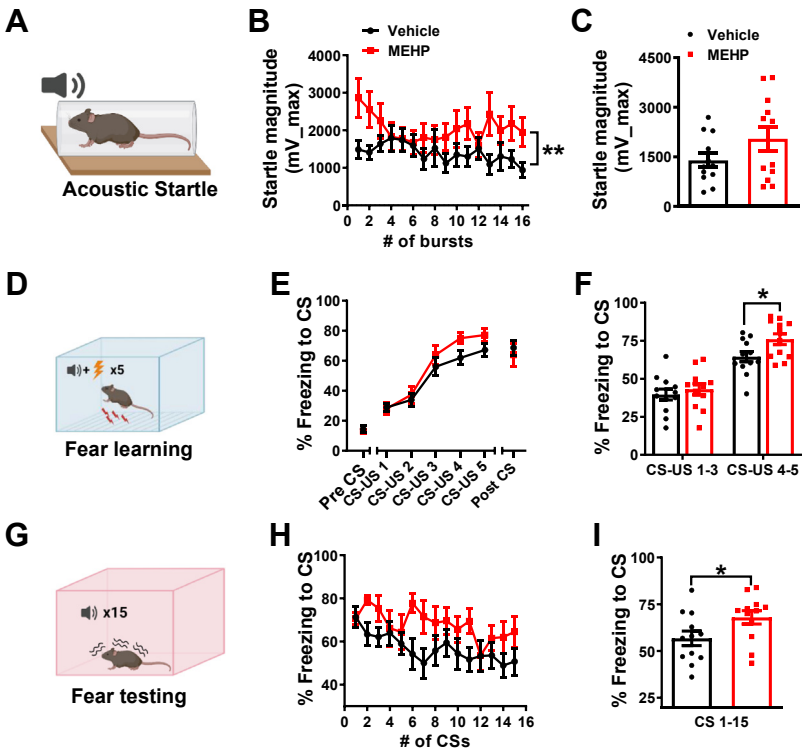

Figure 4. The effects of chronic MEHP exposure on the acoustic startle response and conditioned fear. **(A)** Experimental protocol for acoustic startle test. **(B)** Startle magnitude to 120 dB white noise. Each data point represents 1 acoustic burst. **significant group × burst interaction, $p < .01$, two-way analysis of variance. **(C)** Average startle magnitude across the 16 acoustic bursts. **(D)** Experimental protocol for conditioned fear learning. **(E)** Percentage freezing during fear learning. **(F)** Average freezing percentages of the CS-US 1–3 and CS-US 4–5 during fear learning *$p < .05$, two-tailed Student's t test. **(G)** Experimental protocol for fear testing. **(H)** Percentage freezing during fear testing. **(I)** Average freezing percentages during fear testing. *$p < .05$, two-tailed Student's t test. $n = 12$ mice/group. CS, conditioned stimulus; max, maximum; US, unconditioned stimulus.

conditioned freezing responses were found during the late stage of fear acquisition (Figure 4F) (conditioned stimulus–unconditioned stimulus 4–5: vehicle 64.56 ± 3.36 vs. MEHP 76.08 ± 3.55, $p < .05$). This enhanced conditioned stimulus–dependent freezing in the MEHP group carried over to the next day during fear expression and extinction testing (Figure 4I) (vehicle 56.75 ± 3.89 vs. MEHP 67.98 ± 3.6, $p < .05$).

The Effects of MEHP Exposure on Neuroendocrine and Stress Biomarkers

Anxiety disorders have been linked to dysregulation of the neuroendocrine system, while phthalates can also act to disrupt the endocrine system because they are well-known endocrine-disrupting chemicals. Therefore, next we evaluated the effects of MEHP exposure on changes in plasma corticosterone, adrenaline, noradrenaline, and dopamine concentrations, and inflammatory cytokines. As shown in Figure 5A, MEHP administration significantly increased plasma corticosterone levels (vehicle 67.72 ± 14.21 vs. MEHP 154.5 ± 16.40, $p < .01$), while the concentrations of adrenaline (vehicle 5.89 ± 0.87 vs. MEHP 6.17 ± 0.44, $p > .05$), noradrenaline (vehicle 15.12 ± 2.72 vs. MEHP 11.26 ± 0.8, $p > .05$), and dopamine (vehicle 1.83 ± 0.23 vs. MEHP 1.71 ± 0.11, $p > .05$) remained unchanged (Figure 5B–D). Furthermore, several epidemiological and experimental animal studies have demonstrated a strong association between phthalate exposure and inflammation (27–29). Supporting this, MEHP-treated animals in the current study exhibited a significant increase in circulating proinflammatory cytokine levels (Figure 5E–H) including elevated interferon-γ (vehicle 0.3 ± 0.04 vs. MEHP 0.56 ± 0.11, $p < .05$) and interleukin 2 (vehicle 0.61 ± 0.04 vs. MEHP 1.12 ± 0.12, $p < .01$). Changes in inflammatory biomarkers and immune function can be a cause or consequence of endocrine or glucocorticoid disturbances, and therefore, next we evaluated the effects of MEHP on glucocorticoids and hypothalamic-pituitary-adrenal (HPA) axis function.

The Effects of MEHP Exposure on Glucocorticoid Metabolic Pathways

To test the hypothesis that MEHP alters HPA axis activity (Figure 6 schematic) and consequent increases in plasma corticosterone level, we first examined messenger RNA (mRNA) levels of corticotropin-releasing hormone (*Crh*) and *Fos* (an index of neuronal activity) in the hypothalamus. As shown in Figure 6B–D, despite a trend for a reduction in *Fos* mRNA expression, there were no statistically significant differences in hypothalamic *Crh* mRNA expression or plasma ACTH levels in MEHP-treated animals. These data suggest that the elevated plasma corticosterone levels that we observed (Figure 5A) following MEHP exposure may not be a consequence of enhanced hypothalamic-CRH-ACTH activation but possibly as a result of downstream alterations in glucocorticoid metabolism. Our next studies were designed to examine this possibility.

Corticosterone plasma concentrations, synthesis, and degradation are regulated in part by 11β-hydroxysteroid dehydrogenase (HSD11B) enzyme activity and the

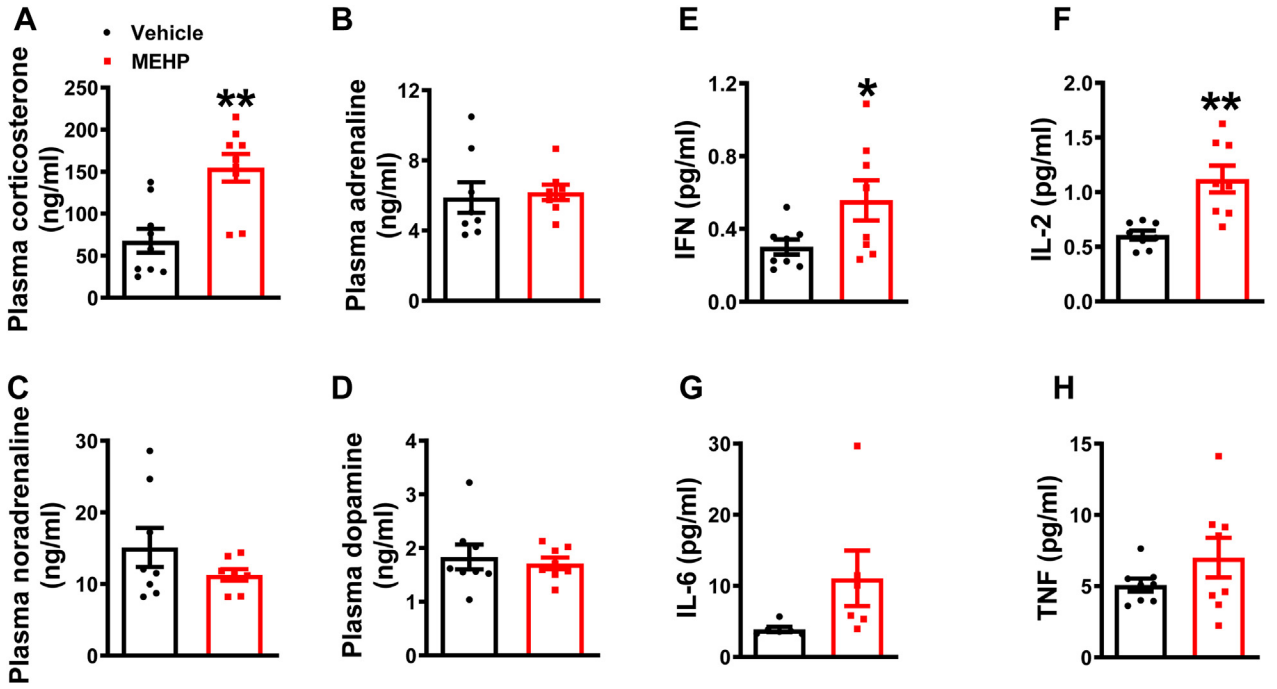

Figure 5. The effects of chronic MEHP exposure on plasma corticosterone, 3-catecholamines, and proinflammatory cytokine levels. Plasma concentrations of **(A)** corticosterone, **(B)** adrenaline, **(C)** noradrenaline, **(D)** dopamine, **(E)** IFN, **(F)** IL-2, **(G)** IL-6, and **(H)** TNF. $^{*}p < .05$, $^{**}p < .01$, two-tailed Student's *t* test. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

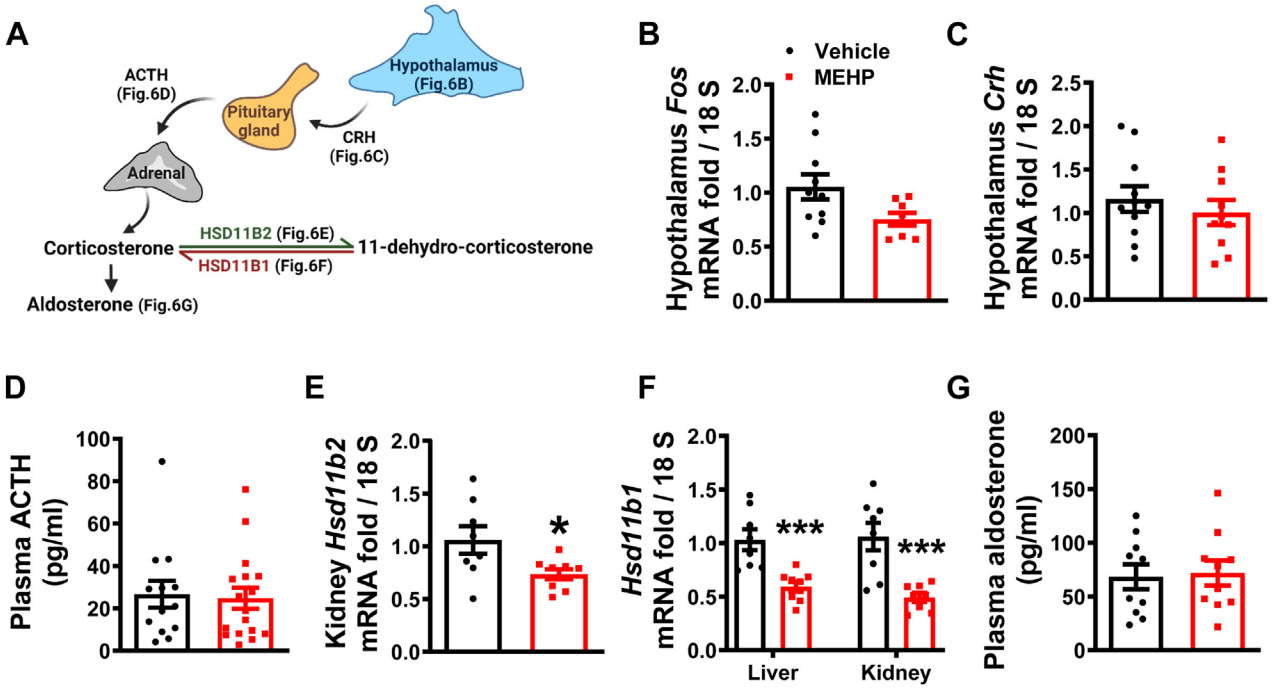

Figure 6. The effects of chronic MEHP exposure on glucocorticoid metabolic pathways. **(A)** Diagram illustrating steps to regulate corticosterone secretion and metabolism that were tested. **(B)** Neuronal activity marker *Fos* mRNA expression in the hypothalamus. **(C)** *Crh* mRNA expression in the hypothalamus. **(D)** ACTH level in plasma. **(E)** *Hsd11b2* mRNA expression in kidney. **(F)** *Hsd11b1* mRNA expression in the liver and kidney. **(G)** Plasma aldosterone concentration. $^{*}p < .05$, $^{***}p < .001$, two-tailed Student's *t* test. *Hsd11b*, 11β-hydroxysteroid dehydrogenase; ACTH, adrenocorticotropic hormone; *Crh*, corticotropin-releasing hormone; mRNA, messenger RNA.

interconversion of active and inactive glucocorticoids (30). For example, corticosterone is metabolized by HSD11B2 to its inactive form 11-dehydrocorticosterone, while regeneration can occur via HSD11B1 [Chapman *et al.* (30) and Figure 6A pathway]. Accordingly, next we investigated the effects of chronic MEHP exposure on HSD11B by quantifying mRNA expression of *Hsd11b1* and *Hsd11b2* in liver and kidney tissue. We hypothesized that either increased *Hsd11b1* or decreased *Hsd11b2* expression could lead to increased plasma corticosterone levels in the MEHP group as a result of increased conversion of 11-dehydrocorticosterone to active corticosterone. As shown in Figure 6E, *Hsd11b2* was lower in kidney samples collected from MEHP-treated animals (vehicle 1.06 ± 0.13 vs. MEHP 0.74 ± 0.05, $p < .05$), while *Hsd11b2* expression was undetectable in liver tissue samples. MEHP-treated animals also showed decreased *Hsd11b1* expression in both liver and kidney tissue (Figure 6F) (liver: vehicle 1.03 ± 0.1 vs. MEHP 0.59 ± 0.04, $p < .001$; kidney: vehicle 1.06 ± 0.13 vs. MEHP 0.49 ± 0.04, $p < .001$), which may suggest a negative feedback mechanism in response to increased plasma corticosterone levels. Furthermore, changes in HSD11B expression can also affect the concentration of the corticosterone catalytic product aldosterone; however, there were no significant differences between groups in circulating aldosterone plasma levels (Figure 6G) (vehicle 68.45 ± 11.56 vs. MEHP 71.82 ± 11.65, $p > .05$).

DISCUSSION

Due to its low cost of production and superior chemical properties, DEHP remains the most widely used plasticizer in PVC products. Once in the body, DEHP is rapidly hydrolyzed to MEHP, which has been shown to exhibit toxicological effects on multiple organ systems—including the liver, heart, testes, and pituitary gland (31–33). Moreover, emerging studies have documented associations between prenatal or early-childhood phthalate exposure and neuropsychiatric and adverse behavioral outcomes (10,11); however, the effects of phthalates on postnatal behavioral outcomes have been studied less often. Using an experimental adult rodent model, we aimed to examine the effects of MEHP exposure on cognition, emotion, and anxiety-like behaviors and their impact on neuroendocrine and inflammatory function. Our results show that MEHP exposure induced anxiety-like behavior, including enhanced fear and startle reactivity, but did not affect short- or long-term memory in adult mice. We also observed that MEHP exposure increased plasma corticosterone levels and decreased peripheral tissue mRNA expression of *Hsd11b2*, which may lead to changes in neuroendocrine and immune homeostasis contributing to enhanced anxiety-like behaviors.

Growing evidence has linked chronic phthalate exposure to neurobehavioral outcomes (34,35), and phthalate exposure can precipitate anxiety-like behavior in rodents (19,20,36,37). For example, mice exposed in utero to DEHP displayed fewer social behaviors, and this effect was transgenerational and persisted to the F3 generation (38,39). Depression and impaired cognitive deficits have also been reported in DEHP- or DINP-exposed mice (37,40,41). In the current study, we found that exposure to MEHP during a postdevelopmental adult period similarly drove abnormal neurobehavioral changes, which likely involve distinct temporally linked biological mechanisms that are unique and distinct from prenatal, gestational, or transgenerational phthalate exposure, as shown in previous studies (42–44). In our study, MEHP-exposed adult mice exhibited increased anxiety-like behaviors, with evidence pointing to an enhanced systemic neuroendocrine and inflammatory response because 3 weeks of MEHP exposure produced a significant basal increase in corticosterone levels and circulating proinflammatory cytokines. Interestingly, this effect was independent of increases in other peripheral stress neuroendocrine biomarkers (i.e., catecholamines, aldosterone).

In addition to the MEHP-induced anxiogenic affects observed in the current study, as determined by open field testing and elevated plus maze testing, MEHP-exposed mice also exhibited increased freezing behavior during conditioned fear learning, as well as increased basal startle reflexes to acoustic bursts of white noise. Conditioned learned fear can also be a way to assess anxiety-like behaviors in mice by assessing physical manifestations of anxiety that can drive physiological responses to fear or threats (e.g., freezing, blood pressure, heart rate, respiration) (45,46). For example, mouse lines that are characterized by high innate anxiety-related behavior exhibit more freezing behavior in response to conditioned stimuli (47,48), indicating that stress-induced anxiety enhances the physiological expression of conditioned fear. Furthermore, the startle reflex is also linked to anxiety because individuals with anxiety disorders show a greater startle reflex (49,50). Thus, the elevated freezing and startle responses observed in the MEHP mice support an anxiogenic phenotype and enhanced conditioned fear-related cardiovascular reactivity (24).

Despite the MEHP-induced anxiogenic effects, we did not observe deficits in measures of cognition or short- or long-term memory following MEHP exposure, which suggests an MEHP-dependent neurobehavioral anxiogenic phenotype that is independent of changes in measures of cognition and memory. These results contrast with some studies that have demonstrated phthalate-induced impairments in rodent learning and memory. However, in those experiments, phthalate administration was given pre- or perinatally, suggesting a developmental phthalate effect on cognition, as opposed to the current study where MEHP was administered to adult mice. Moreover, the inconsistency may be attributed to the duration of phthalate administration because reduced learning and memory were observed after long-term exposure (i.e., 100 days) to DEHP (51). Additionally, it is worth considering whether MEHP-induced neuroendocrine dysregulation and increases in inflammation have long-term effects on cognition later in life. Although some of the defects caused by phthalate can recover after exposure cessation, as previously determined, lesions in organs such as the testes, pituitary gland, or liver cannot be fully reversed, especially in male individuals (52,53). This damage to organ function over time may ultimately lead to more severe consequences that increase the risk to both physiological and cognitive health. Unlike these previous studies, the current study however did not examine long-term MEHP effects on liver or corticosterone metabolism (i.e., HSD11B) and secretion following cessation of MEHP.

Therefore, we cannot exclude the possibility that these MEHP-exposed mice may exhibit cognitive decline later in life.

HPA axis dysregulation is a common neuroendocrine corollary of anxiety and depressive phenotypes (54–56). Phthalates are known neuroendocrine disrupters and therefore can alter the homeostasis of the neuroendocrine system and have significant detrimental neurodevelopmental effects on central nervous system function, specifically the HPA axis. To assess this, following MEHP exposure, we first measured circulating levels of corticosterone and its precursor ACTH (Figure 6). Following 3 weeks of MEHP administration, basal corticosterone levels were significantly elevated, with an absence of a change in an expected corresponding increase in ACTH (55). The dissociation between ACTH levels and corticosterone release is likely due to lag times in the temporal dynamics of glucocorticoid feedback signaling and degradation processes (55). Similarly, at the hypothalamic tissue level, chronic MEHP treatment did not alter HPA axis activity, as indicated by an absence of an increase in hypothalamus *Crh* mRNA expression. However, there was a trend for a reduction in the activity of hypothalamus neurons (*Fos* marker) after MEHP administration, which may be a result of increased negative hypothalamic feedback in response to plasma corticosterone levels. Overall, our neuroendocrine results suggest that MEHP-dependent increases in corticosterone are likely a consequence of activity outside the pituitary at the level of the adrenal gland or other peripheral tissues (e.g., kidney, liver) that regulate peripheral glucocorticoid metabolism and circulating corticosterone levels (Figure 6A).

Previous studies have shown that phthalates can affect glucocorticoid activity by decreasing mRNA levels and enzyme activity of HSD11B2, which in turn converts corticosterone into its receptor-inactive form 11-dehydrocorticosterone (57). Clinical data suggest that increased phthalate exposure is associated with elevated cortisol/cortisone ratio in premature infants, which may be mechanistically linked to the inhibition of HSD11B2 (58). Our current results indicate that chronic MEHP administration decreases mRNA levels of *Hsd11b2*, which could contribute to increased circulating levels of corticosterone. However, contrary to what we expected, the mRNA levels of the type 1 isoform, *Hsd11b1*, were decreased after MEHP administration. Overall, we speculate that the elevated corticosterone levels following chronic MEHP exposure may be due in part to altered negative feedback as a consequence of altered HSD11B enzymatic function and glucocorticoid metabolism.

Glucocorticoid dysregulation is also tightly linked to immune homeostasis and inflammation. For example, the interaction, coordination, and crosstalk between the endocrine system via the glucocorticoid receptor– and immune system–regulating transcription factor nuclear factor-κB is well known for its central role in maintaining this neuroendocrine-immune balance. High corticosterone levels may contribute to decreased glucocorticoid receptor sensitivity and increased levels of proinflammatory signals and cytokines (59) and contribute to the overall MEHP-dependent anxiogenic phenotype independent of any cognitive behavioral effects. Indeed, previous studies have shown that several proinflammatory biomarkers are elevated following phthalate exposure (60–62). In addition, various in vitro studies have reported that acute phthalate exposure increases the production and secretion of inflammatory cytokines from macrophages and neutrophils (e.g., tumor necrosis factor α, interleukin 1β, interleukin 8, and interleukin 6) (63–66). Although less is known about the in vivo effects of phthalate exposure on systemic inflammation, studies suggest that phthalates amplify proinflammatory cytokine production and increase immune cell infiltration to both testicular and cardiac tissue (62,67). Supporting this, we have provided evidence for an increased proinflammatory state in mice chronically exposed to MEHP, which may be a consequence of phthalate-induced cortisol elevations and a driver of anxiety-like behavior observed here. However, additional studies are required to establish this mechanistic link.

In summary, chronic MEHP exposure induces anxiety-like behaviors in adult rodents without altering cognition or memory, which may be due in part to altered HPA axis function and peripheral glucocorticoid metabolism. These results expand the current understanding of the effects of MEHP as an endocrine-disrupting and toxic environmental chemical on neurobehavioral function in adult mice. Future studies are needed to further investigate the link between phthalate exposure in adult mice, glucocorticoid regulation, and the downstream cellular, molecular, and physiological pathways, as well as the reversibility of the MEHP effects. Finally, because MEHP is an endocrine disrupter with known anti-androgenic effects (68–70), the current studies were conducted with male mice. Nonetheless, considering the influence of MEHP exposure on the distribution of glucocorticoid metabolism, it is imperative to conduct future research to more extensively investigate the impact of MEHP exposure on the gender-related variations (71) in the balance of endocrine and immune systems.

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ARTICLE INFORMATION

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