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1. Introduction

Estrogen (E2), as a traditionally known female reproductive hormone, and thyroid hormones (THs), best known as regulators in energy homeostasis, are unique in that they play key roles in the regulation of several other physiological processes as well. For example, E2 and THs play pivotal roles in central nervous system development, including cell division, cell proliferation, cell maturation, apoptosis, and the regulation of the intracellular metabolism, latter which significantly affects most intracellular events on its own. The listed hormonal regulatory effects are mediated by at least three known major mechanisms:

1. Specific (cognate) intracellular receptors (E2-, TH receptors, ERs and TRs) that function as transcription factors when activated by bound hormone ligands (generally considered as genomic effects);
2. Plasma membrane-bound ligand-receptor complexes that activate rapid, non-genomic intracellular signaling cascades; and
3. Crosstalk on multiple levels of genomic and/or non-genomic E2- and TH-activated intracellular signaling.

The numerous trophic effects of E2 and THs that are mediated by ERα,β and TRα,β, are the result of the two hormone's interactive effects on the expression level of each-other's receptors, thereby modulating intracellular mechanisms that depend on the receptor's signal-mediating functions. It is, therefore, obvious that the mechanisms, through which these hormones exert their effects are plentiful and include both intra- and intercellular actions. The referred hormonal mechanisms are versatile, experimental investigation of simultaneous hormone-induced mechanisms is technically extremely difficult.

ERs and TRs are widespread in the brain, however, their expression level depends on the brain region, age and functional-hormonal status of the organism. Thus, it is not known, how ER-TR receptor expression levels correlate with real-time hormonal conditions and to what extent ER-TR gene transcriptional activity correlates

to ER-TR protein synthesis in the developing cerebellum. To address these questions, in the present study we established a primary cerebellar granule cell culture as an i*n vitro* experimental model that is suitable to investigate isolated granule cell responses to various single- or combined hormone treatments and/or glial effects could be experimentally manipulated. Glia-containing (Glia+) or glia-reduced (Glia-) cerebellar granule cell cultures were treated with either E2, T3, T4 or a combination of these hormones, and resulting receptor expression levels were determined by quantitative real-time PCR and Western blot techniques.

Endocrine disrupting chemicals (EDs) are selective hormone receptor modulators and can act as agonists or antagonists of the hormones in question. During development, EDs can influence normal hormonal homeostasis and lead to immediate and/or life-long consequences. With regard to ED effects in the cerebellum, it was previously shown that bisphenol A (BPA) can rapidly activate ERK1/2 in primary cerebellar granule cell cultures and also, after injection of BPA into the cerebella of newborn rat pups. In addition to interactions between BPA and ERs, BPA can alter thyroid-specific gene expression and functions. Our studies indicated that the ratio of THs to E2 in the central nervous system (CNS) is critical for the regulation of nuclear receptor expression.

While a growing body of evidence indicates that EDs, including BPA, interfere with CNS development, the exact mode of BPA action, and how it alters TR expression levels currently is not clear. In the present study, as part of a more extensive study, we examined to what extent BPA alters TRα,β mRNA and protein expression levels in primary cerebellar cell cultures and investigated the effects of combined treatments when cultured cells were co-exposed to a combination of the hormones. We also examined whether the glia could modulate hormone and/or BPA effects on TR mRNA and protein expression levels.

2. Aims of the study

A. Establishing a suitable in vitro experimental model for our experiments by finding and applying specific and goal-oriented modifications to the well-established primary cerebellar cell culture system;

B. Determination of the individual and combined effects of 17-beta-estradiol and thyroid hormones on their own and each other's specific receptors (ERβ, TRα,β);

C. Determination of the potential effects of BPA on the expression of TRα,β;

D. Interpretation of our findings in the context of the cerebellum;

E. Interpretation of our findings integrated into the available relevant literature in the context of cellular and hypothalamic estrogen- and thyroid hormone effects, with special regard to the hypothalamic regulation of feed-intake.

3. Materials and methods

Since neither previous studies nor our own results indicated gender differences in the developing rat cerebellum, both male and female Sprague-Dawley rat pups (body weight: 18–20 g) were used in these studies. Timed pregnant Sprague-Dawley rats were obtained from the vendor at least four days before they gave birth. 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. The date of the pup's birth was considered as postnatal day 0 (P0). Animals were used for granule cell preparation on their P7. Considering the differences between the *in vitro* and the physiological-biological conditions, we found it important that besides the results of the various treatments we also indicate how these results compare to values obtained from age-matched reference samples. Therefore, we also used cerebella taken from P14 rat pups to determine physiological levels of receptor mRNA and protein levels her (*in situ*). Following the guidelines established by the National Institutes of Health, the use of animals was approved by the Animal Welfare Board at Szent István University Faculty of Veterinary Sciences and were approved by the regional animal welfare authority (registry No: 22.1/3947/003/2008).

Primary cerebellar cultures were prepared as described: animals were sacrificed by quick decapitation and the cerebella removed. Cell cultures were prepared without enzymatic treatment and were maintained in serum- and steroid-free conditions. It was our goal to determine isolated cellular responses to the treatments applied. Therefore, to prevent cell-to-cell adherence and thus exclude the masking effects resulting from direct (physical) cellular contact, cerebellar cell suspensions were diluted in culture media until they reached a final cell number of 2300-2700 granule cells/mm^2 after 7 days of incubation. Under such conditions, more than 95% of cells in cultures were granule neurons. Cerebella of rat pups were seeded into separate culture dishes (i.e., 6 dishes per treatment, n=6).

For analysis of mature primary cerebellar granule cells in a glia-reduced environment (Glia-), β-D-arabinofuranoside (AraC) was added 24 hrs after seeding to inhibit the proliferation of non-neuronal cells. In contrast, no AraC was added to the media for analysis of neurons grown in a glia-containing environment (Glia+ experimental groups). Cultures were treated with either of the following hormones (at physiologically relevant concentrations) 7 days after seeding and 6 hours (for qPCR) or 18 hours (for Western blot) before harvesting: 17β-estradiol (E2); 3,3',5-triiodo-L-thyronine (T3); L-thyroxine (T4); E2+T3 or E2+T4; bisphenol A (BPA); BPA+E2; BPA+T3; BPA+E2+T3; BPA+T4; BPA+E2+T4. Reference cultures without any hormone treatments were included in both the Glia- and Glia+ groups (non-treated control; ntC).

After harvesting the cell cultures, the samples were sonicated and cleared by centrifugation for Western blot and qPCR studies. All data that have been presented are representative of at least three independent measurements. Measurement results (including the determination of BPA effects) are normalized to the ntC of the Glia+ cultures.

Statistical analyses were conducted using Excel and GraphPad Prism version 4 by means of one-way ANOVA with Tukey's multiple comparison test.

4. Results and Discussion

Effects of E2 and THs on primary cerebellar cell cultures

In discussing our results from hormone treatment studies, we consider our *in situ* data as a reference, where tissue integrity and the molecular environment, at the time of sampling, were intact. Compared to *in situ* samples, Glia-/+ cultures lost their tissue integrity; in addition, in Glia- cultures the glia is blocked from growing and proliferating; E2 groups are deprived of THs, T3/T4 groups are deprived of E2, and the non-treated controls of Glia-/+ groups are deprived of both E2 and THs.

In the present study, we compare endocrine effects in glia-containing versus glia-reduced cultures. Differences between these groups is clearly the result of the presence or „absence" of glia, since in Glia- cultures granule neurons extremely outnumber sporadic and rudimentary glial cells and, therefore, it seems to be safe to interpret the treatment effects in Glia- as if they were only exerted by neurons.

The applied experimental conditions lead to increases in mRNA expression levels compared to respective *in situ* samples suggesting that loss of tissue integrity generates a need for more E2 and TH action in the cultured cells in order for the cells to adapt to their new environment and to maintain the highest possible cell vitality. Interestingly, characteristic differences in the mRNA and protein patterns (Glia+ vs Glia-) suggest that glial cells play key roles in the regulation of neuronal TRα,β protein biosynthesis. One of the possible glial functions might be the mediation of molecular signals towards granule neurons that may be necessary links between the transcription and translation of TR genes. With regard to ERβ, increased mRNA levels were also accompanied by increased ERβ protein levels, regardless of the presence or absence of glia. This observation indicates that balancing the transcription and translation of ERβ gene is not or is less dependent on glial contribution than that of the TR genes.

Results from the Glia+ subgroup show that loss of tissue integrity, on its own, does not alter the expression level of TRα proteins, with the only exception of the E2 treated Glia+ group. The maintenance of normal levels of TRα protein, however, seems to require higher than normal transcriptional activity, as relevant TRα mRNA expression levels were significantly increased compared to those *in situ*. Additionally/alternatively, the observed increase in transcriptional activity may also reflect a regenerative action on the part of explanted cells.

When cultured cells were deprived of THs (E2 treatment only), our findings suggest that the clear overall glial effect on TRα protein production is dependent on the presence of the hormones used. The overall glial effect was not seen with respect to TH-deprivation, as TRα protein expression values for both E2 treated Glia+ and Glia- subgroups were equally and significantly lower compared to in situ levels. This observation implies that with respect to TRα protein expression, effects of THs, but not those of E2, are conveyed by glial cells.

In case of TRβ protein expression, loss of tissue integrity alone does not lead to a decreased level of expression, as removal of both E2 and THs was necessary to reach the aforementioned significant decrease; addition of any or both of these hormones prevented a loss in TRβ protein expression. In contrast, all subgroups deprived of glia (Glia-) displayed TRβ protein levels significantly lower than those detected in situ, regardless of the presence or absence of physiological amounts of E2 and/or THs. Therefore, it appears that the hormonal effects on the maintenance of normal TRβ protein expression levels are mediated by glial cells. As in the case of TRα, measured TRβ protein levels were backed by significantly higher than normal mRNAs, suggesting that there may be compensatory mechanism(s) in cultured cells on a transcriptional level to maintain the TRβ protein expressions observed.

In ERβ mRNA and protein expression levels, the most salient observation was the overall increase in cultures compared to *in situ* values. This finding suggests that loss of tissue integrity induces an increase in ERβ mRNA and protein expression,

regardless of the presence or absence of glia. It is speculated that such an increase in ERβ (mRNA and protein) might be a reparative/regenerative response on the part of cerebellar cells, and highlights the potential role of ERβ in the regulation of neuronal viability. With regard to ERβ mRNA expression pattern, there are many similarities between ligand-induced TR and ERβ transcriptional activities.

The above described results suggest a E2 and THs are able to regulate the level of their own (cognate) and each other's receptors. A possible mechanism of ligand-dependent ER-TR interactions is that ERs and TRs might be able to bind to hormone response elements (EREs; present in the promoter region of certain genes) either as homo- or as heterodimers. In addition, the existence of an identical half-site at the hormone response elements, possibly shared by ERs and TRs. The latter observation suggests that ERs and TRs may compete for binding to their promoter binding sites and, these findings altogether implicate the possibility of a high degree of co-operation between certain functions of E2 and THs. Such a possibility is supported by a number of other studies as well.

Effects of BPA on TRs

Under in vitro circumstances it is especially interesting that BPA alone suppresses TR mRNA expression. The observation that ntC values in Glia- cultures superseded those measured in ntC Glia+ suggests that the glia plays a role in the regulation of transcription, regardless of the type of receptor examined; even when cells were only treated with BPA alone, consequential decrease in TR transcription was more remarkable if the glia was present in the culture. While these observations are in concordance with previous results, our data also show that BPA's effects on TR transcription is multifactorial and, in addition, differ depending on the presence or the absence of glia.

In contrast to the suppressing effect of BPA on receptor mRNA expression, the combination of BPA with any of the hormones provoked remarkably high transcriptional

activity, regardless of the hormone used or the receptor examined. Such a robust ED effect has been reported earlier, yet, this finding should still be alarming. To our knowledge, currently there is no explanation for this additive effect, although it is likely that the ability of BPA to act on TRs plays a role in the potentiation of transcription.

In our study, it was generally observed that effects of BPA or BPA in combination with E2 and/or THs on translation (receptor protein expression) were less prominent than those found with regard to mRNA expression. Since the specific cellular effects of hormones are mostly mediated by their cognate receptors, this observation can explain why the biological effects of BPA-exposure could have remained masked or even unrecognized for a long time in spite of the dramatically increased transcriptional activity. The unproportional ED effects in transcription versus translation also indicate that regulatory mechanisms that are interposed between transcription and translation, such as microRNA regulation, may also be affected by EDs. These mechanisms apparently play a crucial role in blunting-buffering ED effects downstream of transcription. This idea not only warrants further research of these mechanisms, but also shows that the potential vulnerability of such interposed regulatory mechanisms may determine the severity of ED effects.

While differences between the Glia+ and Glia- groups show comparable trends, it is noteworthy that in Glia+, BPA treatment alone resulted lower TRα mRNA expression than E2 treatment alone, in contrast to the opposite findings in Glia- cultures. In Glia+, TRα receptor protein expression nearly doubled when the cultures were exposed to any of the used ligands, with the exception of E2. E2 treatment alone did not cause a change in receptor protein level when compared to the non-treated control. Thus, the potency of BPA, as a known estrogenic chemical, to influence TRα expression more than E2 underlines the importance of BPA to be considered as a general nuclear receptor modulator, rather than just a chemical with estrogenic or thyroid effect.

In Glia- cultures, major differences were found between groups treated with the hormones only and those exposed to BPA as well. This observation suggests that in the presence of glia, THs must be present for the maintenance of the afore-mentioned double levels of TRα protein expression, and that under such circumstances BPA does not further increase the TH-regulated TRα expression.

The overall pattern of TRβ protein expression values may suggest that there is no glial contribution in the determination of the actual TRβ protein expression levels. It is, therefore, important to consider the role of glia in the regulation of TRβ transcription, since the simple examination of potential glia effects on TRβ protein expression would be misleading. It seems that BPA could influence TRβ protein in a hormone ligand-independent manner, as also indicated with regard to TRα.

We are aware that the *in vitro* conditions, in general may, by themselves, substantially modify physiological parameters. Yet, it is more than likely that the observed BPA effects, combined with numerous biologically linked mechanisms, also occur in vivo, with the notion that in vivo, TRβ expression is restricted to specific ontogenetic states and is highly tissue specific. Altogether, this idea is consonant with results from animal models that have shown that hypothyroidism during critical periods of development causes a variety of abnormalities in the central nervous system, and that TRα and TRβ can compensate for each other's hypofunction. Finally, it should be noted that a growing body of evidence exists to show that BPA and other EDs increase intracellular reactive oxygen species, generate oxidative stress conditions in mitochondria and endoplasmic reticulum, and activate apoptotic processes, such as the caspase 3 and caspase 9 apoptotic pathways, altogether leading to cell death and poor tissue development. The intracellular mediators of these ED effects towards influencing TR expression are currently studied.

5. Conclusions

Results of the present study reveal that, in the developing cerebellum, there is a highly complex interplay between E2 and THs in the maintenance of normal levels of each-other's cognate receptors, and that the hormone effects are most probably mediated by the glia. Our results explain at least some, and raise even more, questions regarding the role and mechanisms of E2 and THs in neurodevelopment, and underscore the importance of the optimal/physiological ratio of E2/THs in the precise orchestration of cerebellar development, memory formation and neuroprotection when necessary. On the other hand, our observations implicate that abnormalities in glial and/or thyroid functions or in tissue E2/TH levels impact, on multiple levels, cerebellar development, cerebellar functions later in life, and the regenerative capability of the cerebellar tissue in case of injury, all of which should be considered in the diagnostics and treatment of relevant clinical conditions. Considering the complexity of interactions between E2 and TH signaling, both intracellularly and intercellularly, it is reasonable to hypothesize that the failure of the integrated ER-TR signaling at one or more points may account, at least in part, for the formation of neuron- or glia-based tumors.

With regard to BPA effects on TR expression levels, some important conclusions can be drawn: 1. BPA alters E2 and TH regulated TR expression; 2. The glia modulates (mediates?) the detected BPA effects. The questions of exactly why and how are BPA-caused actual mRNA and protein levels set at the detected values remain unanswered and warrant further experiments. One of the possible explanations may be that a receptor protein-related negative feedback mechanism limits the intensity of the BPA effects. Alternatively or additionally, the cellular energy and material supplies may set a limit to the intensity of BPA effects. Regardless of which of these explanations may be true, the present results clearly indicate that BPA markedly interferes with the normal hormonal regulation of TR expression and thereby may lead to yet unknown biological consequences, either beneficial or adverse, in the developing cerebellum.

6. New scientific results

In the present series of experiments, we

- established a new in vitro experimental model based on previous experience and applied new, goal-oriented modifications that allowed for the investigation of cellular responses without physical intercellular contacts-pointed out the physiological relevance of the results gained from the applied in vitro experimental model by the comparison of results from cultured cells and in situ tissue;

- provided evidence for the ligand-dependent regulation of ERβ and TRα,β mRNA and protein expression levels;

- implied the existence of a likely regulatory mechanism between ER and TR transcriptional and translational levels through the comparative analysis of qPCR and western blot results;

- provided correlative evidence for the mediating role of the glia in the ligand-dependent regulation of neuronal ERβ and TRα,β mrna and proteins;

- provided in-depth analysis of the potential integrated hormonal mechanisms that govern the hypothalamic regulation of feed-intake based on the present results and the most recent literature data.

7. The author's scientific publications

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