Final report for grant no. 130218

titled as *"Analysis of the effects of endocrine disruptors on the expression of estrogen receptors and thyroid hormone receptors in the developing rat cerebellum"*

Background of the grant topic

Estrogen and thyroid hormone receptors

Thyroid hormones (TH) triiodothyronine (T3) and its prohormone, thyroxine (T4) as well as estrogens (17beta-estradiol, E2) play crucial role in metabolic and developmental processes throughout the mammalian organism. Although studies on animals with disturbed estrogen or thyroid signalling clearly show the decisive impact of E2 and THs on the neurodevelopment, the exact roles that estrogen receptors (ERs) and TH receptors (TRs) play during CNS development are little known, most likely because the effects of these hormones are highly interactive in the living organism. Many of the genes involved in neurodevelopment possess specific sites in their promoter regions (E2- and TH-responsive elements, ERE, TRE) that are able to bind to ERs or TRs. Estrogen and THs, when bound to their cognate receptors, enable these receptors to bind to their EREs and TREs, thus they function as transcription factors to activate or silence relevant gen expression during CNS development.

As earlier results showed that both E2 and THs are required for the precise orchestration of cerebellar development and that alterations in the plasma/tissue concentration of either of the hormones may influence signalling mechanisms that are driven by both E2 and THs through the maintenance of normal levels of each-other's cognate receptors [1]. Insufficient or excess E2 or TH levels, elicited either by disturbed regulatory mechanisms in the hypothalamic–pituitary–gonadal axis or modified on site receptor-ligand interactions, may lead disturbances in the higher cognitive functions particularly in developmental stage.

Endocrine disruptors

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 signalling pathways.

Living organisms 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 by-product 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.

The endocrine disrupting potency of these EDs is a result mostly of their ability to bind to the ERs and TRs. Since they interfere with hormonal signalling mechanisms normally activated by E2 or THs, it is highly probable that exposure to such chemicals represents considerable risks to the normal development of the CNS. 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.

Thus summed up, in the funded experiments we intended to demonstrate how the addition of relevant amounts of well-known ED chemicals influences the expression levels of ERs and TRs under the experimental conditions explained in the proposal, focusing on the aims that were to:

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

B. determine 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. characterize the effect of presence or absence of glial cells on the applied ED agents on the expression of TRα, TRβ and ERβ.

Experimental approach

Cerebellum is a late-developing region of the CNS [2]; therefore investigating of regulatory impact of E2 and TH signalisation on the neurodevelopmental processes can be traced even in the early postnatal period. The postnatal occurrence of cerebellar developmental steps and their E2-TH-driven regulation makes this brain region an excellent model for testing the effects of estrogenic and thyroid disrupting chemicals on distinct neurodevelopmental steps. In addition to this, cerebellar models are widely used for experiments related to hormonal changes, or ligand-receptor interactions, etc. [3]–[6], that make this brain region excellent model considering scientific relevance and experimental reproducibility.

Taken these above in consideration; we decided to use in vitro experimental model applying primary granule cell cultures [7] derived from 7 day old rat pup from both sexes. 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 (AraC) 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) [1]. 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. Harvested cells were tested for changes in ER and TR mRNA and protein levels with quantitative real-time PCR and Western blot method, respectively. Results from treated groups were expressed as fold changes relative to the control group (ntC).

Risks, changes in staff and budget plan

There were no risks, difficulties or major challenges along the experimental procedure, all the methodology was routinely applied in our labs. The budget plan was not significantly altered throughout the grant period. Minor changes in staff didn't notably influence the proposed research plan, since hired members were skilled in all of the methods that were required to fulfil the experiments.

Short evaluation of results funded by this grant

Here we summarize the basic and most important findings classified by the examined EDs.

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 up-regulation, 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 down-regulation in the mRNA levels between ntC and BPA disappeared in both of the TRα, TRβ and ERβ receptors. BPA caused an up-regulation 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 (further details have been published in [8]).

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 down-regulation 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 results 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 (further details have been published in [9]).

Arsenic

A strong glial modulatory effect was detected 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 down-regulation 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.

Arsenic induced a strong down-regulation in all 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 (further details have been published in [10]).

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 up-regulation was found) than in Glia- (strong receptor mRNA down-regulation).

According to the recent literature MBC mostly affects the TH signalling pathways in the cells. In our cell cultures the substance strongly influenced the T3 or T4 treated experimental groups. In Glia+ a potent up-regulatory effect was seen in TRα and ERβ. In TRβ the rate of up-regulation was low or negligible. Without TH-s the overall effect in receptor protein up-regulation 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, 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 seem to highly vary, our results still remain alarming with regard to MBC's influence on neuronal development (further details have been published in [10]).

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 up-regulation of receptor mRNA, but the SEM values were high as well. Combined ED effects were exerted when E2 and T3 levels were low, even in the presence of the glia. It is possible that under physiological circumstances, when young individuals are simultaneously exposed to multiple EDs, their

neural development, 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 up-regulation of the tested receptors. The fold difference compared to ntC cultures were similar to the MBC results in every treatment, indicating that MBC exerts 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 up-regulation 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.

Comparing the results of the ERβ protein expression modulating effects of the different EDs with the combined treatment a strong receptor up-regulation can be observed in all cell cultures. The combined treatment caused a 2.5-fold up-regulation in every ED treated cell culture compared to ntC and the corresponding untreated samples opposed to As or MBC. The difference between the combined treatment and BPA or ZEA was negligible (further details have been published in [10]).

Conclusions

Results clearly indicated 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 was rather heterogeneous although we suspected a somewhat uniform effect due to 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 working 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.

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