Summary of Ph.D. thesis

IMPACTS OF ENVIRONMENTALLY HARMFUL HALOGENATED HYDROCARBONS ON ADAPTATION MEDIATED BY NEUROENDOCRINE REGULATION

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List of abbreviations

5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytriptamin/serotonin
5-HT-erg	serotoninerg
A	adrenaline
A-erg	adrenerg
ACTH	adrenocorticotrop releasing hormone
ANOVA	analysis of variance
COMT	catechol-o-methyltransferase
CRH	corticotrop releasing hormone
DA	dopamine
DDT	dichloro-diphenyl trichloro-ethane
DMEM	Dulbecco's modified essential medium
EDC	endocrine disrupting chemicals
EDTA	ethylenediamine tetra-acetic acid (disodic salt)
EPM	elevated plus maze
FCS	foetal calf serum
GGT	gamma-glutamyl transpeptidaze
HCB	hexachlorobenzene
IARC	International Agency for Research on Cancer
LC	locus coeruleus
LDCV	large dense core vesicle
L-DOPA	L-dihydroxy-phenylalanine
MAO A	monoamine oxidase A
MAO B	monoamine oxidase B
NA	noradrenaline
NA-erg	noradrenerg
OF	open field
OT	oxytocin
OT-erg	oxytocinerg
PAH	policyclic aromatic hydrocarbons
PCB	polychlorinated biphenyls
POP	persistent organic pollutants
PVN	nucleus paraventricularis
RIA	radioimmunoassay
SCN	nucleus suprachiasmaticus
SGOT	serum glutamate oxalacetate transaminase
SGPT	serum glutamate piruvate transaminase
SON	nucleus supraopticus
SSRI	selective serotonin reuptake inhibitor
TCB	trichlorobenzene

VP	vasopressin
AVP	arginine vasopressin
VP-erg	vasopressinerg

Introduction

By homeostasis, living organisms maintain equilibrium with their environments, adapt themselves to the changing external and internal circumstances. Any current state of equilibrium can be interpreted as the change of biocommunication processes emerging under the effect of environmental parameters. Living organisms of effective adaptation abilities first show *neurogenic*, then *neuroendocrine*, *neuroendocrine-immune* and the currently known highest level of *psycho-neuroendocrine-immune* communication accommodation. Chemicals are widely used in industry and agriculture. These include, among others, substances resistant to degradation owing to their stable chemical structure and thereby representing a particularly high environmental risk – such as dichloro-diphenyl-trichloroethane (DDT), or hexachlorobenzene (HCB), for example, known as persistent organic pollutants (POP). The heterogeneous family of POPs are substances interfering with endocrine homeostasis and communication; these are identified as endocrine-disrupting chemicals (EDC). Studies on the impacts of EDC compounds on behaviour have shed light on information relating to sexual behaviour, emotional tone, motivation, communication, anxiety, aggression, dominance and forms of social behaviour. EDC compounds may activate or inhibit the endocrine system, thus the synthesis of hormones, their storage, release, transport and metabolism, as well as the receptor recognition and binding, and furthermore they may interfere in neuroendocrine processes affecting induced responses. In the central nervous system, the structures responsible for endocrine reactions and behavioural functions are common, and therefore hormones often exercise a direct impact on behaviour (and vice versa), or alternatively they may indirectly influence it via the modification of metabolism.

Objectives

(1) In the course of our work, from among EDC compounds and the group of aromatic halogenated hydrocarbons *ClB has been chosen as a model compound to use this represented group of compounds for calling the attention to the threat that may be conveyed by the modification of homeostatic systems*. By now, ClBs have been banned from agriculture, although are still in use in some developing countries. Their industrial use is restricted by exposure limits, imposed by law. ClB's most stabile lipophylic representative is HCB. From this xenobiotic group, there are derivatives of lower chlorine substitution and lipophylic properties present in our environment, while their chemical reactivity is higher than that of HCB.

(2) Living organisms are normally subject to several exposure harms, i.e. stress (effects) simultaneously, and therefore *we have also set the objective to develop and standardize a research protocol that is suitable for the examination of effects triggered by combined chemical loadings*. In our researches, we have tried to work out and standardize the examination model system for the combined effects of two chlorobenzene derivatives, HCB and TCB.

(3) In our studies, we endeavoured *to develop an* in vivo *treatment protocol for modelling chemical exposure*, in order to acquire data suitable for evaluating the preservation of the environmental adaptive potential maintained through accommodation.

(4) Our primary objective was to *evaluate the effects of combined ClB exposure on the neuroendocrine homeostatic control system* by studying the accommodative regulation mediated by the stress hormones OT, AVP, and ACTH.

(5) We endeavoured to *create an* in vitro *model for exploring stress hormone release inducible by the effects of combined exposure*, which

would be suitable also for the standardized monitoring of releasing processes.

(6) In view of the legal regulations pertaining to the limit on ClB exposure, we considered *ascertaining whether extremely low-dose combined ClB load induces changes in the neuroendocrine control of stress hormones* an important assignment.

(7) The hormones involved in neuroendocrine regulation, as well as by the biogenic amines implementing intercellular communication are essential determinants of behavioural patterns. We have investigated the *modifying effect of ClB exposure on OT and VP release mediated by biogenic amines (5-HT, NA).*

(8) We intended to ascertain, *whether the stress responses mediated by hormones and biogenic amines alter the rapid adaptation pattern – that is, animal behaviour.*

(9) Our objectives also included answering the question *whether any combined chlorobenzene treatment of much smaller dosage than the exposure dosage permitted in the relevant legal regulations causes measurable changes in the behaviour of test animals.*

(10) The analysis of our findings focused on identifying the homeostatic mechanisms potentially related to the changes detected in behavioural patterns and/or neuroendocrine regulation by the research protocols outlined above.

(11) Furthermore, we wanted to appraise any additional health risk potentially represented by EDCs, in view of the findings obtained with ClB as their model substance.

(12) Finally, by analyzing our results, we wanted to decide whether the amended regulations should apply to all EDC compounds characterized by or devoid of accumulation.

Methods

For the experiments, we have used male *(180–350 g weight, 6–8 weeks of age at the beginning of the experiments)* Wistar *(Charles River, Isaszeg, Hungary)* rats. The animals were kept under controlled circumstances (temperature, relative humidity, light/dark periods) in cages of 32×40×18 cm (maximum 4 individuals in each cage). The test animals were distributed to the treatment groups described in Section 3.3. The smaller male *(200–250 g weight, 4–6 weeks of age at the beginning of the experiments)* Wistar rats were kept separately, but under identical conditions, and ten used as intruders during the aggression tests. To the animals, food *(CRLT/N, Charles River, Hungary)* and drinking water were supplied *ad libitum*. The treatments were started after the two-week habituation of the animals to the given circumstances and the persons participating in the experiments. The researches conducted with the test animals were carried out with the permission of the Workplace Committee on Animal Research, University of Szeged, with proper respect to the legal regulations pertaining to laboratory test animals and experimental procedures.

Exposures and experimental groups

The animals were treated with the 1:1 mixture of hexachlorobenzene and 1,2,4-trichlorobenzene 1:1 (ClB). The dosing regimen (exposure stress) required to maintain a safe protocol had been determined beforehand in preliminary experiments. The ingestion route and administration through a gastric tube had proven the best for the purposes of the exposure protocol. Thus, the animals received ClB in 1-µg/kg (D2, D2-ClB groups) or 0.1-µg/kg doses (D1, D1-ClB groups) as 1-ml final volume, for 0, 30, 60, or 90 days. We had tested also alternative routes for implementing the exposure, but chose alimentary administration by a gastric tube eventually, as this

made standardizing the experiment possible. The following groups were established upon standardizing the treatment protocol:

1. ClB-treated groups: Ten animals per group and series underwent combined chlorobenzene exposure for 30 days (n=5-5, D1- or D2-ClB-30 groups), 60 days (n=5-5, D1- or D2-ClB-60 groups), or 90 days (n=5-5, D1- or D2-ClB-90 groups) in five series of experiments. Dosing was implemented by the oral route of exposure (which is most prevalent in the literature), according to an alimentary regimen and using a gastric tube.
2. Stressed control groups ('SC'): These animals were treated/stressed over 0 day, 30 days (n=10, SC-30 group), 60 days (n=10, SC-60 group), or 90 days (n=10, SC-90 group) by inserting an empty gastric tube.
3. Absolute control group ('control'): Untreated/unstressed animals (n=10).
4. Positive control group ('+C'): The animals (n=10) received the solvent of ClB (0.001% ethanol solution) as 1-ml boluses through a gastric tube, over 0, 30, 60, or 90 days.
5. Negative control group ('–C'): These animals (n=10) received 1-ml boluses of drinking water of known solute content through a gastric tube, over 0, 30, 60, or 90 days.

Experimental protocol: The animals underwent treatment for 0, 30, 60, or 90 days according to the group scheme set out in Section 3.3. Following treatments with ClB, we evaluated anxiety with the open field (OF) and the elevated plus maze (EPM) tests, and used the modified resident-intruder (RI) test to appraise aggressive behaviour among male animals. Upon concluding the behaviour tests, blood samples (anticoagulated and native) were taken from the animals following decapitation. From these samples, stress hormones (ACTH, OT, VP) were determined with the RIA (radioimmunoassay) or CLIA method. Serum levels of hepatic transferases

(indicators of liver function) such as glutamate oxalacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gamma glutamyl transpeptidase (γGT) were determined as verified markers of the toxicity of ClB exposure. The body weights of the animals, as well as the weights of major organs (liver, spleen, kidney, adrenal glands, etc.) were measured during and/or after treatment. The changes of weights and data from liver enzyme determinations were used to demonstrate the subtoxic effect of the ClB doses (D1, D2) administered.

We prepared primary neurohypophyseal (NH) and adenohypophyseal (AdH) cell cultures from the pituitary glands of treated animals. These monolayer cultures were used to study the real-time kinetics of ACTH, VP, and OT release. The presence of 5-HT and NA receptors, as well as of serotonin- and/or norepinephrine-mediated VP and OT secretion were studied with RIA in NH cultures.

Behavioural tests: During the experiment, each animal was used only once in the specific behavioural tests. The tests were commenced under identical circumstances, after 1 hour of habituation to the room. The animals were involved in the examinations in the groups described in the protocol, but in a random order. The elements of the behaviour shown were recorded with the use of the ceiling-mounted video camera over the test apparatus and the connected behavioural software: Ethovision (v2.3, Noldus Information Technology, Wageningen, Netherlands).

Open Field (OF) test: The OF test is a standardized method for the appraisal of the locomotor activity, exploratory behaviour, curiosity, anxiety, and habituation characteristic of rodents.

Elevated plus maze (EPM) test: EPM is a standard method for evaluating anxiety in rodents. During the tests, the following factors were monitored by the computer and/or the persons conducting the study/follow-up control:

– total duration spent in open and closed arms – preferred area (percentage rate of times spent in the open area and in the apparatus) – number of entries to the zones – frequency and duration of prancing, cleaning, sniffing, freezing. The behaviour of the animals was examined for 3 minutes.

Testing of aggression among males with the resident-intruder (RI) test: In our experimental protocol modified RI tests were performed to study the aggression of animals. The resident animal was placed inside an arena covered with wood chips and left to habituate for 5 minutes. Locomotor/explorative behavioural elements as well as those suggestive of the anxiety of the resident were recorded during the test. After habituation, the intruder animal was placed into the same arena in the 6^{th} minute of the experiment. We observed the behaviour of the animals for 6 minutes.

Neurohypophyseal (NH) monolayer cell cultures: We removed the pituitary glands of the animals subjected to the predefined treatments under sterile conditions, and isolated the neurohypophysis using a dissecting microscope. Following the removal of membranes, pituicytes were separated enzymatically (digestion with trypsin 0.2% for 30 minutes, collagenase 50 μg/mL for 60 minutes, dispase 50 μg/mL for 60 minutes, DNase 30 μg/mL for 60 minutes) and then, mechanically (nylon-blutex filter with 80 μm pore size). The cell suspension was placed into a DMEM + 20% FCS + 30U/ml Pen-Strep culture medium, and then the viability of the cells was determined with the use of the Trypan blue test (≥ 95 %). Thereafter, the concentration of the cell suspension was set to 2×10^5 cell/ml. The final concentration of the cell suspension was adjusted to 2×10^5 cells/mL. The suspended cells were transferred to plastic growth chambers coated with 5% collagen. Adherent cell cultures were flushed with fresh culture medium every 3 days until growth to a confluent state. Then, NH cultures were standardized by determining the relative incidence of OT and VP

production, as well as by inducing specific and non-specific hormone release. Samples with a standard deviation not exceeding 5 to 7% were used to create the groups defined in the study protocol.

Adenohypophyseal (AdH) monolayer cell cultures: We removed the pituitary glands and isolated the AdH as described for NH cell cultures. Following the removal of the membranes, AdH cells were dispersed enzymatically (trypsin 0.2% for 30 minutes, collagenase 50 µg/mL for 40 min, dispase 50 µg/mL for 40 minutes, DNase 30 µg/mL for 20 minutes) and mechanically (nylon-blutex filter with 83 µm pore size). The cell suspension was placed in a culture medium containing DMEM, 10% FCS, and 10 U/mL Pen-Strep. Having checked the viability of cells with the trypan blue test (≥95%), the concentration of the cell suspension was adjusted to 2×10^5 cells/mL. The suspended cells were transferred to plastic growth chambers coated with 5% collagen. Adherent cell cultures were flushed with fresh culture medium every 3 to 5 days until growth to a confluent state. Then, cultures were standardized by determining the relative incidence of ACTH release, as well as by specific and non-specific stimulation (with arginine-vasopressin and 30 mM potassium, respectively). Samples exhibiting ACTH secretion with a standard deviation not exceeding 5 to 7% were used to create the study groups.

In vitro release studies: We replaced the supernatants of monolayer AdH and NH cell cultures with physiological culture medium (DMEM without FCS), and incubated the cultures at 37 °C in an atmosphere containing 5% carbon dioxide. In conformity with the *in vitro* study protocol, samples were obtained at 0.5, 10, 20, 30, 60, and 120 minutes into special plastic tubes, which were frozen immediately at -70 °C. These samples were used to determine ACTH from AdH cultures, as well as OT and VP hormones from NH cultures using RIA or CLIA methods. After registering the kinetics of

the basic hormone secretion, the cultures were washed again, and then kept for 12 hours under physiological circumstances. Then, the kinetics of non-specific hormone release were determined by adding 30 mM potassium. After a 12-hour period of rest, ACTH release was induced with arginine vasopressin, along with the secretion of VP and OT, respectively, with dopamine and histamine as specific agents. The kinetics of hormone secretion were also measured.

NH monoamine receptor studies: Under the established and standardized environmental conditions, cultures were washed first and then, incubated for 120 minutes. Following incubation, samples were obtained from the supernatants and stored at -70 °C, until the determination of VP and OT levels by RIA. Confluent cultures prepared from the animals (controls and ClB-treated) were incubated *in vitro* for 60 minutes in 10^{-6} M 5-HT or NA. At the end of the treatment period, the conditioned media of cell cultures were collected, frozen at -70 °C, and the concentrations of VP and OT released into the media were determined by RIA.

Determination of AVP, OT and ACTH from the supernatant medium: We determined VP and OT levels in the supernatant media of NH cell cultures using protocols as described in the literature. Prior to sampling, media had been conditioned in compliance with protocols for the cell cultures. The supernatant was removed from the cultures and stored at -70 °C until measurement. VP and OT were determined in the samples with a modified RIA method. As shown by the standard curves, the measurement range of the assay was 1 to 128 pg VP/OT, whereas its lower limit of sensitivity was 1 pg/assay tube. The protein content of the samples was determined with the modified method of Lowry. Our results are expressed per milligrams of protein content. ACTH was determined with RIA and CLIA methods, using the equipment (*Immulite 2000, Siemens Healthcare Diagnostic, Deerfield,*

IL, USA, and DPC kit) of the Clinical Endocrinology Unit of the 1st Department of Internal Medicine of the University of Szeged, Faculty of Medicine.

The determination of AVP, OT, and ACTH in serum: We collected the blood samples of decapitated animals into polystyrene tubes containing EDTA. Following centrifugation for 10 minutes at 8000 rpm at 4 °C, the plasma was removed and stored at -70 °C until measurement. The serum levels of VP, OT, and ACTH were measured with the RIA and LIA methods as described earlier.

Statistical analysis: We analyzed the data with v13.0 of the SPSS for Windows statistical software (*SPSS Incorporated, Chicago, IL, USA*). After the examination of the distributions, the data originating from the behavioural tests were analyzed with the use of the ANOVA method.

Summary of results:

(1) Starting on Day 60, the serum level of AVP increased progressively with both (D1 and D2) doses, and the difference vs. control reached statistical significance after 90 days of exposure.

(2) Compared to controls, the increase of serum OT level was statistically significant as early as after 60 days of exposure.

(3) Serum ACTH level increased after both doses. However, while the increase induced by the larger dose became significant after 30 days of exposure, the smaller dose achieved the same only after 90 days of exposure.

(4) In neurophyseal in cell cultures, the in vitro secretion of VP and OT increased in a time-dependent fashion after both (D1 and D2) D1 doses of ClB.

(5) Both 5-HT and NA significantly increased the release of VP and OT by neurohypophyseal cell cultures. Specifically, we found enhanced monoamine-mediated VP and OT release following the treatment of standardized cell cultures prepared from NH monolayer controls and ClB pre-treated systems with 10-6 M 5-HT or NA.

(6) (6) Our findings are in partial agreement with observations published in the literature, which include elevated serum VP level found in psychiatric disorders associated with anxiety or aggression. In our experiments, the quantity of active OT and VP increased depending on the extent of ClB accumulation – both in vitro and in vivo. This suggests that the actual ratio of OT and VP hormones might play a decisive role in behavioural changes. Chronic exposure to ClB administered at two dose levels (D1 and D2) for 30, 60, and 90 days altered the behaviour of the animals – that is, increased both anxiety (as shown by OF and EPM tests) and aggression (as demonstrated by ERI tests) [1-2].

Based on the findings, we grouped our results and conclusions around the topics defined in the objectives of this research.

Owing to their chemical nature, physical-chemical properties, known biological actions (EDC effects), and social implications (chemicalization, social activities, xenobiotic character) ClB compounds are ubiquitous substances [6-9]. We demonstrated their presence in all levels of evolution representing a hierarchy of complexity. Therefore, according to the approach of environmental science, they are suitable for use in model studies of POP/EDC exposure. We intended to investigate multiple chemical exposures by selecting ClB compounds as the exposure agents. Although most exposure studies published in the literature evaluate responses to a single effect only, this is unrealistic, as all systems on Earth

(inanimate objects, living organisms, and the society) are subject to multiple exposures simultaneously. The research methodology studies required before investigating the effects of even the simplest forms of combined exposure led to the development of standardized models for evaluating the impact of exposure to several factors [5]. Methodology studies have thus accomplished the standardization of the experimental system by suitable exposure routes.

Our results with combined, extremely low-dose, long-term, pre-standardized ClB treatment simulating gastrointestinal exposure contribute novel information [3-4].

A. In Wistar rats, treatment with ClB administered through a gastric tube in extremely low, subtoxic doses (0.1 and 1 μg/kg body weight/day) was associated with changes in the serum levels of OT, VP, and ACTH – this confirmed the alteration of neuroendocrine regulation.

B. Additionally, we detected substantial changes in OT and VP release mediated by the monoamine (serotonin and norepinephrine) system suitable for the investigation of neuroendocrine regulation – such changes have not yet been reported from earlier studies.

C. In addition to performing *in vivo* studies, we confirmed the elevation of the serum level of the active fraction of the stress hormones in question (i.e. VP and OT) also by conducting *in vitro* experiments.

D. The ClB-induced enhancement of serotonin- and norepinephrine-mediated VP and OT release correlated with changes in the behavioural patterns of the laboratory animals used in the experimental models. Specifically, we observed a dose-related and time-dependent increase of anxiety and aggression.

E. The abnormalities detected in the neuroendocrine homeostatic control system manifested as behavioural changes. Presenting the results at the

mechanistic level, we have suggested that the ratio of the active forms of OT and VP may have a role in the control of these behaviours. The protocol of our study has contributed data appropriate for confirming this suggestion.

F. The properties of EDCs present in the environment are comparable to those of ClBs. As shown by our results, these may be regarded as significant chemical and biological risk factors.

- These substances represent a chemical risk owing to their xenobiotic nature, represented by persistence, reactive and lipophilic properties, etc.
- They represent a biological risk in addition, owing to their chemical properties, as well as because of their dose-dependent effect, and influence on homeostatic regulatory systems. Aggregately, these result in the changes of behavioural and rapid adaptation patterns. Eventually, this might even increase the risk of psychiatric disease as far as human effects are concerned.

According to our findings obtained in an experimental model, the actions of POPs and EDCs – ubiquitous in the environment as components of the chemical load – as exposure agents generate changes in the homeostasis of biological systems even in extremely low doses, when acting on the long term. In biological systems, these lead to alterations in the potential for homeostatic adaptation, which in turn induce changes in rapid adaptation patterns and behaviour (involving cognitive processes and social behaviour etc.).

In view of our results, reviewing current legal regulations on the use of the POPs/EDCs studied seems justified.

List of publications (without abstracts) relevant to the subject

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