



# Gestational and lactational exposition to di-*n*-butyl phthalate increases neurobehavioral perturbations in rats: A three generational comparative study



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## ABSTRACT

Di-*n*-butyl phthalate (DBP) cause significant deficits in cognition and memory, however the neuroanatomical basis for impairments remain poorly understood. This study evaluates neurobehavioral changes in rats for three successive generations between non-siblings by administering DBP at 500mg/kg bw dose through oral gavage from gestation day-6 to 21 and lactation (3-weeks). Weaning period evaluations and developmental deficits assessed showed variations specific to generation and the toxic potential of DBP was confounded by behavioral deficits that include changes in sensorimotor development reflex response, poor performance, low memory retention and greater latency period. The cytoarchitectural alterations witnessed in hippocampus include condensed nuclei, vacuole formation and remarkable degeneration, shrinkage of pyramidal neurons in CA1 and CA3 regions; disorganized hilar cells and hyperplasia in dentate gyrus. Comparatively, the enlisted changes were high in subsequent generations than preceding and correlates assessed between cognitive impairment(s) and endocrine function confirm a link indicating vulnerability of immature animals as target to disrupt neural and endocrine functions.

## 1. Introduction

Di-*n*-butyl phthalate (DBP) is a ubiquitous environmental contaminant and widely used plasticizer, it is an additive to adhesives or printing inks [1]. Recent findings of De Toni et al. [2] reported the presence of phthalates as well as quantified phthalates namely, DEHP, DEP, DBP in pre-packed coffee capsules. Katsikantami et al. [3] in their review article have reported the maternal exposure to phthalates are able to cross the placental barrier and cause many health issues in humans. Its exposure to food and other materials at higher levels potentially induce abnormal fetal development [4]. Case studies reported by Colón et al. [5] have indicated anomalies such as premature breast development in female subjects while reduced anogenital distance [6], hypospadias [6] and decreased serum testosterone observed in male rats [7]. Arbuckle et al. [8] studies linked adverse reproductive effects and attention deficit disorders upon bisphenol-A exposure. Di (2-ethylhexyl) phthalate exposure shown to cause neurodevelopmental and behavioral deficits in rats [9]. Age-related effects reported upon exposure to phthalates are twice as high in children as adults with 40 % of children (age two to six years) showing higher urinary

concentrations of phthalate metabolites [10]. Findings of Chopra et al. [11] indicated attention deficit disorder and learning disabilities in children of six to fifteen years upon exposure to phthalates. We have previously reported *in utero* and lactational exposure of DBP for three generations brought neuroanatomical perturbations in discrete brain regions and the severity of the effect was higher in subsequent generations [12].

Limited data available on cognitive aspects suggest that higher levels of phthalates adversely affect learning abilities in mice [13] and few studies reported an association between prenatal phthalate exposure and neurological impairments [14], however, the available information on phthalates is biased and inconclusive, especially on aspects of cognitive behavior. The brain weight in rodents was affected by di-2-ethylhexyl phthalate (DEHP) exposure [15,16]; in contrast, Rhodes et al. [17] observed no difference in brain weight upon phthalate-exposure in marmoset monkeys, suggesting changes in brain weight restricted only to rodents, while limited information available on cognitive aspects suggest DBP's toxic potential to cause distinct neurodegenerative changes in the hippocampus of neonatal and immature rats [14]. McIntyre et al. [18] indicated acetylcholine (ACh)

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dependent learning ability in rat hippocampus and results correlated with impaired functions to dysfunction of the cholinergic system in phthalate toxicosis, however the neuroanatomical basis for these impairments remain poorly understood; moreover, the compounded effects that are associated with multi-generational exposures are not explored. In this study, the severity of toxicity, mechanistic differences between generations, as well behavioral deficits that occur upon gestational exposure to DBP was assessed in rats correlating the hormonal and neurochemical changes that prevail during toxic exposures.

## 2. Materials and methods

### 2.1. Chemicals

Di-*n*-butyl phthalate (CAS No: 84-74-2, 99.0 % Purity), AThCh (CAS No: 1866–15-5, 98.0 % Sigma) other AR grade chemicals procured from Merck.

### 2.2. Animals

Healthy, Wistar strain rats acclimatized on a 12-h light/dark cycle and provided food and water *ad libitum* but they have deprived food during the behavioral assessment. The design and protocol of the study approved by the Bangalore University Animal Care Committee (Vide no. CPCSEA No.402, File No.25/525/2009 dated 23.03.2015).

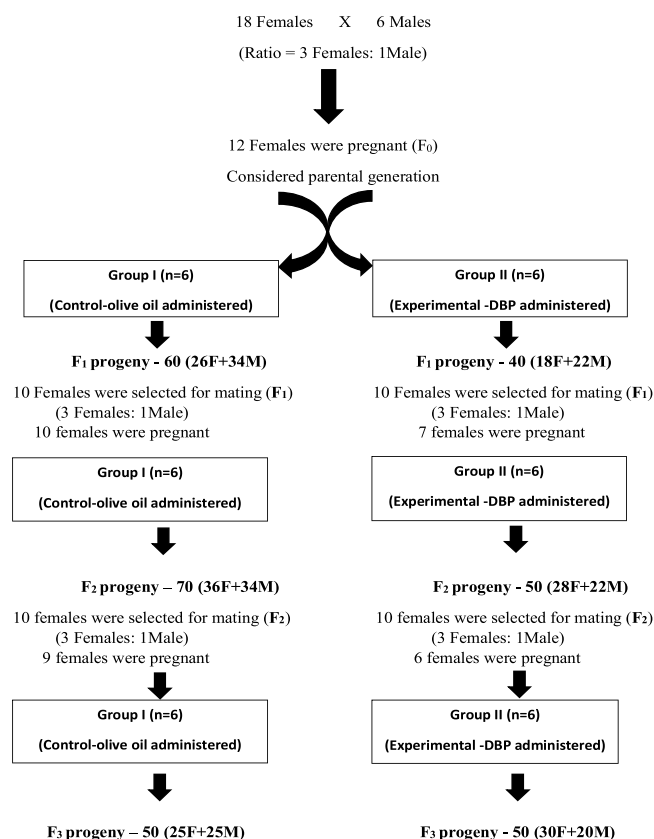
### 2.3. The rationale for dose selection

With regard to DBP, the evaluated LD<sub>50</sub> for Wistar rats is 8012 mg/kg body weight (bw); while 1/16 LD<sub>50</sub> value (i.e., 500 mg/kg bw) was chosen based on previous reports [19] wherein deleterious reproductive outcomes reported following exposure to 1/16 LD<sub>50</sub> dose of DBP during gestation and lactation [20].

### 2.4. Experimental design

The study commenced with 24-healthy adult albino rats (200–250 g) of Wistar strain [18 females and 6 males]. To breed, they were housed in cages (3F:1 M), the next morning the sperm positive females were counted (12 out of 18 were confirmed pregnant) and placed in individual cages; this was considered as GD-0. They were divided into two groups; Group-I comprising control (n = 6), and group II experimental animals (n = 6). From GD-6 onwards until parturition, they were orally gavaged olive oil (0.1 mL/kg bw) to control and DBP to experimental groups at a dose of 500 mg/kg bw/day, dissolved in 0.1 mL olive oil. These rats were labeled as parental generation (F<sub>0</sub>) and were housed separately in cages for littering (F<sub>1</sub> progeny). The litters' count was recorded (60 in control and 40 in experimental) and DBP intoxication continued lactationally till the weaning period of 21-days, further these pups were sexed on PND-21 and recorded. They were allowed to rear separately until sexually mature. Randomly, 10-females (F<sub>1</sub> progeny) were selected from each group (control & experimental) and they were allowed to breed by following the aforesaid regimen to raise F<sub>2</sub> and F<sub>3</sub> progeny. While raising the progeny, utmost care was taken to avoid breeding between siblings of each generation and the recorded census data of each generation is shown schematically, as well the number of rat pups used for respective assays and the adopted procedures was as follows:

The schematic diagram showing the regimen followed to raise F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> progeny



Number of rat pups used in each generation for respective assays

Assessment/ Indices	No. of pups		
	F <sub>1</sub> progeny ♂	F <sub>2</sub> progeny ♂	F <sub>3</sub> progeny ♂
Pre-weaning indices	12	12	12
Neurobehavior assessment (T-maze)	6	6	6
AChE, Thyroid profile, Testosterone	6	6	6
Histology	6	6	6

#### 2.4.1. Pre-weaning indices

The rat pups of each generation were examined to evaluate sensorimotor developmental indices on specific postnatal days (PND) from day-1 to day-21. The sensory-motor development parameters were assessed by adopting the procedure given by Altman and Sudarshan [21] and Pantaleoni et al. [22], and scores were given as good response (+ + + equivalent to 75–100 %), moderate response (+ + - equivalent to 51–74 %), mild response (+ - - equivalent to 26–50 %), and poor response (- - - equivalent to 1–25 %) on respective postnatal days (PND).

**2.4.1.1. Negative geotaxis.** Measured on PND-4; the offsprings were placed in a head-down position on an inclined plane, and a maximum time of 60 s was given for the offsprings to reorient to the head-up position. Following scores were assigned, score 3 if offsprings reorient to a head-up position within 30 s (+ + +), score 2 if offsprings reorient to a head-up position within 60 s (+ + -), score 1 if a response was observed in the stipulated time of 60–120 s (+ - -), and score 0 if offsprings fail to respond after 120 s (- - -).

**2.4.1.2. Surface righting.** Measured on PND-4 and 7; the offsprings were placed on their back on a smooth surface, and the time required to right themselves to a position where all four limbs touch the surface was

recorded; the following scores were assigned: score 3 if offsprings if right within 1 s (+ + +), score 2 if offsprings if right within 1–2 s (+ + -), score 1 if offsprings if right within 2–3 s (+ - -), and score 0 if offsprings if responded beyond 3 s (- - -).

**2.4.1.3. Cliff avoidance.** Measured on PND-7; the offsprings were placed on a platform elevated 10 cm above a table top. The forelimbs and snout of the animals were positioned so that the edge of the platform passed just behind an imaginary line drawn between the eye orbits. A successful response to the retraction of the head was set to 3 for 20 s (+ + +), 2 for 20–60 s (+ + -), 1 for 60–120 s (+ - -), and 0 for beyond 120 s (- - -).

**2.4.1.4. Swimming pattern.** Measured on PND-7, 10, and 14; the offsprings were placed in a water tank wherein water temperature maintained at  $23 \pm 1$  °C; swimming behavior was rated for the direction (if they swim straight, score 3; if they swim by circling, score 2; if they float, score 1) and head angle (ears out of water, score 4; ears half out of water, score 3; nose out of water, score 2; and unable to hold head, score 1). Limb movement was rated as 1 if all the four limbs were used; 2 if only hind limbs were used. Total scoring was assigned as 3 if offsprings swim straight with ears out of water using all four limbs (+ + +), 2 if they swim straight with ears half out of water making use of all four limbs (+ + -), 1 if they swim circling with their nose out of water using two limbs (+ - -), and 0 if they were able to float but unable to hold their head with no limb movement (- - -).

**2.4.1.5. Auditory startle.** A loud, sharp noise created causes an immediate startle response which was seen as a sudden extension of both head and limbs in offsprings, the same was measured on PND-14 and 16; and a score of 3 is given if a sudden extension of head and limbs along with some movement is observed (+ + +), score 2 if a slow extension of head and limbs is observed (+ + -), score 1 if a sound is heard, evidenced by a pinna flick (+ - -), and score 0 if no response is exhibited (- - -).

**2.4.1.6. Hind limb support.** Measured on PND-14, 17 & 21 – for the assessment, a 2 mm thick and 70 cm long wire was tied horizontally between two poles at 50 cm height; when offsprings suspended by the forepaws of the pups on a thin wire and the pups pulled up, they provide support with one or both hind feet. Scoring was given as 3 = pups grasp the wire with both fore and hind feet and try to move along the wire (+ + +), 2 = pups grasp the wire with fore and hind feet (+ + -), 1 = tries to grasp the wire with only forefeet (+ - -), 0 = fails to grasp and immediately fall (- - -).

**2.4.1.7. Visual cliff.** Measured on PND-14, 17, 21; the offsprings were placed on the edge of a cliff, the shallow and deep sides being covered with a glass sheet, the pups stepped into the shallow side. The given scoring of the response was as follows: 3 if response was witnessed within 5 s (+ + +), 2 if response was exhibited within 10 s (+ + -), 1 if pup response is noticed within 15 s (+ - -), and 0 when no response is observed (- - -).

**2.4.1.8. Visual placing.** Measured on PND- 17 and 21; the offsprings were suspended by the tail and lowered towards a scoring was done accordingly: score 3 if they immediately display a raising of the head and extension of forelimbs (+ + +), score 2 if they display slow extension and swaying of the trunk (+ + -), score 1 if they display slow movement of the head along with extension of the forelimbs (+ - -), and score 0 in the absence of extension and movement of head and forelimbs (- - -).

#### 2.4.2. Neurobehavioral assessments

One-month-old male rats (n = 6) from each generation (F<sub>1</sub>-F<sub>3</sub>) were assessed for learning and memory deficits by T-maze according to the

method of Bures et al. [23] and specifications of the behavioral house used and adopted procedure was as follows:

T-maze, a traditional tool was most widely used to measure the cognitive ability of rodents. It consists of sidewalls of 40 cm in height, start box-12 cm x 12 cm, choice area-15 cm x 12 cm, sidearms- 35 cm x 12 cm, goal area-15 cm x 12 cm containing food well and stem- 35 cm x 12 cm. The stem and start box were separated by a sliding door, and a cloth curtain separated the arm and goal area so that the food well from the choice area was not visible to subjects. The set-up was kept in dim light and sound-attenuated room. One-month-old male pups of control and DBP intoxicated groups (F<sub>1</sub>- F<sub>3</sub>) subjected to acquisition and performance rewarded alternation task in T-maze. All experiments were performed between 13:00 and 17:00 h.

**2.4.2.1. Habituation.** The rats were allowed to explore T-maze for 30 min to familiarize themselves. After 30 min, they were returned to their home cages.

**2.4.2.2. Orientation and training session.** Rats deprived food for 24 h were allowed to explore the T-maze for 30 min. After the orientation session of 30 min, the animals were allowed to return to their home cage. Later rats were trained for alternation food reward tasks and were made to run for 10 trials/session/day. In each trial, a rat was placed in the start box, then the sliding door slowly released and the rat was forced to move into one arm that leads to goal area having food pellet as a reward by blocking the other arm that does not have food pellet. After each trial, the walls of the T-maze were cleaned with 5 % ethanol.

**2.4.2.3. Acquisition (learning) test.** The rats were subjected to 10 trials/session until they reach the criteria of making eight correct choices out of 10 trials per day and the acquisition (test) was conducted for 7-days, besides latency period, a number of correct responses and errors (entry into the non-rewarded arm) were recorded.

**2.4.2.4. Memory retention test.** Post-acquisition, rats were subjected to memory retention tests the next day and allowed to perform 30-trials per session and the number of errors committed by each subject was recorded.

#### 2.4.3. Serum hormones and hippocampus tissue AChE assessments

Post-treatment, 30-day-old rats were euthanized by spinal dislocation under 1 % pentobarbital sodium (0.4 mL/100 g) anesthesia to draw blood from cardiac puncture. Serum testosterone and thyroid hormones (TSH, FT<sub>3</sub>, and FT<sub>4</sub>) were measured with the assistance of a diagnostic laboratory, by using fully automated bidirectional interfaced chemiluminescent immunoassay technique (CLIA) located at SRL Diagnostics, Vasanth Nagar, and Bangalore. Acetylcholine esterase activity was estimated spectrophotometrically by the method of Ellman et al. [24] and proteins by the method of Lowry et al. [25].

#### 2.4.4. Hippocampus histology

Neuronal tissue, the hippocampus was isolated from one-month-old rats, stored separately in 10 % buffered formalin and further histologically processed for hematoxylin and eosin staining [26]. Sections containing the hippocampus were float mounted on microscope slides, each slide with mounted sections was submerged in following grades of alcohol for 2 min each: 100 %, 95 %, and 70 % ethanol. Sections were rinsed in distilled water to remove excess ethanol and placed in hematoxylin-eosin staining solution in distilled water for 3 min. Sections were rinsed in distilled water to remove excess stain and immersed in a 0.8 % acetic acid solution in distilled water until fiber tracks became unstained (about 3–5 min). They were then placed in 70 %, 95 % and 100 % ethanol for 2 min each. Sections were placed in clearance solution for a minimum of 15 min before they were cover slipped with mounting medium. Photographic images of the different cell layers were captured using Olympus camera (E-330) and Olympus microscope

(CX-61) with a motorized stage.

### 2.5. Statistical analysis

Statistical analysis for serum thyroid profile, AChE activity were done by one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison *post-hoc* test at  $P < 0.05$  level of significance by using SPSS software version 20.0. The data of the mean number of alternations and the time taken to reach the goal area (latency) in rewarded alternation task were analyzed by ANOVA with repeated measures on one factor followed by Dunnett’s multiple comparison test. Regression analysis and Pearson’s product-moment correlation was run to assess the relationship between errors scored in memory retention test and AChE activity/ FT<sub>3</sub>/FT<sub>4</sub>/TSH in the experimental groups (F<sub>1</sub>-F<sub>3</sub>). In addition, a regression equation was represented and used to construct a regression line on a scatter plot diagram. The data were expressed as mean ± SE,  $P < 0.05$  level of probability was used as the criterion for significance.

## 3. Results

### 3.1. Pre-weaning indices

The sensorimotor reflex development assessed in offsprings by measuring discrete indices such as negative geotaxis, surface righting, cliff avoidance, swimming, auditory startle, hind limb support, visual cliff, and visual placing, etc., and results showed considerable variations (Table 1 and Fig. 1):

#### 3.1.1. Negative geotaxis

Studied on PND-4. Pups showed a good response in both control and F<sub>1</sub> while a moderate response was evident in F<sub>2</sub> progeny and mild response in F<sub>3</sub>. The response observed in DBP intoxicated male progeny being 83.3 % in F<sub>1</sub>; 75.0 % in F<sub>2</sub>, 50.0 % in F<sub>3</sub>.

#### 3.1.2. Surface righting

The ability assessed on PND-4 showed high scores in F<sub>1</sub> and F<sub>2</sub> while

**Table 1**

Sensory-motor reflexes measured in rat pups on respective postnatal days during pre-weaning period: A three-generation comparative study to analyze DBP induced toxicity.

Groups→↓	PND	Control			Experimental		
		F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
Negative geotaxis	4	+++	+++	+++	+++	+++	+-
Surface righting	4	+++	+++	+++	+++	+++	+-
	7	+++	+++	+++	+++	+++	++
Cliff avoidance	7	+++	+++	+++	+++	+++	+++
	7	+++	+++	+++	+++	+++	+-
Swimming	10	+++	+++	+++	+++	+++	+-
	14	+++	+++	+++	+++	+++	+++
	14	+++	+++	+++	+++	+++	+++
Auditory startle	14	+++	+++	+++	+++	+++	+++
	16	+++	+++	+++	+++	+++	+++
	16	+++	+++	+++	+++	+++	+++
Hind limb support	14	+++	+++	+++	+++	+++	+++
	17	+++	+++	+++	+++	+++	+++
	21	+++	+++	+++	+++	+++	+++
Visual cliff	14	+++	+++	+++	+-	+-	+-
	17	+++	+++	+++	+++	+++	++
	21	+++	+++	+++	+++	+++	+++
Visual placing	17	+++	+++	+++	+++	+++	+-
	21	+++	+++	+++	+++	+++	+++

Scores obtained in male pups (n = 12) in each group on respective PND shown against specific index. Score ‘+++’ represents good response equivalent to 76 % - 100 %; score ‘++’ represents moderate response equivalent to 51 % - 75 %; score ‘+’ represents mild response equivalent to 26 % - 50 %; score ‘-’ represents poor response 01 % - 25 %.

F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> represent first, second and third generation.

F<sub>3</sub> progeny comparatively scored low; however, assessments made on PND-7 showed no changes.

#### 3.1.3. Swimming assessments

Evaluated on PND-7 and 10. F<sub>1</sub> progeny showed good response by exhibiting swim in a straight direction with head and ears out of the water and used both limbs, while F<sub>2</sub> and F<sub>3</sub> progeny showed a moderate response and exhibited swim in circles. On PND-14, all offsprings of three generations able to swim straight with head and ears out of the water using both limbs.

#### 3.1.4. Visual cliff activity

Studied on PND-14 and F<sub>1</sub> offsprings showed a moderate response and they stepped on to the shallow side; however, on PND-17, the same offsprings showed a good response. In F<sub>2</sub> and F<sub>3</sub> progeny, a moderate response was evident on both PND-14 and 17; however, on PND-21, all offsprings were able to visualize the cliff reflex as a good response.

#### 3.1.5. Visual placing

Studied on PND-17 and 21. On PND-17, the intoxicated subjects of F<sub>2</sub> and F<sub>3</sub> exhibited moderate response characterized by raising their head and extended their limbs in placing response while on PND-21 all subjects exhibited good response.

Similarly, cliff avoidance (PND-7), auditory startle (PND-14 and 16), and hind limb support (PND-14, 17, and 21) assessed on respective days showed no change intoxicated groups of all the three generations studied.

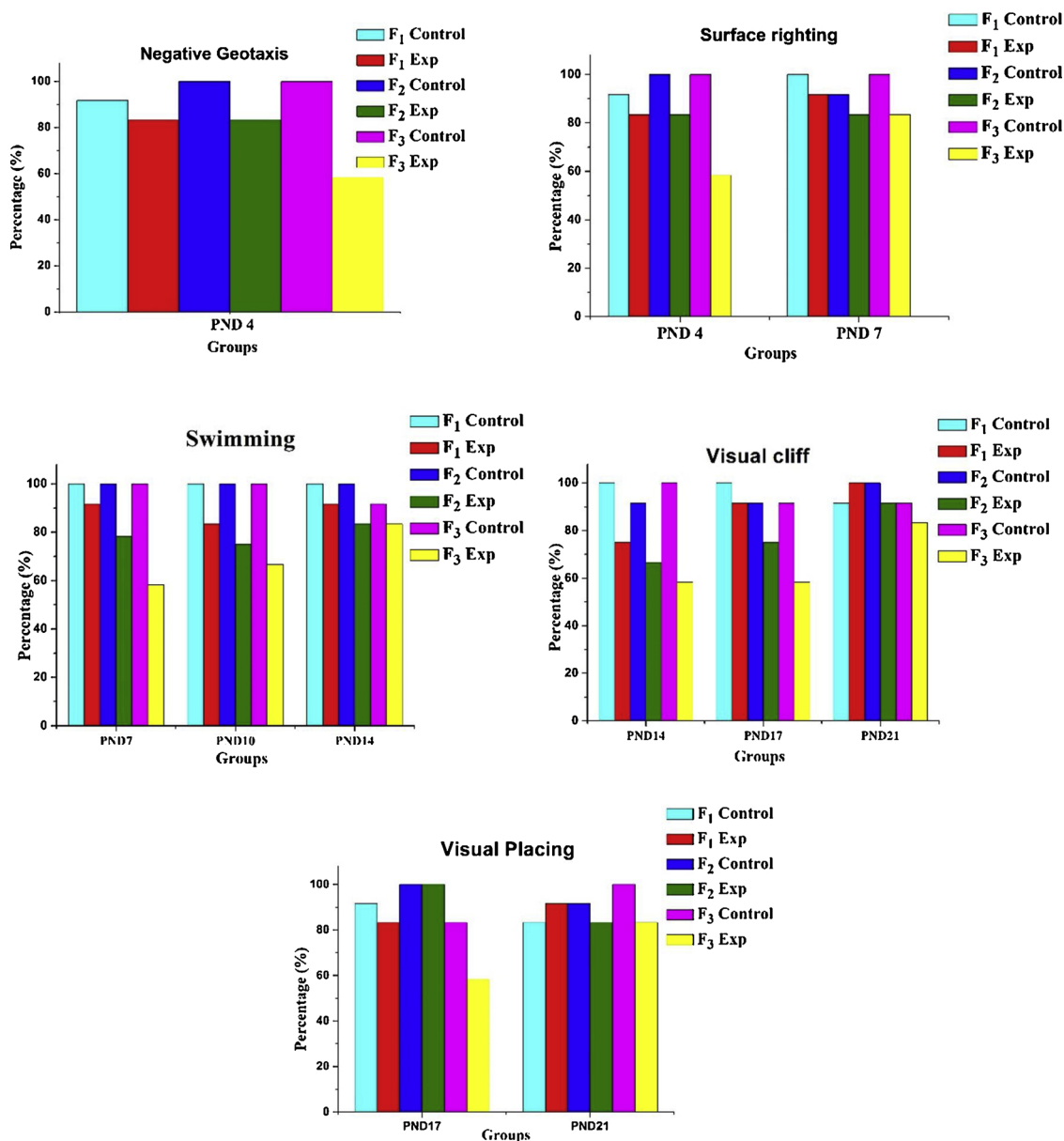
### 3.2. Neurobehavioral assessment

The acquisition and retention data [Fig. 2 (a–e)] subjected to one-way ANOVA with repeated measures revealed a significant difference between groups  $F(5,30) = 60.619, P < 0.005$  and days  $F(6,180) = 42.074, P < 0.005$ ; between groups and days  $F(6,30) = 1.715, P < 0.17$  in learning the task during acquisition, significant difference was observed in the interaction between days and groups. The latency data indicated a significant difference between groups  $F(5,30) = 25.476, P < 0.005$ , however, there was no significant difference observed in the interaction between groups and days  $F(18,120) = 1.715, (P > 0.05)$ . Further, Mauchly’s test of sphericity assumption was violated,  $\chi^2_{(20)} = 79.65, P < 0.0005$ , and therefore, a Greenhouse-Geisser correction was used to analyze the latency. There was a significant effect of DBP on experimental rats as the time is taken to reach the goal was  $F(3,241, 97.245) = 233.621, P < 0.05$ . Errors committed were significant during the 7-days period, between days,  $F(6,180) = 43.324, P < 0.005$ ; between groups was  $F(5,30) = 61.385, P < 0.005$ . In brief, the results indicate that experimental rats on day-7 of performance acquired the task or reached criterion in 3.6, 4.8 and 5.6 sessions (F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> progeny), which was statistically ( $P < 0.05$ ) significant compared to control rats took 2.50, 2.83 and 3.00 sessions to reach the criterion. It is also evident from results that experimental rats of F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> generations were able to achieve 65.0 %, 58.3 % and 50.0 % accuracy in making the correct choice of the arm on day-2 of acquisition training, while on 7-day, experimental rats were able to achieve 86.7 %, 73.3 % and 68.3 % higher accuracy than day-2. Although the latency time to reach the goal area at the beginning of the learning session was different however a steady-state was observed on day-7 by exhibiting 3.9 s, 4.5 s, and 5.3 s in F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> progeny respectively. Further intoxicated subjects committed 9.50 (F<sub>1</sub>), 9.83 (F<sub>2</sub>) and 10.50 (F<sub>3</sub>) errors higher compared to their corresponding control subjects with 6.00 (F<sub>1</sub>), 5.83 (F<sub>2</sub>) and 5.50 (F<sub>3</sub>) errors during their memory retention test.

### 3.3. Serum hormones and hippocampus tissue AChE assessments

The serum thyroid profile (TSH, FT<sub>3</sub>, and FT<sub>4</sub>) and testosterone





**Fig. 1.** Sensory-motor reflexes such as negative geotaxis (on PND-4), surface righting (on PND-4 and 7), swimming (PND-7, 10 and 14), visual cliff (on PND-14, 17 and 21) and visual placing (on PND-17 and 21) measured in male pups during pre-weaning period to evaluate DBP induced multigenerational toxicity. Scores obtained from 01 %-25 %, 26 %-50 %, 51 %-75 %, 76 %-100 % represent poor, mild, moderate and good response respectively. F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> represent the first, second and third generation respectively.

assessments carried out in rat pups upon DBP exposure showed moderate decrements ( $P < 0.05$ ) (Table 3). The serum FT<sub>3</sub> levels were found affected by -29.7, -31.1, and -40.8 % in F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> progeny respectively while FT<sub>4</sub> levels were affected by -18.8, -35.4, and -37.6 %. Further, serum TSH and testosterone levels showed decrements in experimental groups by -33.0, -36.8 and -36.9; - 41.5, -46.6 and -52.5 % respectively. In hippocampus, the AChE levels showed decrements in F<sub>1</sub> (-34.8 %), F<sub>2</sub> (-59.3 %) and F<sub>3</sub> (-71.6 %) offsprings compared to their respective control groups (Fig. 3).

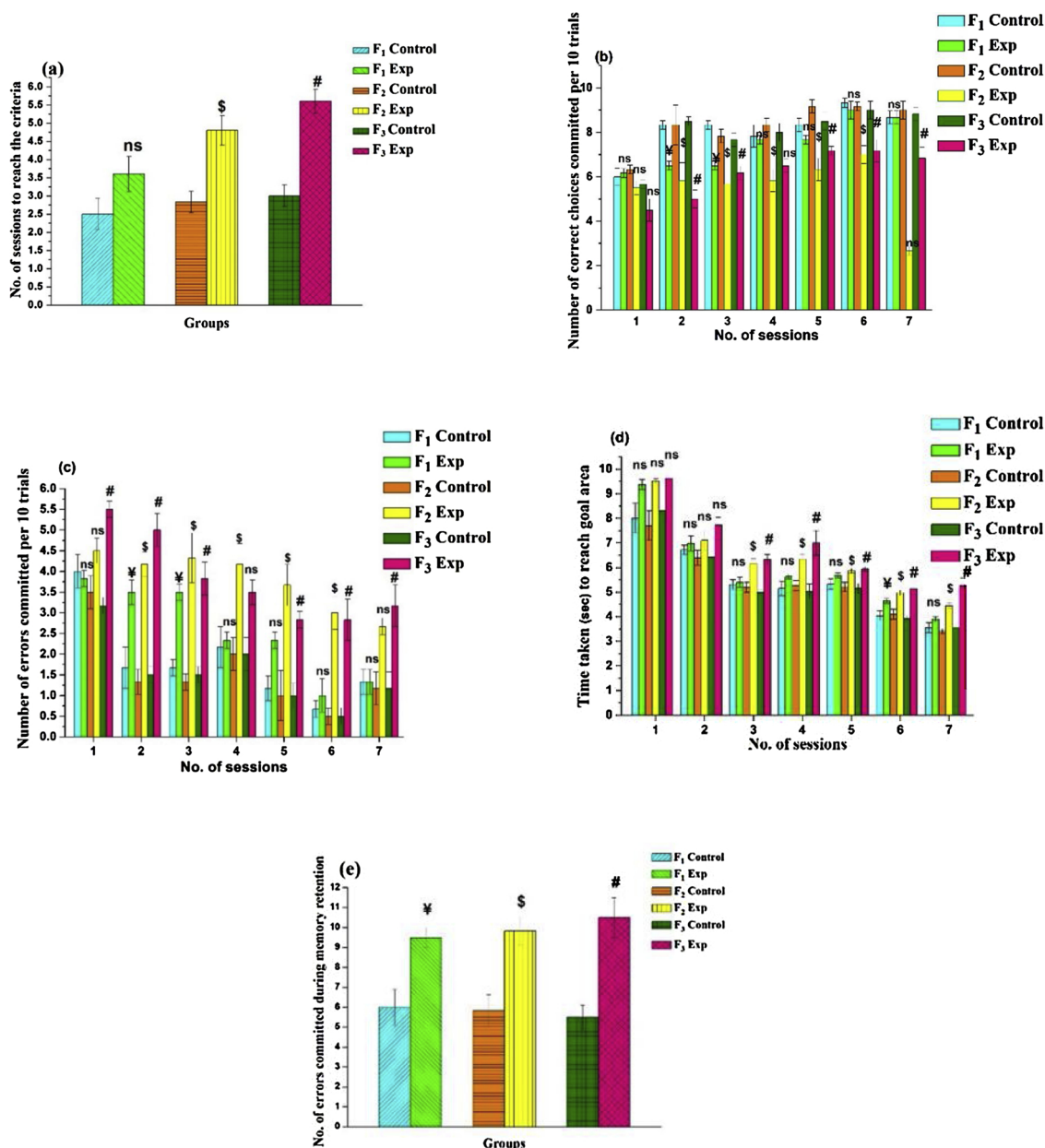
### 3.4. Hippocampus histology

In the hippocampus, disruptions in the formation of neurons followed by reductions in axonal innervations were evident especially in CA1, CA3, and DG regions upon DBP intoxication [Fig. 4(a-r)]. In the control group, neuropil fibers were in clusters with densely packed pyramidal cell layers and intact axonal connections in the CA1 region

while mild variations were evident in the pyramidal layer with vacuolation intoxicated F<sub>1</sub> progeny. Contrarily F<sub>2</sub> progeny showed moderate changes in the pyramidal layer of the CA1 region with enhanced neuronal degeneration, while F<sub>3</sub> progeny showed shrunken and darkened pyramidal cells with vacuolation. With regard to the CA3 region, a prominent degeneration in the pyramidal layer was evident in both F<sub>1</sub> and F<sub>2</sub> offsprings, however, higher fold necrosis was observed in F<sub>3</sub> progeny. Dentate gyrus region having hilar cells with long processes in the control group while F<sub>1</sub> progeny exhibited disturbed hilar cell arrangement and atrophy of neurons besides vacuolation. Likewise, F<sub>2</sub> progeny showed mild hyperplasia followed by severe intracellular edema and severe hyperplasia was evident in F<sub>3</sub> progeny.

### 4. Discussion

The prenatal/fetal period is highly susceptible to epigenomic dysregulation with implications for health, both lifelong and



**Fig. 2.** Neurobehavioral changes in rats upon DBP exposure: A three-generational comparative study. (a) The number of sessions to reach the criteria; (b) Number of correct choices per session; (c) Number of errors committed in each session; (d) Time taken to reach the goal area; (e) Number of errors committed in the memory retention test. Values are mean  $\pm$  SE of six rats. Symbols ‘¥’ (F<sub>1</sub>), ‘\$’ (F<sub>2</sub>) and ‘#’ (F<sub>3</sub>) represent significantly different from their corresponding control, ‘ns’ indicate non-significant from their respective controls as determined by one-way ANOVA with repeated measures followed by Dunnett’s multiple comparison post-hoc test. F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> represent the first, second and third generation.

**Table 2**

DBP exposure caused changes in body weight, feed and water intake in rats: A comparative study of three generations.

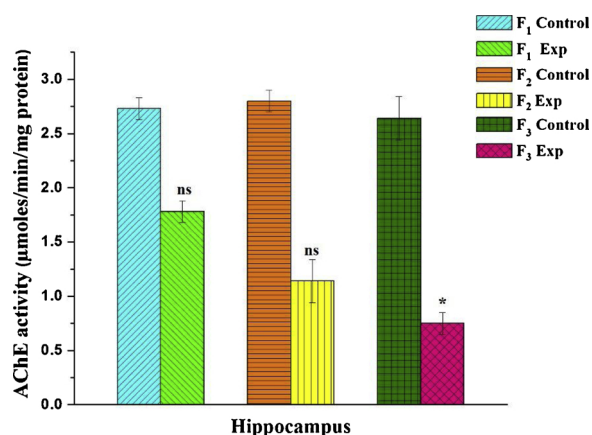
Groups→	Control			Experimental		
	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
Parameters↓						
Feed (g)	14.3 $\pm$ 0.2	13.9 $\pm$ 0.5	14.2 $\pm$ 0.2	11.5 $\pm$ 0.1 (-19.6)	10.2 $\pm$ 0.3* (-26.6)	9.3 $\pm$ 0.2* (-34.5)
Water (mL)	24.7 $\pm$ 0.3	23.7 $\pm$ 0.4	23.5 $\pm$ 0.2	18.8 $\pm$ 0.2* (-23.9)	18.2 $\pm$ 0.3* (-23.2)	17.8 $\pm$ 0.1* (-24.3)
Body weight(g)	62.5 $\pm$ 0.4	61.7 $\pm$ 0.8	63.6 $\pm$ 0.5	59.7 $\pm$ 0.2* (-4.5)	56.3 $\pm$ 0.2* (-8.8)	54.3 $\pm$ 0.6* (-14.6)

Values are mean  $\pm$  SE of 6 observations measured in male pups. \*Significantly different from control,  $P < 0.05$  as determined by one-way ANOVA followed by post-hoc Dunnett’s multiple comparison test. Values in parenthesis represent the percent change and ‘-’ sign indicates decrease over the control group. F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> represent first, second and third generation.

**Table 3**  
Variations in thyroid and testosterone hormone profiles in rats upon DBP exposure for three-generations.

Groups→	Control			Experimental		
	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
Thyroid hormones↓						
FT <sub>3</sub> (pg/mL)	4.38 ± 0.10	4.27 ± 0.13	4.22 ± 0.16	3.08 ± 0.03* (-29.7)	2.94 ± 0.11* (-31.1)	2.50 ± 0.12* (-40.8)
FT <sub>4</sub> (ng/dL)	2.24 ± 0.05	2.37 ± 0.11	2.13 ± 0.13	1.82 ± 0.01* (-18.8)	1.53 ± 0.01* (-35.4)	1.33 ± 0.01* (-37.6)
TSH (ng/mL)	3.36 ± 0.02	3.51 ± 0.07	3.28 ± 0.03	2.25 ± 0.03* (-33.0)	2.22 ± 0.03* (-36.8)	2.07 ± 0.02* (-36.9)
Testosterone (ng/dL)	74.7 ± 0.90	73.8 ± 1.7	71.4 ± 1.9	43.7 ± 1.4* (-41.5)	39.4 ± 0.8* (-46.6)	33.9 ± 0.9* (-52.5)

Values are mean ± SE of six observations (one-month-old male rats). \*Significantly different from controls,  $P < 0.05$  as determined by one-way ANOVA followed by Dunnett's multiple comparison post-hoc test. Values in parenthesis represent the percent change and '-' sign indicates decrease over the control group. F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> represents first, second and third generation. C-Control, E-Experimental. TSH - Thyroid-stimulating hormone, FT<sub>3</sub> - Free triiodothyronine and FT<sub>4</sub> - Free thyroxine.



**Fig. 3.** DBP exposure induced changes in the activity levels of acetylcholinesterase (AChE) in the hippocampus of male rats. Values are mean ± SE of six rats; Symbol '\*' represents significantly different and 'ns' represents non-significant from their respective controls,  $P < 0.05$  as determined by one-way ANOVA followed by Dunnett's multiple comparison post-hoc test. F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> represent first, second and third generation.

transgenerationally. Most of the research to date has focused on the effects of plasticizers yet there is compelling evidence that prenatal environmental exposure to phthalates adversely affects the development and health in later life [27]. Earlier, we have reported gestational DBP exposure induced developmental and teratogenic anomalies in rats wherein risk assessments conveyed potential toxic implications not only on the development of rat fetus (F<sub>1</sub> progeny) but subsequently carried to F<sub>2</sub> and F<sub>3</sub> progeny confirming the embryo-fetal toxic potential of phthalate. The exposures in this study began as early as GD-6 to target a wide window during embryo and fetal development. Followed by a reduction in litter size and sex ratio and the incidence of skeletal and malformation complex involving the face and eye-witnessed significantly in subsequent generations compared to first [20]. As an extension, this study addresses pre- and post-weaning period behavioral impairments in developing rats upon multigenerational DBP exposure.

#### 4.1. Changes in pre-weaning indices (Sensory-motor developmental reflexes)

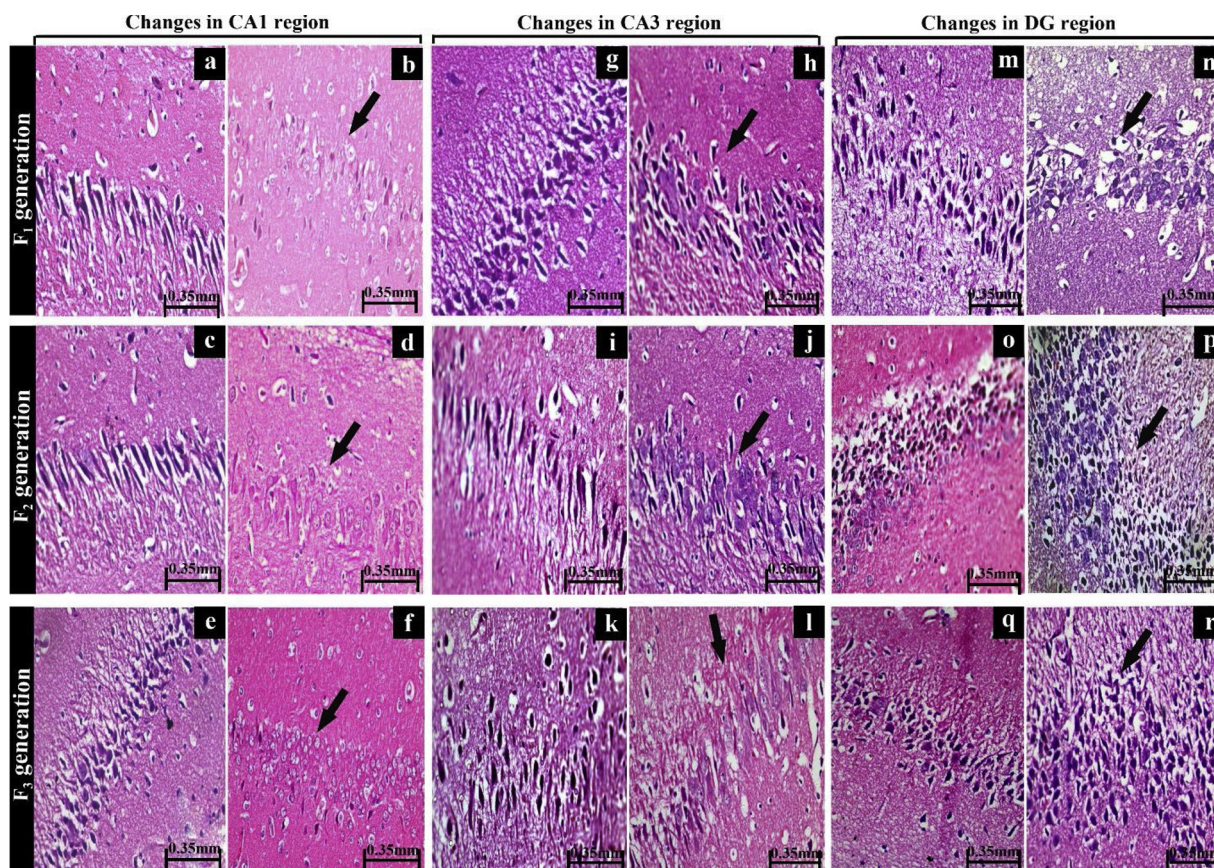
The postural reflex responses indicate sensorimotor development in offsprings. Tanaka [28] reported a delay in surface righting activity in rats on PND-4 and PND-7 upon exposure to high doses of DEHP. Likewise, Li et al. [29] reported variations in sex and dose-specific responses in DBP-intoxicated rat pups, and their results showed low scores in surface righting on PND-7 and shortened forepaw grip time on PND-10 limiting their observations to one generation. In this study, the

sensory-motor reflexes assessed in neonatal pups showed considerable variations which include negative geotaxis (studied on PND-4) exhibiting a moderate response in F<sub>2</sub> progeny and mild response in F<sub>3</sub>, while surface righting ability (studied on PND-4) also exhibited a mild response in F<sub>3</sub>. The swimming performance exhibited a moderate response in F<sub>2</sub> and F<sub>3</sub> progeny upon DBP exposure due to lack of coordination between skeletal muscles and integrative brain domains [30] however other assessments such as cliff avoidance, auditory startle, and hind limb support remain unaffected. Following inferences can be drawn from results that the developing animals are more vulnerable and target neuronal tissue apart from endocrine disruptive actions [31], wherein blocking the differentiation of Wolffian duct, disruption of prostate glands regulating 5 $\alpha$ -di-hydro-testosterone are some of the well pronounced mechanistic actions exhibited upon phthalate exposures. Bodyweight, as well as somatic indices measured in one-month-old rats, has shown considerable decrements (Table 2) and such decrements could attribute to nutritional deprived neurological impairments, the same is reflected in the ontogeny of reflexes and simple behavior patterns (Table 1).

#### 4.2. Behavioral assessments

Learning and memory are unique cognitive abilities that are essential for many types of behavior. Rat hippocampus matures in the first few weeks after birth and implicated as a key brain region for spatial learning and memory [32]. In this study, the histology of the hippocampus ascertained degenerative changes in CA1, CA3 and DG regions (Fig. 4) leading to impaired behavioral functions in F<sub>1</sub>-F<sub>3</sub> rats (Fig. 2). It is evident that the F<sub>3</sub> progeny committed errors in memory retention test (10.5/30 trials) and selected the correct baited arm with higher accuracy on day-7 when compared to day-1. In the acquisition test, F<sub>3</sub> rats took 5.6 sessions and F<sub>1</sub> and F<sub>2</sub> rats took 3.6 and 4.8 sessions to learn the task, accordingly the latency period was 5.3 s in F<sub>3</sub> rats. The aforesaid changes could be due to reductions in axonal connections between neurons of CA3 and DG regions suggesting variation in dopaminergic pathways. The findings of this study corroborate with the results of Smith et al. [33] wherein similar reductions in hippocampus axonal connections (between neurons of CA3 and DG regions) were pronounced in male rats upon exposure to DEHP resultantly memory got affected and altered behavioral outcome was witnessed including hyperactivity in experimental rats. Holahan et al. [34] drew similar inferences upon DEHP exposure indicating perseverative behaviors and hyperactivity in rats and results advocate the involvement of dopaminergic system to elevate locomotor activity throughout the operant conditioning task in both genders. Involvement of other factors cannot be overruled out, for instance, Ma et al. [35] indicated a significant increase in caspase-3 and glial fibrillary acidic protein (GFAP) concentrations in hippocampal CA1 region and cerebral cortex as a consequence of exposure to di-isononyl phthalate (DINP) [36].





**Fig. 4.** Coronal section through CA1 region (a-f), CA3 region (g-l), DG region (m-r) of hippocampus showing DBP exposure caused cytoarchitectural alterations in one-month-old male rats. a, g, m- F<sub>1</sub> control, b, h, n- F<sub>1</sub> intoxicated, c, i, o- F<sub>2</sub> control, d, j, p- F<sub>2</sub> intoxicated, e, k, q- F<sub>3</sub> control and f, l, r- F<sub>3</sub> intoxicated. Impression (I): b- slight degeneration of pyramidal cells with vacuolation and condensed nuclei; d- degeneration of neurons; f- apparent decrease in pyramidal cells and shrunken eosinophilic neurons; h and j - decrease in pyramidal cells and degeneration of neurons, l- necrosis of neurons; n- hilus containing hilar cells with disturbed arrangement and atrophy of neurons with vacuolation, p-slight hyperplasia and r-intracellular edema and hyperplasia. (H & E staining; scale bar- 0.35 mm).

#### 4.3. The discrepancy in the cholinergic system

Acetylcholine is a marker in different neural systems [37]. It plays a crucial role in synaptogenesis, axonal growth and brain maturation during fetal development and such processes are integral to normal behavior and cognitive development in postnatal life. In rodents, learning and memory-related to spatial navigation involve the dorsal hippocampus [38] wherein cholinergic transmission plays a key role [39]. As a neuromodulator, ACh plays a prominent role in learning involving neocortex and hippocampus, and enhance the amplitude of synaptic potentials following long-term potentiation in domains like dentate gyrus, CA1, piriform cortex, and neocortex. Memory impairments associated with the dysfunction of the hippocampal cholinergic system indicated in exposures of bisphenol-A. Liu et al. [40] showed the involvement of phthalate metabolites in affecting the memory and learning process. In addition, Liu et al. [41] reported inefficient nicotinic acetylcholine receptors and suppression in the activity levels of AChE in subjects upon exposure to phthalates. In this study, inhibition in the activity levels of AChE was observed in experimental groups (F<sub>1</sub> - F<sub>3</sub>) upon DBP exposure, leading to the accumulation of ACh. Corroborating the above findings, Jee et al. [42] indicated similar inhibitions in AChE activity in a more pronounced way in bagrid catfish upon exposure to DBP and DEHP in a concentration-dependent manner.

#### 4.4. Changes in serum hormones relevance to cognition

Androgens influence the cognitive performance as they can alter the structure of the hippocampus by induction of spines and spine synapses

on the dendrites of CA1 pyramidal neurons, also causing changes in the long-term synaptic plasticity [43]. Testosterone is converted to estradiol in several areas of the brain, including the hippocampus [44]. In rodents, suppression in testosterone cause variations in hippocampus-dependent behavior and aberrant axon outgrowth and also affects mossy fiber synaptic transmission and some forms of plasticity [45]. The results of this study are consistent with findings of Lin et al. [46] wherein authors reported, similar decrements in serum testosterone levels on PND-49 in male Long-Evan rats upon gestational and lactational exposure to DEHP. *In utero* exposure to DBP (400 mg/kg bw) caused a significant decrease in serum testosterone in rabbits affecting development [47]. Parks et al. [48] explored the plausibility that phthalates being anti-androgens mask the actions of testosterone during fetal development. The changes evident in hippocampal function resulting from suppression in androgen levels may reflect the outcome of DBP consequently rats committed more errors and the same was evident in F<sub>3</sub> progeny. Thyroid hormones are vital for brain development and functioning throughout life, which explicit their action at a particular time [49]. Thyroid hormone deficiency, even for short duration may lead to irreversible brain damage, the consequences of which depend not only on the severity but also on the specific timing of onset and duration of the deficiency. The prenatal TH loss contributes to difficulties in visual processing and visual-motor abilities, as per Zoeller and Rovet [50] early neonatal TH insufficiency impair visuospatial abilities while in late neonatal period TH deficiency initiate sensorimotor deficits in causing deficits in fine motor, auditory processing, attention and memory skills [50]. Thereby the deficits observed in the learning process in DBP-intoxicated (F<sub>1</sub>-F<sub>3</sub>) rats could be correlated to



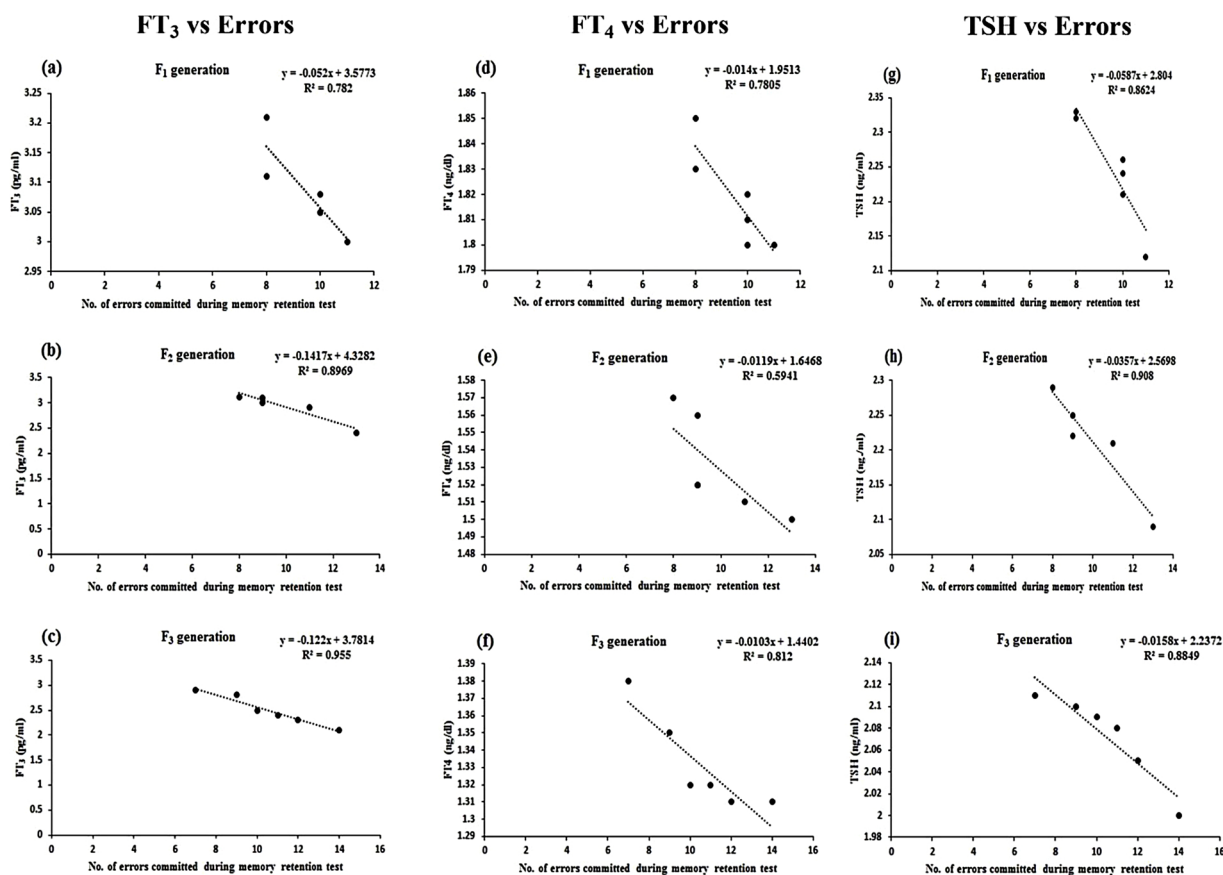


Fig. 5. Scatter plots analyses showing correlates between errors committed during memory retention test and FT<sub>3</sub> (a-c)/ FT<sub>4</sub> (d-f) and TSH (g-i) of one-month-old male rats (n = 6) upon DBP exposure: A multigenerational assessment.

decrements found in thyroid hormones evidenced by cytoarchitectural alterations. Furthermore, the hypothyroid brain caused structural defects in granular layers of the hippocampus, which affected the learning and memory, resultantly, F<sub>3</sub> progeny committed more errors in memory retention test (10.5/30 trials) and selected the correct baited arm with higher accuracy on day-7 when compared to day-1. In the acquisition test, F<sub>3</sub> rats took more sessions than F<sub>1</sub> and F<sub>2</sub>.

In rats, phthalate exposures alter sexual differentiation by altering fetal Leydig cell development and hormone synthesis which in turn, results in a “Phthalate Syndrome” which includes abnormalities of the testis and other androgen-dependent tissues. In this study, only male progeny (F<sub>1</sub>-F<sub>3</sub>) were chosen for detecting serum testosterone and thyroid hormones (TSH, FT<sub>3</sub>, FT<sub>4</sub>) upon DBP toxicity and female sex hormones were not taken into account, the reason being that the thyroid hormones get fluctuate with the estrous cycle, which is to the disadvantage of detection. In addition, F<sub>1</sub>-F<sub>3</sub> male progeny were chosen according to our prior study [20], which showed that the estrogen-like effect of DBP on reproductive and nerve systems. Thyroid hormones also play an important role in maintaining the link between neural development and reproduction [51,52]. A thyroid hormone insufficiency later in postnatal development is linked with sensorimotor deficits [50]. Normally, testosterone levels rise gradually from PND-20 to 30. Ackermann et al. [53] investigations have shown the involvement of testosterone on memory performance and variations in testosterone, as well as thyroid levels, may alter the behavior in postnatal life [54]. Thereby, the sensorimotor deficits witnessed in the present study could be due to testosterone and thyroid hormone deficiency.

#### 4.5. Correlates between cognition v/s AChE activity/serum hormones

To ascertain whether thyroid/testosterone discrepancy results in

hippocampal dysfunction leading to behavioral change or whether changes in the cholinergic system causes relative cognitive dysfunction, relative correlates were assessed. Scatter plots (Regression equation and R<sup>2</sup>) drawn between errors committed in the retrieval test by intoxicated progeny and their thyroid hormone levels (FT<sub>3</sub>/FT<sub>4</sub>/TSH) indicated a strong negative correlation [Fig. 5(a-i)] signifying the effect of DBP on the susceptibility of progeny and this could also possibly mediate some changes in cognitive function.

Liu et al. [41] and Jee et al. [42] have suggested disruptions to the cholinergic system as one of the possible mediating factor(s) in DBP caused the cognitive change in rats. In context to present study findings cells in the hippocampus are affected upon DBP exposure leading to a decline in sensory, motor and cognitive functions. The correlates assessed between hippocampal AChE activity and errors scored in memory retention test showed a negative correlation and projected ‘r’ values among progeny being -0.856 ( $P < 0.05$ ) in F<sub>1</sub>, -0.917 ( $P < 0.01$ ) in F<sub>2</sub>, -0.923 ( $P < 0.01$ ) in F<sub>3</sub> respectively [Fig. 6 (a-c)]. However, a non-significant and moderate negative association was evident among F<sub>1</sub> and F<sub>2</sub> progeny between their serum testosterone levels and committed errors excepting F<sub>3</sub> progeny wherein the existence of a negative correlation was evident among the aforesaid variable [Fig. 6 (d-f)]. Given the associations above, a significant negative relationship was nonetheless observed between AChE values and behavioral indices, confirming the applicability of the latter in the hippocampus for cognitive dysfunction.

#### 4.6. Exposures in the real-life context

Understanding responses to plasticizers that are representative of real-life scenario is paramount. Several phthalates, including DEHP, BBP, DBP, and DINP act, at least in part, through a common mechanism

### Acetylcholinesterase vs Errors

### Testosterone vs Errors

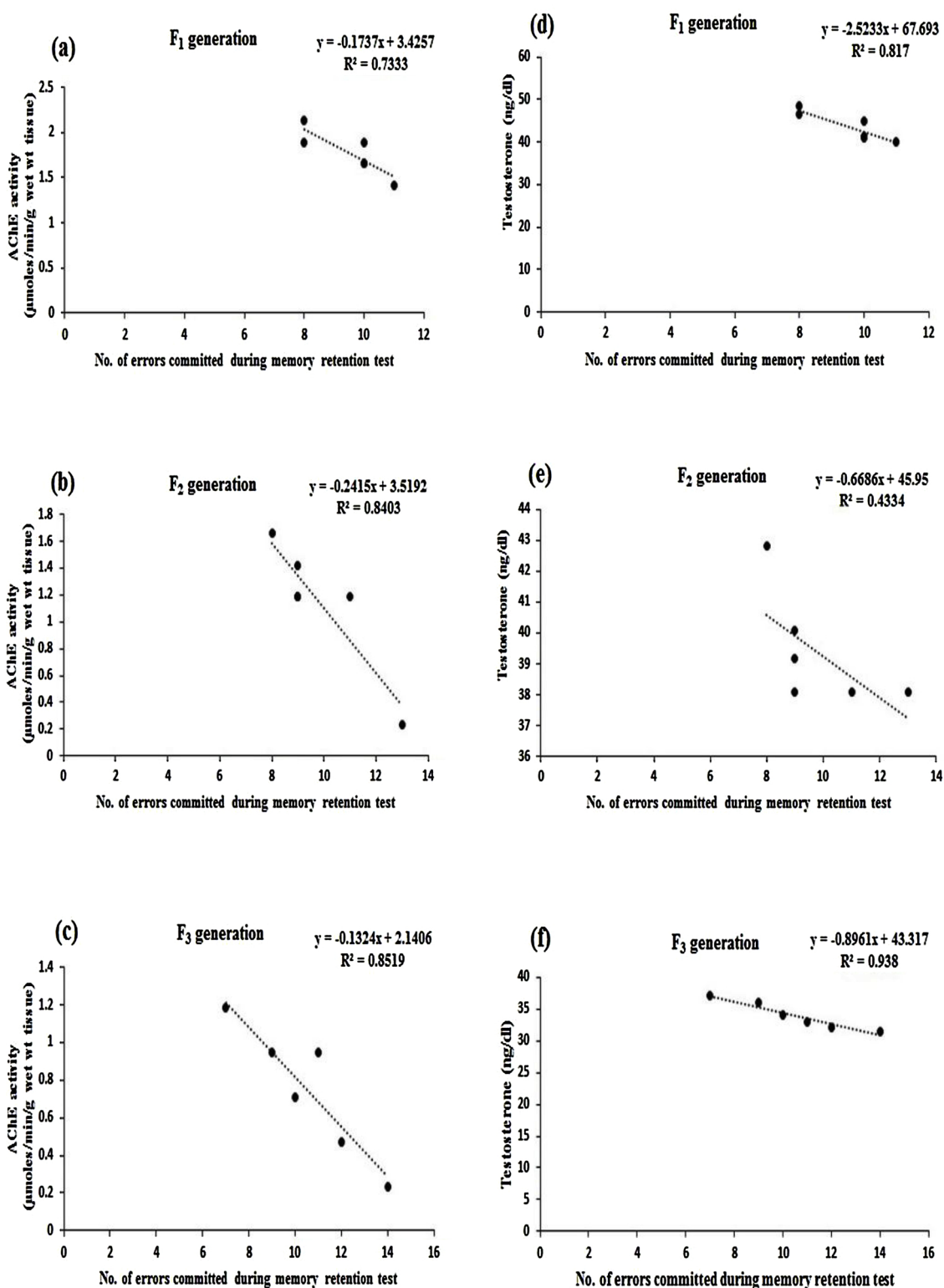


Fig. 6. Scatter plots analyses showing correlates between errors committed during memory retention test and AChE activity (a-c)/ testosterone (d-f) of one-month-old male rats (n = 6) upon DBP exposure: A multigenerational assessment.

when they independently cause harmful effects on the developing male reproductive system, which appears to be their most sensitive endpoint [27,42,54]. It appears that exposure to these phthalates should be considered in aggregate with exposures to other chemicals that are also

anti-androgenic through androgen receptor blockade or interference with testosterone synthesis [55]. Currently, inadequacies upsurge in each model system to find the appropriateness in mixture toxicity studies concerning additivity, synergism, potentiation, or antagonism

[56]. Estimating the relative potency of individual chemicals and adding them in order to estimate the total potency of a mixture is one way to evaluate the impacts associated with aggregate exposures.

This study sheds light on the exquisite vulnerability of the fetal period and early postnatal life to DBP exposure for three generations. It is increasingly clear that the timing is even more important than the dose. Phthalate exposures that occur at critical periods of development in utero or early life alter the development, whereas the same levels of exposure in fully developed brains may have a differential effect. One general mechanism by which pre and postnatal exposures could be linked to neurodevelopmental changes later in life is through the alteration of epigenetic markers, which have a central role in determining the functional output of the information that is stored in the genome. The explication of this relationship could provide causal information about the importance of the timing, duration, and pattern of exposures to neurodevelopment. However, one of the main limitations of this study design is certainly the inability to assess epigenetic mechanisms. This having been said, further studies assessing the toxicity of multiple doses of phthalates and mixtures are essential to authenticate or validate the suggested mixture effects.

## 5. Conclusion

Phthalate exposures during the early embryonic stage cause various adverse effects. During organizational stages of development, endocrine responses are unlike the typical one in adulthood. The results and inferences presented here are far from being conclusive but highlight the critical responses exhibited by rats upon multigenerational exposure to DBP. The findings ascertain the neurotoxic potential of DBP involving cellular changes in the hippocampus and the altered hormonal milieu facilitating neuronal impairment. These effects are likely multi-factorial in origin but the net result presumably disrupting hippocampus leading discrepancies in neurobehavior and severity of effects evidenced in subsequent generations if the toxic regimen is continued.

## Authors statement

Both authors equally involved in the execution of experimental work, and preparation of the manuscript and have no competing and conflict interests to declare.

## Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

## Declaration of Competing Interest

Both the authors equally contributed to the assessments and preparation of the manuscript and declare no conflict of interest.

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## Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2020.03.006>.

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