Prenatal Exposure to Diethylhexyl Phthalate Impairs the Recovery of Spatial Memory Post-traumatic Brain Injury in a Sex-specific Manner

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Research Article

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Abstract

Background

Diethyl hexyl phthalate (DEHP) is a chemical plasticizer that causes significant neurological impairments after prenatal exposure. The impact of these exposures on cognitive recovery after severe traumatic brain injury (sTBI) has never been studied. We hypothesized that prenatal exposure to DEHP will impair cognitive recovery after a sTBI in a sex-specific manner.

Methods

To test this hypothesis, six-month-old male and female offspring that were prenatally exposed to DEHP were subjected to controlled cortical impact injuries, and their spatial memory was tested weekly using a Morris Water Maze (MWM) test for 4 weeks post-sTBI.

Results

We observed that DEHP-exposed male rats demonstrated significantly impaired spatial memory up to four weeks post-sTBI when compared to controls. In contrast, DEHP-exposed female rats showed no detectable impairment in spatial memory recovery compared to injured controls. These observations were accompanied by marked increases in neurotransmitter levels in the cortex and dopamine levels in the hippocampus of female, but not in male rats. No significant differences in lesion area and number of hippocampal neurons were observed in both sexes.

Conclusion

These results suggest that prenatal exposure to DEHP may have long lasting effects in the brain that could lead to impaired recovery specifically in male subjects following post-sTBI.

Introduction

Motor and cognitive functions progressively decline after severe traumatic brain injury (sTBI) causing chronic disability\(^1\). Only 26% of patients who sustained moderate-to-severe TBIs report improved outcomes 5 years after the injury due to the complex cascade of pathophysiological events and comorbidities\(^3\). Neuroendocrine dysfunction is a common comorbidity among TBI patients as nearly two-thirds of the patients experience such a disorder\(^4\). Furthermore, neuroendocrine dysfunction could further contribute to stress-induced memory and executive function impairments\(^5\).

Di-2-ethylhexyl phthalate (DEHP) is an endocrine disrupting chemical (EDC) that has transgenerational effects on neuroendocrine function\(^6\)–\(^8\). Since human exposure to phthalates is ubiquitous\(^9\),\(^10\), it is important to investigate if prenatal exposure of DEHP could contribute to neuroendocrine dysfunction, such as interference with the hypothalamic-pituitary-adrenal (HPA) axis, leading to impaired recovery from sTBI.

The HPA axis or the stress axis is activated in response to injury, inflammation and infection\(^11\). Activation of the HPA axis is essential for maintaining homeostasis and for timely recovery from TBI\(^12\). The HPA axis is made up of corticotrophin releasing hormone neurons located in the hypothalamus, corticotrophs in the anterior pituitary and the adrenal cortex that secretes glucocorticoids (corticosterone (CORT) in rodents and cortisol in humans)\(^13\). Glucocorticoids act through a negative feedback mechanism inhibiting further HPA axis activation\(^11\). The hippocampus contains glucocorticoid receptors (GR) and plays a critical role in this feedback inhibition by binding glucocorticoids\(^14\) and directly influencing neuronal excitability and restructuring neural connectivity\(^15\). In addition, uninhibited elevation in glucocorticoid levels impairs memory tasks associated with the hippocampus\(^16\). Glucocorticoid levels post-sTBI can be elevated or decreased depending on the injury type, severity, time of the injury, and sex; however, there is a consensus that an inverted U-shaped relationship between functional performance and glucocorticoid secretion.
exists. Neither hyper-secretion nor hypo-secretion of glucocorticoids is beneficial for learning and memory\textsuperscript{17}. Hence, stress axis disruption post-TBI can have a direct impact on learning and memory dysfunction.

Recent data from our laboratory suggests that prenatal exposure to 7.5mg/kg BW of DEHP can increase CORT levels in adult female offspring, but not male offspring (Mohankumar et al., Athens, 2021. Unpublished). These offspring also exhibit certain behavioral abnormalities. However, the impact of sTBI on animals prenatally exposed to DEHP has never been studied. Considering the evidence presented above, we hypothesized that prenatal exposure to DEHP could negatively impact spatial memory recovery post-TBI in a sex-specific manner. To test this, rats prenatally exposed to DEHP were subjected to controlled cortical impact (CCI), and their spatial memory was tested for 4 weeks (Fig. 1). At the end of the observation period, CORT levels were measured in the serum, extent of tissue injury in the brain in terms of neuronal loss was determined by histological assessment, and neurotransmitter levels were measured in specific brain areas.

**Methods**

**Animals.** All animal work and procedures were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC, A3437-01) and complied with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. All experiments were carried out in compliance with the ARRIVE guidelines.

7 pregnant Sprague Dawley dams (F0) were randomly assigned to be treated with either 10 µl of saline (control) or 10 µl of saline containing 7.5mg/kg bis(2-ethylhexyl) phthalate (DEHP) by oral gavage from days 6-21 of gestation. A total of 20 Sprague-Dawley rats (F1) offspring were obtained: 6 control males, 6 control females, 4 DEHP-exposed males, and 4 DEHP-exposed females. F1 rats were housed with their group mates. They were subjected to controlled cortical impact (CCI) at 6 months of age, and single housed post-CCI. All animal handlers were blinded to assigned groups post-CCI.

**Controlled Cortical Impact Injury.** A CCI injury to the motor cortex was induced using a custom-built pneumatic impactor that has been characterized by us previously\textsuperscript{18}. Briefly, animals was anesthetized with 2-3% isoflurane, and received a craniotomy to expose the left hind limb motor cortex region in the right hemisphere (bregma 0, 1.5mm lateral from the midline as the center of the impact)\textsuperscript{19}. A 3mm diameter tip affixed to a pneumatically actuated piston was driven at an average velocity of 2.17m/s and an average cortical dwell time of 250 msec to create an injury to a depth of 2mm. The craniotomy was covered with 0.5% sterile seakem agarose (Lonza, NH) after cleaning out excess blood and debris surrounding the lesion. The incision was closed using nylon fiber sutures (ACE, MA), and the animals were returned to their home cage for recovery. All animals were guillotined at study endpoint, and brain tissue and blood collected for further analyses. All females received CCIs and were sacrificed at diestrus.

**Behavioral Tests.** The Morris Water Maze (MWM) task was used to measure spatial learning and memory following sTBI\textsuperscript{20}. Briefly, training was performed in 3 phases each day: 1) in clear water with exposed platform (motor control), 2) repeated platform search in milk-opacified water and 3) free exploration without platform. All experiments were performed in a round pool (183 cm in diameter and 91 cm deep) that was filled with clear water (phase 1) or opaque water (containing powdered non-fat milk; phase 2 and 3). A square platform (10 cm x 10 cm) was placed in the south corner of the pool either visible (2 cm above water; phase 1), submerged 2 cm below water level (phase 2) or completely removed (phase 3). Visual cues were permanently affixed to walls on three sides of the pool. The platform was left exposed during all trials for the first 2 days of training. Subsequently, the rat would perform one trial in phase 1 conditions, three in phase 2, and one in phase 3 per experiment day. The rat was released from either the north, east, or west corners in a random order. To evaluate spatial memory, the platform was removed, and the rat was released from the north corner facing the wall of the pool. Once released, the rat was allowed 2 minutes to search for the platform location. Test sessions were recorded, and movement was tracked offline using a custom Bonsai workflow\textsuperscript{21}. Time spent in each quadrant was calculated using MATLAB\textsuperscript{®} (Mathworks, inc.). At the end of the observation period, animals were sacrificed by rapid decapitation. Females were subjected to vaginal cytology and sacrificed when they were in the state of diestrus. Serum was separated from trunk blood and used for CORT radioimmunoassay. The brain was quickly harvested, frozen on dry ice and stored at -80°C until further analysis.

**CORT Measurement.** Serum CORT was measured in duplicate using a double antibody radioimmunoassay as described previously\textsuperscript{22}.
Monoamine Measurement.

Brain sectioning and microdissection. A cryostat (Slee, London, UK) maintained at -10°C was used to section brain tissue at 300 μm thickness. Following this, the PVN, right (ipsilesional) cortex, contralesional cortex, ipsilesional hippocampus, and contralesional hippocampus were microdissected on a cold stage using the Palkovits’ microdissection procedure with a stereotaxic brain atlas as a reference 23. Microdissections were completed using a 500 μm diameter punch, and the brain punches were stored at -80°C until required for HPLC analyses.

Neurotransmitter analysis by HPLC-EC. HPLC-EC was used to analyze brain punches for norepinephrine (NE), dopamine (DA), and serotonin (5-HT). Brain punches were briefly homogenized in 0.05 M perchloric acid on ice and an aliquot was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). The remaining homogenate was centrifuged at 18,000 × g for 8 min at 4°C. The supernatant was injected with an internal standard (dihydroxybenzylamine, 0.05 M) into the autoinjector for HPLC analysis. The HPLC-EC system comprised of a 5-μm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C and a LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase was maintained at 1.8 ml/min using a LC-20AD pump (Shimadzu, Columbia, MD). Protein levels in tissue punches were measured using the micro bicinchoninic acid assay (Pierce, Rockford, IL). Samples were assayed in duplicate according to the manufacturer’s protocol. Neurotransmitter concentrations in tissue samples were expressed as pg/µg of protein.

Immunohistochemistry. Non-consecutive coronal brain tissue sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) solution for ten minutes, rinsed with PBS three times and incubated in blocking buffer (PBS with 0.5% Triton-X100 containing 4% goat serum) for an hour. Sections were then incubated in blocking buffer containing primary antibody against NeuN (ABN91; 1:500) overnight at 4°C. The next day, sections were rinsed with washing buffer (PBS with 0.5% Triton-X100) three times followed by an hour of blocking. Sections were incubated in blocking buffer containing AlexaFluor 488 (Life Technologies, CA), for secondary binding. The sections were rinsed again with washing buffer, counterstained with NucBlue (Life Technologies, CA), and mounted with fluoromount-G (SouthernBiotech, AL). Images were obtained using the Leica DM microscopy system (Leica Microsystems Inc., IL). Fiji (NIH) was used to process the raw images obtained for the presence of neurons24. Coronal sections between bregma +0.5mm and -0.5mm was used to evaluate the presence of neurons post-CCI.

Statistical Analysis. For all group comparisons, Kolmogorov-Smirnov or Shapiro-Wilk tests were performed to test for normality. When sample size and normality were verified, student t-test was performed for two group comparisons, and Wilcoxon rank sum test was used for multiple pairwise comparisons. Two-way Analysis of Variance was used to compare two independent variables between groups. All statistical tests, except neurotransmitter data, were performed using SigmaPlot (Systat Software, Inc., CA) and R-studio (RStudio Inc.,MA). All neurotransmitter data were analyzed using unpaired t-tests to identify differences between the control group and DEHP group using GraphPad prism software 9.0.0. A p-value < 0.05 was considered to indicate a statistically significant difference. All values are reported as mean ± standard error of mean (s.e.m.).

Results

Spatial memory recovery was impaired in male DEHP-exposed rats. Spatial memory recovery was tested weekly on the Morris water maze (MWM) task over a period of 4 weeks post-sTBI (Fig. 2). Longer time spent in the target quadrant (South; initially trained with escape platform) indicated a stronger spatial memory20. Both control and DEHP-exposed male rats demonstrated decreased preference for the target quadrant, 1-week post-sTBI (Control male: 32.5±3.3%, DEHP male: 24.0±4%) in comparison to baseline (Control male: 41.3 ±3.3%, DEHP male: 34.7± 4%; p=0.04; Fig. 2a). Four weeks post-TBI, control rats were able to recover to baseline performance (41.7±3.3%), while DEHP rats showed a sustained deficit (29.2±4%, p=0.024). The preferred quadrant was once again confirmed by average tracing of male control and DEHP rats, with hyper-intense regions in the heatmap representing highly overlapping paths (Fig. 2b). Traces of male control and DEHP rat activity before TBI displayed hyper-intense regions localized in the target quadrant as marked with red lines. Locomotor activity was reduced 1-week post-TBI in both control and DEHP rats. 4 weeks post-TBI, the intensity of activity in the target quadrant was increased in male control rats but remained low in male DEHP rats. No
significant differences in activity were detected between control and DEHP-exposed females (Fig. 2c). Both control female rats and DEHP female rats maintained a preference for the target quadrant 1 week and 4 weeks post TBI (Fig. 2c, d).

**Effects of pre-natal exposure to DEHP on lesion area, and hippocampal neuronal number.** To investigate the physiological damage caused by CCI, we quantified the cortical lesion area, as well as the number of neurons in the hippocampus (Fig. 3). No statistically significant differences in lesion area (male, p=0.411; female, p=0.461) or the number of hippocampal neurons (male, p=0.0774; female, p=0.293) between DEHP vs control animals were observed in both sexes.

**Neurotransmitter changes in the cortex following sTBI in rats prenatally exposed to DEHP:** There were no significant differences in neurotransmitter levels (pg/µg protein; Mean±SEM) in the ipsilesional or contralesional cortex between control and DEHP-exposed male rats 4 weeks post TBI (Table 1). In contrast, DEHP exposed female rats demonstrated significantly higher norepinephrine (NE), dopamine (DA) and serotonin (5-HT) levels (6.14±0.93, 1.7±1.09 and 1.64±0.95 respectively) compared to control (2.26±0.44, 0.322±0.14 and 0.39±0.1 respectively) in the ipsilesional cortex (Fig. 4). DA levels were moderately elevated only in females in the contralesional cortex in DEHP animals (0.3±0.04) compared to control (0.06±0.001; p<0.05). There were no significant differences between the two groups in the levels of NE or 5-HT in the contralesional cortex (Table 1).

**Neurotransmitter changes in the hippocampus following sTBI in rats prenatally exposed to DEHP:** Similar to observations recorded in the cortex, there were no significant changes in neurotransmitter levels (pg/µg protein; Mean±SEM) in the ipsilesional or contralesional hippocampus between control and DEHP-exposed male rats 4 weeks post-sTBI (Table 1). However, in female rats, we observed a marked increase in DA levels in DEHP exposed rats (1.13±0.09) compared to control (0.017±0.004; p<0.01). There were no other changes in NE or 5-HT levels in female rats (Table 1).

**Changes in stress axis activity in male and female rats following sTBI.** There were no significant changes in NE, DA or 5-HT levels in the paraventricular nucleus of the hypothalamus in both male and female rats post sTBI. Nor did we observe any changes in serum CORT levels between control and DEHP rats in both male and female rats (Table 2).

**Discussion**

This study demonstrates that prenatal exposure to DEHP, a ubiquitous endocrine disrupting chemical, can impact spatial memory in a sex-specific manner following sTBI. Prenatal DEHP-conditioned adult male rats displayed significantly reduced spatial memory, while control rats successfully recovered spatial memory deficits 4 weeks post-TBI (Fig. 5). In contrast to adult male subjects, prenatal-DEHP-conditioned adult females showed little spatial memory impairment following TBI. This was accompanied by increased monoamine levels in the ipsilesional cortex of DEHP-exposed female rats. DA levels were also elevated in the contralesional hippocampus of DEHP-exposed females. Although we suspected that changes in stress axis activity as measured by monoamine levels in the PVN and serum CORT would contribute to the sex-specific recovery of spatial memory, we did not observe any changes in these parameters.

The sTBI injury in this experiment was confined to the right cortex. We did not observe any significant differences in the lesion area or the number of NeuN staining neurons between the control and DEHP-exposed groups suggesting that prenatal DEHP exposure did not contribute to exaggerated post-sTBI degenerative responses (Fig. 3). However, it appeared to have impacted neurotransmission (Table 1, 2). We have previously observed that prenatal DEHP exposure significantly decreases monoamine levels in the cortex of uninjured male rats but increases them in female animals (Mohankumar et al., Athens, 2021. Unpublished). The monoamine levels observed after sTBI in the present study were about 4-fold lower than what we had observed in uninjured animals, but the pattern observed after prenatal DEHP exposure was preserved. This effect was apparent even 4 weeks after sTBI indicating that monoamine transmission was severely compromised by the injury in male rats and probably requires a longer recovery period to return to baseline. Interestingly in female rats, there was a significant increase in all three monoamines in the ipsilesional cortex suggesting a possible compensatory mechanism that contributes to their improved performance on the MWM. A similar compensatory increase in neurotransmission has been observed in the early phases of other degenerative disorders involving the cortex such as Alzheimer’s disease. This could be part of a wide spectrum of biological processes that contribute to brain function and homeostasis. While some clinical reports indicate that female TBI patients tend to have a higher percentage of physiological abnormalities, leading to neurosurgical interventions and mortality, other studies suggest that females recover faster than males from TBI. The inherent differences in white matter architecture, synaptic connections, differences in hormonal
milieu and the related divergence in gene expression between males and females\textsuperscript{32} could all be contributing factors and need further investigation.

The hippocampus is a major center for spatial memory in both rodents and humans\textsuperscript{33,34}. Previous studies have demonstrated that an impact to the rodent cortex can result in spatial learning deficits despite an intact hippocampus\textsuperscript{35,36}. Reduced synaptic integrity and apoptotic dentate gyrus granule cells are largely responsible for possible hippocampal memory impairments\textsuperscript{37,38}. We examined the number of neurons present in the hippocampus to confirm that the injury was limited to the cortex and did not find any differences between control and DEHP-exposed rats in both sexes (Fig. 3). However, we found increased levels of DA in the contralesional hippocampus in female DEHP-exposed rats that could be linked to their better performance on the MWM. DA in the hippocampus is important for various types of learning and memory\textsuperscript{39}. Moreover, photostimulation of DA neurons in the midbrain has been shown to enhance memory persistence and improve hippocampal network dynamics\textsuperscript{40}. Therefore, the increase in DA levels in DEHP-exposed females could have probably contributed to the better recall of the platform location on the MWM. A point to consider is that both control and DEHP-exposed females had the same performance on the MWM, yet DA levels in the hippocampus were only increased in DEHP-exposed females. Although the reason for this is unclear, there could be underlying processes that are taking place in DEHP-exposed animals that makes them over-compensate after sTBI. A recent study indicates that female rats are able to upregulate the lipid content in the hippocampus, which in return makes them resistant to DEHP, while males could not\textsuperscript{41}. Moreover, intrinsic sex differences in hippocampal neurogenesis and function have long been addressed to emphasize how male and female brains react differently under the same injury/stress conditions\textsuperscript{42,43}. Additional investigation is necessary to understand the mechanism in which the hippocampus is protected following sTBI despite pre-exposure to DEHP in female rats. Detailed examination of hippocampal integrity including synaptic plasticity in prenatal-DEHP-treated rats will facilitate better understanding of the effect of prenatal DEHP exposure on hippocampus-related learning and memory processes post-TBI.

The HPA axis is an essential regulator of stress and injury responses\textsuperscript{11}. NE and 5-HT are believed to be stimulatory and DA inhibitory or without effect on the HPA axis\textsuperscript{13}. Dysregulation of the HPA axis results in detrimental outcomes including poor memory function\textsuperscript{44}. About 65% of moderate-to-severe TBI patients suffer from long-term cognitive impairments\textsuperscript{45}, which could be exacerbated due to CORT imbalance. In this study, we did not observe any differences in NE, DA or 5-HT levels in the PVN between control and DEHP-exposed rats post TBI. Moreover, there were no differences in serum CORT between the two treatments or between the sexes. The levels of neurotransmitters in the PVN and CORT observed in this study are markedly lower than what has been observed in these rats without TBI (Mohankumar et al., Athens, 2021. Unpublished). Although we did not detect any significant differences in CORT or neurotransmitter levels, we cannot exclude the potential role HPA-axis plays in the prolonged memory impairment. Altered glucocorticoid receptor expression in the hippocampus post-TBI causes hippocampal neuron apoptosis and aggravates spatial memory impairment\textsuperscript{46,47}. This could be a possible mechanism that is in play in male rats which hinders recovery post-TBI.

**Conclusion**

This study demonstrated that prenatal exposure to DEHP led to impaired recovery of higher cognitive function post-TBI in male, but not in female rats. These results also suggested that the sex and environmental exposures can significantly affect injury progression and recovery from TBI of individuals and accentuates the need for precision medicine.

**Declarations**

**Ethical Approval:** All animal work was performed in accordance with the UGA institutional animal care and use committee’s (IACUC) guidelines.

**Consent to Publish:** We confirm that this manuscript complies with all instructions to authors, has not been published elsewhere and is not under consideration by another journal, and that authorship requirements have been met and the final manuscript was approved for publication by all authors.

**Consent to Participate:** Not applicable. No human studies were performed.
**Author contributions:** L.K. and S.M.J.M. designed, supervised, and wrote the manuscript. S.M.J.M and A.K. handled prenatal programming of the animals. M.K.S., C.E.L., and R.F. performed animal surgeries, behavioral testing, and serum/brain collection. A.K. did microdissection. Monoamine and corticosterone measurements and analysis were done by A.K. and S.M.J.M. M.K.S. performed immunohistochemistry, imaging, analysis, and writing. C-F.V.L. contributed to analysis and writing. P.V.H. provided behavioral testing methodology expertise. We declare that all authors read and approved the manuscript, all data were generated in-house, and no paper mill was used.

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**Competing Interests:** All authors confirm no conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Availability of Data and Materials:** Materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes.

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### Tables

**Table 1.** Neurotransmitter levels in male and female rats prenatally exposed to DEHP and subjected to sTBI. * indicates p<0.05; ** p<0.01. NE: Norepinephrine, DA: Dopamine, 5-HT: Serotonin.

| Neurotransmitter | Ipsilesional cortex | Contralesional cortex | Ipsilesional hippocampus | Contralesional hippocampus |
|------------------|---------------------|-----------------------|--------------------------|---------------------------|
|                  | Male                | Female                |                          |                           |
|                  | Control  | DEHP       | Control | DEHP       | Control  | DEHP       | Control     | DEHP       |
| NE (pg/µg protein) | 10.13±5.57  | 2.65±0.8 | 1.58±0.51  | 6.51±2.84  | 2.66±0.8 | 2.63±0.07  | 3.39±1    | 2.37±0.69 |
| DA (pg/µg protein)  | 1.92±0.74  | 3.44±2.9 | 0.4±0.24   | 0.16±0.08  | 0.29±0.15 | 0.71±0.05  | 0.72±0.31 | 0.73±0.04 |
| 5-HT (pg/µg protein) | 1.97±1    | 0.24±0.11 | 0.49±0.39  | 0.28±0.08  | 0.63±0.31 | 0.37±0.24  | 0.83±0.35 | 0.64±0.48 |
|                  | Female           |                       |                          |                           |
| NE (pg/µg protein) | 2.26±0.44  | 6.14±0.93** | 2.98±0.92  | 2.29±0.66  | 2.49±0.46 | 4.37±1.11  | 3.1±0.69  | 12.35±9.6 |
| DA (pg/µg protein)  | 0.322±0.14 | 1.7±1.09*  | 0.06±0.001 | 0.3±0.04*  | 0.97±0.76 | 1.27±0.46  | 0.017±0.004 | 1.13±0.09**|
| 5-HT (pg/µg protein) | 0.39±0.1   | 1.64±0.95* | 0.25±0.07  | 0.94±0.92  | 0.82±0.23 | 1.95±1.53  | 0.488±0.09 | 1.85±1.06 |

**Table 2.** Neurotransmitter levels in the paraventricular nucleus and serum CORT in male and female rats prenatally exposed to DEHP and subjected to sTBI. NE: Norepinephrine, DA: Dopamine, 5-HT: Serotonin.

| Parameter          | Male                     | Female                  |
|--------------------|--------------------------|-------------------------|
|                    | Control | DEHP | Control | DEHP | DEHP | Control | DEHP |
| NE (pg/µg protein) | 22.04±6.25 | 7.63±2.23 | 3.88±1.02 | 6.166±0.96 |
| DA (pg/µg protein) | 11.39±8.5   | 8.39±5.19 | 16.46±9.21 | 2.4±0.36   |
| 5-HT (pg/µg protein) | 9.82±7.4  | 3.99±1.25 | 3.76±1.89  | 1.57±1.07  |
| Serum CORT (ng/ml) | 109±34.5 | 93.4±35 | 68.5±10.6 | 43.2±16    |

### Figures

Figure 1
Experiment Schedule. Pregnant Sprague Dawley rats were treated with saline (control) or 7.5 mg/kgBW of DEHP by oral gavage from day 6-21 of gestation. When male and female offspring were 6 months old, they were subjected to Morris water maze (MWM) training for 5 days and subsequently subjected to a controlled cortical impact (CCI)-induced severe traumatic brain injury (sTBI). Spatial memory was monitored at weekly intervals over 4 weeks post-TBI. Serum and brain were collected at the end of 4 weeks and processed for further analysis.

**Figure 2**

Spatial memory tested using the Morris water maze (MWM) post-TBI. DEHP treatment impaired the recovery of spatial memory in male rats post-TBI. (a) Percent time spent by DEHP male rats in the target quadrant (south quadrant) remained consistent between weeks 1-4 post-TBI when compared to control males, which recovered to pre-TBI levels at 4-weeks post TBI. '*' indicates p<0.05. (b) Heat maps of the average tracing of male control rats and male DEHP rats pre-TBI, week 1 post-TBI and week 4 post-TBI. Red lines denote the target quadrant (south quadrant). In contrast, no difference in MWM performance was detected between treatment groups in female rats post-TBI. (c) DEHP treatment did not affect spatial memory recovery post-TBI in females. (d) Heat maps of the average tracing of female control rats and female DEHP rats pre-TBI, week 1 post-TBI and week 4 post-TBI demonstrates that female DEHP and control rats exhibit similar swimming tracks. Red lines mark the target quadrant (south quadrant).

**Figure 3**

CCI induced consistent tissue lesion, and the number of hippocampal neurons was unaffected by the treatment. (a) Representative coronal section of a male control brain used in lesion quantification. White dashed line outlines the lesion area. Blue, DAPI; red, NeuN. Scale bar: 1mm. (b, c) Lesion area is represented as % cortex missing when compared to the contralesional cortex. (d-g) Representative images of hippocampal neuronal staining. Blue, DAPI; red, NeuN. Scale bar: 100 µm. (h, i) No significant difference in the average number of hippocampal neurons observed between treatments 4 weeks post-TBI.
Figure 4

Monoamine neurotransmitters were measured in the ipsilesional cortex 4 weeks post-CCI. No difference was detected between control and DEHP-treated male rats in (a) norepinephrine, (b) dopamine or (c) serotonin levels. DEHP-treated female rats had higher levels of (a) norepinephrine, (b) dopamine and (c) serotonin compared to control rats 4 weeks post TBI. * indicates p<0.5. **indicates p<0.01.

Figure 5

Summary of findings. Prenatal exposure to DEHP impaired spatial memory recovery in males following sTBI, while DEHP-conditioned adult females increased monoamine levels in the ipsilesional cortex, attributing to minimal spatial memory deficit. In addition, increased dopamine level in the contralesional hippocampus was observed. Figure created with BioRender.com.

Supplementary Files

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- Fig234RawData.xlsx