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

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2.1. a,

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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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V. Sepp K, Laszlo AM, Molnar Z, Serester A, Alapi T, Galfi M, Valkusz Z, Radacs M: The Role of Uron and Chlorobenzene Derivatives, as Potential Endocrine Disrupting Compounds, in the Secretion of ACTH and PRL. International Journal of Endocrinology 2018 Paper: 7493418. (2018)

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Abbreviations

ACTH: adrenocorticotropic hormone

AdH: adenohypophysis

anti-TG: anti-thyroglobulin antibody

anti-TPO: anti-thyroid peroxidase antibody

ATPase: adenosine triphosphate-ase enzyme

AVP: 8-arginine vasopressin

ATA: American Thyroid Association

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

LIA: luminescence immunoassay

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

MU: monuron

NE: norepinephrine

NH: neurohypophysis

OT: oxytocin

PU: phenuron

PRL: prolactin

PRLOMA: prolactinomas adenohypophysis

RIA: radioimmunoassay

TSH: thyroid-stimulating hormone

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 ED in the medical histories of the patients.

1. Introduction

Factors generating disruption in the endocrine system are called endocrine disruptors (ED). Such ED factors can be environmental burdens: soil, air, water, food, etc. Exposure should be explored in terms of duration, volume, cumulative risk for social, economic, geographical, occupational, genetic and health factors.

Cellular ED effects are diverse, e.g. they disturb the physiological fundament of the binding process of specific receptors and hormones as 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 ED agent may bind to the receptor, so that it interferes with normal signal transduction (e.g. in space, time), thus causing an atypical response. The electromagnetic field (EMF) can also lead to similar consequences;

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

- modifying the transport of specific hormones by interfering with the transport protein functions (ED, 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.

The mechanisms outlined may play a role in obesity, diabetes mellitus, cardiovascular disease, male and female reproductive abnormalities, hormone sensitive tumors (in women: breast, ovarian, endometrial; in male: prostate), thyroid diseases, neuro-developmental disorders, as well as other yet unexplained ED effects.

2. Objectives

Disruption of the endocrine system, which actually affects the unity of the psycho-neuro-endocrine-immune system, may play a role in the pathogenesis 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, which may also facilitate the development of preventive solutions.

2.1.

A very high incidence of cellular abnormalities was found in the endocrinological disorders in the last half century, which caused functional abnormalities in the endocrine glands, especially in the thyroid and the pituitary. Thus, in the case of cell proliferative diseases of the thyroid and the pituitary, it is a major health and therapeutic question whether the benign alterations and/or malignancies of these glands should be investigated in connection with certain pathogenic factors.

a, - Therefore, in this work, one of the basic questions was whether ED could cause a disease in the thyroid via transformation disorders and processes. To find an answer, recognition of the disease, diagnostic classification and exploration of anamnestic relationships became necessary.

b, - Whether ED effects induce hypophyseal cell transformation disturbances, by generating endocrine regulatory disorders?

2.2.

The release of hormones into the blood flow through exocytosis is a prerequisite for maintaining a healthy homeostasis; the disruption of this process is a major factor in the pathogenesis of many diseases. The question arises whether hormone (adrenocorticotropic hormone (ACTH), prolactin (PRL), oxytocin (OT), 8-arginine vasopressin (AVP)) secretions change as a result of ED effects in the neuro- and adenohypophysis; furthermore whether ED cause adenohypophysis cell transformation?

a, - The clarification of the role of uron (phenuron) and halogenated hydrocarbon (monuron, diuron, chlorobenzenes (ClB)) compounds used in agriculture has been the focus of our investigations for this purpose in terms of ED effects. These compounds represent continuous environmental exposure, not only through industrial and agricultural activities, but also through burdening the nutritional chain, making them important from an environmental hygienic point of view.

b, - In the investigation of EMF effects on the cell membrane, ED effects were studied through the changes observed in membrane receptors. It was uncertain whether EMF could modify the functions of major monoamine (norepinephrine) receptors in neuroendocrine regulation.

Studying it through the disruption in the hormone secretion regulating circuit (attractor) coupled with the given receptor function, obviously required the development of a new research model.

3. Methods

A, *Human studies*

Characteristics of the patients

Thyroid carcinoma was observed in 341 patients, of whom 78% were women /average age: 58 years (20-87 years)/ and 22% were men /average age: 56 years (26-89 years)/.

Incidence of histological types of thyroid carcinomas: 188 papillary (55%; ♀, ♂ age: 56 years), 58 follicular (17%; ♀ age: 62 years, ♂ age: 69 years), 35 papillo-follicular (10%), 27 medullary (8%; ♀ age: 54 years, ♂ age: 52 years), 18 unknown (5%), 9 Hurthle cell (3 %) and 6 anaplastic (2%; ♀ age: 74 years, ♂ age: 64 years). The risk of recurrence for differentiated thyroid carcinoma was classified by European Thyroid Association (ETA) and American Thyroid Association (ATA) methods. 35 patients (whose anti-thyroid peroxidase (anti-TPO) and/or anti-thyroglobulin (anti-TG) factors were also known in addition to the thyroid-stimulating hormone (TSH) values) from the above group were recruited (as a pilot study), based on the results of our ED experiments. The protocol was complemented for this purpose with environmental factor (ED) expositions (occupational, residential, electrical devices within and outside the home, plastics and other chemical agents - from industry, agriculture, household, etc.). In our clinical hormone laboratory, a polyclonal antibody assay was used for anti-TPO (Elecsys Anti-TPO assay), a monoclonal antibody assay for anti-TG (Elecsys Anti-Tg assay), a monoclonal antibody assay for TSH (Elecsys TSH assay), to perform electrochemiluminescence immunoassay (ECLIA) on Modular E170 analyzer (Roche, Mannheim, Germany). 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).

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

I. Experimental models:

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

Female Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from various litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for hypophysis cell cultures. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary (relative air humidity of 55-65%, 22±2°C

ambient temperature, 12 h dark and 12 h light periods) in groups of 10 animals each. Standard pellet food and tap water were available *ad libitum.*

2. AdH cell cultures

After pentobarbital anaesthesia (4.5 mg/bodyweight kg (bw. kg) Nembutal, Abbott, USA) the rats were decapitated and adenohypophyses were separated under a preparative microscope. For the primary monolayer cell cultures, 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), and mechanically dispersed 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 installed 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 μg/ml/). The cells were cultured at 37 °C in a CO_2 incubator that provided a humidified athmosphere of 95% air and 5% CO_2, and cells were washed every 3 days. AdH cultures were functionally standardized as well by the measurement of PRL and ACTH hormones (by luminescence immunoassay (LIA) and radioimmunoassay (RIA) methods) from the supernatant medium, followed by basic hormone-depletion kinetics and assay tracking.

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 rats were decapitated and adenohypophyses were separated under a preparative microscope. For the primary monolayer cell cultures, 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), and mechanically dispersed 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 installed 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 μg/ml/). The cells were cultured at 37 °C in a CO_2 incubator that provided a humidified athmosphere of 95% air and 5% CO_2, and cells were washed every 3 days. PRLOMA cultures

were functionally standardized as well by the measurement of PRL and ACTH hormones (by LIA and RIA methods) from the supernatant medium, followed by basic hormone-depletion kinetics and assay tracking.

4. Neurohypophysis (NH) cell culture model

Male Wistar rats were kept in conditions described in point 3.B.I.1. They were decapitated (3.B.I.2.) and the NHs were digested enzymatically (0.2% trypsin /Sigma, Germany/ in PBS, and in 0.05% collagenase /Sigma, Germany/) and cells mechanically triturated (pore sizes 100, 80 and 48 μm in series), the cell viability was controlled, and the density was installed ($2x10^5$ cells/ml). The dispersed cells were placed onto 24-well plastic plates (Costar, USA) coated with 5% rat tail collagen /DMEM (Dulbecco's Modified Eagle's Medium; Sigma, Germany) + 20% FCS (fetal calf serum - Gibco, USA) + 0.1 μg/ml PenStrep (Sigma, Germany) in supplemented media/. 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 with fresh DMEM. Cultures were standardized functionally by immunohistochemical procedures according for OT and AVP content (by RIA method) and release.

II. ED treatments

5. Investigation model for effects of extracellular ionic milieu changes in AdH and PRLOMA cells

Standardized AdH and PRLOMA cultures were conditioned in Tyrode medium, then time kinetic experiments were performed for low Ca^{2+} milieu experiments ([Ca^{2+}]: 0; 0.5; 1.0; 1.5 mM; n=12/group, repeated in 10 independent cases), where hormones were measured from the supernatant media at 10, 20, 30, 60, 90. min of the experiment. ACTH and PRL were determined by LIA and RIA methods. Protein content of the hormone releasing cells was assayed by a modified Lowry-method, and Pierce BCA Protein Assay Kit was used (Thermo Fisher Scientific Inc., Rockford, USA). SAS was applied for the statistical analysis (Version 9.3 SAS Institute Inc., Cary, NC, USA).

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

After functional and viability standardization of AdH and PRLOMA cell cultures carried out as described in points 3.B.I.2. and 3.B.I.3., the ED effects were evoked by uron derivates (PU [10^{-6} M], MU [10^{-6} M], DU [10^{-6} M]) and by chlorobenzenes (1,4-dichlorobenzene (dClB): [0.1 ng/ml]; chlorobenzene mix (mClB [0.1 ng/ml]: hexachlorobenzene+1,2,4-

trichlorobenzene=1:1). The controlled cycle of ACTH hormone secretion was set using AVP [10^{-6} M] and corticosterone (B, [1μg / ml]) separately, as well as combined. ED agents were added to the experimental system alone and in the presence of AVP, from which samples of the supernatant medium were taken after 60 minutes of treatment. ACTH and PRL were measured by LIA and RIA. Protein content of the hormone releasing cells was assayed by modified Lowry-method, and Pierce BCA Protein Assay Kit was used (Thermo Fisher Scientific Inc., Rockford, USA). The Mg^{2+}-dependent adenosine triphosphate (ATP)ase activity was measured by the modified method of Martin and Dotty. SAS was applied for the statistical analysis (Version 9.3 SAS Institute Inc., Cary, NC, USA).

7. Treatment of Wistar rats with ED agents in vivo

In vivo treatment of male Wistar 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 done 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 drinking water was injected, in the positive controls, the solvent (0.015 % ethanol/distilled water) 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. AdH cell cultures were made and ACTH release was followed. There was no significant difference between the results of the various control groups when analyzing measured ACTH values, thus our results list the absolute control data only. For the statistical analysis IBM SPSS Statistics, Version 21 program was used.

8. Investigation of ED Effects on NH Cultures

Standardized monolayer NH cell cultures (3.B.I.4.) were treated *in vitro* with 10^{-6} M concentration of phenuron (PU, CAS registry number: 101-42-8), diuron (DU, CAS registry number: 330-54-1) and monuron (MU, CAS registry number: 150-68-5; Sigma, Germany) as ED factors. Condition cycles of standardized monoamine receptor-coupled OT and VP exocytosis were used as experimental models. For this, epinephrine (E), norepinephrine (NE), serotonin (5-HT), histamine (HA), dopamine (DA) (Sigma, Germany) monoamines were used at a concentration of 10^{-6}M. To follow the combined monoamine + EDC effect, we created the following experimental layouts: 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; in which the same dose (10^{-6} M) of monoamines and ureaherbicides were used. Samples were

taken from the supernatant media after the experimental treatments. OT and AVP were determined by RIA. Protein content of the hormone releasing cells was determined by modified Lowry-method, and Pierce BCA Protein Assay Kit was used (Thermo Fisher Scientific Inc., Rockford, USA). SAS was applied for the statistical analysis (Version 9.3 SAS Institute Inc., Cary, NC, USA).

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

We established a turkey (*Meleagris gallopavo*) model in which we used their nucleated red blood cells to monitor the effects of in vivo ELF-EMF exposure. The turkeys were kept in accordance with the animal welfare requirements of the University of Szeged. In setting up the experimental system, groups of female turkeys were formed according to treatment regimens (absolute control /no treatment/, negative control /the treatment positions are carried out/, positive control /treatment protocol is carried out while electromagnetic machine is on, but at position 0/ and treated /according to treatment protocol ν=50 Hz, B=10 μT intermitted ELF-EMF, 3 times a day /every 8 hours/ for 20 min, for 3 weeks, then without treatment for 5 weeks/. In accordance with the treatment protocol, blood samples were taken from the clavicular vein and red blood cells were separated, then with standardized cell count, beta-adrenergic receptor functions were followed by determining the NE-activated (also with the antagonist: propranolol) intracellular 3'-5'cyclic adenosine monophosphate (cAMP) level (Amersham cAMP Biotrak EIA system, GE Healthcare, UK). SAS program package was used to statistically process results from treated and untreated groups (Version 9.3 SAS Institute Inc., Cary, NC, USA).

4. Results

In accordance with objective 2.1.a, we examined whether endocrine disruptor compounds (EDC) can be a causative agent in thyroid tissue transformation processes. When diagnosing the 341 thyroid malignant tumor patients, the respective thyroid transformation disorders were classified according to the American and European diagnostic recommendations and ratings. When selecting patients for further examination (35 people), the TSH, anti-TG and/or anti-TPO parameters determined at the time of diagnosis were decisive. The autoimmune biomarkers of the thyroid gland showed a substantial increase in 10 patients and were consistent with ED effects. Thus, there was a correlation between the presence of thyroid tumor malignancy and the elevation of these biochemical markers and ED effects.

According to objective 2.1.b, we investigated whether hypophyseal endocrine regulatory disorders can be induced with hormone mimetic ED effects. If so, are they able to induce or disrupt cellular transformation?

Chronic *in vivo* estradiol treatment of ♀ Wistar rats induced a benign adenohypophysis cell transformation disorder, in the form of prolactinoma, from which viability and functionality (specific and non-specific) standardized monolayer cell cultures as *in vitro* models were made. The PRL release of prolactinoma cell cultures (PRLOMA) were enhanced significantly when compared to the untreated, healthy AdH cell cultures. ACTH secretion stimulated by AVP was also strongly enhanced in the PRLOMA cell cultures when compared to similar treatment patterns in AdH cultures.

The differentiated and general cell functions of hormone-producing cells are ensured by the controlled extracellular ionic milieu, maintained by homeostasis. The results of the effects of extracellular hypoionic environment, specifically, the appearance of the low calcium condition in normal AdH and PRLOMA cells, have clearly shown alterations. ACTH and PRL release kinetics followed in time showed a maintained character in low calcium milieu close to physiological levels in AdH cell cultures. However, in the case of PRLOMA, the course of ACTH and PRL release kinetics was highly divergent and significantly different from control and AdH patterns.

In the 2.2.a objective point, we wanted to clarify the EDC role of phenylurea (PU) and other halogenated hydrocarbons (ClB, MU, DU), widely applied in agriculture. For this purpose, we used our cell culture models (NH, AdH and PRLOMA) and followed their hormone release functions (OT, VP, ACTH, PRL). According to our results, the control cycles of AVP activated ACTH release of AdH models were significantly altered by the urons and the ClB derivatives. In the case of PRLOMA, the basic release, its activated and inhibited release parameters also changed. The baseline PRL release of the AdH model did not show any appreciable change in the presence of uron and ClB derivatives. However, the same agents led to a significant difference in the PRL release from PRLOMA.

In order to study the regulation of OT and AVP secretion in NH cell culture models, (monoamine-activated) regulatory neuroendocrine cycles were developed and standardized.

In this system, uron derivatives caused changes in OT release; these changes were strong in the case of norepinephrine, moderate with histamine, epinephrine and dopamine, and weak with

serotonin. In the same test model, all monoamines had an effect on AVP secretion (dopamine, norepinephrine: strong; epinephrine, serotonin: moderate; histamine: weak effect).

The dose (0.1; 1,0; 10 μg/bw. kg) and time dependence (0. day: control, treatment periods: 30, 60, 90 days) of mClB was tested *in vivo*. According to our experiments, serum ACTH levels showed a significant increase as a result of ClB treatments in a time- and dose-dependent manner.

The ACTH release of AdH cultures derived from *in vivo* experimental ClB exposures also exhibited significant increases in a time- and dose-dependent manner. In this protocol, we simultaneously followed the energy transfer mediated by magnesium-dependent ATPase in AdH cultures, which was significantly altered by the ClB treatments in a time- and dose-dependent manner in comparison with control values.

In the 2.2.b. objective point we investigated that the ED effects can be generated not only by chemical inducers but also by physical effects. ELF-EMF may be a challenging factor in this respect. To investigate this effect we developed an *in vivo* turkey exposure test model (treated: with ELF-EMF: ν=50 Hz, B=10 μT; three times/day treatment for 20 minutes/8 hours; absolute control: untreated; negative control: using experimental protocol without ELF-EMF; positive control: ELF-EMF equipment in stand-by mode) in which the noradrenaline-activated beta-receptor function of the cell membrane was monitored in a time-dependent manner by measuring intracellularly synthesized cAMP levels. According to our results, the rate of ELF EMF influenced cAMP synthesis decreased significantly compared to the untreated state. However, we also demonstrated that this process was reversible in our model, as cAMP parameters improved to control levels after 5 weeks of a no-treatment experimental setup.

5. Summary

1.

Exploring the pathomechanisms of health problems that emerge from exposure to ED factors is important both in the diagnostic procedure and the therapeutic medical practice. Therefore, it seems to be relevant to thematically explore possible ED exposure (occupational, residential, lifestyle, electric 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 (ATA, ETA) should certainly be complemented from this aspect. When exposure to ED is high, it looks desirable to determine the amount of anti-TG and/or anti-TPO in the blood serum. Compared to the ATA and ETA results, all malignant thyroid tumour (follicular cell

origin) patients I examined and were 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.

If characterization and classification of ED factors is accessible in a big-data system, preventive solutions may reduce the risk of incidence and progression of endocrine malignancies (Objective 2.1.a)

2.

A benign tissue proliferative disease of the hypophysis is prolactinoma, an endocrine disorder whose pathological regulatory factor was experimentally mimicked in our laboratory. ED that evoke structural changes similar to those seen in our *in vitro* PRLOMA model by their primary or secondary effects all cause cell transformation disturbances (e.g. estrogen mimetics). We have managed to standardize a model which enables one to study chronic ED effects leading to benign endocrine disorders, which in turn can be examined according to the requirements of the BOOLE network algorithms. (Objective 2.1.b)

3.

It has been proven that in the presence of ED the AVP-induced ACTH release attractor is shifted both in normal AdH and PRLOMA, where the regulating disturbance in PRLOMA is even more pronounced. If these two models are burdened with ED exposures classified as primary (direct) and/or secondary (indirect) ED factors, the PRLOMA becomes even 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 presumably pass into a new system cycle. It was found that both uron compounds (PU, MU, DU) and chlorobenzenes (mClB, dClB) have a primary ED effect on PRL and ACTH release of both the healthy adenohypophysis and the one with PRLOMA. The primary ED effect of the uron agents was also verified by examining the regulatory attractor of monoamine (DA, NE, E, 5-HT, HA) activated OT and 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 high degree of instability toward secondary EDC effects compared to healthy AdH. (Objective 2.2.a)

4.

Biological effects caused by EDC factors have been standardized to the effects of 1,4-dichlorobenzene by the ISO 14040 standard packet (- which prefers life cycle studies -) for eco-toxicological potential (ETP) and human toxicity potential (HTP). This ISO 14040 standardisation method was used in our work for the characterisation and classification of ED effects. Our results may be useful in opening up a new algorithm for the diagnostics and therapy of certain endocrine disorders. (Objective 2.2.a)

5.

In addition to the ED effects induced by chemical agents, we also began to clarify the ED role of physical factors. The effects of ELF-EMF were studied *in vivo*. We found that the functional activity of the cell membrane was significantly altered by chronic treatment with ELF-EMF radiation. In our research system, the receptor-agonist/antagonist attractor was set up as a test model, similarly to the previous hypophysis release models. The EMF effect can be regarded as an ED factor for probably all the beta-receptor-linked hormone releases.

We also developed a reversibility test model of the ELF-EMF effect, which could provide important information for the design of the planned data system with the determination of the strength and duration of the periods of ED effects. (Objective 2.2.b)

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