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1. Introduction

The term “endocrine disruptor” (ED) refers to a group of substances, which – even in small doses – alter the physiological regulatory pathways of endogenous hormones, and thus, disorganize the normal neuroendocrine functions of the body. The hormonal imbalance caused by these foreign substances is a result of dysregulated feedback loops and/or disturbed cellular signaling pathways.

EDs are found almost everywhere. The animals and human beings are continuously surrounded by a multitude of EDs from different origin. Many of the EDs are able to breach the physical barriers through different mechanisms. For example, absorption through the skin or through wounds or sometimes by simultaneous exposures: one of the EDs weakens the protective mechanisms of the organism helping the other substance to enter the body. Some substances may get into the bloodstream through mucous membranes (oral, nasal, eye, respiratory tract, etc.), while orally incorporated EDs might be absorbed through the intestines.

Altering the balance of the neuroendocrine regulation will lead to serious developmental, medical and even agricultural consequences. Depending on the point of disruption in the organism, EDs have a serious impact on the cellular components of the blood; the quality, and cellular quantity of the immune response; the homeostasis and the functions during detoxification of the liver and kidneys; the neuroendocrine organ functions (disrupting the regulative characteristics of specific parts of the hormonal milieu in the animals) and even on the central nervous system. Addition to the adverse health effects, EDs interfere with the reproductive physiology of animals, thus lower the possible productivity of the livestock causing major economic losses.

In the experiments in this thesis we used four well-known and widespread EDs of different origins: Bisphenol-A (BPA), an industrial byproduct during plastic synthesis, banned in the European Union since 2011, currently under re-evaluation; Zearalenone (ZEA), an exogenous mycotoxin well-known for its adverse effects in livestock animals, causing major losses in both animal counts and agricultural profit in Europe; Camphor (4-methylbenzylidene camphor, MBC) a natural phytoestrogen found as a component of most cosmetic and medical product worldwide, and Arsenic (As), a simple but especially strong substance with an ED characteristic, occurring naturally or after contamination in groundwater and drinking water.

Thyroid hormones (TH) triiodothyronine (T3) and its prohormone, thyroxine (T4) are mainly metabolic regulator hormones produced by the thyroid gland. TH production is regulated by the neuroendocrine system: the hypothalamus in the hypothalamic–pituitary–thyroid axis (HPT) actively monitors the T4 and T3 serum levels. Due to the feedback system a given

substance – e.g. an ED – can alter the TH balance by affecting the HPT axis on a number of points. Inadequate T3 amount in the brain is associated with neurological diseases.

Estrogen hormones estrone (E1), estradiol (E2), and estriol (E3) are gonadal steroids, and part of the sex hormones in animals. Mainly, estrogens are responsible for the development of the female reproductive system and the secondary sex characteristic, and they have a regulatory role on the developed systems as well. Similarly to the HPT axis the estrogen levels are maintained and regulated by the hypothalamic–pituitary–gonadal axis (HPG).

Main functions of estrogens are the development and regulation of almost all of the mammalian tissues. Disturbances in estrogen regulation might lead to tumor development, mostly in the primary and secondary sexual organs, e.g.: breast cancer and prostate cancer. Estrogens play a critical role during the development of the CNS, and they are important regulators of the neuron and glial function. Insufficient estrogen levels are connected with disturbances in the higher cognitive functions of the brain by altering the physiology of the synapses, a phenomenon which even leads to secondary behavioral or psychological problems.

The majority of E2 and TH actions are mediated through the binding of hormones to their respective hormone receptors located in the cell nucleus. The receptors act as ligand-modulated transcriptional factors, after activation they modify the targeted gene expression (e.g.: the gene coding their respective receptor proteins). The endocrine disrupting potency of the above mentioned EDs is a result mostly of their ability to bind to the estrogen (ER) and thyroid receptors (TR). Available data suggest that E2 and THs are equally important regulators of neuronal development. Based on the previous studies by our research group and the existing literature it is strongly possible that the disturbances caused by EDs will alter the physiological synchrony between the hormones of the neuroendocrine system (on the activational and modificational level).

Considering the lack of experiments on the intracellular mechanisms of specific EDs, and the absence of specified antagonistic/agonistic roles of the substances, it is necessary to conduct an experiment with an “ED cocktail” – a mixture of different EDs. This may shed light on the possibly cumulative physiological effects of the adverse substances in our environment.

2. Significance and aims of the study

Animals and human beings make contact with endocrine disruptors frequently. EDs can be found almost everywhere in our environment, and after incorporation it is a strong possibility that the different substances can strengthen the effect of each other. The exact method of disruption on the neuroendocrine system is not known due to the complexity of the signalization pathways between the hypothalamus, hypophysis, thyroid gland, gonads and the target cells. One of the route of disruption happens through the disruption of the hormone–receptor complex.

The hormonal imbalance caused by EDs is a result mostly of their ability to bind to estrogen receptors (ERα and ERβ) and TH receptors (TRα and TRβ) causing dysregulated feedback loops and/or disturbed cellular signaling pathways. Based on the existing literature and our studies, ED induced disturbance of the physiological synchrony between E2 and THs during CNS development could be more pervasive and far-reaching than currently appreciated, and merits investigation considering that the possible outcome of prenatal exposure to a diverse selection of EDs is not known.

The aim of this thesis is **to prove additive adverse effects of different xenoestrogen on the differentiating neuron cells**. Therefore, in the experiments, we used Bisphenol-A (BPA), Zearalenone (ZEA), Arsenic (As) and 4-methylbenzylidene camphor (MBC), four widespread EDs of different origins, individually and simultaneously. To shed some light on this scientific puzzle the following aims were established:

A. Determination of the potential effects of the different endocrine disruptors (BPA, ZEA, As, MBC) alone and in combination on the expression of TRα, TRβ and ERβ;

B. Determination of the individual and combined effects of 17-beta-estradiol (E2) and thyroid hormones (THs) parallel with the applied ED substances on the expression of TRα, TRβ and ERβ;

C. Characterization of the effect of presence or absence of glial cells on the applied ED agents on the expression of TRα, TRβ and ERβ.

3. Materials and methods

Due to the receptor availability on the cerebellar granule cells, we decided to use the postnatal rat cerebellum as a source of primary cells and as a cell culture model. Male and female Wistar rat pups were used in the experiments of this thesis. Timed pregnant Wistar rats were obtained from the vendor at least four days before pre-partum. Animals were kept under standard laboratory conditions, with tap water and regular rat chow *ad libitum* in a 12-h light, 12-h dark cycle. Attention has been paid to sort the siblings into different treatment groups. Following the guidelines established by the National Institutes of Health, the use of animals was approved by the Animal Welfare Board at Szent Istvan University Faculty of Veterinary Science and were approved by the regional animal welfare authority (registry No: XIV-I-001/2201-4/2012).

Cerebella were obtained on the postnatal day 7. The removed cerebella were dissociated without enzymatic treatment applying gentle trituration. Cells were maintained in a serum and steroid free culture for 7 days. For analysis of primary cerebellar granule cells in a glia reduced environment, cytosine β-D-arabinofuranoside) was added to half of the samples 24 hours after seeding to inhibit the proliferation of non-neuronal cells (Glia- experimental groups). In contrast, no AraC was added to the media for analysis of neurons grown in a regular glia containing environment (Glia+ experimental groups). Cultures were simultaneously treated with the hormones (E2, T3 and/or T4 at physiologically relevant concentrations) and/or endocrine disruptors (BPA, ZEA, As and/or MBC) 7 days after seeding. The cells were harvested 6 hours after the treatment. Changes in ER and TR mRNA levels were determined with quantitative real-time PCR method. Western blot method was used 18 hours after the treatment to determine the changes of receptor protein levels.

In both cases in the control group (ntC) mRNA and protein value was arbitrarily set to 1 and results from other groups were expressed as fold changes relative to the control group. Statistical analyses were conducted using Excel and GraphPad Prism version, by means of one-way ANOVA with Tukey’s multiple comparison test.

4. Results and Discussion

Bisphenol-A

Regardless of the presence or absence of glial cells, all of the experimental groups showed suppressed receptor mRNA expression in BPA vs. ntC groups, but hormone treatment resulted in increased mRNA expression in every BPA treated culture. It should be noted, however, that such E2 and T3-deprived conditions cannot be considered as physiological. It is clear from our results that BPA alone suppresses TRα, TRβ and ERβ mRNA expression. However, BPA in combination with the hormones in this thesis will elevate the receptor expression. To our knowledge, currently there is no explanation for this massive upregulation, although it is likely that as an indirect BPA-linked mechanism, the ED effect on TRs and ERs may potentiate the transcription.

It was generally observed that the effects of BPA were less prominent on the receptor protein expression, than those found with regard to mRNA expression. The downregulation in the mRNA levels between ntC and BPA disappeared in both of the TRα, TRβ and ERβ receptors. BPA caused an upregulation in all of the expression levels of the measured receptors in every cell culture and treatment. Our results – the differences between BPA effects on the receptor transcription and translation – indicate that some of the regulatory mechanisms interposed between the mentioned processes (e.g. microRNA regulation) may also be affected by BPA.

We found a notable difference between Glia+ vs. Glia- in case of TRα receptor protein expression. According to our results it is safe to say that BPA elevates the TRβ and ERβ protein expression, but doesn't affect the TRα levels when glial cells are present *in vitro*, possible *in vivo* as well. Without the supporting effects from astroglia cells BPA elevated the protein expression in every experimental group. Due to the increased expression levels we can confirm our hypothesis: BPA acts as an ED on the neuronal function.

Zearalenone

ZEA inhibited the mRNA expression in every experimental groups compared to their ZEA untreated pairs. We found only minor differences between Glia+ vs. Glia-, however the differences between the strength of the downregulation were not significant. From the EDs used in the experiment of this thesis, ZEA exerted a strong global inhibitory effect on the mRNA expression levels. The mycotoxin is a mixed agonist-antagonist of specific receptors (e.g.: ERα and ERβ). Process of activation and/or inhibition covers a broad range of intracellular processes thus the method of disruption is not clear yet. Our result are especially relevant to

shed light onto the mechanisms in the above phenomenon due to the inhibition found in every case on the end-point (the transcription) of the affected intracellular pathways.
The main difference found between the effect of ZEA on mRNA and protein expression was the direction of the effect. ZEA inhibited the mRNA expression in all examined receptors however the protein levels were elevated after ZEA treatment vs. the untreated pair in every experimental group.
Regarding the effect of applied culture conditions on TRα translation, we observed an increased protein expression after ZEA treatment in every Glia- culture. In comparison in Glia+ this phenomenon was only detectable in the ntC and the E2 group. From the results we might assume a protective or modulatory TH effect, if the glial cells weren't suppressed and T3 or T4 was present in the experimental groups. In case of TRβ and ERβ we found no notable differences between Glia+ vs. Glia-. After ZEA treatment the receptor expression level increased in every experimental group, showing a strong modulatory effect on the receptor proteins thus indicating an ED characteristic.

Arsenic

A strong glial modulatory effect was visible both on the change of the TRα TRβ and ERβ mRNA expression levels. The results from the cell culture containing granule cells co-cultured with glia shows no significant change between the experimental pairs. The difference in the pairs – between As treated and untreated cell cultures – were also negligible. In all of the glia- cultures however we found a strong inhibition vs. the As untreated pairs in every experimental group.
On the protein expression level As induced a strong downregulation at mRNA level in most of our cell cultures. Compared to the changes in the mRNA expression, in Glia+ As lowered the expressed protein levels in ERβ vs. the ED untreated pairs. In case of TRα and TRβ the changes were inconsistent, only a minor alteration was found similar to the results from the TRα and TRβ mRNA values from PCR experiments. In Glia- the mRNA and protein changes were similar though the strength of the inhibition was reduced during the translation.
As induced a strong downregulation in all of the cell cultures without astroglia. The glial cells likely reduce the apoptotic effects of As on the nerve cells. Under physiological conditions (In Glia+) the effect of As was strong on the ERβ protein expression, but the translation in TRα and TRβ was negligible.

4-methylbenzylidene camphor

Adding to the afore-listed knowledge, probably the most prominent effect of MBC on our experimental model, among the other EDs used in this study, was its potency to increase ERβ mRNA expression in Glia+ cultures as compared to its respective ntC. We found notable differences between Glia+ vs. Glia- in the TRα and TRβ receptors as well, the direction of the disruption was different in Glia+ (mostly a receptor mRNA upregulation was found) than in Glia- (strong receptor mRNA downregulation).

According to the recent literature MBC mostly affects the TH signaling pathways in the cells. In our cell cultures the substance strongly influenced the T3 or T4 treated experimental groups. In Glia+ a potent upregulatory effect was seen in TRα and ERβ. In TRβ the rate of upregulation was low or negligible. Without TH-s the overall effect in receptor protein upregulation was significantly less in TRα. In comparison with Glia+, in Glia- the results were similar in TRα, TRβ produced a visible pattern in the receptor expression after MBC treatment. In ERβ differences between the majority of MBC treated groups were not found.

Altogether, of the EDs tested in this study, MBC appears to influence TRα, TRβ and ERβ mRNA and protein expression via a mechanism distinct from that/those involved in BPA-Zea-As effects, nevertheless it is evident that MBC effects are also mediated by the glia. Since, however, MBC effects on different cell types seems to highly vary, our results still remain alarming with regard to MBC's influence on neural development.

Endocrine disruptors in combination

The most conspicuous trend concerning the effects of the combined treatment on the **receptor mRNA levels** were found in Glia+ TRα, TRβ and ERβ cultures, in samples with reduced E2 or T3 levels. To a lesser extent the same phenomenon was observable in the Glia- cultures as well. In all of the mentioned pairs we found a robust upregulation of receptor mRNA, but the SEM values were high as well. All of the groups produced inconclusive data, possibly because of the summarized effect of the different mechanisms of disruption (e.g.: agonist – antagonist interaction between EDs or blocking different parts of the receptor signalization pathways). Combined ED effects were exerted when E2 and T3 levels were low, even in the presence of the glia. Since co-administration of E2 and T3 substantially reduced the robust effect of the mixture of EDs on receptor transcription, it is possible that under physiological circumstances, when young individuals are simultaneously exposed to multiple EDs, their neural development (in probably any CNS areas with the appropriate receptors), is more affected by environmental EDs when secretion of T3 and E2 is insufficient.

Comparing the different ED treatments to those of the samples undergone combined treatment, our findings clearly show a strong MBC influence on the **TRα protein** expression. When glial cells were co-cultured with neuron cells, combined treatments caused a strong upregulation of the tested receptors. The rate of upregulation was higher than the effect of BPA, ZEA or As. The fold difference compared to ntC cultures were similar to the MBC results in every treatment, indicating that MBC exert the strongest effects on the TRα protein expression modulating processes from the different EDs of the experiment. Another possibility is that MBC masks the other EDs effects.

The effects on the **TRβ receptor** expression after combined treatment showed a strong similarity to the As or MBC treatment. The TRβ fold difference compared to ntC was negligible. The outcome of this experiment is exceptionally interesting because the combined treatment contained BPA and ZEA with MBC and As. BPA or ZEA alone caused a strong upregulation of the TRβ receptor protein. Hypothetically due to the lack of modulatory effects of MBC or As on receptor expression, BPA and ZEA should have an impact on different modulatory functions leading to protein translation, furthermore the influenced functions shall compensate for one another. Another possibility is that MBC and/or As masks or inhibits the effects of BPA or ZEA, thus maintaining a normal protein expression.

Comparing the results of the **ERβ protein** expression modulating effects of the different EDs with the combined treatment a strong receptor upregulation can be observed in all cell cultures. It is clear that both in the Glia+ and Glia- samples the combined ED effect on protein translation was distinct from the effects of MBC and As. The combined treatment caused a 2.5-fold upregulation in every ED treated cell culture compared to ntC and the corresponding untreated samples opposed to As (the fold difference was halved compared to the untreated samples) or MBC (6-7 fold upregulation compared to the untreated samples). The difference between the combined treatment and BPA or ZEA was negligible. Interpreting the results can be problematic because the final result of regulation during protein translation of the “ED cocktail” could have happened due to different processes: 1.) Simply different effects of regulation may lead to a summated fold difference value. 2.) Possibly effects of MBC and As are weak compared to BPA and/or ZEA, or their influence are masked by the BPA and/or ZEA action in the cell culture. 3.) Hypothetically it is also possible that robust increases in translation may potentially exhaust cellular energy resources, thereby influencing other energy-dependent cellular processes, e.g.: the unknown “interposed mechanisms” or the translation as well.

Nevertheless, the „ED cocktail” did alter the receptor expression of TRα, TRβ and ERβ of the nerve cells, and there was a notable change in the receptor upregulation.

Due to different results measured in Glia+ and Glia- cultures, and the disparity of ED effects between the receptor transcription and translation indicate that not just a specific, but a

complex group of regulatory processes might be disrupted by the tested substances. We suspect that in addition to the ligand-dependent mediating effect of the glia (type 2 deiodinase activity (conversion of T4 to T3), glial presentation of T3 to neurons or modulating the signals between the astroglia and neurons), at least another modulatory method – possibly the microRNA regulation – is targeted by EDs. Changes measured from the qPCR experiments (mRNA levels) were 2- or 3-magnitudes higher than the Western-blot results (protein levels). The mentioned mechanisms between the aforementioned processes apparently play a crucial role in directing ED effects towards the lowered level of transcription measured in our experiments. It is also possible that robust increases in transcription (caused by BPA, ZEA or MBC) may potentially exhaust cellular energy resources, thereby influencing other energy-dependent cellular processes, e.g.: the unknown “interposed mechanisms” or the translation as well.

Nevertheless, disrupting the physiology of the neurons and the glia by the exposure of the test substances may lead to yet unknown – either beneficial or adverse – biological consequences in the CNS, and in the neuroendocrine organs of the organism.

5. Conclusions

Results in this thesis clearly indicate that all of the EDs – alone or in combination – interfere with the physiological hormonal regulation of TRα, TRβ and ERβ mRNA and protein expression. Due to different results measured in Glia+ and Glia- cultures, and the disparity of ED effects between the receptor transcription and translation indicate that not just a specific, but a complex group of regulatory processes will be disrupted by the tested substances. The method of disruption depends on the type of ED, hypothetically all biochemical processes may be altered between the receptor activation and the gene transcription. EDs can act as an agonist, or an antagonist of the target receptor, depending on the structure and biochemical traits of the substance.

The impact of different EDs on the receptor expression were rather heterogeneous although we suspected a somewhat uniform effect due their properties to affect the thyroid and estrogen hormone system. From the experiments three important conclusions can be drawn: 1.) EDs' influential effects on TRα, TRβ and ERβ transcription and translation depends on the specific ED; 2.) THs and E2 affect the interference caused by endocrine disruption. Combined ED effects are exerted when E2 and T3 levels are low; 3.) Glia modulates ED effects on the mRNA and protein level of receptors in cultured cell populations.

Contrary to our hypothesis the effects of the combined ED exposure on the receptor protein expression of the target cells were not additive, even though the effect robustly altered the physiology of the neurons. It seems that specific EDs might mask each other's influence or inhibit the effect of each other or they might exhaust the energy storage of the cells, preventing further receptor expression.

This thesis clearly proves that all of the used EDs alone or in combination disrupt the neuroendocrine system. Disrupting the physiology of neurons and glia by the exposure of the test substances may lead to yet unknown – either beneficial or adverse – biological consequences in the CNS, and in the neuroendocrine organs.

6. New scientific results

A/1. TRα, TRβ and ERβ mRNA and protein expression were disrupted by different levels due to bisphenol-A (BPA), zearalenone (ZEA), arsenic (As) and 4-methylbenzylidene camphor (MBC) alone and in combination.
Compared to the ntC **mRNA levels** BPA greatly enhanced and ZEA halved the transcription; when the glia is limited in the cell cultures, mRNA levels are lowered by As; The effect of MBC on the transcription is strongly modulated by co-administered hormones
The **receptor protein levels** are doubled by BPA and ZEA in case of TRβ and ERβ (TRα protein expression was only influenced by BPA when the glial effect was not potent). As reduced ERβ the expression in the presence of Glia. In glia reduced cultures expression level of TRα, TRβ and ERβ was decreased. MBC greatly elevated TRα and ERβ but TRβ was unaffected in the presence of glia.

A/2. The combined effects of disruption are receptor specific in the cell cultures, but these effects were not additive. On the mRNA levels the combined treatment resulted in inconclusive data. MBC dominated in the disruption in TRα. Only As and MBC influenced the TRβ expression. ERβ was mainly modulated by BPA and ZEA.

B/1. 17-beta-estradiol (E2) and thyroid hormones (T3, T4) altered the individual effect of investigated endocrine disruptors (BPA, ZEA, As, MBC) in a receptor specific, and an endocrine disruptor specific way. T3 lowered the TRα, TRβ and ERβ receptor mRNA expression in BPA treated cultures. The effects of As were not influenced by the hormones. T3 inhibited the effect of MBC on the protein expression.

B/2. Toxic interaction of BPA, ZEA, As and MBC was highly increased in the presence of low lewel of E2 and T3.

C. The presence of glial cells reduced the influence of endocrine disruptors on the receptor mRNA and protein expression levels. The modulatory effects of EDs were more potent in the absence of glial cells.

7. The author's scientific publications

Publications related to the topic of the present dissertation

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Jocsak G., Kiss DS., Toth I., Goszleth G., Bartha T., Frenyo LV., Horvath TL., Zsarnovszky A.: **Comparison of Individual and Combined Effects of Four Endocrine Disruptors on Estrogen Receptor Beta Transcription in Cerebellar Cell Culture: The Modulatory Role of Estradiol and Triiodo-Thyronine**, *Int J Environ Res Public Health*, 13(6). pii: E619. doi: 10.3390/ijerph13060619., 2016. (IF: 2.035)

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