EXAMINATION OF ENDOCRINE DISRUPTOR EFFECTS IN NEUROENDOCRINE SYSTEMS, *IN VIVO* AND *IN VITRO*

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II. K. Sepp, A. Serester, Zs. Molnár, M. Radács, Zs. Valkusz, M. Gálfi: Environmental effect on thyroid dysfunction. In: Tünde, Alapi; István, Ilisz (szerk.) Proceedings of the 24th International Symposium on Analytical and Environmental Problems. Szeged, Magyarország: Szegedi Tudományegyetem, pp. 397-401. 5 p. (2018)

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III. K. Sepp, A. László; M. Radács; A. Serester; Zs. Valkusz; M. Gálfi; Zs. Molnár: The Hormone Exocytosis in Prolactinoma and Normal Adenohypophysis Cell Cultures by the Effects of Hypocalcaemia. Cell and developmental biology 6: 1 paper: 1000182, 7 p. (2017)

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Abbreviations

ACTH: adrenocorticotropic hormone

AdH: adenohypophysis

anti-TG: anti-thyroglobulin antibody

anti-TPO: anti-thyroid peroxidase antibody

AVP: 8-arginine vasopressin

ATA: American Thyroid Association

ATPase: adenosine triphosphate-ase enzyme

B: corticosterone

bw. kg: bodyweight kilogram

cAMP: 3'-5'cyclic adenosine monophosphate

ClB: chlorobenzene

dClB: 1,4-dichlorobenzene

DA: dopamine

DU: diuron

E: epinephrine

ED: endocrine disruptors

EDC: endocrine disruptor compounds

ELF-EMF: extreme low frequency and dose electromagnetic field

EMF: electromagnetic fields

ETA: European Thyroid Association

HA: histamine

5-HT: serotonin

IR: immunoreactive

LIA: luminescence immunoassay mClB: chlorobenzene mix (hexachlorobenzene+1,2,4-trichlorobenzene=1:1)

MU: monuron

NE: norepinephrine

NH: neurohypophysis

OT: oxytocin

P: propranolol

PU: phenuron

PRL: prolactin

PRLOMA: prolactinomas adenohypophysis

RBC: red blood cells

RIA: radioimmunoassay

SUSP: suspension

TSH: thyroid-stimulating hormone

wk: week

Examination of endocrine disruptor effects in neuroendocrine systems *in vivo* and *in vitro*

The effects of environmental loads (physical, chemical, biological) interfere with human homeostatic psycho-neuroendocrine-immune mechanisms. Clarifying the role of the triggered effects and their impact factors became an acute task for the 21st century. The aim of the present study was to investigate the effects of chemical (aromatic/halogenated hydrocarbons) and physical (extreme low frequency and dose electromagnetic fields: ELF-EMF) environmental loads as endocrine disruptors (ED). In addition, we have investigated the role of these factors in cell and individual exposure to clarify the mechanisms induced. We considered it particularly important to study the relationship between indirect and direct effects in cell transformation events associated with endocrine regulatory disorders.

In our work, we developed in vivo (Wistar rat chlorobenzene treatment through gastric tube; ♀ Wistar rats treatment by subcutaneous estrogen implantation; treatment of turkey with ELF-EMF), and in vitro (neurohypophysis, adenohypophysis monolayer cell culture) exposure models and standardized them for general viability and/or specific functional attractors (mechanism cycles).

The chemical agents tested were hexachlorobenzene: 1,2,4-trichlorobenzene = 1:1 (mClB); 1,4-dichlorobenzene (dClB); phenuron (PU), monuron (MU) and diuron (DU) as ED investigated for dose and time dependence). It has been found that said agents have ED effects on OT, AVP, ACTH and PRL release. Furthermore, we have determined the human toxicity potential (HTP) values for dClB in the chemical exposures studied.

We have demonstrated that ELF-EMF is a cellular ED which modifies the functions of cell membrane receptors (G proteins) involved in regulatory mechanisms. We developed an adenohypophyseal prolactinoma (PRLOMA) model by estrogen stimulation through a positive feed-back mechanism. We have experimentally demonstrated that the functional derangement of the PRLOMA-like adenohypophysis with ED agents often triggers a non-compensable event cascade when compared to normal cell function.

Based on our in vitro results, we also investigated the role of ED effects in the background of thyroid cell transformation disorders diagnosed in medical practice. We found a correlation between ED exposures and anti-TG or anti-TPO-labeled thyroid malignant tumors, respectively. Our findings reveal that mClB, PU, MU, DU, and ELF-EMF can be regarded as ED. In addition, we have demonstrated that chronic endocrine regulatory disorders may induce cell transformation so that the target cells of which show different behavior in their regulation compared to healthy cells. We have found a relationship between the real thyroid cell transformations (malignant tumors), the presence of anti-TG/anti-TPO markers and EDs in the medical histories of the patients.

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1. Introduction

1.1 The environment and the endocrine system

Over the last few decades, nearly 100,000 artificial molecules have been introduced into everyday use in our society through chemization, affecting the functionality of the psycho-neuro-endocrine-immune system, i.e. influencing the maintenance of homeostasis. This is an environmental burden through the use of soil, air, water, food and consumer products. Exposure should be explored in terms of duration, volume, cumulative risk for social, economic, geographical, occupational, genetic and health factors (1). While medicinal products for human use are subjected to extensive human *in vitro* and *in vivo* toxicity studies for the approval procedure, even limited human and/or eco-toxicological, etc. effects studies are seldom required for the application of already existing or newly synthesized chemicals, even though these substances may contaminate the environment directly as well as indirectly (2). During exposure, the concentrations of the chemicals as well as the duration of the effects and their presence in the human life cycle are all important factors. The most critical time-span is the prenatal period for the development of abnormal hormonal imprinting (early effect is more persistent), but the years of early childhood are also highly sensitive (3). Physiological and structural alterations induced by the chronic presence of specific chemicals, are the results of epigenetic changes, which, like mutations, may be passed on from one generation to the next (4). The resultant of the exposure to the given chemicals may be modified by the length of their biodegradation time, together with a combination of several external chemical factors, as well as the chronic presence of a low dose (5). The peripheral essential functions of the neuroendocrine system are predominantly mediated by the hormones of the adrenal cortex and the thyroid, any changes in their function may lead to essential pathophysiological events (6, 7). The endocrine system is involved in the coordination of cell functions not only through the direct effects of hormones, but also through ionic milieu, for example the regulation of calcium level by the parathyroid gland (8). Chemical agents are not the only culprits causing endocrine function disorders, changes of physical energy transmission (electromagnetic fields (EMF)) can do the same (9). The agents that generate endocrine disruption are called endocrine disruptors (ED). ED effects at the effectorial (cellular) level interfere with the physiological bases of the binding between the specific receptor and its hormone due to external environmental factors. By classifying the interfering effects mentioned, the following mechanisms can be distinguished according to the pathways involved in mediating the ED effects:

- the endocrine disruptor compounds (EDC) may bind to the receptor, which interferes with the normal transduction (e.g. in space, time), thus causing an atypical response.

An EMF factor can also cause similar results;

- the factors (chemicals, EMF) interacting with the receptor do not induce activity but inhibit physiological hormone binding, which consequently obstructs the endocrine response to be regulated;

- modifying the transport of specific hormones by interfering with the transport protein function (EDC, EMF effects);

- ED factors may interfere structurally and functionally with the enzyme pool of hormone synthesis (e.g. binding and/or biochemical transformation of specific hormones);

- ED effects may interfere with the expression of specific hormone receptors (5, 10, 11, 12, 13). The mechanisms outlined above may play a role in obesity, diabetes mellitus, cardiovascular diseases, male and female reproductive abnormalities, hormone sensitive tumors (in women: breast, ovarian, endometrial, in males: prostate), thyroid diseases, neuro-developmental disorders (14, 15).

1.2 The environment and the thyroid (diseases, cancer, medical history)

Environmental factors can alter thyroid function in many ways. Such a mechanism may be brought about through the modulation of the binding process of thyroid hormones and thyroid-stimulating hormone (TSH) to the receptor; formation of autoantibodies blocking thyroid function, inhibition of iodine uptake; interfering with binding to hormone transporting proteins, altering thyroid hormone synthesis; inhibition of deiodinase enzyme; or direct thyroid damaging effect (16, 17). The outlined factors appear not only in the development of various functional disorders (e.g. thyroid dysfunction, autoimmune thyroid diseases), but also in the development of a significantly increasing number of thyroid carcinomas in the last few decades (18). In an animal model, the decrease in thyroid hormone production and the consequently elevated level of TSH can lead to the development of thyroid tumors (hypertrophy, hyperplasia, adenoma, carcinoma) (19, 20). In humans, higher TSH levels may have also thyroid cell transformation inducing activity (21, 22) and there are data between the connection with autoimmune thyroid disease (Hashimoto’s thyroiditis) and thyroid cancer (23, 24). Recognizing the relationship between exposure to the environment and the illness it creates is not easy, because of the long latency (up to several decades), and therefore, thorough history taking during the medical

examination can provide important information from this aspect as well. Only few data are available for thyroid carcinomas associated with proven ED effect (25, 26).

1.3 Effects of Endocrine Disruptors (regulatory disturbances, ionic milieu, homeostasis)

Increasing number of physical and chemical burdens have triggered environmental health risks (27) and have become known as ED effects (chemical (EDC) and physical (EMF)). ED exposures generate changes at the level of the organism, affecting e.g. the human homeostatic complexity. They may alter the potential of the psycho-neuro-endocrine-immune regulation network (10), its capacity (28), and may bring about changes leading to regulatory disturbances. These effects may be a heavy homeostatic regulatory burden even in young healthy individuals, but in elderly healthy people they may cause changes really difficult to compensate for (29). The human adaptation patterns induced by environmental burdens are obviously more characteristic when the exposure takes place while some functional and/or structural disorder is also present (30). An open dynamic system (such as a living human organism) is able to maintain equilibrium with its direct environmental conditions by the aid of its genetic and functional adaptation potential (31). Hormones are creative elements of the neuroendocrine regulation in the human homeostasis (32). In neuroendocrine regulation, peripheral endocrine functions are maintained by neuronal and hormonal control (33). In the outlined context, human neuroendocrine regulation can be interpreted as a network of open, dynamic biological systems. This is accomplished by the human body via ensuring its essential processes, ionic milieu, pH balance, volume control, osmolality requirements and appropriate partial O_2/CO_2 tensions, etc. which are regulated parallel in **n** dimensions [- located in space and continuous in time -]. So if an ED effect disrupts the ionic balance (e.g. adrenal, pituitary, parathyroid disorders), other homeostatic regulatory elements need to change their processes with immediate compensatory mechanisms because maintaining homeostasis is sine qua non of healthy life and adaptation itself. In the maintenance of healthy, general cellular functions, the extracellular concentrations of the monovalent Na^+/K^+ and the bivalent of Ca^{2+}/Mg^{2+} cations may be of particular importance in ED effects (34, 35), as they have vital roles in the regulation of membrane potential, ionic balance, extra- and intracellular transductions and information transmission, energy balance, exocytosis, synthesis processes, etc. The study of effects in

connection with electric energy usage (EMF effects as ED effects) is markedly interesting, as this is essential in the use of energy for humans. The membrane potential of cells is controlled by the homeostatically maintained inequal distribution of ions. This is the electrical and magnetic space maintained in the living system which clearly changes when utilizing other forms of energy (extra environmental load) in EMF (which can be interpreted as electrosmog). In an altered EMF background, the orientation, movement of the dipoles, the expression and/or functional security of the receptors may undergo vast changes. All these can obviously generate ED effects (e.g. breast cancer, infertility) (36). Environmental burdens having ED effects on the neuroendocrine functions may affect the individual, their life functions, quality of life and characters of their progeny in subsequent generations (14).

2. Objectives

The problem area of endocrine disruption alluded to in the introduction suggests that today's medical science, including the challenges of endocrinology, has to face numerous environmental health risks. Disruption of the endocrine system, which actually affects the unity of the psycho-neuro-endocrine-immune system, may play a role in the development of many diseases. Thus, studying the changing environmental conditions in the living spaces provided by society and exploring the associations between exposures and consequent health problems can help us to reveal the pathomechanisms of certain systemic diseases. The most significant results of our data may be, however, that they should provide an aid in the formation of preventive measures.

2.1.

Endocrinological diseases connected to disturbances in cell transformation, particularly those leading to functional lesions in the thyroid (37) and pituitary (38), have become especially common. Thus, in the case of cell proliferative diseases of the thyroid (39) and the pituitary (40), it is a major health and therapeutic question whether the benign and/or malignant hypophysis and/or thyroid diseases should be investigated in connection with their pathogenic factors.

a, - In this work, one of the basic questions was whether EDC could cause a disease in the thyroid via disordered transformation processes. To find an answer, identification of the disease, its diagnostic classification and exploration of case histories became necessary;

b, - Whether ED effects could induce hypophysis cell transformation disturbances by generating endocrine regulatory disorders.

2.2.

The release of hormones into the blood flow through exocytosis provides active communication in the neuroendocrine regulation which is a prerequisite for maintaining a healthy homeostasis; the disruption of this process is a major pathogenetic factor (41). The questions arise whether hormone secretions do change due to ED effects in the neuro- and adenohypophysis; whether EDC cause adenohypophysis cell transformation; furthermore when reasonable ED effects can be detected, in which neuroendocrine diseases they may play a role; and finally whether the exploration of such pathomechanic factors have any significance from the viewpoint of environmental health.

a, - For this purpose, the clarification of the role of uron and halogenated hydrocarbon compounds used in agriculture has been the focus of our investigations in terms of EDC effects. These compounds exert continuous environmental exposure, not only via industrial and agricultural activities, but also through burdening the nutritional chain, and thus making them important from an environmental hygienic point of view.

b, - In the investigation of EMF effects on the cell membrane, the ED role was studied through the changes observed in membrane receptors. It was uncertain whether EMF could modify the functions of major monoamine receptors in the neuroendocrine regulation. Studying it through the disruption in the hormone secretion regulatory circuit (attractor) coupled with a given receptor function, obviously required a new research model.

3. Methods

A. Human studies

Characteristics of the patients

Gender distribution: 78% (266 persons) of the 341 thyroid carcinoma patients are female, 22% (75 persons) are male. The incidence of the disease is 3.5 times higher among women.

Age distribution: the average age of women is 58 years, the youngest being 20 years old, the oldest 87. The average age of men is 56 years, the youngest is 26, the oldest is 89 years old.

Histological distribution: occurrence of histological types of thyroid carcinomas: 188 papillary (55%), 58 follicular (17%), 35 papillo-follicular (10%), 27 medullary (8%), 18 unknown (5%), 9 Hürthle cell (3 %) and 6 anaplastic (2%). In women and men, the average age in papillary carcinoma patients is equally 56 years; 62 and 69 years for follicular carcinoma, 54 and 52 years for medullary, 74 and 64 for anaplastic tumors.

The risk of recurrence for differentiated thyroid carcinoma was classified by European Thyroid Association (ETA) and American Thyroid Association (ATA) methods (Table 1).

	1	**2**	**3**
ETA *2006 guidelines*	very low *the tumor is unifocal T1 (≤1 cm) N0M0 and there is no extension beyond the thyroid capsule*	low *the tumor is T1 (>1 cm) N0M0, or T2N0M0, or multifocal T1N0M0*	high *the tumor is any T3; any T4; any T with N1 or M1*
ATA *2009 guidelines*	low risk *no local or distant metastases; no tumor invasion of local regional tissues; no aggressive histology or vascular invasion*	intermediate risk *microscopic invasion of the tumor into the perithyroidal tissue; cervical lymph node metastases are present*;	high risk *macroscopic tumor invasion; incomplete tumor resection; distant metastasis; thyroglobulinemia*

Table 1 Risk classification systems for differentiated thyroid carcinoma

In the anamneses the effects of environmental (exposure to ED compounds) medic status were not examined by these classifications of thyroid differentiated carcinoma. In the endocrine regulation network, the linkage of TSH, anti-thyroglobulin antibody (anti-TG) and/or anti-thyroid

peroxidase antibody (anti-TPO) factors were not studied. In the present work, the diagnostic protocol was supplemented with environmental health issues in which we studied occupation, workplace, place of residence, number of electric devices inside and outside the home, plastic items and exposure to chemicals. We investigated (n=35) patients from the aspect of the relationship between the laboratory results (TSH, anti-TPO, anti-TG) and environmental health factors. Statistical analyses were carried out in SPSS (SPSS, Inc., Chicago, IL, USA, version 22.0) and in Statistical software (Statistica 9.0, Statsoft, Tulsa, OK).

Determination of hormone and antibodies

Whereas the usual microsomal antibody tests employ unpurified microsomes as antigen preparation, the anti-TPO tests use a purified peroxidase. The two procedures are of comparable output in terms of clinical sensitivity, but better lot-to-lot consistency and higher clinical specificity can be expected from anti-TPO tests due to the superior quality of the antigen used. Recombinant antigen and polyclonal anti-TPO antibodies are used in the Elecsys Anti-TPO assay. Measuring range is 5.00-600 IU/mL (defined by the lowest detection limit and the maximum of the master curve). Values below the lowest detection limit are reported as <5.00 IU/mL. Values above the measuring range are reported as >600 IU/mL.

The anti-TG determination is used as an aid in the detection of autoimmune thyroid diseases. The Elecsys Anti-TG assay uses human antigen and monoclonal human anti-TG antibodies. Measuring range is 10.0-4000 IU/mL (defined by the lowest detection limit and the maximum of the master curve). Values below the lowest detection limit are reported as <10.0 IU/mL. Values above the measuring range are reported as >4000 IU/mL.

The Elecsys TSH assay employs monoclonal antibodies specifically directed against human TSH. The antibodies labeled with ruthenium complex consist of a chimeric structure from human and mouse-specific components. As a result, interfering effects due to HAMA (human anti-mouse antibodies) are largely eliminated. Measuring range is 0.005-100 µIU/mL (defined by the lowest detection limit and the maximum of the master curve). The functional sensitivity is 0.014 µIU/mL. Values below the lowest detection limit are reported as <0.005 µIU/mL. Values above the measuring range are reported as >100 µIU/mL (or up to 1000 µIU/mL for 10-fold diluted samples).

TSH, anti-TPO and anti-TG were measured from serum using electrochemiluminescence immunoassay (ECLIA) on Modular E170 analyzer (Roche, Mannheim, Germany).

B. *In vivo* and *in vitro* experimental methods

I. Experimental models

1. Adenohypophysis (AdH) and prolactinomas adenohypophysis (PRLOMA) experiments

Female Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for hypophysis cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept at a controlled relative air humidity of 55-65% and 22±2°C ambient temperature. Experimental animals lived on an automated diurnal cycle (12 h dark and 12 h light) in groups of 10 animals for 6 months. Standard pellet food and tap water were available *ad libitum.*

2. AdH cell cultures

After pentobarbital anaesthesia (4.5 mg/bodyweight kilogram (bw. kg), Nembutal, Abbott, USA) the normal animals were decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2 % /Sigma, Germany/ for 30 min; collagenase /Sigma, Germany/: 30 μg/ml for 40 min; dispase /Sigma, Germany/: 50 μg/ml for 40 min in phosphate-buffered saline /PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (∅: 83 and 48 μm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2x10^5/cm^3$. The dissociated cells were placed onto 24-well plastic plates (5% collagen coated /Nunc., Germany/; Dulbecco's Modified Essential Medium /DMEM/ + 20% Fetal Calf Serum /FCS/ + antibiotics /Penicillin+Streptomycin: 1.0 IU/ml/). The cells were cultured at 37 °C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for prolactin (PRL) and adrenocorticotropic hormone (ACTH) release.

3. Induction of prolactinoma and PRLOMA cell culture

Female Wistar rats (n=20) were treated subcutaneously with estrone-acetate (CAS registry number: 901-93-9, Sigma, Germany; 150 μg/bw. kg/week) for 6 months to induce adenohypophyseal prolactinomas. After pentobarbital anaesthesia (4.5 mg/bw. kg, Nembutal, Abbott, USA) the treated (prolactinomas adenohypophysis: PRLOMA) animals were decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell

cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2 % /Sigma, Germany/ for 30 min; collagenase /Sigma, Germany/: 30 μg/ml for 40 min; dispase /Sigma, Germany/: 50 μg/ml for 40 min in phosphate-buffered saline /PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (Ø: 83 and 48 μm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2x10^5/cm^3$. The dissociated cells were placed onto 24-well plastic plates (5% collagen coated /Nunc., Germany/; Dulbecco's Modified Essential Medium /DMEM/ + 20% Fetal Calf Serum /FCS/ + antibiotics /Penicillin+Streptomycin: 1.0 IU/ml/). The cells were cultured at 37 °C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for PRL and ACTH release.

4. Neurohypophysis (NH) cell culture model

Male Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for hypophysis cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of the University of Szeged, Hungary. During the research period, rats were kept at a controlled relative air humidity of 55-65% and 22±2 °C ambient temperature. Experimental animals lived on an automated diurnal cycle (12 h darkness and 12 h light system) in groups of 10 animals. Standard pellet food and tap water were available *ad libitum*. After pentobarbital anaesthesia (4.5 mg/bw. kg, Nembutal, Abbott, USA) the animals were decapitated. NH tissues were separated under a preparative microscope. The NH tissue was digested enzymatically with 0.2% trypsin (Sigma, Germany) in phosphate-buffered saline for 60 min, and with 0.05% collagenase (Sigma, Germany) for an additional 60 min at 37 °C. The enzymatic hydrolysis was stopped by the addition of 100 μg/ml trypsin inhibitor (Sigma, Germany). Mechanical disintegration of the tissue was performed on nylon blutex sieves (pore sizes 100, 80 and 48 μm in series). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2x10^5$ cells/mL. The dispersed cells were placed onto 24-well plastic plates (Costar, USA) coated with 5% rat tail collagen (Sigma, Germany). The starting cell density was $2x10^5$ cells/mL of medium (Dulbecco's Modified Eagle's Medium; Sigma, Germany) supplemented with 20% foetal calf serum (Gibco, USA), 0.1 μg/L PenStrep (Sigma, Germany). The cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed every

3 days. The 14 day old neurohypophysis primary cell cultures were standardized by immunohistochemical methods (by relative incidence/unit plate-area of immunoreactive (IR) content oxytocin (OT) or 8-arginine vasopressin (AVP) cells), and the OT and AVP time-release kinetic activity was determined. After these procedures the basal OT and AVP levels were measured by radioimmunoassay (RIA) methods in the neurohypophysis cell cultures.

II. ED treatments

5. Investigation model for effects of change extracellular ionic milieu by AdH and PRLOMA cells

After functional standardization, the basal ACTH and PRL levels were determined in both normal primary adenohypophysis cell cultures and primary monolayer PRLOMA cell cultures (Tyrode's medium /Sigma, Germany/). In the medium, only the [Ca^{2+}] was modified; all other essential anions and cations were under homeostatic (e.g. isoionic) conditions. The hormone release of primary cell cultures was detected under hypocalcaemic conditions of varying degrees ([Ca^{2+}]: 0; 0.5; 1.0; 1.5 mM; n=12 in each group). Samples were taken at 10, 20, 30, 60 and 90 minutes after treatment to measure hormone kinetics. The PRL and the ACTH contents were detected in the supernatant media. ACTH and PRL were determined by luminescence immunoassay (LIA) and RIA methods (3.B.II.6). Protein content of the hormone releasing cells was assayed by a modified Lowry-method (42), and Pierce BCA Protein Assay Kit was used (Thermo Fisher Scientific Inc., Rockford, USA).

Statistical analysis

Repeated measurements of ACTH and PRL hormone release in different treatment groups on cell cultures (time points at 10, 20, 30, 60 and 90 minutes; n=12 in each group) were compared using marginal models (also known as population average models). Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied. In the case of ACTH, unstructured covariance matrix, for PRL data, the heterogeneous first order autoregressive covariance matrix resulted in the best fit among different structures (variance components, compound symmetry, first order autoregressive, toeplitz, unstructured and their heterogeneous versions), based on Akaike's information criterion (AIC) statistic. Pairwise comparisons were estimated by least squares means using Sidak p-value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions. In the case of extreme values, winsorization technique was applied by shifting the strongly outlying data toward the center to protect parameter estimation against the emergence of

unexpectedly large errors. Statistical analyses were performed in SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), where p-values of <0.05 were considered to indicate statistical significance (43).

6. Investigation of EDC Effects on AdH and PRLOMA Cell Culture Models

EDC treatment groups were set up with the primary monolayer AdH and PRLOMA. Functional standardization was performed to verify specific and non-specific ACTH hormone release functions. In the constant ionic concentration medium, non-specific hormone release was induced by +30 mM [K^+] for 20 min /control AdH sample: 1528 pg ACTH/mg protein (AdH); +30 mM [K^+] AdH sample: 3871 ACTH/mg protein (AdH); PRLOMA sample control: 2198 pg ACTH/mg protein (PRLOMA); +30 mM [K^+] PRLOMA sample: 5923 pg ACTH/mg protein (PRLOMA)/. Specific functional ACTH release control was regulated by 1μg/ml corticosterone (B) and 10^{-6} M AVP treatments.

Experimental protocol

For our experiments, the time and dose kinetic assays determining the effects of uron and chlorobenzene (ClB) derivatives were performed on standardized AdH and PRLOMA *in vitro* cultures. AdH and PRLOMA cell cultures were first treated for 60 minutes with 1,4-dichlorobenzene (dClB: [0.1 ng/ml]), chlorobenzene mix (mClB: [0.1 ng/ml]: hexachlorobenzene + 1,2,4-trichlorobenzene=1:1) and uron derivatives (phenuron [PU]: 10^{-6} M; monuron [MU]: 10^{-6} M; diuron [DU]: 10^{-6} M). At the end of the EDC treatment periods samples for hormone determination were obtained from the supernatant media of the cell cultures. Basal ACTH and PRL hormones release were determined. When studying the ACTH mediated role of EDC agents in the hypophysis/adrenal cortex regulation, the EDC agents were co-administered with AVP [10^{-6} M] and after the 60 minute treatment period, the samples were taken from the supernatant media of both AdH and PRLOMA for the determination of released ACTH. In the regulation cycle of AVP/corticosterone (B) feedback studies in Figure 1, ED were added together with B. AVP was administered after a 20 minute pre-treatment with EDC + B, and at the end of the treatment period, the supernatant media was sampled.

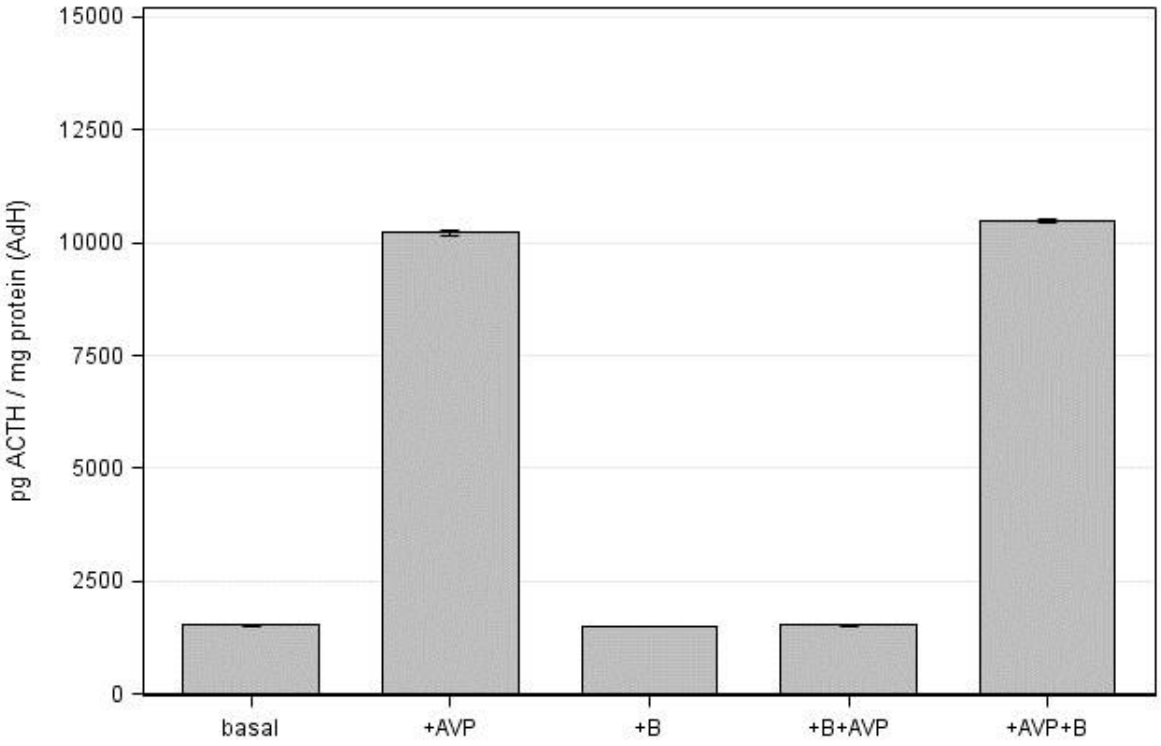

Figure 1 Functional assay for ACTH release in normal rat adenohypophysis cultures, *in vitro*

(n = 8-12 in each group); mean level ± standard error of the mean (SEM) (pg ACTH / mg protein): AVP significantly increases ($p<0.001$), whereas corticosterone alone does not alter ACTH release when compared to control; preincubation with + B inhibited AVP activation. (basal: control; AVP: 8-arginine vasopressin; B: corticosterone)

Statistical analysis – in AdH/PRLOMA EDC effect protocol

Measurements (n=8-12 per group on 24 lots: pooled samples on AdH cell cultures) of ACTH and PRL hormone release by disease (PRLOMA, AdH) in different EDC groups (Control, dClB, mClB, PU, MU, DU) by regulation (base, +AVP, +B, +B+AVP, +AVP+B) were compared using mixed models on rats (44). The regulation cycle was verified in a mixed model for the comparison of the control groups of EDC for ACTH in the 5 regulation phases, using disease and regulation as fixed effects and random intercept for the lots. For ACTH data a mixed model was applied with disease, EDC and regulation (only base, +AVP, and +B+AVP) as fixed factors and random intercept for the lots. For PRL measurements a mixed model was applied with disease and EDC as fixed factors and random intercept for the lots for basal regulation data. In the analysis models the reference group was the normal (healthy AdH) control (no EDC treatment) base (no regulation) group. Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied in all models with unstructured covariance matrix. Pairwise comparisons were estimated by least squares means

using Sidak p-value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions. Statistical analyses were performed in SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), where p-values of <0.05 were considered to indicate statistical significance (43).

Determination of hormone content and adenosine triphosphate (ATP)ase activity

The rat PRL RIA KIT (Institute of Isotopes Ltd., Budapest, Hungary) was used to determine the supernatant PRL content; all components were stored at 2-8 ºC, where they were stable. Non-specific binding, defined as the proportion of tracer bound in the absence of antibody, was determined to be <5%. The sensitivity of the RIA procedure was 0.07 ng/tube. The intra-assay precision obtained was 0.92 ± 0.03 ng. PRL data are given in ng PRL / mg protein. The Mg^{2+}-dependent ATPase activity was measured by the modified method of Martin and Dotty (62).

From the supernatant media, 500 µL samples were removed by Gilson pipette at appropriate times and stored at -80°C until LIA was performed. The ACTH levels of supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and Diagnostic Product Corporation kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK). ACTH data are given in pg ACTH / mg protein.

7. Treatment of Wistar rats with ED agents in vivo

In vivo treatment of Wistar male rats with mClB (dose of mClB: 0.1, 1.0 and 10 µg/bw. kg; n=10/group; duration of exposure: 0 day control, treatment: 30, 60 and 90 days) was made using a gastric tube, in 1ml of 0.015% ethanol in distilled water. In the control system, no treatment was used for absolute controls; in the stress controls, empty stomach tubes were used; in the case of negative controls, 1 ml of final drinking water was injected; in the positive controls, the solvent for ClB was injected in the same volume. In the experimental protocol, ACTH was determined from the serum samples (Immulite 2000 ACTH test kit, Siemens Healthcare Diagnostic, Deerfield, IL, USA). According to 3.B.I.2. cell cultures were made and the ACTH release was followed. There was no significant difference between the control group results when analyzing ACTH measured values, thus our results only show absolute control data. To the statistical analyse IBM SPSS Statistics, Version 21 programbag was used.

8. Investigation of ED Effects on NH Cultures

NH cell culture treatment protocol (Figure 2)

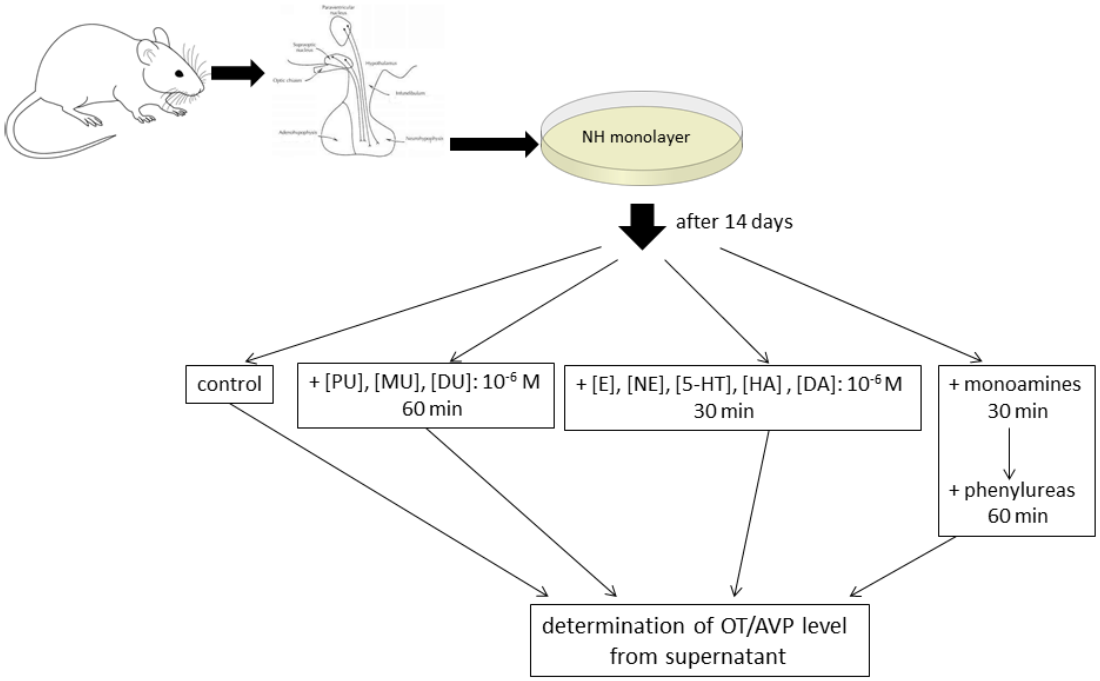

Figure 2 The *in vitro* treatment protocol

PU: phenuron, DU: diuron, MU: monuron, E: epinephrine, NE: norepinephrine, 5-HT: serotonin, HA: histamine, DA: dopamine, OT: oxytocin, AVP: 8-arginine vasopressin

The control samples were untreated and served as self-controls, which showed the basal release (60 min) of OT and AVP in NH. The effects of PU, MU and DU added to the NH for 60 minutes at a concentration of 10^{-6} M were examined one by one (45) /PU (CAS registry number: 101-42-8), DU (CAS registry number: 330-54-1) and MU (CAS registry number: 150-68-5) (Sigma, Germany)/. In our earlier studies dose dependent kinetics of phenylurea agents were determined, and the present experimental doses were selected in accordance with our previous results, because the saturation of receptor binding sites depends on the affinity and number of receptor binding molecules. Monoamine activated G-protein receptors in NH were treated with 10^{-6} M (epinephrine: E, norepinephrine: NE, serotonin: 5-HT, histamine: HA, dopamine: DA) (Figure 3) for 30 min (46, 47, 48, 49, 50) and the same concentration (10^{-6} M) of phenylurea agents.

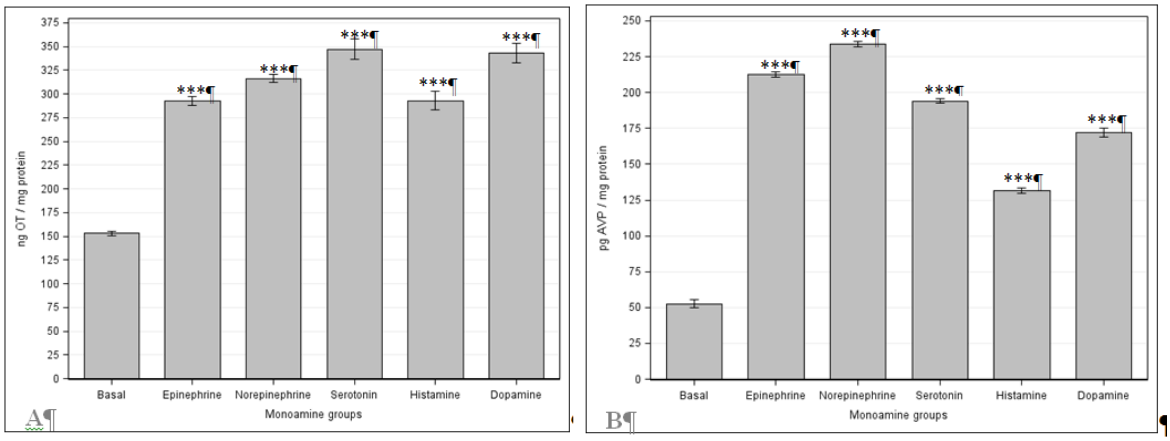

Figure 3 The effect of monamines on OT (3A) and AVP (3B) release in neurohypophysis cell cultures

Pairwise comparisons verified the regulation cycle: all monoamine 10^{-6} M groups increased significantly (***p<0.0001) compared to the basal regulation in control OT (n=12) and AVP (n=10). All data presented as mean±SEM

The combined treatment groups of the NH were treated firstly with monoaminergic compounds (for 30 min), then phenylurea compounds (for 60 min) (1. E+PU, NE+PU, 5-HT+PU, HA+PU, DA+PU; 2. E+MU, NE+MU, 5-HT+MU, HA+MU, DA+MU; 3. E+DU, NE+DU, 5-HT+DU, HA+DU, DA+DU).

The OT and the AVP contents were detected in the supernatant media. From the supernatant media, 500 µl samples were removed by Gilson pipette at appropriate times and stored at -80 °C until peptide RIA was performed to measure OT and AVP (51, 52).

A modified Lowry Method (42) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

Statistical analysis

Pooled samples of neurohypophysis cell cultures (12 lots) were measured for OT and AVP hormone release in different EDC groups (control, PU, MU, DU) by monoamine regulation (basal, E, NE, 5-HT, HA, DA) in rats (n=10 or 12 per group). Data were analyzed using mixed models (44, 53). The monoamine regulation cycle was verified in mixed models for the comparison of 6 monoamine levels in the control groups for OT and for AVP. In the random intercept model monoamine was used as the fixed factor and the lots as the intercept. For the two investigated hormone data (OT, AVP) mixed models were applied with EDC and monoamine as fixed effects and random intercept for the lots. In the analysis models the reference group was the control (no EDC treatment) basal (no monoamine) group.

Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied in all models with unstructured covariance matrix. Pairwise comparisons were estimated by least squares means using Sidak p-value adjustment. Model residuals were checked for normality assumptions. Statistical analyses were performed in SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), where p-values of <0.05 were considered to indicate statistical significance (43).

9. ED impact assessment of in vivo extremely low frequency and dose electromagnetic field (ELF-EMF)

Female adult turkeys (from state farm, 5000-5200 g) with veterinary certificate were used as the model system. The animal care and research protocols were in full accordance with the guidelines of the University of Szeged, Hungary.

Four animals served as control (absolute control (AC) – untreated; positive control (+C) – equipment was in standby mode; negative control (-C) – the machine was switched off; sham control (SC) – the bird went through the protocol without receiving any EMF exposure). Forty animals were treated with intermittent ELF-EMF. The animals were individually numbered.

Experimental Conditions

The turkeys were kept together during the experimental process except the treatment time. For the treatment, they were put separately in metal-free cages. At the beginning of the experiment, the animals were conditioned for 1 week (adaptation period) to eliminate the stress effects (possibly caused by the new surroundings, animal caregiver, food, etc.).

ELF-EMF Treatment

The treatment was performed using a special unit designed for the generation of pulsed ELF-EMF (8 ms energy exposure – 2 ms energy free pause; these were controlled by an electromagnetic equipment: ME3951A, Gigahertz Solutions, Germany). During treatment, the cages were covered by a 200 cm × 80 cm "magnetic blanket" that was operated by the Hungarian electric regulation (U=220 V, ν=50 Hz). The turkeys were exposed to ν=50 Hz, B=10 μT intermittent ELF-EMF treatment for 20 minutes every 8 hours for the 3 weeks of the experimental period.

In Vivo experiments (Figure 4a)

Monitoring of Behavior

The animals were regularly observed for physical activity (relaxation, play, behavior, competition, aggression) and food and water consumption (54).

Blood Samples

Heparinized blood samples were taken from the subclavian vein at the same time every week during the experiment. All measurements were performed from 4-6 technical parallels. Blood samples were separated by centrifugation for 5 min at 2000 rpm. After removing the blood plasma, the red blood cells (RBC) were washed 3 times with 0.9% saline solution. At the end of the procedure, the erythrocytes were suspended in Ringer solution buffered by 10 mM TRIS/HCl; pH 7.4 and hematocrit value was 20%.

Toxicological Monitoring from Turkey Blood-serum Enzyme Measurements

During the experiment, the following biochemical enzyme parameters were measured from the blood: serum glutamic oxaloacetic transaminase (SGOT) (55), serum glutamic pyruvic transaminase (SGPT) (56), alkaline phosphatase (AP) and gamma-glutamyl transpeptidase (γGT) with Dialab methods (Dialab Production, Austria). Lactate dehydrogenase (LDH) activity was also measured (55).

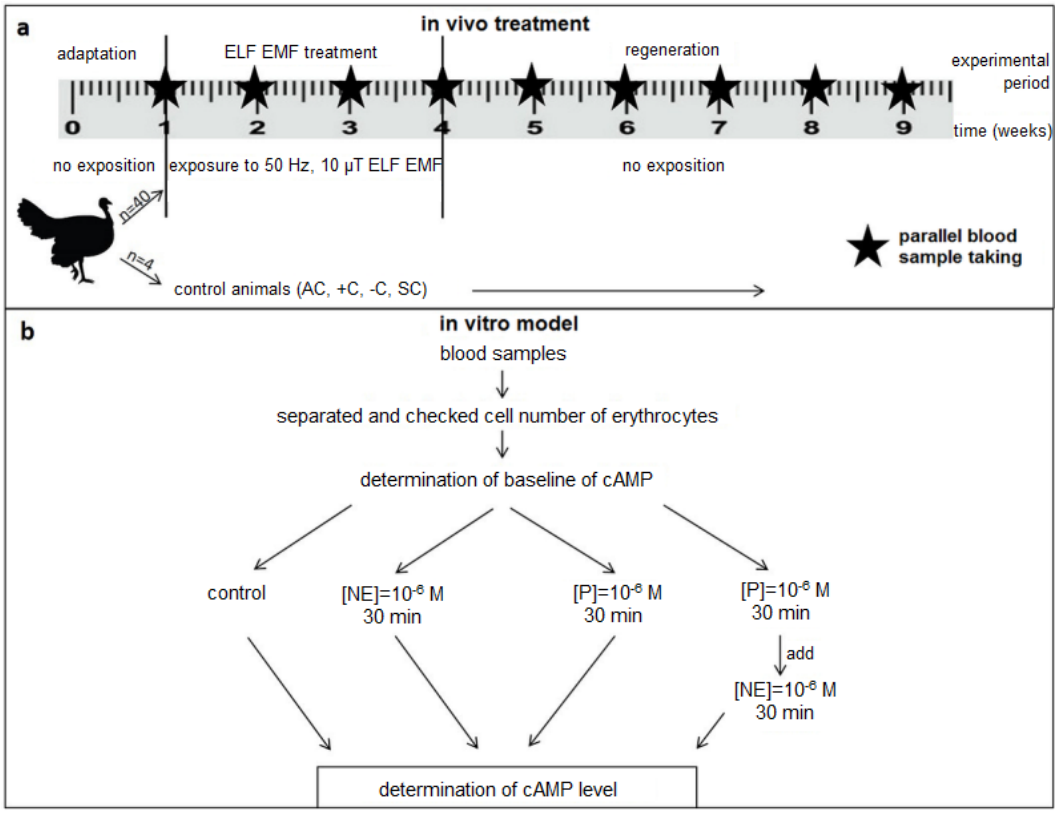

Figure 4 Structure of the ELF-EMF experimental model

In Vitro Experiments (Figure 4b)

Incubation Protocol

In the first *in vitro* experimental step, the baseline intracellular 3'-5'cyclic adenosine monophosphate (cAMP) was determined from all blood samples. For the study of the activation of β-adrenergic receptor, an agonist ([NE]=10^{-6} M; t=30 min) as well as an antagonist (propranolol (P): [P]=10^{-6} M; t=30 min) were used both separately and combined. Combination means that the agonist was added at a concentration of 10^{-6} M to the erythrocytes after a 30 min pre-incubation with the antagonist at a concentration of 10^{-6} M. In all procedures, the intracellular cAMP level was measured after incubation. (Figure 5)

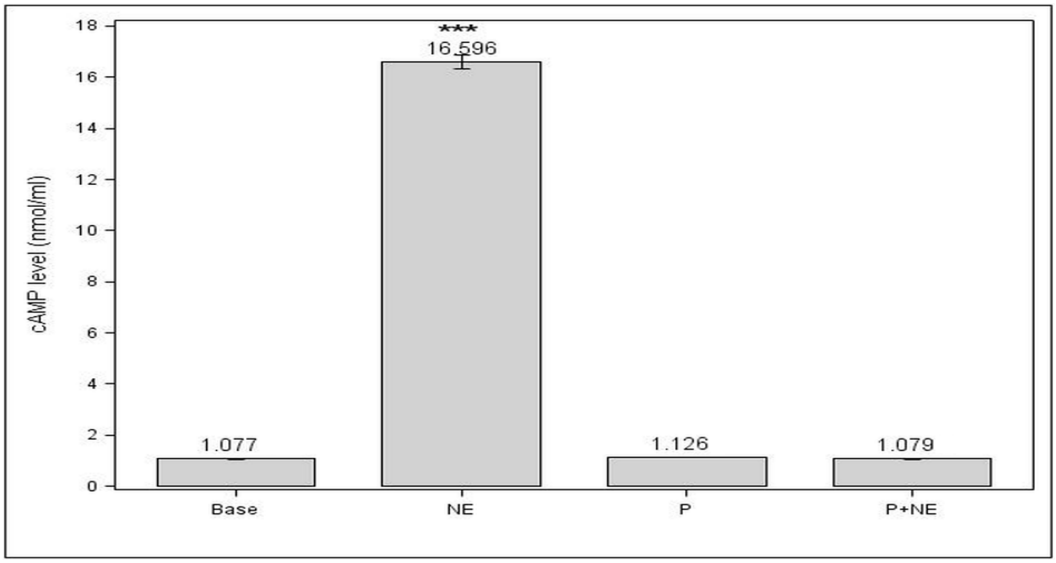

Figure 5 Verification of *in vitro* experimental model for NE-activated beta-adrenoceptor function at adaptation (week 1, no ELF-EMF exposure)

Mean (cAMP levels) ±SEM at adaptation (week: wk 1). Mean and SEM are calculated on n = 40 animals (treated group from wk 2). Notation: ELF, extremely low frequency and dose; EMF, electromagnetic field; cAMP: 3'-5'cyclic adenosine monophosphate; Base: baseline; NE: norepinephrine; P: propanolol; nmol/mL, nmol cAMP/mL RBC SUSP: RBC: red blood cells; SUSP: suspension. *** indicates difference between pairs of NE and all other groups with Type I error α=0.001.

Hemoglobin Determination: the hemoglobin was transformed into cyanohemoglobin, which was detected photometrically (λ=540 nm) (57).

Determination of cAMP Level: in vitro samples were taken from RBC suspension (SUSP) of turkeys. These were denatured at 100 °C for 3 minutes, and kept at -20 °C until further use. Before cAMP determination, the samples were defrosted and precipitated (at 0 °C, 1600 rpm, t=1 min). The cAMP content of samples was quantitated by competitive binding assay (Amersham cAMP Biotrak EIA system, GE Healthcare, UK).

Statistical Analysis

Means were calculated from technical replicates for each subject (experimental unit: turkey), and used for analyses of cAMP levels (as a marker of β-adrenoceptor function). Descriptive statistics (mean, standard deviation (SD), n, minimum, median, maximum) were calculated to identify distribution of cAMP levels by treatment groups and time points. To verify the β-adrenoceptor function in the *in vitro* experimental model during the adaptation period (week: wk 1), cAMP levels were compared between groups of base, NE, P and P+NE, based on linear mixed model using random intercept for the subjects (43, 53). Mean cAMP levels of n=40 animals (selected for ELF-EMF treatment from wk 2) and standard errors (SE)s are presented. To compare NE-activated β-adrenoceptor function of control animals (n=4) to the average cAMP levels of turkeys (n=40 and n=44) at the adaptation period (wk 1), one-sample t-tests were applied. 95% confidence intervallum (CI)s are presented with means and *P*-values (*P*). Effects of ELF-EMF treatment on NE-activated β-adrenoceptor function were analyzed using a linear mixed model where cAMP levels of the treatment period (wk 2-4) as repeated measures of treated animals (n=40) were compared to the cAMP levels at the adaptation period (wk 1). To estimate means in ELF-EMF treated and untreated groups over time in the whole experimental period (wk 1-9) considering between-subject differences and within-subject correlation, marginal model was applied using unstructured covariance structure (53). Differences of least squares means are calculated according to Sidak's adjustment. To characterize the reversible nature of NE-activated β-adrenoceptor function after ELF-EMF treatment by the rate of change in time, piecewise linear mixed model was used, which could describe the linear trajectories of cAMP levels in the treatment and in the regeneration periods (53). An intraclass correlation coefficient was calculated to describe variation. In marginal and mixed models, the restricted maximum likelihood estimation method was used with unstructured covariance structure. Model residuals were checked for normality. All statistical analyses were performed using SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), with Type I error α=0.05 (43).

4. Results

In connection with 2.1.a objective, screening of patients, their diagnostic and therapeutic approach were made with regard to the possible endocrine disruptor effects.

For better understanding the thyroid diseases, ED effects are to be revealed during the endocrinological examination of the patients, as they may be crucial in the regulation of metabolism in thyroid cell transformation. We examined 341 thyroid cancer patients whose therapeutic results are presented below.

These 341 patients were treated according to the therapeutic protocol as follows: 244 cases (72%) with total thyroidectomy, 44 patients (13%) with subtotal thyroidectomy and the remaining 53 patients (15%) had other types of surgery (lobectomy, debulking) or no surgery was performed. After surgical intervention, I-131 radioiodine therapy was applied in 199 (59%) patients, whereas 142 patients (41%) were not given I-131 treatment or information is missing in this respect. Among the radioiodine-treated patients, 66 (34%) had one, 59 (30%) two, and 30 (15%) received three doses of therapeutic radioiodine. In two cases extremely multiplied (ten or eleven) radioiodine treatments were needed. Average dose per treatment: ablative: 1850-3700 megabecquerel (MBq), therapeutic: 3700-7600 MBq. Eighteen patients (5%) were treated with external radiation. In the background of the increasing occurrence of thyroid tumors, we have started to clarify the role of ED effects by extended diagnostic procedures (biochemical markers and detailed case history data).

Table 2 shows that, in addition to the TSH values at the establishment of diagnosis, the elevations of anti-TG and/or anti-TPO levels demonstrated a relationship between malignant thyroid tumor and autoimmune thyroid disorder. Into the present ED effect study, the tabulated individuals were enrolled with markedly increased antibody levels (Table 3).

	data code	age	ATA	ETA	TSH (mIU/l)	anti-TG (IU/ml)	anti-TPO (IU/ml)
control					0,27-4,29	<115	<34
1	**AE**	29	1	2	4.67	**3298**	**>600**
2	**BA**	18	2	3	1.59	**1125**	**242**
3	BB	18	2	3	2.32	24.51	10.35
4	CSB	44	1	2	1.8	-	12.44
5	**CP**	66	2	3	2.44	**855**	-
6	DA	60	1	2	3.14	20.29	-
7	DI	64	1	2	2.46	34.1	-
8	DM	36	2	3	2.94	56.3	-
9	FI	38	1	2	3.32		23.59
10	HE	52	1	2	5.15	45.46	-
11	HL	76	1	2	1.30	21.55	8.24
12	HB	29	2	3	2.61	15.53	7.31
13	JA	23	1	2	0.85	<10.10	-
14	**KS**	43	2	3	3.28	**704**	-
15	**KG**	54	2	3	0.96	**238**	-
16	**KAN**	18	1	2	1.35	**304.40**	-
17	KI	59	1	2	0.72	28,72	-
18	MZS	50	1	2	1.38	46,16	-
19	**MA**	42	2	3	1,81	**367.80**	-
20	NN	32	1	2	1.11	-	17.87
21	NBA	22	1	2	0.97	18	-
22	**RV**	55	2	3	1.36	**458.3**	**78.39**
23	**SA**	35	2	3	11.13	**-**	**282**
24	SR	39	2	3	2.24	10.20	-
25	SZJ	61	2	3	0.45	-	8.65
26	SZI	77	1	2	0,68	10.47	-
27	SZT	27	1	2	1.52	22.48	-
28	TKM	40	1	2	1.44	38.4	-
29	TFP	48	1	2	1.05	19.71	-
30	TI	54	1	2	1.42	12.94	-
31	TT	21	1	2	0.80	-	10.76
32	TGYL	84	1	2	2.44	13.44	-
33	**VSG**	38	2	3	6.24	-	>**600**
34	VM	59	1	2	3.2	13.72	-
35	ZK	64	2	3	1.46	34.81	-

Table 2 Parameters and classification (ATA, ETA) of thyroid cancer patients (58, 59)

	data code	age	TSH (mIU/l)	anti-TG (IU/ml)	anti-TPO (IU/ml)	TSH/anti-TG x 10^{-6}	TSH/anti-TPO x 10^{-6}	environmental factors
control			0.27-4.20	<115	<34	<4.29/115	<4.29/34	
median						*0.0353475*	*0.057794*	
1	AE	29	4.67	3298	>600	**0.00141***	**< 0.007789***	9
2	BA	18	1.59	1125	242	**0.00141***	**0.00657***	8
5	CP	66	2.44	855	-	**0.002853***	**-**	8
14	KS	43	3.28	704	-	**0.004659***	**-**	8
15	KG	54	0.96	238	-	**0.004033***	**-**	9
16	KAN	18	1.35	304.40	-	**0.004434***	**-**	6
19	MA	42	1.81	367.80	-	**0.004921***	**-**	8
22	RV	55	1.36	458.3	78.39	**0.002967***	**0.017349***	7
23	SA	35	11.13	-	282	**-**	**0.039468***	8
33	VSG	38	6.24	-	>600	**-**	**< 0.0104***	6

TSH: thyroid-stimulating hormone; anti-TG: anti-thyroglobulin antibody; anti-TPO: anti-thyroid peroxidase antibody,
**p<0.01 relation to the median*

Table 3 Increased autoimmune parameters (anti-TG, anti-TPO) in thyroid cancer (58)

TSH data related to thyroid autoimmune parameters (TSH/anti-TPO, TSH/anti-TG) were always lower than the calculated median of the controls. These parameters were correlated with the presence of additional ED factors in the medical history.

The AVP-induced ACTH release of normal AdH cells can be inhibited by corticosterone (B) pretreatment *in vitro*. The basic ACTH release was significantly increased in the presence of 10^{-6}M AVP (60 min), which was prevented by (20 min) pre-administration of 1 µg / ml B prior to AVP (Figure 1). We also investigated the ACTH-release stimulating effect of AVP on experimentally produced PRLOMA cells, where a significantly increased ACTH-response was found when compared to normal AdH; increased basal ACTH release and increased AVP activated ACTH release (Figure 6).

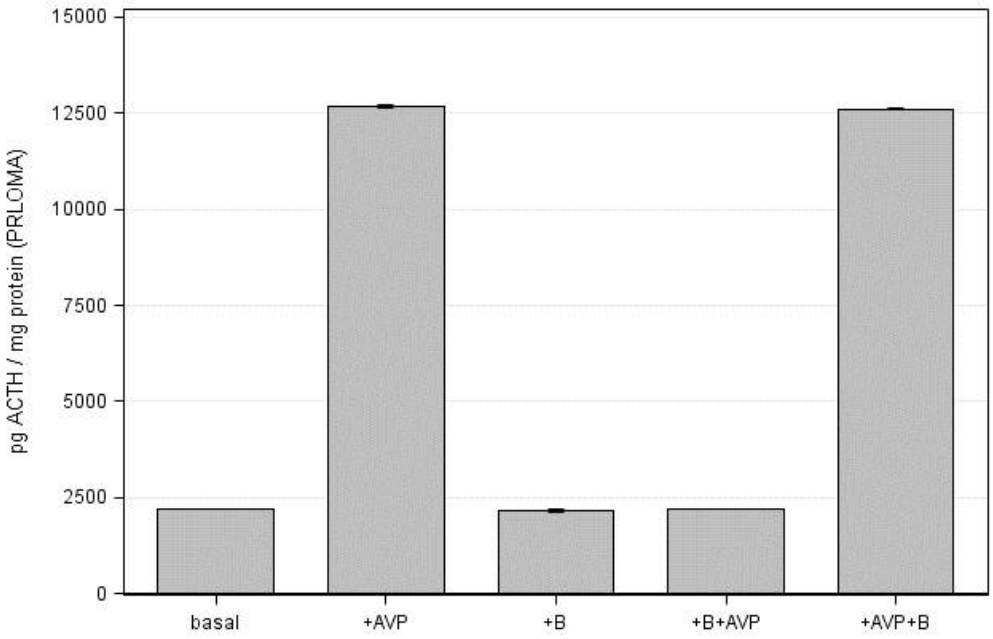

Figure 6 Functional assay for ACTH release in rat prolactinomas adenohypophysis cell cultures (PRLOMA), *in vitro*

(n=11-12 in each group); mean level ±SEM (pg ACTH/mg protein): Compared with normal AdH cells (Figure 1) ACTH release always shows a similarly significant ($p<0.001$) increase in PRLOMA cells. (basal: control; AVP: 8-arginine vasopressin; B: corticosterone) (45)

Transformed cells induced by disturbance of the endocrine regulatory cycle, PRLOMA cells, whose cell cultures showed a significant increase in PRL secretion compared to AdH cell cultures (Table 4).

	PRL release (ng PRL/mg protein)	
control	**normal AdH**	**PRLOMA**
	7.13±0.04	17.14±0.02

Table 4 Basal PRL release from normal AdH and PRLOMA cell cultures, *in vitro*
(mean level±SEM; n=12) (45)

Changes in the behavior of the cells of healthy adenohypophysis versus transformed prolactinomas adenohypophysis have also been examined at low extracellular calcium ion concentrations [Ca^{2+}]. Their membrane function was evaluated by exocytotic activity (ACTH, PRL). Figure 7 shows the ACTH release of both the normal AdH control systems and the treated groups. Our experiments revealed statistical differences between treated and untreated groups. In Figure 7 the ACTH content of the absolute control group in its supernatant media was compared with that of the groups treated with 0 and 0.5 mM [Ca^{2+}]. During this experiment, the ACTH release of the treated groups decreased appreciably when compared to controls. It

was found that at 60 and 90 min of the experiment the hormone content in the supernatant medium was higher at the 0 mM groups than at the 0.5 mM groups.

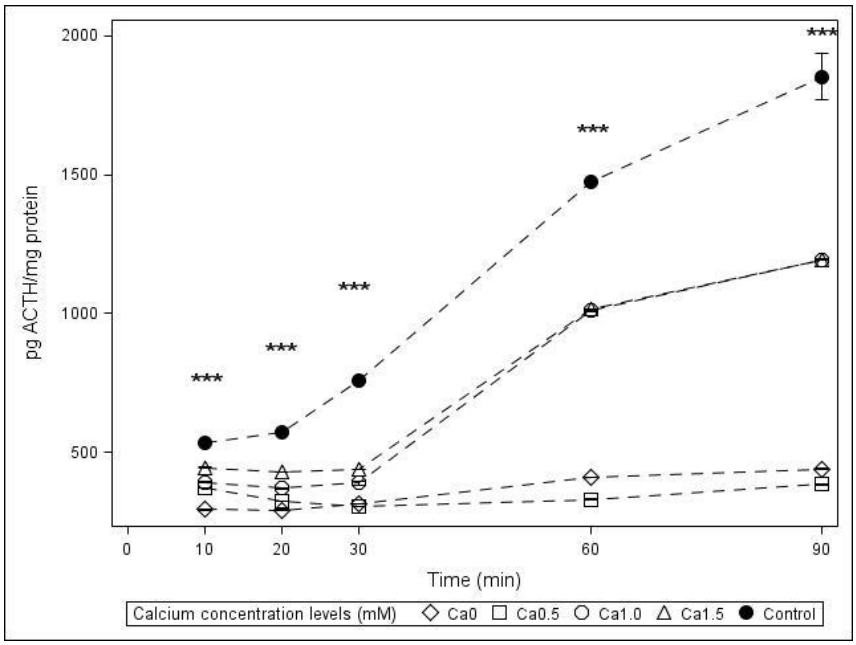

Figure 7 The effects of low Ca^{2+} milieu on ACTH release from AdH cell cultures

Cell cultures (n=12) were treated as follows [Ca^{2+}]: 0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken at 10, 20, 30, 60, 90 minutes. *** indicates the significant differences between the hormone release from various treated groups versus the ACTH release from normal AdH as the control group. The level of significance was chosen as $p<0.0001$. (60)

The basal PRL release from the control AdH systems and the treated primary cell cultures are represented in Figure 8. The hormone levels in the medium of cell cultures treated with 1.0 mM Ca^{2+} (Figure 8) were reduced significantly depending upon the duration of exposure, in contrast to the control groups. The hormone levels of the supernatant media in 1.5 mM Ca^{2+} treated groups decreased significantly. As shown in Figure 8, the PRL release was reduced significantly by the effects of 0 and 0.5 mM Ca^{2+} depending upon the duration of exposure. Figure 8 also shows that the levels of PRL were reduced significantly by the effects of 1.0 mM Ca^{2+} compared with the controls. PRL release at the cardinal points of the research protocol decreased significantly.

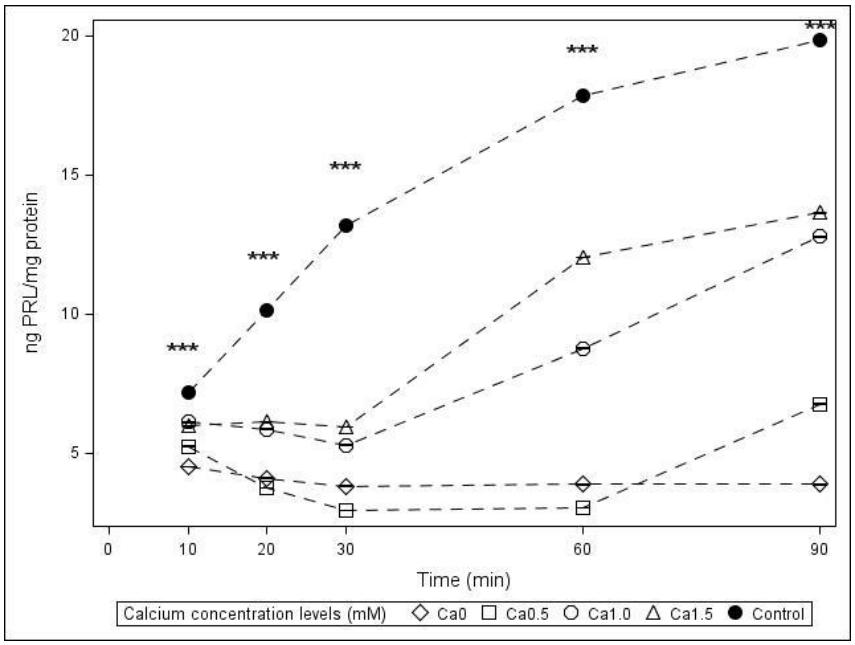

Figure 8 The effects of low Ca^{2+} milieu on the PRL release from adenohypophysis (AdH) monolayer cell cultures

Cell cultures (n=12) were treated as follows [Ca^{2+}]:0, 0.5, 1.0, 1.5 mM. After treatment, samples from the supernatant media were taken at 10, 20, 30, 60, 90 minutes. *** indicates the significant differences between the PRL release of various treated groups versus the PRL release of normal AdH as the control group. The level of significance was chosen as $p<0.0001$. (60)

Figure 9 shows the ACTH release from the PRLOMA cell culture systems: non-treated control and low Ca^{2+} treated PRLOMA groups.

As shown in Figure 9, at 10 min of the experiment the ACTH of PRLOMA cell cultures treated with 0 mM Ca^{2+} was enhanced compared with that of the control groups. In the supernatant media, the ACTH levels decreased significantly in both treated groups compared with the controls depending upon the duration of exposure. It was observed that the ACTH release of PRLOMA cell cultures treated with 0 mM Ca^{2+} increased significantly at 90 min of the experiment.

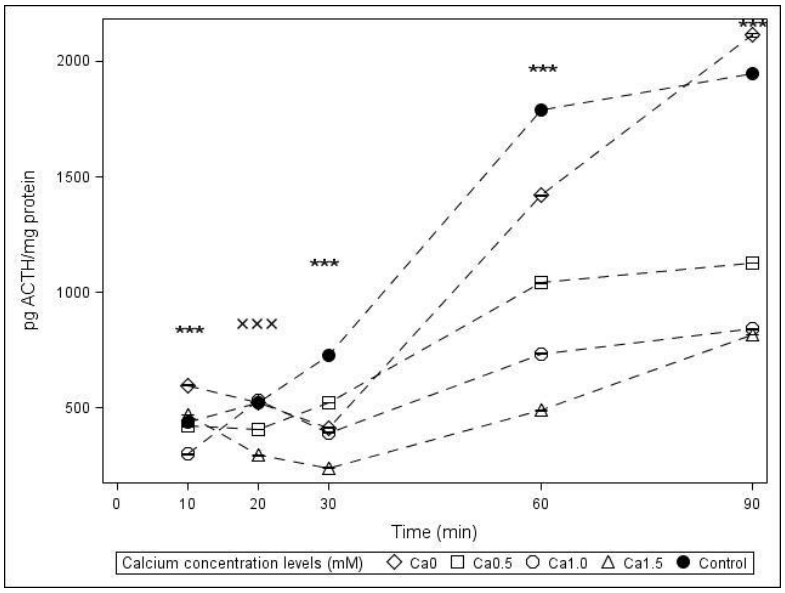

Figure 9 The effects of low Ca^{2+} milieu on the release of ACTH from PRLOMA cell cultures

Cell cultures (n=12) were treated as follows [Ca^{2+}]: 0, 0.5, 1.0, 1.5 mM. After treatment, samples from the supernatant media were taken at 10, 20, 30, 60, 90 minutes. *** indicates the significant differences between the ACTH release of various treated PRLOMA groups versus the ACTH release of PRLOMA control group. The level of significance was chosen as p<0.0001. **xxx** indicates that the control is not significantly different from the data of PRL release at 0mM [Ca^{2+}]. (60)

In Figure 10, it can be seen that the PRL release from PRLOMA cell cultures, under different hypocalcaemic conditions, decreased significantly depending upon the duration of exposure compared with the control group. Astonishingly, notable enhancement was detected in the PRL release from the groups treated with 1.5 mM Ca^{2+}. At 90 min of the experiment, the PRL content of the media increased significantly as a consequence of 0 mM Ca^{2+} against the control.

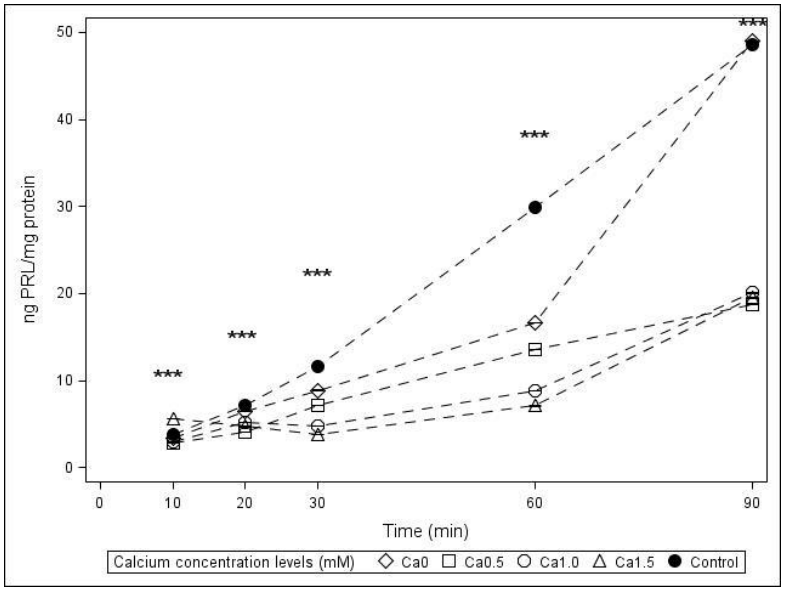

Figure 10 The effects of low Ca^{2+} milieu on the PRL release from PRLOMA cell cultures

Cell cultures (n=12) were treated as follows [Ca^{2+}]: 0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken at 10, 20, 30, 60, 90 minutes. *** indicates the significant differences between the PRL release of various treated PRLOMA groups versus the PRL release of PRLOMA control group. The level of significance was chosen as p<0.0001. (60)

According to objective 2.2.a, Figure 11 shows the effect of various ED compounds (dClB, mClB, PU, MU, DU) on ACTH release from AdH cultures under the following circumstances: basal, AVP activated (+AVP) and the corticosterone-inhibited AVP activation (+B +AVP) in AdH cultures. It can be seen that ACTH release was not altered by ED agents compared to the control group. In the AVP activated samples, ACTH release showed a significant increase compared to the control group even in the presence of each EDC. Under regulatory effects (+B +AVP-feedback), large deviations could not be detected even in the presence of each EDC.

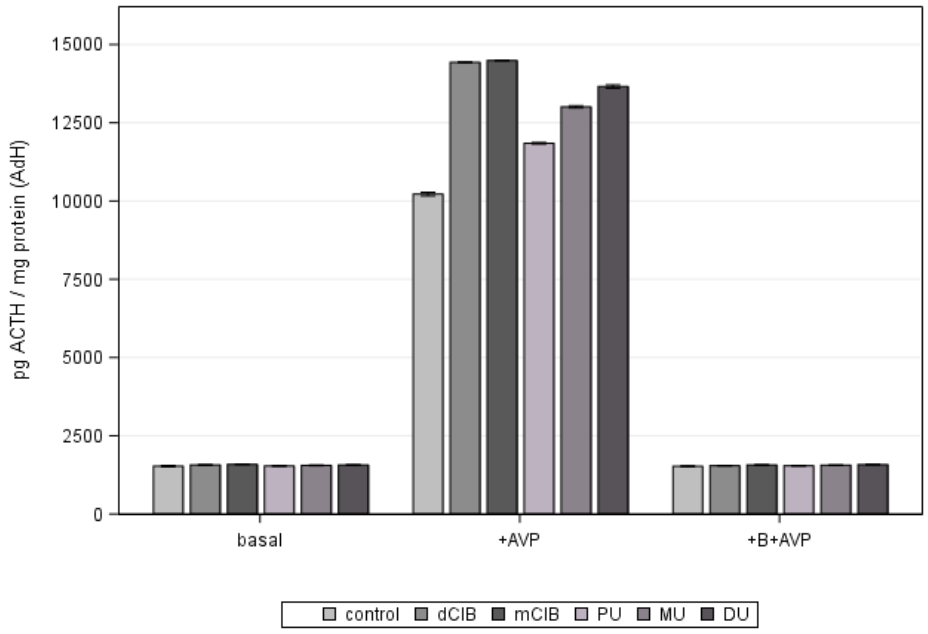

Figure 11 EDC effects in the regulation of ACTH release from normal rat AdH cultures, *in vitro*

mean (ACTH level) ± SEM; The mean and SEM are calculated from n=12. Abbreviations: corticosterone [B]: 1 µg/ml; [AVP] (8-arginine vasopressin): 10^{-6} M, + B + AVP: in combination therapy B precedes AVP administration by 20 minutes; [dClB] (1,4-dichlorobenzene): 0.1 ng/ml; [mClB] (chlorobenzene mix): 0.1 ng/ml]; [PU] (phenuron): 10^{-6} M; [MU] (monuron): 10^{-6} M; [DU] (diuron): 10^{-6} M; AdH: adenohypophysis; ACTH: adrenocorticotropic hormone. All EDC groups differ significantly from the control ($p<0.001$) for +AVP. (45)

Figure 12 shows the effects of dClB, mClB, PU, MU, DU on ACTH release from PRLOMA cultures under the following circumstances: basal, AVP activation (+AVP) and corticosterone inhibited AVP activation (+B+AVP). It can be seen that ED agents modulate ACTH release compared to the control basal group. AVP activated ACTH release from PRLOMA cultures shows a significant increase for each ED compound used. EDC effects were detected in the regulation model (+B+AVP-feedback) as well.

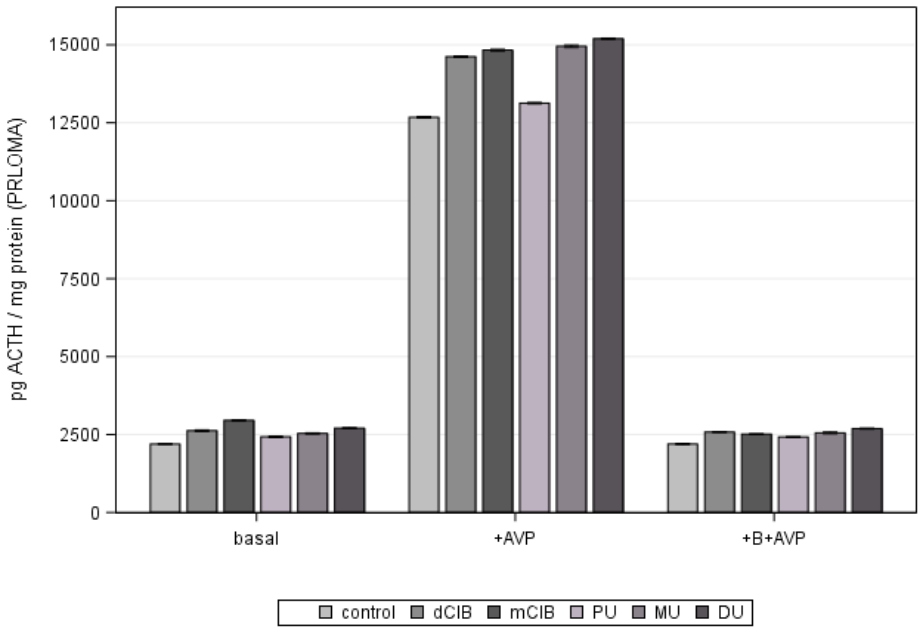

Figure 12 EDC effects on the regulation of ACTH release from rat PRLOMA cultures, *in vitro*

mean (ACTH level) ± SEM; The mean and SEM are calculated from n=12. Abbreviations: [corticosterone] [B]: 1 µg/ml]; [AVP] (8-arginine vasopressin) 10^{-6} M, +B+AVP: in combination therapy B precedes AVP by 20 minutes; [dClB] (1,4-dichlorobenzene): 0.1 ng/ml; [mClB] (chlorobenzene mix): 0.1 ng/ml; [PU] (phenuron): 10^{-6} M; [MU] (monuron): 10^{-6} M; [DU] (diuron): 10^{-6} M; PRLOMA: prolactinomas adenohypophysis ; ACTH: adrenocorticotropic hormone. All EDC groups differ significantly from the control ($p<0.001$) for basal, +AVP and +B +AVP. (45)

Figure 13 shows the PRL release from AdH cultures in the presence of the ED compounds tested. It can be seen that the ED compounds did not cause relevant differences in PRL release.

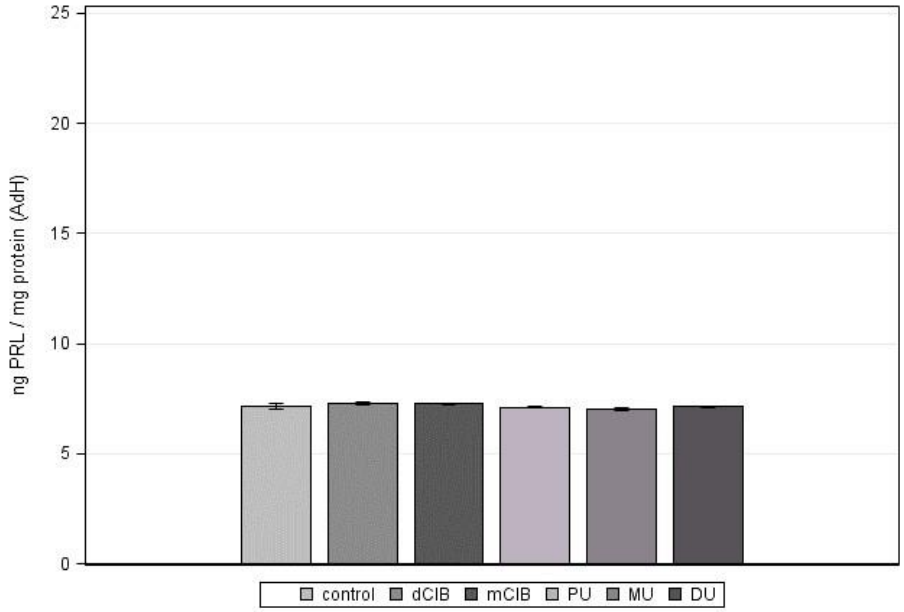

Figure 13 Effects of EDC on PRL release from normal rat AdH cultures, *in vitro*

mean (PRL level) ± SEM; The mean and SEM are calculated from n=12. Abbreviations: [dClB] (1,4-dichlorobenzene): 0.1 ng/ml; [mClB] (chlorobenzene mix): 0.1 ng/ml; [PU] (phenuron): 10^{-6} M; [MU] (monuron): 10^{-6} M; [DU] (diuron): 10^{-6} M; PRL: prolactin; AdH: adenohypophysis. (45)

Figure 14 shows the PRL release from rat PRLOMA cultures in the presence of ED chemical agents enumerated in our experimental protocol. All the examined compounds showed a significant stimulating effect on the release of PRL from PRLOMA cultures compared to that of the control.

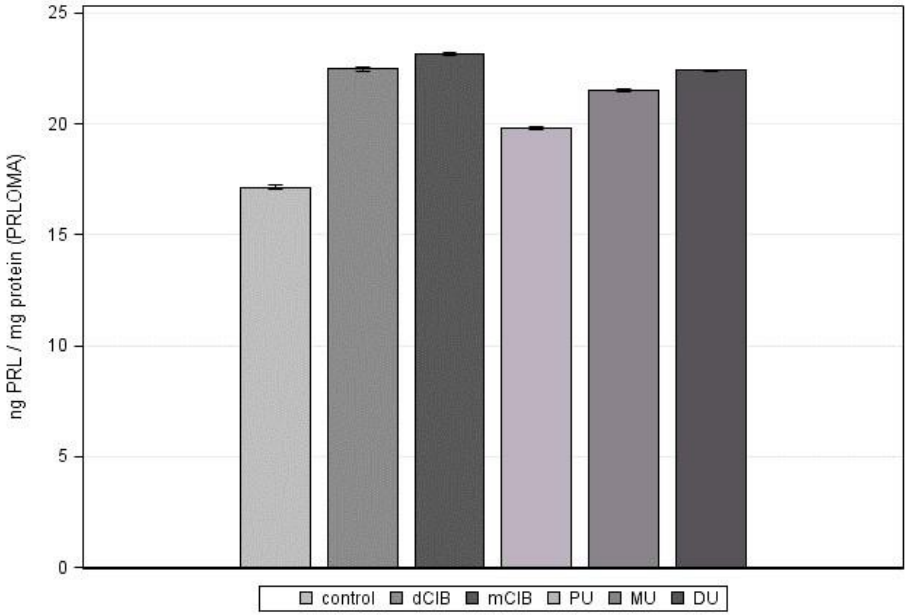

Figure 14 Effects of EDC on PRL release from rat PRLOMA cultures, *in vitro*
mean (PRL level)±SE; The mean and SEM are calculated from n=12. Abbreviations: [dClB] (1,4-dichlorobenzene): 0.1 ng/ml]; [mClB] (chlorobenzene mix): 0.1 ng/ml; [PU] (phenuron): 10^{-6} M; [MU] (monuron): 10^{-6} M; [DU] (diuron): 10^{-6} M; PRL: prolactin; AdH: adenohypophysis. All EDC groups differ significantly from the control ($p<0.001$) for basal release. (45)

We also studied the effects of uron ED on NH cell culture models by measuring oxytocin and AVP release as membrane function markers. OT and AVP release are indicators for the monoamine activated receptor cycle of neurohypophysis cells. The changing of these mechanisms was investigated using phenylurea agents (Figure 15 and Figure 16). E activated OT release (Figure 15 A) was significantly suppressed by PU and DU. Figure 15 B shows the effects of phenylureas on NE activation. MU and DU significantly reduced the effect of NE. OT exocytosis induced by 5-HT is shown on Figure 15 C. The applied phenylurea agents caused only a modest change in OT release from the NH. In the case of HA, only the combination with DU reduced the OT release significantly as seen in Figure 15 D. Figure 15 E shows OT release triggered by DA and again, only the combination with DU decreased it significantly.

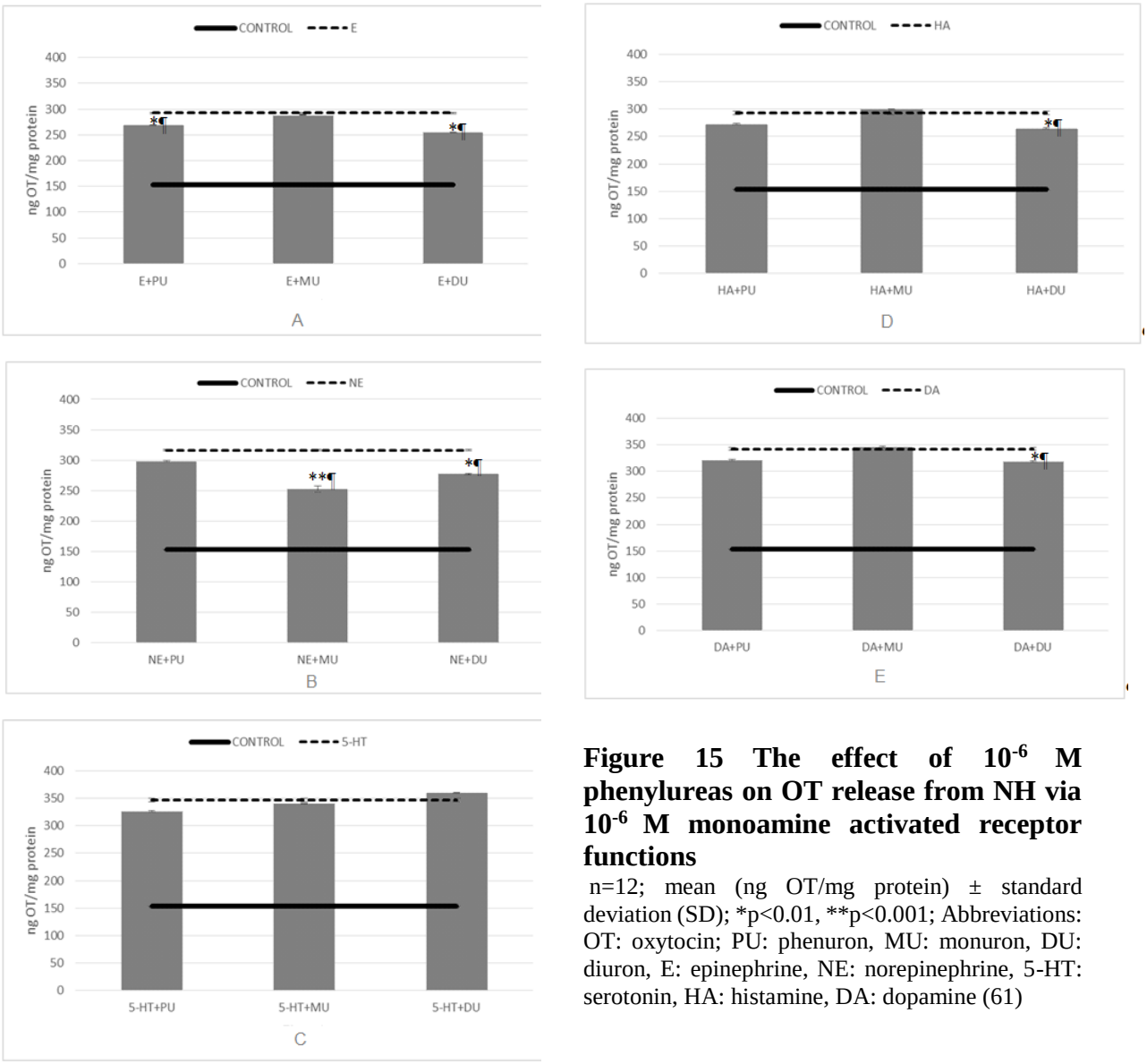

Figure 15 The effect of 10^{-6} M phenylureas on OT release from NH via 10^{-6} M monoamine activated receptor functions

n=12; mean (ng OT/mg protein) ± standard deviation (SD); *p<0.01, **p<0.001; Abbreviations: OT: oxytocin; PU: phenuron, MU: monuron, DU: diuron, E: epinephrine, NE: norepinephrine, 5-HT: serotonin, HA: histamine, DA: dopamine (61)

As to the AVP release, the E activation was modified significantly by PU, DU and MU (Figure 16 A). NE induced AVP release (Figure 16 B) was reduced by PU and DU. The stimulatory effects of 5-HT on the AVP release from NH cells (Figure 16 C) were decreased by PU and MU, but increased by DU. In Figure 16 D, it can be seen that the HA induced AVP release was diminished by PU and DU. The DA receptor mediated AVP release (Figure 16 E) from NH was significantly suppressed by PU, MU and DU.

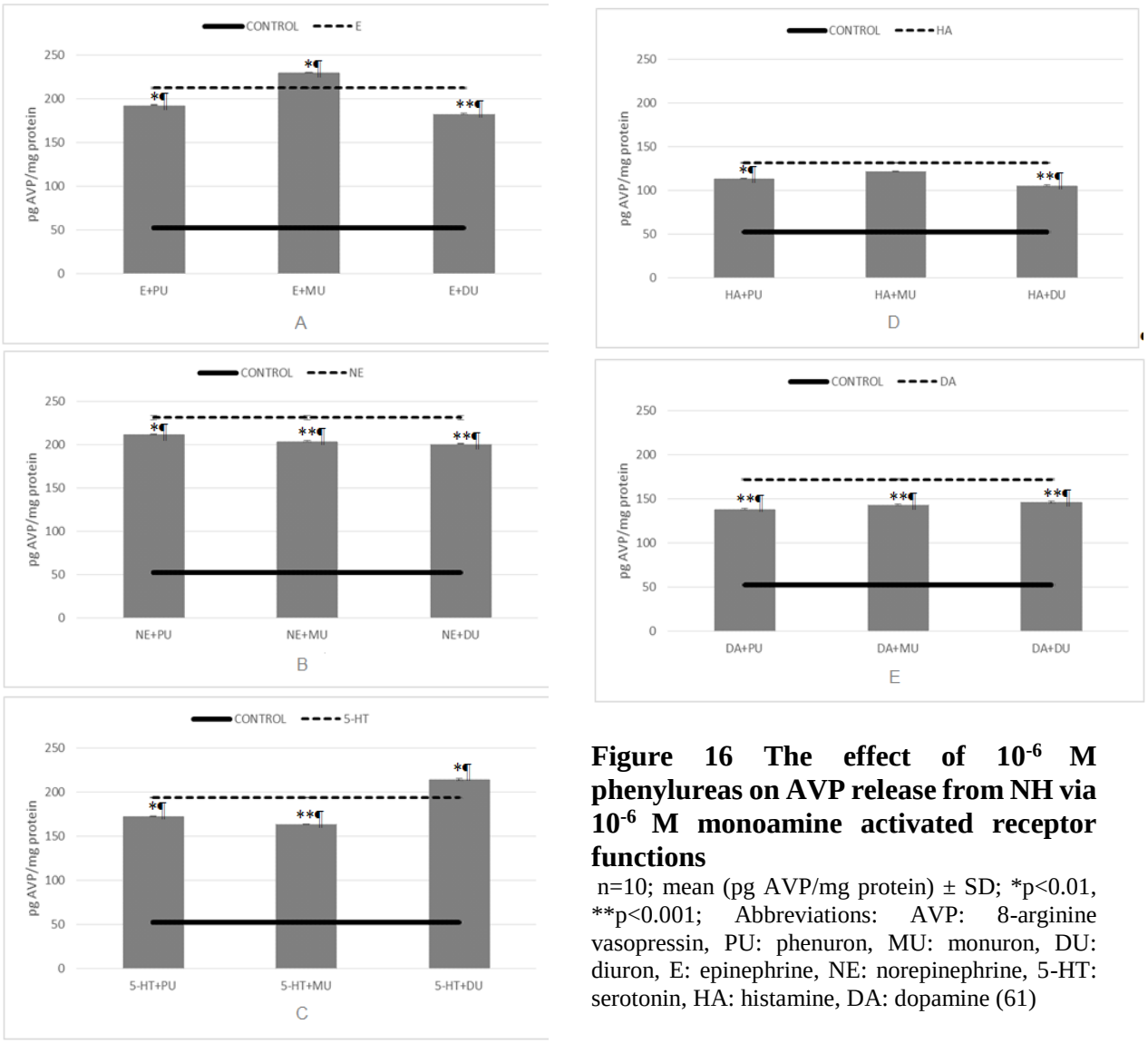

Figure 16 The effect of 10^{-6} M phenylureas on AVP release from NH via 10^{-6} M monoamine activated receptor functions

n=10; mean (pg AVP/mg protein) ± SD; *p<0.01, **p<0.001; Abbreviations: AVP: 8-arginine vasopressin, PU: phenuron, MU: monuron, DU: diuron, E: epinephrine, NE: norepinephrine, 5-HT: serotonin, HA: histamine, DA: dopamine (61)

The effects of polyhalogenated hydrocarbon chlorobenzenes (mClB, dClB) are particularly important in the determination of human toxicity potential in environmental toxicity studies. The endocrine effects of the duration of treatment and the exposure dose of the chlorobenzene mix were monitored by the ACTH release from the adenohypophysis into the blood serum collected from the *in vivo* rat treatment experiments (Figure 17) and from the supernatant medium of the *in vitro* AdH model (Figure 18). We also investigated whether the ClB mix modified the energy transfer (Mg^{2+}-dependent ATPase activity) necessary for the hormone release (Figure 19).

Our experiments revealed statistical differences between treated and untreated groups. In Figure 17, the serum ACTH concentration of the absolute control groups was compared to that of the groups treated with 0.1, 1.0 and 10.0 µg/bw. kg ClB. The serum ACTH levels of the groups

treated with 0.1 µg/bw. kg mClB increased appreciably compared with the control group. In the serum, the hormone concentration increased significantly after treatment with 1.0 µg/bw. kg ClB. Notable differences were detected between the hormone secretions of the control groups and those of the 10.0 µg/bw. kg manipulated groups.

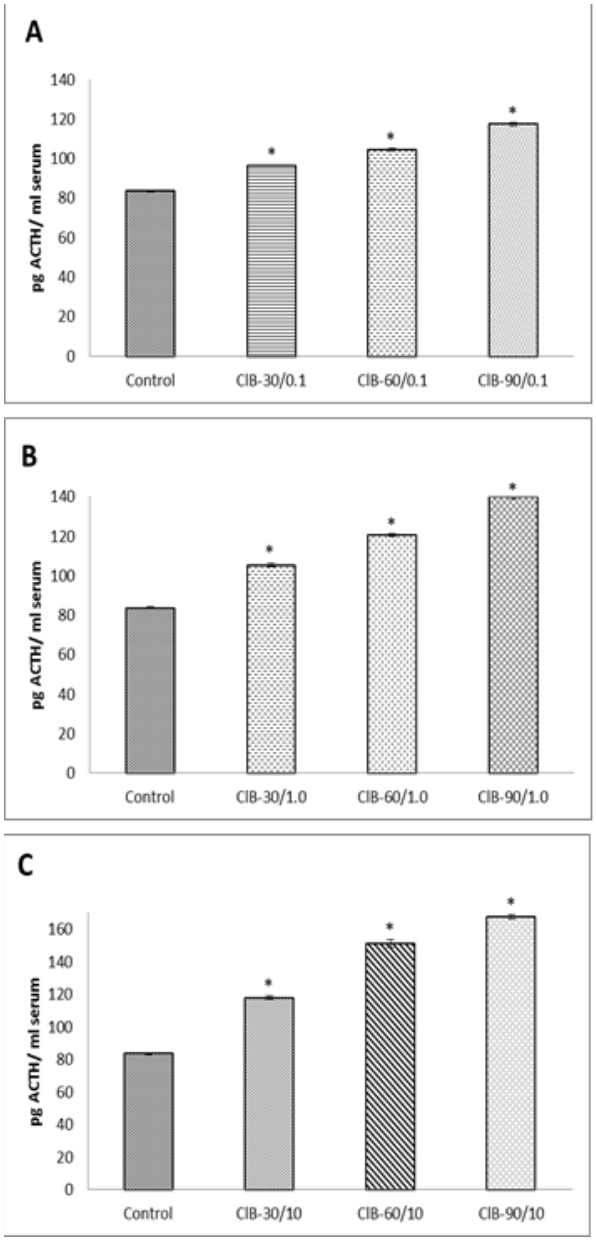

Figure 17 ACTH concentration of rat serum after *in vivo* exposition to chlorobenzene mix. A: Experimental groups (n=10) exposed to 0.1 µg/bw. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/bw. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/bw. kg dose of ClB. The asterisks indicate the statistically significant differences to control ($p<0.001$). ACTH: adrenocorticotropic hormone; ClB: chlorobenzene (62)

As shown in Figure 18, the ACTH release from cultured adenohypophysis increased significantly depending upon the dose and the duration of exposure. The release of ACTH increased significantly as a consequence of *in vivo* 0.1 µg/bw. kg mClB treatment compared with the control. The hormone levels of the supernatant media in 1.0 µg/bw. kg ClB treated groups showed an increasing tendency. As depicted in Figure 18, the hormone levels in the 10.0 µg/bw. kg ClB treated groups were elevated significantly compared with the control group.

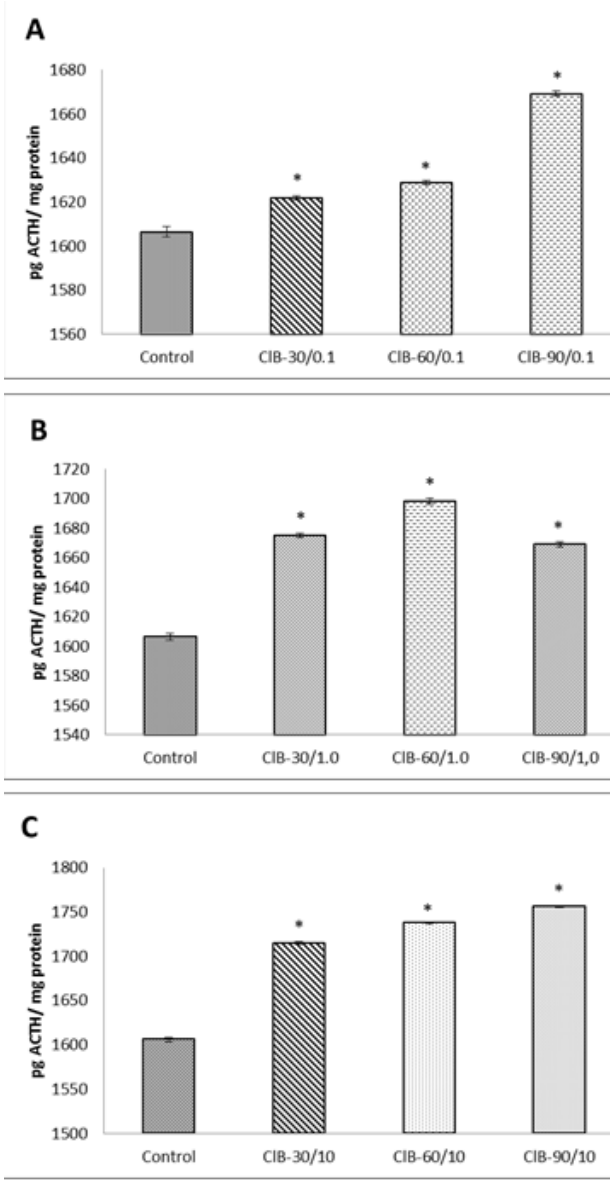

Figure 18 ACTH release from adenohypophysis cell cultures after *in vivo* exposition of the rats to chlorobenzene mix. A: Experimental groups (n=10) exposed to 0.1 µg/bw. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/bw. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/bw. kg dose of ClB. The asterisks indicate the statistically significant differences to control ($p<0.001$). ACTH: adrenocorticotropic hormone; ClB: chlorobenzene (62)

As shown in Figure 19, Mg^{2+}-ATPase activity was elevated significantly due to 0.1 µg/bw. kg mClB treatment compared with the control (100%). Notable enhancements were also observed in the enzyme activities after 1.0 µg/bw. kg and 10 µg/bw. kg ClB treatments.

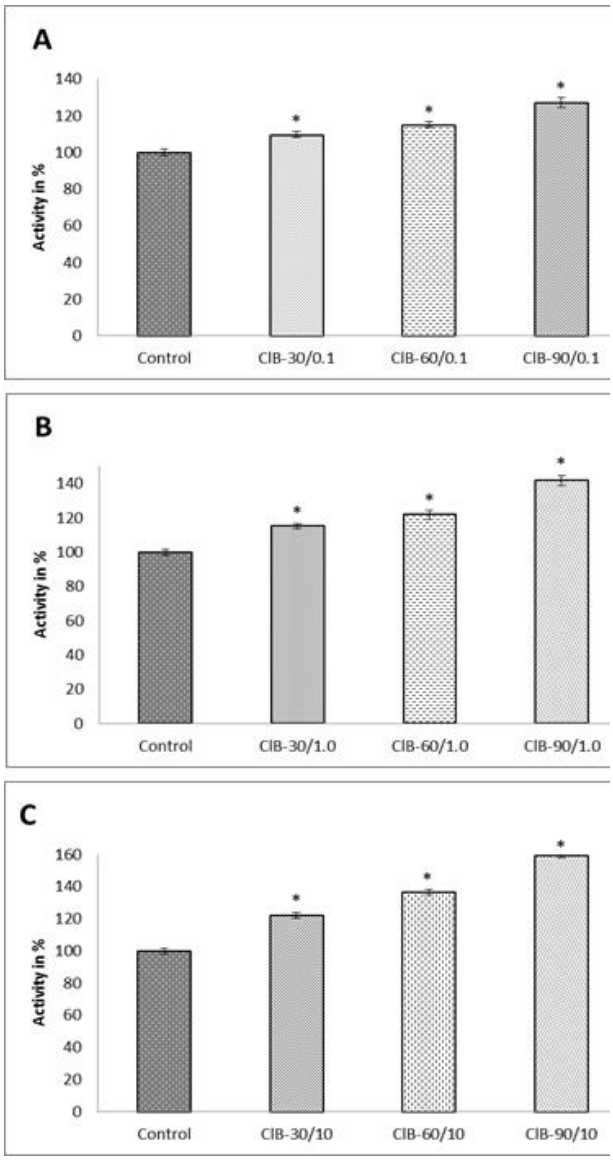

Figure 19 The effects of *in vivo* subtoxic doses of chlorobenzene mix on Mg^{2+}-dependent ATPase activity in rats

A: Experimental groups (n=10) exposed to 0.1 µg/bw. kg dose of ClB. B: Experimental groups exposed to 1.0 µg/bw. kg dose of ClB. C: Experimental groups exposed to 10.0 µg/bw. kg dose of ClB. The asterisks indicate the statistically significant differences to control (P<0.001). (62)

When studying the effects of ED, we also included the direct physical exposure to EMF and its action on membrane functions in connection to 2.2.b objective. As an environmental factor, we used EMF doses present in our daily life (low frequency, extreme low dose, chronic treatment, regular prions). The effects of of the EMF factors on the cell membrane were monitored by the β-adrenergic receptor cycle using *in vitro* red blood cell models after an *in vivo* exposure protocol. β-adrenergic receptor functions were monitored by measuring intracellular levels of the secondary messenger cAMP.

The effects of *in vivo* ELF-EMF exposure were detected by the *in vitro* β-adrenoceptor functional model. The effect of 10 µT ELF-EMF *in vivo* treatment on the NE-activated and β-adrenoceptor mediated intracellular level of cAMP is depicted on Figure 20. Compared to the

untreated response of the adaptation period, the β-adrenoceptor mediated cAMP levels decreased ($P < 0.001$) during the ELF-EMF treatment (Figure 20).

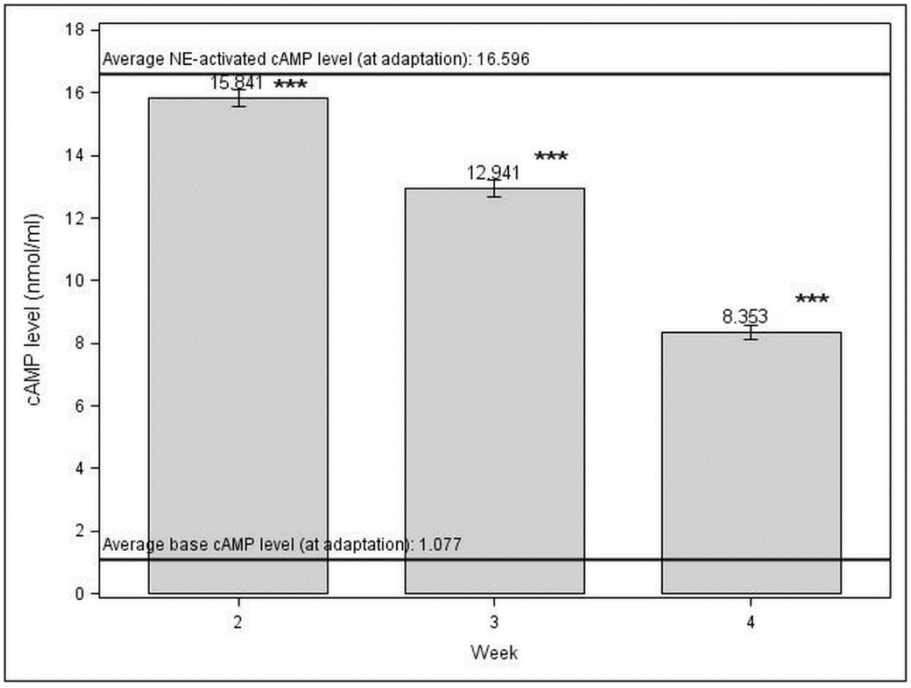

Figure 20 Effects of *in vivo* ELF-EMF treatment (n=40) **on the NE-activated β-adrenoceptor function**

Mean NE-activated cAMP levels ± SEM during treatment period (wk 2-4). Reference lines represent mean cAMP (nmol/ml RBC SUSP) level at the adaptation period (wk 1) for baseline (lower) and NE-activation (upper). ELF: extremely low frequency and dose, EMF: electromagnetic field, NE: norepinephrine, base: baseline, cAMP: 3'-5'cyclic adenosine monophosphate, RBC: red blood cell, SUSP: suspension. *** indicates significant difference from NE-activated cAMP level at the adaptation (wk 1) with Type I error α=0.001. (63)

Figure 21 shows the characterization of ELF-EMF treatment on NE-activated β-adrenoceptor functions in time. The effect of ELF-EMF was significant over time compared to the control group during weeks 3-5 measured in nmol cAMP/ml in RBC SUSP. The values of the control group did not change throughout the whole experiment (Figure 21)

The cAMP level of subjects in the ELF-EMF treated group had a decrease in the treatment period, while there was an increase in the regeneration period (Figure 21). At the intercept of the two fitted linear pieces (wk 4), the mean cAMP level was 9.6 nmol/ml RBC SUSP in the treated group, which was 7.1 nmol/ml RBC SUSP less than that of the control group (Figure 21). As 60% of the variation of cAMP levels comes from individual characteristics of turkeys, only 40% is from within subject effects.

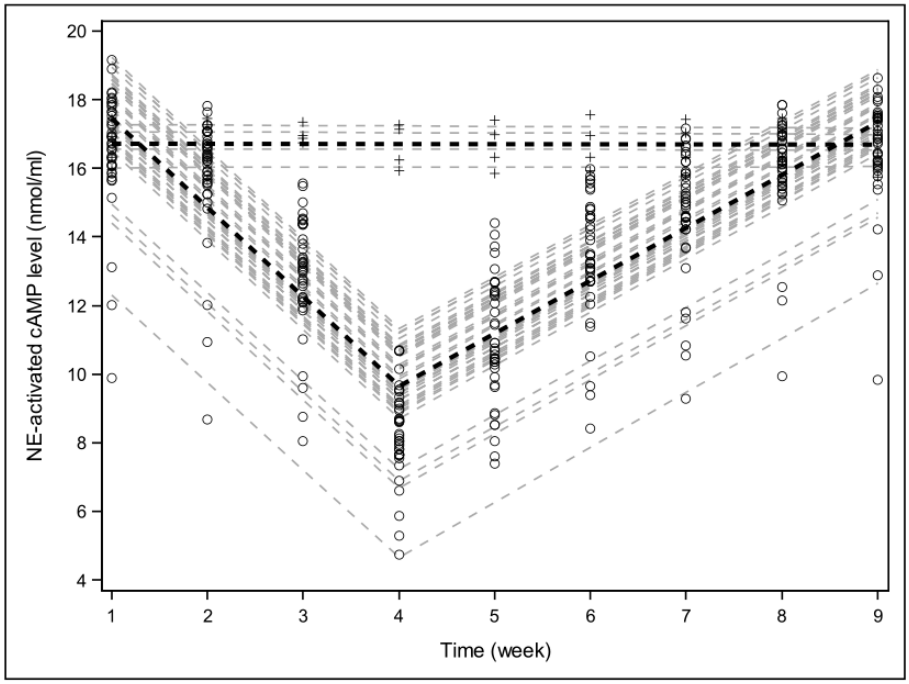

Figure 21 Effects of the complete ELF-EMF experiment on turkeys

Result of piecewise linear mixed model. Dashed thick, black lines represent predicted means. Dashed dark grey lines show individual trajectories. + control (n=4), ∘ treated (n=40) subjects, cAMP: 3'-5'cyclic adenosine monophosphate, NE: norepinephrine, nmol/ml: nmol cAMP/ml RBC SUSP, RBC: red blood cell, SUSP: suspension (63)

The Reversible Nature of the Biological Experimental Model

After the treatment with ELF-EMF, a major question was whether the biological system (NE-activated β adrenergic receptor function) was capable of returning to the starting state. Furthermore, a relevant question was whether, and if so, how this capacity was modified during the treatment. For this reason, the NE-activated β-adrenoceptor function was investigated weekly, over the regeneration period. The alterations of the β-adrenoceptor function are demonstrated during the experimental period (adaptation, ELF-EMF treatment, regeneration) on Figure 21. The measured NE-activated levels of cAMP were in compliance with the normal parameters at the end of the adaptation period. The piecewise linear mixed model revealed a decreased effectiveness of β-adrenoceptors, as the NE-activated cAMP level was getting lower and lower during the treatment with ELF EMF. After the end of the ELF-EMF treatment, the β-adrenoceptor functions returned to the starting out state in 5 weeks. As mentioned above, the recovery (increase) of β-adrenoceptor functions in the treated group was slower in the regeneration period (slope of cAMP: 1.5 nmol/ml RBC SUSP) as compared to the rate of decrease during the treatment period (Figure 21). The treated group did not differ from the control at the end of the regeneration period.

5.Discussion, conclusions

Objective 2.1.a

Nowadays, the functioning of societies generates numerous environmental burdens that represent a high health risk. Physical and/or chemical and/or biological environmental conditions alter the adaptive potentials of living systems, including those of higher genera, resulting in disturbances in their operation (64). In humans, the development of a number of diseases may be induced by changes in environmental conditions (65), which force the functioning of the human body to change. Biological system functioning disturbances caused by short-term, weak environmental effects are classified as reversible „small disturbances" by environmental science. Acute, but strong environmental effects, however, can alter the biological structure suddenly by causing malfunctioning; these are called „structural disturbances" (66). When a physician detects the result of an environmental effect causing a small disturbance in the human body, rapid and easily curable (with appropriate treatment) forms may be faced (e.g. sterile inflammation, infection). Therefore, these are reversible life sustaining process shifts (i. e. local characteristic changes). However, chronic, subtoxic, very low dose environmental exposure (lasting a period of several years) may lead to structural disturbances (e.g. cancer, metabolic disorders, cardiovascular diseases, mental disorders) (67). When subjected to chronic exposure, the living system (human organism) initially enters a period that can be followed by chronic inflammatory processes and it already can be interpreted as disease; further presence of the exposure forces the biological pattern to change its structure (e.g. cell transformation), and the disruption of cell division may be the consequence. At this point benign cell proliferations can be found which are relatively easily detectable (e.g. as a tumor by diagnostic imaging). If the environmental effect continues to persist, the biological pattern that has already been structurally disturbed may respond by turning malignant. The system state cycle, when this process stabilizes, will result in a previously unknown, both structurally and functionally new system state (68). In medical practice, these are interpreted as fixations of malignant diseases (69). Environmental exposures are classified as physical (e.g. radiation) and/or chemical (e.g. agents) and/or biological (e.g. infectious) stressors. Humans, as the evolutionarily most complex organisms, respond by making a shift in their homeostatic regulation. The optimal homeostatic system for humans is the psycho-neuro-endocrine-immunity, which is interpreted as health. When the attraction range changes, i.e. the environmental condition of a person's quality of life will be different, human adaptation patterns will also be modified, depending on the strength of the effectors and the duration of

the effect. From an environmental hygienic point of view, therefore, a new approach would be justified in medical practice as regards taking the medical history and in diagnostic approach.

Environmental loads that cause homeostatic disturbances can be identified as homeostatic disruptor (HD) effects; among them endocrine disruptor (ED) effects can be also found. In accordance with this classification, I have formed psycho-neurodisruptors (PNDs) (e.g. psychotropic substances) and immunological disruptors (IDs) (e.g. allergens), as sub-classes with direct effect area characteristics.

EDC can be found as pathogenic factors in endometriosis, obesity, type2 diabetes mellitus, cryptorchidism, male infertility, decreased testosterone levels, thyroid functional disorders, etc. Since the thyroid gland can also be viewed as a regulator of metabolic functions in the biological control of the human body, it is continuously exposed to environmental stressors (physical, chemical). In the elimination and neutralization of chemical agents, shifts in metabolic processes may generate inflammatory responses. Therefore, the demonstration of the thyroid autoimmune inflammatory processes (anti-TPO, anti-TG) has been proved useful for diagnostic purposes. Additionally, when medical case history is taken, environmental stressors are systematically displayed for working conditions, households, lifestyles, and so on, i.e. the possibility for contact contamination is explored (58). Results suggest (59) that thyroid carcinomas can be evaluated as irreversible structural disorders, the frequency of which has increased according to literature data in recent decades (70). However, according to further vertical studies (Table 2, 3) (58), all individuals with autoimmune inflammation and elevated anti-TG or anti-TPO levels continuously encountered an environmental stressor that can facilitate a malignant structural thyroid disorder. The increasing frequency of thyroid carcinomas may warrant screening for thyroid disease (by determining the possible effects of environmental risk factors and thyroid function tests complemented by autoimmune inflammatory factors: anti-TPO, anti-TG), similarly to the possible suggestion to evaluate the ED effects for breast cancer risk (71).

Objective 2.1.b

From the viewpoint of the ED effect, it is desirable to clarify the role of some effectors in the cell-transformation disorders by model experiments. For this purpose, an endocrine benign cell proliferation model was constructed with induced feed-back disturbance in rats. Specifically, adenohypophysis prolactinoma was induced by treatment with an estrogen mimetic for 26 weeks, with weekly subcutaneous implantation (estrone-acetate / CAS registry number: 901-

93-9, Sigma, Germany; 150 µg / bw. kg/week). The hormone release characteristics of the thus induced prolactinoma (PRLOMA) were studied. For this purpose, we have developed a model in which the functional response of endocrine cells already showing benign changes can be traced after direct and/or indirect environmental stimuli. The model was standardized by determining the relative frequency of PRL and ACTH producing cells in healthy adenohypophysis (AdH) cell cultures versus prolactinomas adenohypophysis (PRLOMA) cultures based on their IR hormone contents (result Fig 1, 6, 7, 8, 9, 10, table 4) (60).

In the AdH model, PRL secretion was significantly lower than in the PRLOMA culture ($control_{AdH}$: 7.13 ± 0.04 ng PRL/mg protein; $control_{PRLOMA}$: 17.14 ± 0.02 ng PRL/mg protein). With regard to ACTH secretion, PRLOMA cells, again, showed a significantly elevated level ($control_{AdH}$: 1528.25 ± 6.14 pg ACTH/mg protein; $control_{PRLOMA}$: 2193.64 ± 1.92 pg ACTH/mg protein) in comparison to normal AdH cells. Adenohypophysis cells with non-malignant transformation certainly have different endocrine functions in the homeostatic system since their spontaneous release activity was different for ACTH and PRL. The ACTH release control cycle is described by "AND" function, and is under hypothalamic control; it is regulated by adrenocortical negative feedback. Biological cycles that can be described with the "AND" function are those essential for life (e.g. human hypothalamus-adenohypophysis-adrenal cortex axis functional disorders). The "OR" function-related control systems are not essential at the organizational level of the given individual (e.g. PRL), life functions can be maintained even in their absence. In the "OR" cycle it can be seen that PRL release is centrally controlled (brain) by DA. DA is a monoamine mediator that can modify AVP synthesis as well as release. The disturbance of the control processes of the "OR" cycle will affect the "AND" cycles. Chronic dysregulation of the "OR" cycle may lead to structural disturbance, e.g. cellular proliferation, which is sustained by the continuity of the feedback information (e.g. PRL release is induced by the continuous presence of estrogen receptor, which increases cell proliferation as a cellular level mechanism).

In the endocrine regulatory system, we selected an attractor for functional stability assay, which was studied with activation and inhibition cycles for the given model cells (Fig 1). Activation was induced with AVP, which significantly increases ACTH release in healthy endocrine regulation (+ AVP_{AdH}: 10220.88 ± 20.36), and is inhibited by corticosterone (B) (+ B_{AdH}: 1502.17 ± 3.25) (45). Studying the same control cycle with PRLOMA (+ AVP_{PRLOMA}: 12674.50 ± 7.23; + B_{PRLOMA}: 2155.50 ± 7.04), we found that the regulatory pattern persists but with slightly different ratios. The effects mediated by changes in hormone levels in the regulation

prevail in both healthy and PRLOMA cells. At the same time, the basic regulation of the adenohypophyseal-adrenocortical axis shows a shift in PRLOMA. This may be important for environmental hygienic reasons because it implies a shift in the regulation of essential functioning of the hypophyseal-adrenal axis, which, if further induced by, for example, environmental exposure, may enhance the regulatory disturbance (6).

Objective 2.2.a

The ED effect was modeled by indirect and direct load on AdH and PRLOMA cells with the aim of identifying *primary* and/or possibly *secondary* endocrine disrupting effects. When studying a direct effect, the action of the respective agent on endocrine function is investigated, and as a primary biological effect, the ED action may be determined for that substance. If an ED effect is detected where the primary effect is not endocrine in nature, but the response mechanism results in an endocrine disorder, then we talk about a secondary ED effect. I considered it important to create this subclassification because this way even additive effect spectra can be identified, which may have relevance in therapeutic practice (72).

When studying the primary ED effect, we investigated the action of halogenated aromatic hydrocarbons on the exocytotic activity of the hypophysis cells. In the case of AdH cells, the subtoxic doses of 1,4-dichlorobenzene, hexachlorobenzene + 1,2,4-trichlorobenzene, phenuron, monuron and diuron agents in the functional model of ACTH release (Fig 1) did not significantly alter the basic and the AVP/corticosterone influenced ACTH release when compared to the control (Fig 11). However, in the AVP activated state, each agent showed a significant increase in ACTH release relative to the control level. When these tests were carried out on cells showing a transformation in their proliferating ability (PRLOMA) (Fig 6, Fig. 12.), a difference was found in the baseline levels (with the exception of phenuron) compared to the controls, which was also seen in AVP stimulation inhibited by corticosterone, and was maintained in the AVP stimulated AdH cells. For the ED effect, this result shows that cell transformation has sensitized the cells and the system has become unstable, and no longer able to compensate for some effects with its own vital potential. In medical practice, this may be of importance as it may support the flipping of healthy state cycle cells from the already unstable (e.g. PRLOMA) state to a new system cycle, i.e. the malignant attractor (computed tumor) model. In the outlined *in vitro* model, a primary ED effect has been demonstrated for the functioning of healthy and benign adenohypophysis cells in an essential function of ACTH release. As regards non-essential prolactin production, different secreted hormone levels were

detected in the baseline interpretation of AdH versus PRLOMA cells (Table 4) (45). Compounds with ED activity disrupted PRL secretion in PRLOMA, but not in normal AdH cells (Figure 13, 14).

According to *in vivo* data of mClB treatments, depending on the duration of treatment and the dosage, the amount of serum ACTH (Fig 17) increased; and this was in correlation with ACTH secretion of *in vitro* AdH cells after the same treatment and energy transfer /Mg^{2+}-dependent ATPase / (Fig 18, 19). According to the coherence of the effects of independent *in vitro* and *in vivo* treatments, these compounds can be considered as primary ED agents (62).

The neurohypophysis also plays an essential role in homeostasis. Our investigations were extended to uron agents for OT and AVP release functions in order to clarify further ED effects. (Our group clarified the effect of ClB on these hormone secretions earlier (52)). Our results (Fig 2) show that AVP release attractants activated by monoamines (epinephrine, norepinephrine, histamine, serotonin, dopamine) were significantly altered by uron compounds. With respect to OT release, basal release values were significantly altered only by phenuron when compared to control. However, in the case of different monoamine attractors, only monuron (containing a single chlorine substituent) did not deviate from the significant effects of the other agents. Monuron changed the activated state after the administration of norepinephrine significantly, and this phenomenon was not observed in the case of any other monoamines. The essential AVP regulatory processes in case of each monoamine activated AVP release attractor were altered by the members of the halogenated uron group, which makes these agents a clear primary ED factor (Fig. 15, 16)(61). The role of OT today is not considered to be essential in homeostasis – thus, after hypophysectomy, it is not routinely substituted, but its role in vasoconstriction, cerebral plasticity, immunomodulation, etc. (73) seems to be obvious, so it may be important for the organism to function optimally.

Secondary ED effects were studied by simulating the isoforms. In this model, an indirect effect, which is caused by an environmental agent, was modeled by maintaining and shifting the physiological optimum in the ionic milieu. In this respect, our team has already studied the effects of potassium changes in the extracellular ionic milieu on hormone release (74). As a continuation of this, we investigated the effects of extracellular hypocalcemia on normal ADH and PRLOMA cells. When hypocalcaemia moves towards the physiological range, ACTH release approaches the control values in normal AdH cells (Figure 7). Release characteristics are still regulated for time and dose dependencies within the first 30 minutes, only after 60

minutes do extreme effects and exocytosis separate from each other. In PRLOMA, the time-traced curves of the extracellular hypocalcemic characteristics of ACTH release (Figure 9) follow no particular order. As to prolactin release, AdH cells in calcium deprived milieu secrete normal quantities which were not compensated for their control range. As the dose of calcium approaches the physiological value, AdH cells present compensated prolactin secretion patterns (Figure 8). In PRLOMA, the hypocalcemia dose curves are time-decompressed (chaotic), compared to those of normal AdH cells (Figure 10)(60). It can be seen, that the changing environmental impacts can quickly shift PRLOMA cells from stability, as they cannot compensate for these changes. This also demonstrates that transformed cells characterized by a significant increase in their rate of division are not stable. In these cells, the cell attractor cannot adequately compensate for the shifted condition, which can be modeled and characterized by „AND" functions (conditions for maintaining isoosmosis, isoionia, isovolemia, etc.).

Secondary ED effects may be pivotal because they are expressed through the extracellular parameter changes which are responsible for the homeostasis of the cells; these changes may generate more serious processes than it would be expected in unstable, diseased organisms. It is particularly important to keep in mind in the case of individuals whose ability to adapt is not yet, or no longer of a high potential (e.g. newborns, infants, aging organisms, etc.). In medical practice, therefore, it is essential to know about and to assess the risk of secondary ED effects.

To accomplish this, databases need to be created; adequate data sources are available, e.g. electronic patient record system, but they should be structured in a big data system. Previously, the display and classification of primary and secondary ED agents with effect factors were not separated. The significance of this need has already been recognized, but its use not regularly seen in therapeutic practice. Our work presents an initial version, since according to the ISO 14040 standard for Life Cycle Analysis (LCA), when determining human health potential, environmental factors must be standardized for 1,4-dichlorobenzene. This standard is also used to evaluate ecotoxicological events, which will greatly support extrapolation from animal experiments. The primary ED effects presented in this paper have already been reported, and are therefore classified as ED components. Based on the numeric data of the effect, it would be worth considering the effect of the standard compound as a unit, and relating the effect of other EDC to this value. This is what our group is working on at the moment. It would also be useful to do the same with secondary effects, specifying various „AND" systems.

Objective 2.2.b

From an environmental hygienic point of view, we also wished to examine the role of physical environmental exposures in addition to EDC (75). Thus, our research also focuses on studying the role of EMF effects in neuroendocrine regulation. The EMF influences are present quite dominantly in societies through the use of electricity (transformation, transportation) (76) and via the operation of communication systems (77). The effects of electric fields can be warded off by electrostatic shielding (Faraday cage) (78), but this does not solve the problem of magnetic exposure factors (79). Its significance is different from that of EDC because the latter have a homeostatic compensating mechanism (organ, organ system, buffer system, etc.) which, for example, provides adaptation in humans. It is probable that the attraction range (environmental conditions) can be described by „AND" function modified biological patterns. For this reason, only those living attractors were able to survive which were able to operate compensatory mechanisms (regulatory cascades) with thresholds (80). No organic regulation to compensate for the effects of magnetic field ever developed in biological evolution. It can therefore be assumed that in the environment that constitutes the background for evolution, no changes have occurred that may have disrupted the life functions of the biological material (81). There is no homeostatic mechanism (no magnetic organ) for the present living biological material patterns (for organisms on Earth) to compensate for the magnetic field induced effect. Society, as the present evolutionary scene, uses such magnetic field modifying technical and technological tools which generate these changes continuously. The behavior of electrically-charged structures and charged molecules changes (82) in the magnetic field, which, if it affects the living organism, generates threshold-dependent but safe state changes. In the present theses, we investigated the mechanism of norepinephrine activation, which is of significance in neuroendocrine regulation. The NE membrane receptor acts on a specific activation pathway as an agonist, via the cAMP messenger system. The transduction process starting with the NE + receptor binding from the extracellular space on the β-adrenoceptor, it highly depends on the activation (83) and integrity of the membrane (84), the membrane potential (85), the rate of movement of the receptor subunits (86) and its direction (87).

Since the mentioned physical parameters (potential, velocity, displacement) are vector quantities, the changes in the force fields that are experimentally simulated by the EMF effect, can generate changes in the outlined events. These can only be detected when they are threshold effects at the level of a particular mechanism. Our results show that the β-adrenoceptor function was disrupted by *in vivo* EMF treatment (Fig 20), which resulted in a marked decrease of

receptor activity. When the treatments were suspended, we observed that the induced direct and/or indirect ED effect was still apparent as a minor disorder. All this was confirmed by the appearance of regeneration (Fig 21), as the investigated monoamine activated attractor returned to its stable (control) state. In our further course of our work we also continue to investigate the monoamine receptor involved in the OT and AVP release in the experimental model presented. In this regard, we can evaluate the results as an indirect ED effect. However, monoamines also have a direct ED effect due to their hormone-like properties. In another context, we can say that as there is no direct body and/or regulatory system for adaptation to EMF effects, these effects can only be interpreted as indirect (63).

6. Summary

1.

Exploring the pathomechanisms of health problems that emerge from exposure to ED factors is significant in the diagnostic and therapeutic practice as well. Therefore, it is important to thematically explore possible ED exposure (occupational, residential, lifestyle, electrical appliances/devices in the apartment and the living environment, the workplace and its environment) when taking medical history, and the recommendations of the Endocrine Societies should certainly be complemented with them. Among the biochemical parameters, when exposure to ED is high, it seems to be desirable to measure the amount of anti-TG and/or anti-TPO in the serum. Compared to the ATA and ETA results, all malignant thyroid tumour (follicular cell origin) patients, whom I examined, and had been exposed to ED, all of them had elevated levels of anti-TG and/or anti-TPO. Accordingly, chronic ED exposure may be a causative factor in triggering malignant effects via autoimmune inflammatory processes.

When characterization and classification of ED factors are accessible on a broader scale, preventive solutions may reduce the risk of developing and progressing endocrine disorders. (Objective 2.1.a)

2.

Our benign pituitary PRLOMA model was experimentally generated in rats. ED that stimulate structural changes similar to those seen in our model all cause cell transformation disturbances (e.g. estrogen mimetics). We managed to standardize a model which enables one to study chronic ED effects in addition to the triggering ED effects in benign endocrine disorders, which can be examined according to the requirements of the Boolean network algorithms. (Objective 2.1.b)

3.

It has been proven that the AVP-induced ACTH release attractor is shifted both in AdH and PRLOMA, which enhances regulating disturbance in PRLOMA. When these two models are further burdened with ED exposures classified as primary (direct) and/or secondary (indirect) ED factors, the PRLOMA becomes more unstable because it cannot present the compensatory mechanisms characteristic for a healthy state cycle. Due to its regulatory instability in the presence of EDC agents in subtoxic doses, PRLOMA may easily migrate into a new system cycle. It was found that both uron compounds (phenuron, monuron, diuron) and chlorobenzenes

(1,2,4-trichloro-benzene+hexachlorobenzene=1:1; 1,4-dichlorobenzene) have a primary ED effect on PRL and ACTH release of both the healthy adenohypophysis and the one with prolactinoma. The primary ED effect of the uron agents was also verified by following the regulatory attractor of monoamine (DA, NE, E, 5-HT, HA) activated OT, AVP secretion of neurohypophysis cells. Those EDC effects which do not interfere with the endocrinium directly, but through disruption of one of the „AND" functions (in our case, ionic milieu - Ca^{2+}) can be viewed as secondary ED activities. Regulatory behavior of PRLOMA also has a higher degree of instability toward secondary EDC effects compared to healthy AdH. (Objective 2.2.a)

4.

Biological effects caused by EDC factors are standardized relative to the effects of 1,4-dichlorobenzene by the ISO 14040 standard packet, which prefers lifecycle studies, eco-toxicological potential (ETP) and human toxicity potential (HTP). We conducted the study of the known standard effect in our work accordingly, and we consider it important to measure and record the effect(s) for a significant EDC in various endocrine disorders. (Objective 2.2.a)

5.

In addition to the ED-induced effects of the chemical agents, we also began to clarify the ED role of physical factors. The effects of ELF-EMF (as a factor) were studied *in vivo*. We found that the functional activity of the cell membrane was significantly altered by the use of extreme low dose electromagnetic radiation by a chronic treatment scheme. In our research system, the receptor-agonist/antagonist attractor (endocrine regulation cycle) was set up as a test model, similarly to the previous hypophysis release models. The EMF effect can be labeled as an ED factor for all beta-receptor-linked hormone releases. The reversibility test model of the ELF-EMF was also developed, which could provide important information in the design of the planned data system due to the strength and periods of its effect. (Objective 2.2.b)

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