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Study of endocrine disruptor effects in AVP and OT mediated behavioral and reproductive processes in female rat models

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ABSTRACT

Environmental exposures may have endocrine disruptor (ED) effects, e.g., a role for halogenated hydrocarbon chlorobenzenes in increasing vasopressin (AVP), oxytocin (OT) secretion and, in association, anxiety and aggression in male rats has been shown. Our aim is to investigate whether 1,2,4-trichlorobenzenehexachlorobenzene= 1:1 (mClB) treatment of female rats also shows ED effects and reproductive biology differences, and whether AVP may have a mediator role in this? Female Wistar rats were treated (0.1; 1.0; 10.0 μg/bwkg/day) with mClB (by gastrictube) and then 30; 60; 90 days after treatment anxiety (open field test) and aggressive (resident intruder test) behaviors AVP, OT concentrations from blood plasma samples were detected by radioimmunoassay on 30; 60; 90 days. Treated female rats were mated with untreated males. Mating success, number of newborn and maternal aggression on the neonates were monitored. Results showed that AVP, OT levels; and anxiety, aggressive behaviors; and mothers' aggression towards their offspring increased significantly in relation to the duration and the dose of mClB treatment. But mating propensity and number of offspring decreased. Patterns of AVP, OT release and anxiety, aggression behaviors, and reproductive-related behaviors were correlated. Consistent with the literature, our studies confirmed the role of AVP and OT in different behavioral effects.

1. Introduction

The chemicals present in our environment can alter the behavioral patterns of higher vertebrates [1-3]. These environmental stresses can disrupt the homeostatic functions of organisms, causing disruptive effects. Healthy homeostasis is based on the permanent and complex functioning of the psycho-neuro-endocrine-immune system, which can generate various pathological processes in response to disruptive events [4-6]. Such potential pathogenic factors include agents of endocrine disrupting (ED) (e.g., halogenated hydrocarbons [7], phthalates [8], microplastics [9], etc.) present in all elements of the environment (soil [10], water [11], air [12]). Their effects interfere not only with physiological events in individuals, but also with reproductive processes (e.g., through reproductive behaviors and/or reproductive biological events [13-15]).

One group of endocrine disruptors of interest are chlorobenzenes (ClB) have been shown (in previous studies) to significantly altere plasma levels of arginine vasopressin (AVP) and oxytocin (OT) in male rats. These effects were strongly associated with changes in certain behavioral patterns e.g., aggression and anxiety in male Wistar rats [16, 17].

Hexachlorobenzene (HClB) interferes with thyroid function, and other physiological effects caused impaired liver, kidney, bone, and skin functions, etc., in a dose-dependent manner [18]. Its neurotoxic effects can also be associated with anxiety and aggression based on certain cognitive and locomotor behavioral determinants, which is

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demonstrated by relatively few research data [19,20]. Chronic prenatal and / or postnatal maternal exposure to HClB has been shown to cause hyperactivity and hyperexcitability in rodent offspring, as well as other abnormal movement patterns associated with tremors and tonic twitching. It has caused discrete alterations in experimental models of certain cognitive functions such as learning and memory [21,22].

It is known that the nervous system and hormonal balance of sexually mature higher vertebrates are ready for reproduction and the behavioral motivations to do so. Thus, the reproductive process, which has been established by reproductive behaviors (paced mating, lordosis behavior, maternal care, mate selection, hopping-darting-ear-wiggling, courtship) [13,23-25], can now be interpreted as a complex biological cascade of mechanisms.

Since physiological regulatory mechanisms, through instinctive and/or learned feedforward response mechanisms of behavior, are also involved in the functioning of the homeostatic system [23,26], the environmental effects of ED that perturb these may clearly be the cause of pathogenic events. Thus, the individual's external-internal physical, instinctive and/or learned behaviors (e.g., activity or lack thereof, anxiety, fear, desire, inclinations, emotions, quality of peer relations, aggressiveness, and dominance) and the biological mechanism system become integrated [26-28]. Research findings on ClB exposure related to reproductive events (paced mating, maternal care, infertility) are also under-represented in the literature [29,30].

If ClB exposures interfere with the normal presence of stress hormones for whatever reason, the behavior of the individual will be disturbed by the display of a pattern that is different from the healthy one (e.g., the natural anxiety expressed in threatening situations, which is essential for the individual to meet environmental challenges, will be over- or under-activated). However, in interpersonal relationships, this change in the behavioral pattern can be particularly detrimental [16,27]. If, in addition to anxiety, other behavioral patterns such as aggression are modified by the same environmental exposure (ClB), this may further impair the existence of interpersonal relationships and, consequently, the transmission of preferred traits [17,24].

AVP is known to be an essential stress hormone [31] and, in addition to its homeostatic physiological functions, it is also responsible for the expression of behavioral patterns (e.g., avoidance behavior, memory, learning, stress response, social behaviors) [32]. Increased levels of AVP have been measured in plasma and/or cerebrospinal fluid (CSF) in patients with anxiety disorders [33]. Decreased aggression and memory impairment were observed in $V1_b$ receptor knockout mice [34]. A positive correlation was also found between AVP concentrations in CSF and aggressive acts committed by patients with personality disorders [35]. Similar results have been obtained in experiments in which the anterior hypothalamus of animals with increased aggression towards unknown conspecifics showed increased density of AVP-ergic neurons [36].

The role of central OT has also been demonstrated in many aspects of behavior, e.g., maternal behavior, inhibition of learning and memory, stress-induced behavior, anxiety, and aggression [37]. OT also plays a dominant role in the development of trust [38]. It is generally accepted that OT reduces anxiety. Female rats treated with intracerebroventricular OT showed reduced anxiety on the elevated plus maze test compared to animals treated with "artificial" cerebrospinal fluid [39]. Acute and chronic OT treatment had an anxiolytic effect in rats. Female OT-knockout mice exhibited increased anxiety [40]. In humans, intranasal OT reduces anxiety [41]. OT gene knockout mice show increased aggressive behavior, and OT concentrations of CSF are inversely related to aggression [36]. In humans, it reduces the activity of the amygdala [42], so its absence leads to hostility, fear, and mistrust, which are conducive to aggression.

When a behavioral disturbance is induced by changes in plasma concentrations of these hormones in response to environmental exposures, the question is whether other physiological functions regulated by these hormones are also affected.

In view of the above, an important question is whether ED-acting ClB compounds can induce hormone secretion response mechanisms associated with behavioral and reproductive biological abnormalities by the appearance of complex neuroendocrine disruption.

To study these issues, we wanted to investigate in vivo gastrointestinal exposures to ClB mixtures (1,2,4-Trichlorobenzene: Hexachlorobenzene= 1:1 (mClB)) in littermate ♀ rats of the Wistar strain whether 30, 60, 90 days of mClB treatments are sub-toxic alter plasma AVP and OT concentrations.

If so, can AVP and OT be interpreted as one of the possible mediators between these biological regulatory levels as a regulatory node?

2. Materials and methods

2.1. Materials

The materials used in our experiments were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) or Invitrogen Corporation (Carlsbad, CA, USA). Equipment used in cell culture techniques (petri dish, plate, etc.) was obtained from BD Biosciences (San Jose, CA, USA). For culturing, cells were suspended in the following medium: Dulbecco's Modified Essential Medium (DMEM, Sigma, Germany) + 20 % Fetal Calf Serum (FCS, Sigma, Germany) + 100 IU/ml Penicillin + 100 IU/ml Streptomycin (Sigma, Germany). During the experiment, cell cultures were incubated at 37 °C with a CO_2 content of 5 %.

2.2. Animals

Certified healthy female rats were used in our experiments (Wistar strains weighing 100–120 g, at the beginning of in vivo treatment) (Charles River, Isaszeg, Hungary). During the experimental period, the animals were kept in a controlled environment (relative humidity, temperature, diurnal cycle) in 32 × 40 × 18 cm cages (3 animals/cage). The experimental animals were kept separately, but under the same conditions, and additional smaller, untreated female rats were used as intruders during the aggression tests. Food and drinking water were available ad libitum for the animals. The treatments were started after two weeks of habituation of the animals to the conditions and the persons who participated in the experiments. Experiments on the animals were carried out with the permission (No: XX./594/2018; date of release: 05/29/2018) of the University of Szeged's Workplace Animal Experimentation Committee and in compliance with the legislation on laboratory animals and experimental procedures (EU Directive 2010/63/EU for animal experiments).

2.3. In vivo examination

2.3.1. Applied exposures and experimental groups

During the experiments, the animals were treated with a 1:1 mixture of hexachlorobenzene and 1,2,4-trichlorobenzene.

As a gastrointestinal route of exposure, the mClB treatment was administered by gastric tube at 0.10 μg/kg body weight (D1); 1.0 μg/kg body weight (D2); and 10.0 μg/kg body weight (D3)/dose; 1 ml in final volume; for 30, 60 and 90 days. When the treatment protocol was standardized, the following mClB treatment and reference control groups were formed:

1. ClB treated groups: 30-day ($n = 8$ in each ClB-30 group: D1, D2, D3), 60-day ($n = 8$ in each ClB-60 group: D1, D2, D3) and 90-day ($n = 8$ in each ClB-90 group: D1, D2, D3) mClB in vivo exposure groups, which were treated by feeding via gastric tube.
2. Stress control groups: (SC) Animals treated / stressed with empty gavage for 0, 30 ($n = 5$, SC-30 group), 60 ($n = 5$, SC-60 group) and 90 ($n = 5$, SC-90 group) days.
3. Absolute control group: Control animals (C) not treated / stressed ($n = 5$).

4. Positive control group: (+C). During the treatment protocol, the animals received ClB solvent (0.001 % ethanol solution) in a final volume of 1 ml by gavage for 30, 60 and 90 days. ($n = 5$).
5. Negative control group: (—C) In the treatment scheme, the experimental animals received drinking water through a stomach tube in a final volume of 1 ml for 30, 60 and 90 days ($n = 5$).

2.3.2. Experimental protocol

The animals involved in the experiment were treated with mClB for 30, 60 and 90 days. After ClB treatments, anxiety was assessed by open field (OF), and aggressive behavior by modified resident-intruder (RI) tests.

After behavioral tests, native and anticoagulated blood samples were collected from the experimental animals. Stress hormones (OT, AVP) were determined from these samples by radioimmunoassay (RIA) and immunochemiluminescence assay (LIA).

Liver transferase enzyme levels - serum aspartate aminotransferase (AST; the normal range of AST in our laboratory: 30–250 IU/dm^3 serum), serum alanine aminotransferase (ALT; the normal range of ALT in our laboratory: 50–200 IU/dm^3 serum), and gamma-glutamyl transpeptidase (GGT; the normal range of GGT: 2–20 IU/dm^3 serum in our laboratoty) - were determined from serum, which are proven markers of toxicity from chlorobenzene exposure [43]. According to the literature, enzyme concentrations that vary from laboratory to laboratory are known as normal liver enzyme ranges [44-46]. During and/or after treatments, body weight and weight of major organs (liver, spleen, kidneys, adrenal glands, etc.) of the animals in the treatment regimen were also measured. Data from body weight changes and liver enzyme concentrations were used to confirm the subtoxicity of the chlorobenzene doses (D1, D2, D3) we used. Toxic ranges appear at mg/kg body weight exposures according to the literature [47-49]. In our laboratory, we established the sub-toxic range with μg/kg body weight oral exposure. The AVP and OT content of the blood samples of treated rats was measured using RIA and LIA methods [17,50].

After exposure intervals according to the experimental protocol, treated female rats were mated and the success of mating was monitored. We also tracked the number of offspring born from successful pregnancies and the number of fatal attacks on newborn offspring (0–3 days old) that represent maternal aggression.

2.3.3. Behavior studies

In the experiments, each animal was used once in each behavioral test. The tests were performed under identical conditions. The tests were started after 1 hour of habituation to the room. The animals were randomly assigned to groups according to the protocol. The experimental apparatus was cleaned with 70 % ethanol before each test to eliminate any possible effects of the odors generated on the animal / behavior. Tests were performed after complete exiccation. The behavioral elements displayed were recorded above the test apparatus using a ceiling-mounted, light sensitive video camera (PTZOptics 20X ZCam NDI), associated behavioral software: *Ethovision (v2.3, Noldus Information Technology, Wageningen, Netherland)* [16,17]. Maternal aggression was monitored by bloody attack and/or killing of newborn in the litter and by recording the number of surviving offspring.

2.3.3.1. Open field (OF)studies. The OF test is a standardized method for assessing locomotor activity, exploratory behavior, curiosity, anxiety, and habituation in rodents [16,17]. The test animal is placed in the middle of a standard-sized (45 cm high, 80 cm in diameter, circular) gray, non-slip floored, empty arena with an open top. During the test, the distance travelled is observed and recorded by the computer and/or the test person who performs the post-test checks. Behavior was monitored for 5 min for each animal.

2.3.3.2. Resident intruder (RI) tests - aggressiveness test. In our experimental protocol, we used modified RI tests to test the aggression of the animals [17,51]. The resident animal was placed in wood chip-covered area and allowed to habituate for 5 min. During the test, specific locomotor / exploratory and anxiety behavior measures were recorded for the resident animal. After habituation, at minute 6 of the experiment, the intruder animal was placed next to the resident. During the test, the rapid chase of the intruder is observed and recorded by the computer and/or the test person who performs the post-test checks. Animal behavior was monitored for 5 min.

2.3.4. Hormone assays

Blood samples taken from experimental animals were collected in Na_2EDTA-coated polystyrene tubes. Plasma was removed from the tubes after centrifugation (4 °C, 8000 rpm, 10 min), plasma aliquots were separated and stored at −70 °C until measurements. Following extraction on Amprep C8 minicolumns (Y2-VW-RPN1902; Amersham, UK) with a recovery of ≥95 %, plasma AVP and OT levels were assayed by RIA. The sensitivity of both AVP and OT assay was 1 pg per tube. All samples (in the case of both OT and AVP) were run in one assay, in duplicate, with 50 μl of plasma per well. Synthetic AVP (Organon, Oss, The Netherlands; antidiuretic activity: 408 IU/mg) was used as a reference preparation for antibody production and radiolabelling. AVP antibody was generated against the AVP-(ε-aminocaprionic acid)-thyroglobulin conjugate in sheep. The immunization regimen consisted of injections every 2 weeks for 12 weeks. The final antibody dilution used in the assay tube was 1:350.000. The cross-reactions were 23.3 % with lysine-8-AVP, <0.01 % with oxytocin, and <0.03 % with vasotocin. Synthetic OT was used after radiolabelling. OT antibody was generated against the OT-(ε-aminocaproic acid)-thyroglobulin conjugate in rabbits of the New Zealand strain. The cross-reaction with oxypressin was 92.7 %. ^{125}I labeling of OT was performed by the chloramine-T method. The standard curve covered the range 1.0–128 pg per assay tube in case of both AVP and OT.

2.4. Statistical analysis

2.4.1. Analysis of enzymes

After checking distributions of continuous variables liver enzymes (AST, ALT, GGT; $n = 60$) by descriptive statistics and revealed medium-strong monotone positive relationship between them with Pearson correlation and scatterplots, two-way MANOVA [52,53] was applied to investigate mean differences between groups of treatment (D1, D2, D3) and duration of time (30, 60, 90 days and control). Equal variances between the subgroups were ckecked by Levene's test and boxplots, homoscedasticity assumption was verified using the robust Box's M test. Normal distribution of model residuals was monitored for the separate dependent variables with Shapiro-Wilk tests and graphically by histogram and QQ-plot. Multivariable normality was checked by Mardia's skewness and kurtosis. When the Wilk's tests were significant in the overall MANOVA model, separate two-way ANOVA models were run for the dependent variables. In the case of significant ANOVA models, Sidak post hoc tests were applied to analyze multiple comparisons between the investigated pairs of groups (30, 60 and 90 days vs. Control for each treatment).

2.4.2. . Analyses of the control groups

In the analyses of the control groups in the case of count variables (chasing, newborn), as the data were not overdispersed, Poisson regression analyses [54-56] using logarithmic link function were used to compare counts between four control categories (absolute: reference category, stress, negative, positive) by duration of time (0 day: reference category, and 30, 60, 90 days) on $n = 80$ cases (for each model). To compare the continuous dependent variables (plasma AVP, plasma OT, distance) between the four control categories by duration of time (4 groups) on $n = 80$ cases, two-way ANOVA models [50,57] were used. Normality of model residuals were checked using d'Agostino test and

skewness, kurtosis statistics [58] besides histogram and QQ-plot. In case of not normal distribution, Aligned Rank Transformation (ART) ANOVA [59,60] was applied instead of two-way ANOVA. Homogeneity of variance was verified by boxplot and Levene's test.

2.4.3. Analyses of mClB treatments in plasma AVP and OT, behavior change, and mating behavior

Control data was randomly selected from the control system data for chasing and newborn counts, plasma AVP and OT and distance for each duration category, as no significant differences were found among them. All applied statistical models and analysis methods to check their assumptions were the same as in the analyses of control systems with some addition. In case of count variables (chasing: $n = 96$, newborn: $n = 120$, newborn attacked: $n = 120$) the Poisson regression model fit was verified by goodness-of-fit tests based on Pearson Chi-square and AIC criteria. Overall Chi-square tests were applied for the mClB treatment (4 groups: D1, D2, D3, control) and duration of time (3 groups: 30, 60, 90 days) effects in the Poisson models. To compare each group to the reference group for the count variables Wald Chi-square tests based on maximum likelihood estimation, and incident rate ratios (IRR) with 95 %CIs were calculated. To compare each treatment group (D1, D2, D3) to the control category for each duration of time (30, 60 and 90 days), contrast tests followed by Holm p-value adjustment were run as pairwise comparisons in two-way ANOVA models for the continuous variables (plasma AVP: $n = 96$, plasma OT: $n = 96$). To compare each treatment group (D1, D2, D3) to the control category for each duration of time (30, 60 and 90 days), ART-C contrast tests [61] followed by Holm p-value adjustment were run as pairwise comparisons in ART ANOVA model for the continuous variable distance ($n = 96$), because residuals of two-way ANOVA model were not normally distributed. To analyze binary dependent variable mating, logistic regression analysis [62] was applied on $n = 195$ cases to compare mating success between four treatment groups (D1, D2, D3 and control) and duration of time (30, 60, 90 days) using Fisher's scoring as optimization technique. Since quasi-complete separation of data points was detected in the logit model for predictor treatment (all matings ended in pregnancy for the control) and validity of the model fit was questionable, Firth's bias-correction as a penalized likelihood estimation method [63] was applied in the logistic regression analysis. Logistic regression model fit was verified by likelihood ratio and Hosmer and Lemeshow tests, and AIC criteria was also calculated. Overall Wald tests were applied for the treatment and day effects. To compare each treatment group (D1, D2, D3) to the reference group (control) for mating success Wald Chi-square tests based on penalized maximum likelihood estimation with odds ratios (OR) with 95 %CIs were calculated.

All statistical tests were two-sided with the significance level set at 5 % (except for the d'Agostino test, where alpha=0.001). The statistical software SAS Studio (SAS OnDemand for Academics, Release 3.81, SAS Institute Inc., Cary, NC, USA) was applied for analyses.

3. Results

Mean and standard deviation (SD) of liver enzymes (columns: AST /serum aspartate aminotransferase/, ALT /serum alanine aminotransferase/, GGT /gamma-glutamyl transpeptidase/) by mClB treatment duration categories (columns: 30, 60, 90 and control days) and treatment groups (rows: D1, D2, D3; $n = 5$ in each subgroup) can see on the Table 1. All enzymes are in the valid range (AST: [43.17, 181.77]; ALT: [52.23, 125.77]; GGT: [4.35, 15.41]). Liver enzyme levels were the healthy ranges set by our laboratory for AST (30–250 IU/dm^3 serum), ALT (50–200 IU/dm^3 serum), and GGT (2–20 IU/dm^3 serum). Post hoc test results showed significant ($p < 0.0001$) differences in AST for all investigated pairs of groups (9 pairs: separately for D1, D2 and D3 treatments: 30 days vs. Control, 60 days vs. Control and 90 days vs. Control). In the pairwise comparisons of ALT, all 9 pairs of groups resulted significant difference ($p < 0.001$) in means except day 30 and

Table 1
Evolution of liver enzyme toxicity after exposure to mClB.

Treatment	mClB treatment duration																					
	30 days						60 days						90 days						Control			
	AST		ALT		GGT		AST		ALT		GGT		AST		ALT		GGT		AST		ALT		GGT	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
D1	71.76	4.17	61.70	1.92	5.93	0.30	97.81	2.73	68.37	2.18	5.96	0.78	118.17	2.64	77.90	2.16	6.68	0.54	47.92	3.89	59.84	6.12	5.20	0.66
D2	123.90	5.53	71.93	2.88	6.06	0.25	132.47	3.29	80.79	2.29	7.19	0.27	142.69	2.43	85.92	1.54	7.59	0.43	48.00	2.36	60.44	3.46	4.95	0.46
D3	140.65	5.33	78.93	4.99	7.20	0.62	161.94	4.91	88.40	6.12	12.53	0.69	173.99	7.19	116.98	5.95	14.67	0.50	48.10	1.93	60.55	3.70	5.30	0.46

60 vs. Control in treatment D1. In the pairwise comparisons of GGT, the results of all 9 pairs of groups showed a significant difference ($p < 0.001$) in means except 30 and 60 days vs. Control in treatment D1, and day 30 vs. Control in treatment D2. After statistical verification, we may state that subtoxic doses were set for the experiment. The levels of liver enzyme increased with time and dose of chlorobenzene treatment compared to controls, but remained within the normal range.

Mean and standard deviation (SD) of the continuous variables of the control system (columns: Plasma AVP, Plasma OT, Distance) by each duration group (columns: 30, 60, 90 days and control) to compare control categories (rows: Absolute control, Stress control, Negative control, Positive control; $n = 5$ in each subgroup) in Table 2/a. None of the investigated variables in the control system showed a significant difference between the four investigated control groups by duration of time (for all tests: $p > 0.9$).

Tables 2/b_1 and b_2 show median (Me) with minimum and maximum of binary and count variables of the control system (columns: mating, chasing count, newborn count) by each duration group (columns: 30, 60, 90 days and control) to compare control categories (rows: Absolute control, Stress control, Negative control, Positive control; $n = 5$ in each subgroup). None of the investigated variables in the control system resulted in a significant difference between the four investigated control groups by duration of time (for all tests: $p > 0.7$).

Fig. 1/a shows mean ± standard error of mean (SEM) for plasma AVP (pg AVP/ml plasma) presented on bars (mean) with error bars (SEM) comparing the four investigated treatment groups (Control, D1, D2, D3) by three time duration categories (30, 60 and 90 days). Asterisks indicate a significant (** $p < 0.01$) difference (elevation) from the control group within each time duration category.

Mean ± standard error of mean (SEM) for plasma OT (pg OT/ml plasma) are presented on bars (mean) with error bars (SEM) comparing the four investigated treatment groups (Control, D1, D2, D3) in Fig. 1/b. by three time duration categories (30, 60 and 90 days). Asterisks indicate significant (*** $p < 0.001$) difference (increase) from the control group within each time duration category.

Mean ± standard error of mean (SEM) for anxiety (distance taken in cm) are presented on bars (mean) with error bars (SEM) comparing the four investigated treatment groups (Control, D1, D2, D3) in Fig. 2. by three time duration categories (30, 60 and 90 days). Asterisks indicate significant (*** $p < 0.001$) difference (decrease) from the control group within each time duration category.

Median (Me) with minimum and maximum of chasing count (aggression) by each duration category (columns: 30, 60, 90 days) to compare mClB treatment groups (rows: Control, D1, D2, D3; $n = 8$ in each subgroup) can be seen Table 3. All three treatments (D1, D2, D3) significantly ($p < 0.0001$) increase the risk of the number of chasings comparing to the control group. Incident Rate Ratios (IRR) are given with 95 % Confidence Intervals (CI; Low: lower level of CI, Up: upper level of CI) for each treatment group (D1, D2, D3) compared to the control. The risk of the number of chasings is 2.29 (95 %CI for IRR: [1.65, 3.17]) times higher in the D1, 3.21 (95 %CI for IRR: [2.35, 4.38]) times higher in the D2 and 5.31 (95 %CI for IRR: [3.95, 7.14]) times higher in the D3 treatment groups compared to the control regardless of treatment duration.

Percentages of mating success (diminution) of mClB treatment groups (D1, D2, D3; $n = 20$ in each subgroup) compared to control (100 %, $n = 5$ for each treatment: D1, D2, D3) by each duration category (30, 60, 90 days) on the Fig. 3. The odds of mating success is 25 times smaller in treatment D3 group comparing to control (OR=25.00, 95 %CI: [1.31, 500.00]).

Table 4/a shows Median (Me) with minimum and maximum of newborn count by each duration category (columns: 30, 60, 90 days) to compare mClB treatment groups (rows: Control, D1, D2, D3; $n = 10$ in each subgroup). All three treatments (D1, D2, D3) significantly ($p < 0.05$) decrease the risk of the number of newborns comparing to the control group. IRR are given with 95 % Confidence Intervals (CI; Low: lower level of CI, Up: upper level of CI) for each treatment group (D1, D2, D3) compared to the control. The risk of the number of newborns is

Table 2/a
Control systems for chlorobenzene exposure experiments consisting of control categories AVP, OT and anxiety data.

Control categories	30 days					60 days					90 days					Control				
	Plasma AVP		Plasma OT		Anxiety (distance taken)	Plasma AVP		Plasma OT		Anxiety (distance taken)	Plasma AVP		Plasma OT		Anxiety (distance taken)	Plasma AVP		Plasma OT		Anxiety (distance taken)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Absolute control	4.25	0.60	6.01	0.32	1975.95	5.59	4.60	0.54	5.97	0.57	1975.82	4.79	4.48	0.65	5.76	0.54	1976.30	6.03	4.44	0.50	5.93	0.47	1976.88	5.38
Stress control	4.67	0.51	5.91	0.51	1975.17	5.69	4.47	0.63	5.89	0.46	1976.24	4.73	4.53	0.65	6.05	0.62	1976.70	5.00	4.48	0.54	5.82	0.49	1969.35	13.58
Negative control	4.57	0.55	5.80	0.52	1975.77	5.56	4.57	0.66	6.00	0.48	1975.54	5.05	4.48	0.71	6.00	0.31	1976.01	5.42	4.43	0.56	5.92	0.47	1975.92	4.83
Positive control	4.50	0.68	5.96	0.55	1976.35	5.81	4.50	0.67	6.04	0.51	1976.51	6.16	4.39	0.70	5.98	0.58	1976.14	6.12	4.55	0.70	5.93	0.43	1975.32	4.98

Table 2/b_1
Control systems for chlorobenzene exposure (30, 60 days) experiments consisting of control categories for aggressive and reproductive behavior data.

Control categories	30 days								60 days								Control							
	Mating			Chasing count			Newborn count		Mating			Chasing count			Newborn count		Mating			Chasing count			Newborn count	
	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max
Absolute control	1.0	1	1	2.0	2	3	11.0	8	13	1.0	1	1	2.0	1	4	12.0	9	13	1.0	1	1	2.0	1	3	11.0	8	12
Stress control	1.0	1	1	2.0	1	4	11.0	9	13	1.0	1	1	2.0	2	3	12.0	8	13	1.0	1	1	2.0	1	3	10.0	8	13
Negative control	1.0	1	1	2.0	1	3	11.0	9	13	1.0	1	1	2.0	1	3	11.0	10	13	1.0	1	1	2.0	1	3	10.0	8	12
Positive control	1.0	1	1	3.0	1	4	12.0	8	13	1.0	1	1	3.0	1	4	11.0	9	13	1.0	1	1	2.0	1	4	10.0	9	12

0.85 (95 %CI for IRR: [0.731, 0.998]) times higher in the D1, 0.70 (95 % CI for IRR: [0.60, 0.83]) times higher in the D2 and 0.48 (95 %CI for IRR: [0.40, 0.58]) times higher in the D3 treatment groups compared to the control regardless of treatment duration.

Table 4/b shows Median (Me) with minimum and maximum of newborn attack count by each duration category (columns: 30, 60, 90 days) to compare mClB treatment groups (rows: Control, D1, D2, D3; $n = 10$ in each subgroup). Treatments D2 and D3 significantly ($p < 0.01$) increase the risk of the number of newborn attacks compared to the control group. IRR are given with 95 % Confidence Intervals (CI; Low: lower level of CI, Up: upper level of CI) for each treatment group (D1, D2, D3) compared to the control. NS indicates Not Significant IRR for treatment D1 compared to the control. The incident rate for treatment D1 is not significantly ($p = 0.220$, 95 %CI for IRR: [0.60, 9.02]) different from the control group holding treatment duration constant. The risk of the number of newborn attacks is 6.67 (95 %CI for IRR: [1.98, 22.43]) times higher in the D2 and 19.67 times higher in the D3 (95 %CI for IRR: [6.17, 62.74]) treatment groups compared to the control regardless of treatment duration.

4. Discussion

An increased presence of AVP has been detected in the CSF of patients with anxiety [33] and an increase in aggression in patients with personality disorders [35]. Experimentally, it has been shown that when aggression towards conspecifics increases, the neuronal density of AVP in the anterior hypothalamus is intensified [36]. OT has a general anxiolytic effect [39-41] and the concentration of OT is inversely related to aggression [36]. In organisms, the psycho-neuroendocrine events determined by the regulation of AVP and OT are not isolated processes [24,27], but represent a general context for the maintenance of a homeostatic balance of living quality, where movement, metabolism, and reproduction are stably regulated.

When we wanted to study the effects of in vivo environmental exposure to mClB on changes in plasma AVP and OT concentrations in female Wistar rats and to look for a link between possible hormone level differences and the onset of anxiety and agnosia, we also wanted to go further with this knowledge to study reproductive events related to reproductive/sexual behaviors. We hypothesized that altered levels of AVP and OT hormones in plasma could generate anxiety and aggressive behavioral disorders. However, if this is the case, can we detect differences in reproductive processes (as essential life events) already at the subtoxic concentrations of mClB exposure that we used? Furthermore, can a correlation be detected between changes in AVP, OT plasma concentrations, behavioral changes, and reproductive events?

To safely evaluate our research results, we first demonstrated the subtoxicity of the exposure doses (Table 1). In this follow-up, the results of liver toxicity enzymes (AST, ALT, GGT) at the applied doses (D1, D2, D3) of μg/kg bwt during treatment time courses (30, 60, 90 days) did not exceed the normal, physiologically permissible range of enzyme levels at any time during the dietary route of exposure.

To statistically assess the effects in the experimental protocol, a control design (stress/absolute/positive/negative control groups) was needed that could be used as a validated reference to monitor the effects of mClB on plasma AVP/OT concentrations, anxiety (Table 2 /a), aggressive behavioral elements and reproductive processes (Tables 2/b_1 and b_2).

Plasma AVP (Fig. 1/a) and OT (Fig. 1/b) values determined with mClB exposures showed a significant increase as a function of the dose and time applied.

This means that mClB does have an ED effect in Wistar females. This has previously been described only for males [16,17]. But a very important fact to answer our research questions is that the effects of central OT and AVP can overlap, but can also be independent and opposite [32]. OT is anxiolytic and antidepressant, AVP increases anxiety and can enhance depressive-like behavior [64]. If ED-acting mClB has altered concentrations of plasma AVP, OT, its consequences may

Table 2/b_2
Control systems for chlorobenzene exposure (90 days) experiments consisting of control categories for aggressive and reproductive behavior data.

Control categories	90 days									Control								
	Mating			Chasing count			Newborn count			Mating			Chasing count			Newborn count		
	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max	Me	Min	Max
Absolute control	1.0	1	1	2.0	1	3	12.0	8	13	1.0	1	1	2.0	1	3	11.0	8	12
Stress control	1.0	1	1	3.0	1	4	11.0	9	13	1.0	1	1	2.0	1	3	10.0	8	13
Negative control	1.0	1	1	2.0	1	3	12.0	9	13	1.0	1	1	2.0	1	3	10.0	8	12
Positive control	1.0	1	1	2.0	1	3	11.0	9	13	1.0	1	1	2.0	1	4	10.0	9	12

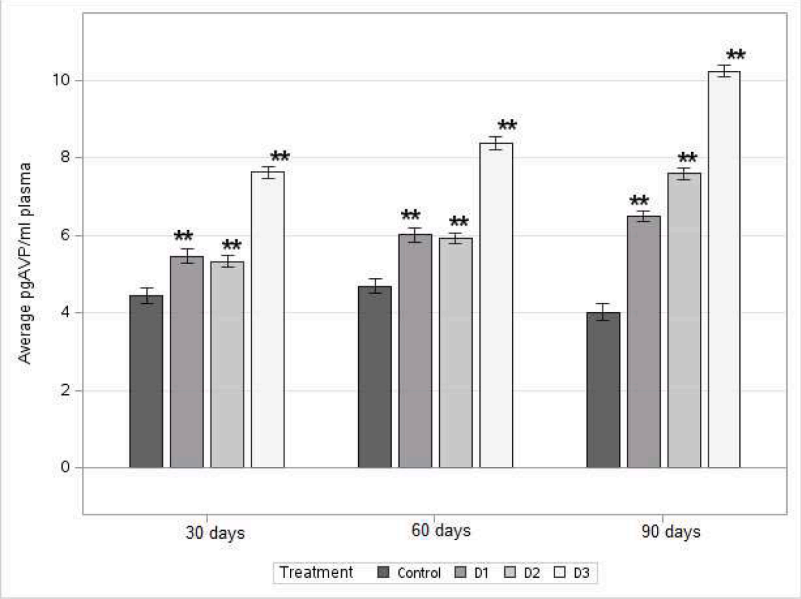

Fig. 1/a. Changes in plasma AVP levels after mClB treatment.

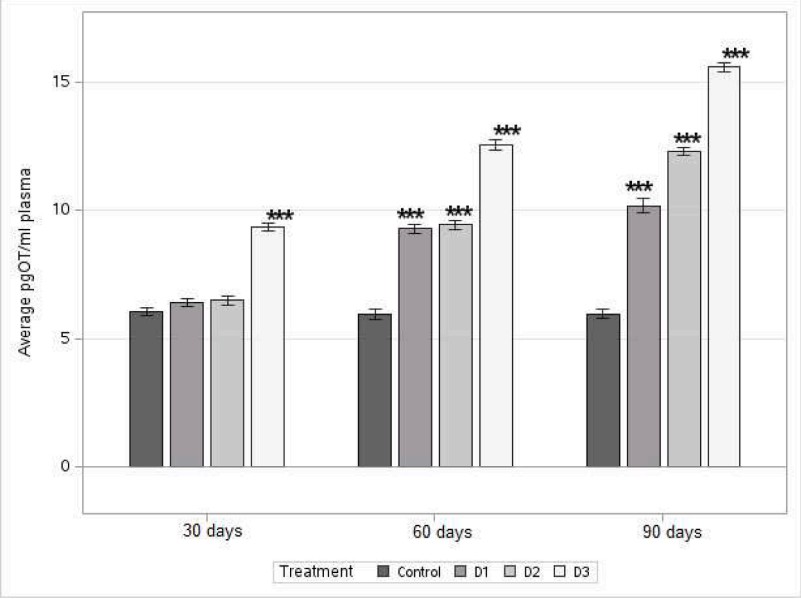

Fig. 1/b. Effect of mClB treatment on plasma OT levels.

also be reflected in behavior.

The result showing behavioral patterns (anxiety, aggression) revealed a dose- and time-dependent decrease in the distance travelled by exposed animals in the arena (Fig. 2), an increase in the number of interindividual contacts and a much higher frequency of chasing by treated females against foreign intruders (Table 3). These results are in agreement with those of previous OF and RI tests in male mClB treated individuals [16,17], although they are more pronounced in females.

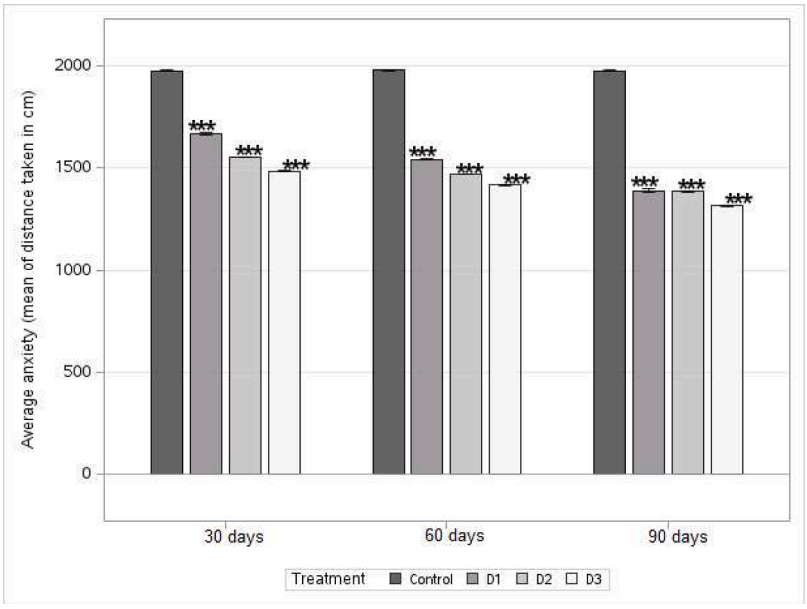

Fig. 2. Effect of mClB exposures on the pathway in the OF test.

Table 3
Behavioral consequences of mClB treatment in resident–intruder tests.

mClB treatment	30 days			60 days			90 days			IRR	95 % CI	
	Me	Min	Max	Me	Min	Max	Me	Min	Max		Low	Up
Control	2.0	1	3	2.0	1	3	2.5	1	4	–	–	–
D1	4.0	3	6	5.0	3	6	6.0	5	7	2.29	1.65	3.17
D2	5.0	3	6	8.0	4	9	8.5	7	11	3.21	2.35	4.38
D3	7.5	6	9	10.0	8	11	17.0	14	20	5.31	3.95	7.14

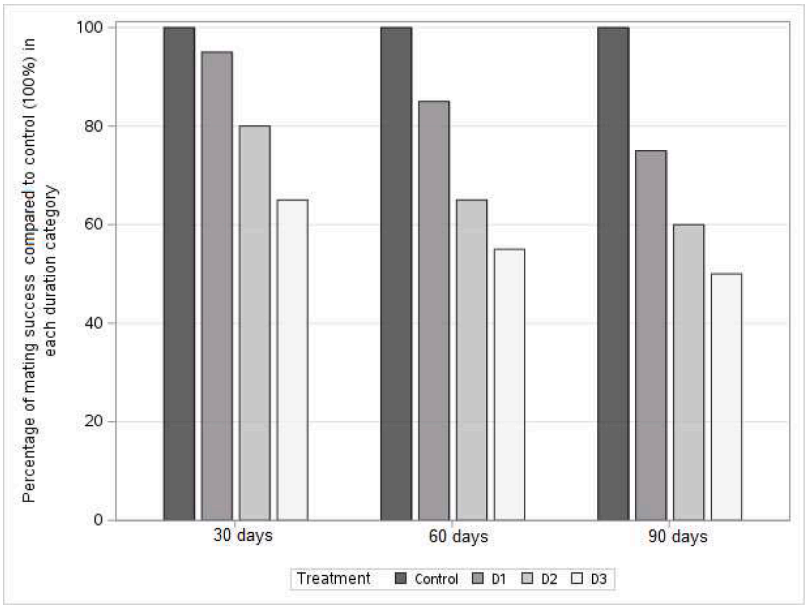

Fig. 3. Changes in mating success after mClB treatment in Wistar females.

Our results on reproductive behavior showed that mating propensity (Fig. 3) and number of offspring (Table 4/a) significantly decreased with increasing mClB exposure time and dose. Maternal aggression towards newborns was also confirmed in our experiments, which also increased in a dose- and time-dependent manner (Table 4/b). Maternal offspring care actions of female rats were reduced based on aggressive behaviors towards offspring.

OT and AVP are key neuropeptides involved in maternal behavior and anxiety, showing increased activity in different regions of the brain (for example, around birth, increased expression and release of mRNA, and

Table 4/a
Effects of different doses and durations of mClB treatment in female Wistar rats on the number of newborns.

mClB treatment	30 days			60 days			90 days			IRR	95 % CI	
	Me	Min	Max	Me	Min	Max	Me	Min	Max		Low	Up
Control	12.0	9	13	12.0	9	13	11.5	9	13	–	–	–
D1	11.0	8	12	10.0	8	11	9.0	7	11	0.85	0.731	0.998
D2	9.0	8	10	8.5	7	10	7.0	5	9	0.70	0.60	0.83
D3	7.0	4	8	5.5	4	7	4.5	3	6	0.48	0.40	0.58

Table 4/b.
Effect of mClB treatment on maternal attacks in female Wistar rats.

mClB treatment	30 days			60 days			90 days					95% CI
Control	0.0	0	1	0.0	0	1	0.0	0	1	–	–	–
D1	0.0	0	1	0.0	0	1	0.0	0	1	NS	0.60	9.02
D2	0.0	0	1	0.5	0	2	1.0	0	2	6.67	1.98	22.43
D3	1.0	0	2	2.0	1	3	3.0	2	4	19.67	6.17	62.74

increased expression of receptor mRNA, receptor density, and/or binding have been described) [65]. OT and AVP are also known to be important mediators of social recognition [66]. But sex differences in social cognitive behavior draw attention to the relationship between OT, AVP, and sex hormones.

Increased of OT and AVP levels results in anxiety-like behaviors and, therefore, induces changes in reproductive behavior [37,67].

The role of female sex hormones in reproductive processes is known. The other possible roles of these hormones in behavior can be further investigated through the suitability of the experimental model we have set up. However, the present work also draws attention to the fact that the subtoxic doses of mClB (as ED compounds) used cannot provide an exemption from the risks arising from their exposure, as they are depositable compounds whose effects may lead to the development of various diseases due to long exposure times, compromising homeostasis and interpersonal relationships necessary for reproduction. A particular merit of this work is the focus on a model system to study the environmental exposures created by human society in relation to maternal behavior and endocrinological events.

5. Conclusions

ED-acting mClB is deposited in living organisms and, therefore, long exposures disturb neuroendocrine regulation, e.g., plasma hormone levels of AVP and OT, which are significantly increased depending on treatment duration and doses in female rats. As stress hormones, AVP and OT have been shown to be associated with behavioral patterns of anxiety and aggression, which were also significantly increased by exposure to ED in a dose- and time-dependent manner. But the AVP effect was also dominant in reproduction-related behaviors, which were fine-tuned by OT. The literature suggests that AVP and OT in different brain territories are responsible for different behavioral effects, which can be detected in the results of in vivo experiments as a result of altered hormone presence in response to both ED effects.

CRediT authorship contribution statement

Krisztián Sepp: Writing – original draft, Methodology, Investigation, Funding acquisition. **Anna László:** Visualization, Software, Formal analysis, Data curation. **Márta Gálfi:** Writing – review & editing, Methodology, Investigation. **Marianna Radács:** Writing – review & editing, Project administration, Methodology, Investigation. **Miklós Mózes:** Methodology, Investigation. **Péter Hausinger:** Methodology, Investigation. **Regina Pálföldi:** Methodology, Investigation. **Médea Veszelka:** Validation, Methodology, Investigation. **Zsuzsanna Valkusz:** Validation, Supervision, Investigation. **Zsolt Molnár:** Writing – original draft, Methodology, Investigation, Conceptualization.

Data availability

Data will be made available on request.

References

[1] S. DeLeon, R. Halitschke, R.S. Hames, A. Kessler, T.J. DeVoogd, A.A. Dhondt, The effect of polychlorinated biphenyls on the song of two passerine species, PLoS. One 8 (2013) e73471, https://doi.org/10.1371/journal.pone.0073471.

[2] N.S. Fraga, A.S. Martins, D.R. Faust, H. Sakai, A. Bianchini, C. Carneiro da Silva, A. A. Aguirre, Cadmium in tissues of green turtles (Chelonia mydas): a global perspective for marine biota, Sci. Total. Environ. 637-638 (2018) 389–397, https://doi.org/10.1016/j.scitotenv.2018.04.317.

[3] O. Faroon, D. Jones, D. de Rosa, Effects of polychlorinated biphenyls on the nervous system, Toxicol. Ind. Health 16 (2000) 305–333, https://doi.org/10.1177/074823370001600708.

[4] S. Fragki, H. Dirven, T. Fletcher, B. Grasl-Kraupp, K.B. Gützkow, R. Hoogenboom, S. Kersten, B. Lindeman, J. Louisse, A. Peijnenburg, A.H. Piersma, H.M.G. Princen, M. Uhl, J. Westerhout, M.J. Zeilmaker, M. Luijten, Systemic PFOS and PFOA exposure and disturbed lipid homeostasis in humans: what do we know and what not? Crit. Rev. Toxicol. 51 (2021) 141–164, https://doi.org/10.1080/10408444.2021.1888073.

[5] A. Nadal, I. Quesada, E. Tuduri, R. Nogueiras, P. Alonso-Magdalena, Endocrine-disrupting chemicals and the regulation of energy balance, Nat. Rev. Endocrinol. 13 (2017) 536–546, https://doi.org/10.1038/nrendo.2017.51.

[6] A. Mokarizadeh, M.R. Faryabi, M.A. Rezvanfar, M. Abdollahi, A comprehensive review of pesticides and the immune dysregulation: mechanisms, evidence and consequences, Toxicol. Mech. Methods 25 (2015) 258–278, https://doi.org/10.3109/15376516.2015.1020182.

[7] A. Covaci, A.C. Gerecke, R.J. Law, S. Voorspoels, M. Kohler, N.V. Heeb, H. Leslie, C. R. Allchin, J. De Boer, Hexabromocyclododecanes (HBCDs) in the environment and humans: a review, Environ. Sci. Technol. 40 (2006) 3679–3688, https://doi.org/10.1021/es0602492.

[8] F. Pallotti, M. Pelloni, D. Gianfrilli, A. Lenzi, F. Lombardo, D. Paoli, Mechanisms of testicular disruption from exposure to bisphenol a and phtalates, J. Clin. Med. 9 (2020) 471, https://doi.org/10.3390/jcm9020471.

[9] A.K. Priya, A.A. Jalil, K. Dutta, S. Rajendran, Y. Vasseghian, J. Qin, M. Soto-Moscoso, Microplastics in the environment: recent developments in characteristic, occurrence, identification and ecological risk, Chemosphere 298 (2022) 134161, https://doi.org/10.1016/j.chemosphere.2022.134161.

[10] J.N. Möller, M.G.J. Löder, C. Laforsch, Finding microplastics in soils: a review of analytical methods, Environ. Sci. Technol. 54 (2020) 2078–2090, https://doi.org/10.1021/acs.est.9b04618.

[11] S. Esteban, L. Moreno-Merino, R. Matellanes, M. Catalá, M. Gorga, M. Petrovic, M. López de Alda, D. Barceló, A. Silva, J.J. Durán, J. López-Martínez, Y. Valcárcel, Presence of endocrine disruptors in freshwater in the northern Antarctic Peninsula region, Environ. Res. 147 (2016) 179–192, https://doi.org/10.1016/j.envres.2016.01.034.

[12] E.C. Plunk, S.M. Richards, Endocrine-disrupting air pollutants and their effects on the hypothalamus-pituitary-gonadal axis, Int. J. Mol. Sci. 21 (2020) 9191, https://doi.org/10.3390/ijms21239191.

[13] D. López-Rodríguez, C.F. Aylwin, V. Delli, E. Sevrin, M. Campanile, M. Martin, D. Franssen, A. Gérard, S. Blacher, E. Tirelli, A. Noël, A. Lomniczi, A.S. Parent, Multi- and transgenerational outcomes of an exposure to a mixture of endocrine-disrupting chemicals (EDCs) on puberty and maternal behavior in the female rat, Environ. Health Perspect. 129 (2021) 87003, https://doi.org/10.1289/EHP8795.

[14] A.C. Gore, A.M. Holley, D. Crews, Mate choice, sexual selection, and endocrine-disrupting chemicals, Horm. Behav. 101 (2018) 3–12, https://doi.org/10.1016/j.yhbeh.2017.09.001.

[15] S. Mhaouty-Kodja, L. Naulé, D. Capela, Sexual behavior: from hormonal regulation to endocrine disruption, Neuroendocrinology. 107 (2018) 400–416, https://doi.org/10.1159/000494558.

[16] G. Nagyeri, Z. Valkusz, M. Radacs, T. Ocsko, P. Hausinger, M. Laszlo, F.A. Laszlo, A. Juhasz, J. Julesz, M. Galfi, Behavioral and endocrine effects of chronic exposure to low doses of chlorobenzenes in Wistar rats, Neurotoxicol. Teratol. 34 (2012) 9–19, https://doi.org/10.1016/j.ntt.2011.09.011.

[17] Z. Valkusz, G. Nagyéri, M. Radács, T. Ocskó, P. Hausinger, M. László, F.A. László, A. Juhász, J. Julesz, P. Pálföldi, M. Gálfi, Further analysis of behavioral and endocrine consequences of chronic exposure of male Wistar rats to subtoxic doses of endocrine disruptor chlorobenzenes, Physiol. Behav. 103 (2011) 421–430, https://doi.org/10.1016/j.physbeh.2011.03.016.

[18] L. Reed, V. Buchner, P.B. Tchounwou, Environmental toxicology and health effects associated with hexachlorobenzene exposure, Rev. Environ. Health 22 (2007) 213–243, https://doi.org/10.1515/reveh.2007.22.3.213.

[19] J. Fu, Q. Shi, X. Song, X. Xia, C. Su, Z. Liu, E. Song, Y. Song, Tetrachlorobenzoquinone exhibits neurotoxicity by inducing inflammatory responses through ROS-mediated IKK/IκB/NF-κB signaling, Environ. Toxicol. Pharmacol. 41 (2016) 241–250, https://doi.org/10.1016/j.etap.2015.12.012.

[20] G.E. Seralini, G. Jungers, Endocrine disruptors also function as nervous disruptors and can be renamed endocrine and nervous disruptors (ENDs), Toxicol Rep. 31 (2021) 1538–1557. 10.1016/j.toxrep.2021.07.014.

[21] Toxicological Profile for Hexachlorobenzene. Atlanta (GA): agency for toxic substances and disease registry (US); 2015 Aug.

[22] B. Starek-Świechowicz, B. Budziszewska, A. Starek, Hexachlorobenzene as a persistent organic pollutant: toxicity and molecular mechanism of action, Pharmacol. Rep. 69 (2017) 1232–1239, https://doi.org/10.1016/j.pharep.2017.06.013.

[23] S.H. Meerts, R.S. Schairer, M.E. Farry-Thorn, E.G. Johnson, H.K. Strnad, Previous sexual experience alters the display of paced mating behavior in female rats, Horm. Behav. 65 (2014) 497–504, https://doi.org/10.1016/j.yhbeh.2013.12.015.

[24] G.T. Taylor, J. Weiss, R. Rupich, Male rat behavior, endocrinology and reproductive physiology in a mixed-sex, socially stressful colony, Physiol. Behav. 39 (1987) 429–433, https://doi.org/10.1016/0031-9384(87)90368-4.

[25] E.M. Brandling-Bennett, M.E. Blasberg, A.S. Clark, Paced mating behavior in female rats in response to different hormone priming regimens, Horm. Behav. 35 (1999) 144–154, https://doi.org/10.1006/hbeh.1998.1507.

[26] A. Kumar, R.K. Narayan, V. Pareek, C. Kumari, S.K. Ghosh, M.A. Faiq, Neural Control of Behavior, in: J. Vonk, T. Shackelford (Eds.), Encyclopedia of animal cognition and behavior, Springer, Cham, 2019, pp. 1–19.

[27] I.D. Neumann, Alterations in behavioral and neuroendocrine stress coping strategies in pregnant, parturient and lactating rats, Prog. Brain Res. 133 (2001) 143–152, https://doi.org/10.1016/s0079-6123(01)33011-x.

[28] E.M. Byrnes, R.S. Bridges, Reproductive experience alters anxiety-like behavior in the female rat, Horm. Behav. 50 (2006) 70–76, https://doi.org/10.1016/j.yhbeh.2006.01.006.

[29] H.A. Dhaibar, H. Patadia, T. Mansuri, R. Shah, L. Khatri, H. Makwana, S. Master, P. Robin, Hexachlorobenzene, a pollutant in hypothyroidism and reproductive aberrations: a perceptive transgenerational study, Environ. Sci. Pollut. Res. Int. 28 (2021) 11077–11089, https://doi.org/10.1007/s11356-020-11278-x.

[30] J.O. Bustnes, B.J. Bårdsen, B. Moe, D. Herzke, S.A. Hanssen, K. Sagerup, C. Bech, T. Nordstad, O. Chastel, S. Tartu, G.W. Gabrielsen, Temporal variation in circulating concentrations of organochlorine pollutants in a pelagic seabird breeding in the high Arctic, Environ. Toxicol. Chem. 36 (2017) 442–448, https://doi.org/10.1002/etc.3560.

[31] D. Zelena, O. Pintér, D.G. Balázsfi, K. Langnaese, K. Richter, R. Landgraf, G. B. Makara, M. Engelmann, Vasopressin signaling at brain level controls stress hormone release: the vasopressin-deficient Brattleboro rat as a model, Amino Acids. 47 (2015) 2245–2253, https://doi.org/10.1007/s00726-015-2026-x.

[32] H.B. Patisaul, Endocrine disruption of vasopressin systems and related behaviors, Front. Endocrinol. (Lausanne) 8 (2017) 134, https://doi.org/10.3389/fendo.2017.00134.

[33] V. Kormos, B. Gaszner, Role of neuropeptides in anxiety, stress, and depression: from animals to humans, Neuropeptides. 47 (2013) 401–419, https://doi.org/10.1016/j.npep.2013.10.014.

[34] J.T. Winslow, T.R. Insel, Neuroendocrine basis of social recognition, Curr. Opin. Neurobiol. 14 (2004) 248–253, https://doi.org/10.1016/j.conb.2004.03.009.

[35] E.F. Coccaro, R.J. Kavoussi, R.L. Hauger, T.B. Cooper, C.F. Ferris, Cerebrospinal fluid vasopressin levels: correlates with aggression and serotonin function in personality-disordered subjects, Arch. Gen. Psychiatry 55 (1998) 708–714, https://doi.org/10.1001/archpsyc.55.8.708.

[36] O.J. Bosch, Maternal aggression in rodents: brain oxytocin and vasopressin mediate pup defence, Philos. Trans. R. Soc. Lond. B Biol. Sci. 368 (2013) 20130085, https://doi.org/10.1098/rstb.2013.0085.

[37] S. Sabihi, N.E. Durosko, S.M. Dong, B. Leuner, Oxytocin in the prelimbic medial prefrontal cortex reduces anxiety-like behavior in female and male rats, Psychoneuroendocrinology. 45 (2014) 31–42, https://doi.org/10.1016/j.psyneuen.2014.03.009.

[38] A. Ebert, M. Brüne, Oxytocin and Social Cognition, Curr. Top. Behav. Neurosci. 35 (2018) 375–388, https://doi.org/10.1007/7854_2017_21.

[39] P. Mak, C. Broussard, K. Vacy, J.H. Broadbear, Modulation of anxiety behavior in the elevated plus maze using peptidic oxytocin and vasopressin receptor ligands in the rat, J. Psychopharmacol. 26 (2012) 532–542, https://doi.org/10.1177/0269881111416687.

[40] R.C. Mantella, R.R. Vollmer, X. Li, J.A. Amico, Female oxytocin-deficient mice display enhanced anxiety-related behavior, Endocrinology 144 (2003) 2291–2296, https://doi.org/10.1210/en.2002-0197.

[41] D.C. Gomes de Oliveira, M.H.N. Chagas, L.V. Garcia, J.A.S. Crippa, A.W. Zuardi, Oxytocin interference in the effects induced by inhalation of 7.5% CO(2) in healthy volunteers, Hum. Psychopharmacol. 27 (2012) 378–385, https://doi.org/10.1002/hup.2237.

[42] M. Kanat, M. Heinrichs, G. Domes, Oxytocin and the social brain: neural mechanisms and perspectives in human research, Brain Res. 1580 (2014) 160–171.

[43] S.S. Tamber, P. Bansal, S. Sharma, R.B. Singh, R. Sharma, Biomarkers of liver diseases, Mol. Biol. Rep. 50 (2023) 7815–7823, https://doi.org/10.1007/s11033-023-08666-0.

[44] S.T. Wolford, R.A. Schroer, F.X. Gohs, P.P. Gallo, M. Brodeck, H.B. Falk, R. Ruhren, Reference range data base for serum chemistry and hematology values in laboratory animals, J. Toxicol. Environ. Helath 18 (1986) 161–188, https://doi.org/10.1080/15287398609530859.

[45] Q. He, G. Su, K. Liu, F. Zhang, Y. Jiang, J. Gao, L. Liu, Z. Jiang, M. Jin, H. Xie, Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method, PLoS. One 12 (2017) e0189837, https://doi.org/10.1371/journal.pone.0189837.

[46] O. Boehm, B. Zur, A. Koch, N. Tran, R. Freyenhagen, M. Hartmann, K. Zacharowski, Clinical chemistry reference database for Wistar rats and C57/BL6 mice, Biol. Chem. 388 (2007) 547–554, https://doi.org/10.1515/BC.2007.061.

[47] L. Giribaldi, F. Chiappini, C. Pontillo, A.S. Randi, D.L. Kleiman de Pisarev, L. Alvarez, Hexachlorobenzene induces deregulation of cellular growth in rat liver, Toxicology. 289 (2011) 19–27, https://doi.org/10.1016/j.tox.2011.07.004.

[48] S. Hadjab, D. Maurel, Y. Cazals, P. Siaud, Hexachlorobenzene, a dioxin-like compound, disrupts auditory function in rat, Hear. Res. 191 (2004) 125–134, https://doi.org/10.1016/j.heares.2003.12.017.

[49] H. Lilienthal, C. Benthe, B. Heinzow, G. Winneke, Impairment of schedule-controlled: behavior by pre- and postnatal exposure to hexachlorobenzene in rats, Arch. Toxicol. 70 (1996) 174–181, https://doi.org/10.1007/s002040050257.

[50] Z. Molnár, R. Pálföldi, A. László, M. Radács, K. Sepp, P. Hausinger, L. Tiszlavicz, Z. Valkusz, G. Gálfi, Effects of chronic and subtoxic chlorobenzenes on adrenocorticotrophic hormone release, J. Environ. Sci. (China) 34 (2015) 165–170, https://doi.org/10.1016/j.jes.2015.03.014.

[51] É. Mikics, M.R. Kruk, J. Haller, Genomic and non-genomic effects of glucocorticoids on aggressive behavior in male rats, Psychoneuroendocrinology. 29 (2004) 618–635, https://doi.org/10.1016/S0306-4530(03)00090-8.

[52] R. Khattree, D.N. Naik, Applied Multivariate Statistics With SAS Software, 2nd ed., SAS Press John Wiley & Sons, Cary, 1999.

[53] C.J. Huberty, S. Olejnik, Applied MANOVA and Discriminant Analysis, 2nd ed., John Wiley & Sons, New Jersey, 2006.

[54] A.J. Dobson, An Introduction to Generalized Linear Models, 2nd ed., Chapman & Hall/CRC, Boca Raton, 2002.

[55] A. Agresti, Categorical data analysis, 2nd ed., John Wiley & Sons, New York, 2002.

[56] A.C. Cameron, P.K. Trivedi, Regression analysis of count Data, 2nd ed., Cambridge University Press, New York, 2013.

[57] R. Cody, Biostatistics by Example Using SAS® Studio, SAS Institute, Cary (2016).

[58] R.B. D'Agostino, A. Belanger, R.B. D'Agostino Jr., A suggestion for using powerful and informative tests of normality, Am. Stat. 44 (1990) 316–321, https://doi.org/10.1080/00031305.1990.10475751.

[59] J.O. Wobbrock, L. Findlater, D. Gergle, J.J. Higgins, The aligned rank transform for nonparametric factorial analyses using only ANOVA procedures, in: Proceedings of the ACM conference on human factors in computing systems (CHI '11) 2011, ACM Press, Vancouver, CanadaNew York, 2011, pp. 143–146, https://doi.org/10.1145/1978942.1978963. May 7-12.

[60] E. Durner, Effective analysis of interactive effects with non-normal data using the aligned rank transform, ARTool and SAS® University edition, Horticulturae 5 (2019) 57, https://doi.org/10.3390/horticulturae5030057.

[61] L.A. Elkin, M. Kay, J.J. Higgins, J.O. Wobbrock, An aligned rank transform procedure for multifactor contrast tests, in: Proceedings of the ACM Symposium on User Interface Software and Technology (UIST '21). Virtual Event 2021, ACM Press, USANew York, 2021, pp. 754–768. October 10-14, doi.org/10.1145/3472749.3474784.

[62] D.W. Hosmer, S. Lemeshow, Applied logistic regression, 2nd ed., John Wiley & Sons, New York, 2000 https://doi.org/10.1002/0471722146.

[63] G. Heinze, M. Schemper, A solution to the problem of separation in logistic regression, Stat. Med. 21 (2002) 2409–2419, https://doi.org/10.1002/sim.1047.

[64] I.D. Neumann, R. Landgraf, Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors, Trends. Neurosci. 35 (2012) 649–659, https://doi.org/10.1016/j.tins.2012.08.004.

[65] O.J. Bosch, I.D. Neumann, Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action, Horm. Behav. 61 (2012) 293–303, https://doi.org/10.1016/j.yhbeh.2011.11.002.

[66] D. Aspesi, E. Choleris, Neuroendocrine underpinning of social recognition in males and females, Neuroendocrinol 34 (2022) e13070, https://doi.org/10.1111/jne.13070.

[67] Y. Yamamoto, B.S. Cushing, K.M. Kramer, P.D. Epperson, G.E. Hoffman, C. S. Carter, Neonatal manipulations of oxytocin alter expression of oxytocin and vasopressin immunoreactive cells in the paraventricular nucleus of the hypothalamus in a gender-specific manner, Neuroscience 125 (2004) 947–955, https://doi.org/10.1016/j.neuroscience.2004.02.028.