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BISPHENOL A INFLUENCES OESTROGEN- AND THYROID HORMONE-REGULATED THYROID HORMONE RECEPTOR EXPRESSION IN RAT CEREBELLAR CELL CULTURE

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Thyroid hormones (THs) and oestrogens are crucial in the regulation of cerebellar development. TH receptors (TRs) mediate these hormone effects and are regulated by both hormone families. We reported earlier that THs and oestradiol (E_2) determine TR levels in cerebellar cell culture. Here we demonstrate the effects of low concentrations (10^{-10} M) of the endocrine disruptor (ED) bisphenol A (BPA) on the hormonal (THs, E_2) regulation of TRα,β in rat cerebellar cell culture. Primary cerebellar cell cultures, glia-containing and glia-destroyed, were treated with BPA or a combination of BPA and E_2 and/or THs. Oestrogen receptor and TH receptor mRNA and protein levels were determined by real-time qPCR and Western blot techniques. The results show that BPA alone decreases, while BPA in combination with THs and/or E_2 increases TR mRNA expression. In contrast, BPA alone increased receptor protein expressions, but did not further increase them in combination with THs and/or E_2. The modulatory effects of BPA were mediated by the glia; however, the degree of changes also depended on the specific hormone ligand used. The results signify the importance of the regulatory mechanisms interposed between transcription and translation and raise the possibility that BPA could act to influence nuclear hormone receptor levels independently of ligand–receptor interaction.

Key words: Endocrine disruptors, thyroid receptor, transcription, translation, bisphenol A, rat cerebellum

Thyroid hormones (THs) and oestrogens (mostly 17β-oestradiol, E_2) play critical roles in the regulation of central nervous system (CNS) development, in-

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cluding the cerebellum. Effects of these hormones are mediated by numerous mechanisms, including, but most probably not limited to, cognate receptors (oestrogen receptors alpha and beta [ERα,β] and TH receptors alpha and beta [TRα,β]) (Belcher and Zsarnovszky, 2001; Fan et al., 2010), membrane-linked ligand-receptor complexes (Belcher, 2008; Leonard, 2008) and crosstalk between genomic and/or non-genomic E_2- and TH-activated intracellular signalling pathways (Vasudevan et al., 2001; Zhao et al., 2005). Previously we demonstrated that E_2 and THs alone or in combination determine TRα,β and ERβ mRNA and protein expression levels in primary cerebellar cell cultures (Scalise et al., 2012). In addition to hormone effects, we also showed that the astroglia (glia) may be an additional site of these actions. Based on the available literature and our studies, endocrine disruptor- (ED) induced disturbance of the physiological synchrony between E_2 and THs during cerebellar development could be more pervasive and far-reaching than currently appreciated, and merits investigation (Somogyi et al., 2011).

Oestrogenic ED chemicals, such as phytoestrogens or environmental pollutants are selective ER 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 lifelong consequences (e.g., Zsarnovszky et al., 2007; Miodovnik et al., 2014). With regard to ED effects in the cerebellum, we have previously shown that bisphenol A (BPA) rapidly activates ERK1/2 in primary cerebellar cell cultures (Wong et al., 2003) and also after injection of BPA into the cerebella of newborn rat pups (Zsarnovszky et al., 2005). These effects were concentration dependent, with a U-shaped concentration–response curve, which could indicate compound actions of BPA. In support of these findings, Mathisen et al. (2013) described that perinatal BPA exposure increased Pax6 (transcription factor playing a role in granule cell development and migration) in newborn mice cerebella and in cerebellar cell cultures. In the hippocampus, BPA modulated dendritic morphogenesis via effects on ER (Xu et al., 2014). Likewise, BPA also promoted dendritic growth in maturing cerebellar Purkinje cells (Shikimi et al., 2004). This is consonant with our previous results (Wong et al., 2003; Zsarnovszky et al., 2005).

In addition, BPA can alter thyroid-specific gene expression (Gentilcore et al., 2013) and functions (Zoeller et al., 2005; Iwamuro et al., 2006; Delfosse et al., 2014). Our studies indicated that the ratio of THs to E_2 in the CNS is critical for the regulation of nuclear receptor expression (Scalise et al., 2012).

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, is not clear. In the present study we examined to what extent BPA alone or in combination with THs and/or E_2 alters TRα,β mRNA and protein expression levels in primary cerebellar cell cultures. We also examined

whether the glia could modulate hormone and/or BPA effects on TR mRNA and protein expression levels.

Materials and methods

Hormones and BPA were obtained from Sigma Aldrich Ltd., Hungary.

Animals

Since neither previous studies nor our own results indicated sex differences in the developing rat cerebellum in any of the parameters examined in this study, both male and female Sprague-Dawley rat pups (body weight: 18–20 g) were used. Timed pregnant Sprague-Dawley rats (Charles River Laboratories, Germany; n = 60) were obtained from the vendor at least four days before they gave birth. Animals were kept under standard laboratory conditions (temperature: 24–25 °C; humidity: 55–60%; room ventilation rate: 17 air changes per hour, with the animals protected from draughts; noise reduced to the possible minimum in the human hearing range, as well as in the ultrasonic range), with tap water and regular rat chow *ad libitum* in a 12-h light, 12-h dark cycle. The date of the pups' birth was considered as postnatal day 0 (P0). Animals were used for granule cell preparation on their P7 (n = 576, i.e., n = 6 per each treatment group).

Following the guidelines established by the National Institutes of Health (NIH), the use of animals was approved by the Animal Welfare Board at Szent István University, Faculty of Veterinary Science and were approved by the regional animal welfare authority (registry no.: XIV-I-001/2201-4/2012).

Preparation of primary cerebellar cell cultures

Primary cerebellar cultures were prepared as described earlier (Wong et al., 2001) with modifications, as follows. 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 as previously described (Wong et al., 2001). Cerebellar cell suspensions were diluted with culture medium to obtain 2300–2700 granule cells/mm^2 prior to testing. Cerebella of rat pups were seeded into separate culture dishes (i.e., 6 dishes per treatment, n = 6). Astroglia in the cultures was identified by immunohistochemical labelling for the specific astroglia marker glial fibrillary acidic protein (GFAP), as we previously described and illustrated (Scalise et al., 2012).

Treatments

For analysis of mature (post-mitotic) primary cerebellar granule cells in a glia-destroyed environment, a final concentration of 10^{-5} M cytosine β-D-arabinofuranoside (AraC; Sigma Aldrich Ltd., Hungary; Cat. # 147-94-4) was

added 24 h after seeding to inhibit the growth and proliferation of non-neuronal cells (Glia– cultures). In contrast, no AraC was added to the media for analysis of neurones grown in a glia-containing environment (Glia+ cultures). The presence or absence of glia in the cultures was verified and illustrated as shown earlier (Scalise et al., 2012). Cultures were treated with either of the following hormones (at physiologically relevant concentrations, as described below) and/or bisphenol A, 7 days after seeding and 6 h (for qPCR) or 18 h (for Western blot) before harvesting: 17β-oestradiol (E_2, $1.16x10^{-10}$ M, Sigma Aldrich Ltd., Hungary, Cat. # E2758); 3,3',5-triiodo-L-thyronine (T3, 0.92×10^{-9} M, Sigma Aldrich Ltd., Hungary; Cat. # T2877); L-thyroxine (T4, 65×10^{-9} M, Sigma Aldrich Ltd., Hungary; Cat # T1775); E_2 + T3 or E_2 + T4 (at concentrations described above); bisphenol A (BPA, 10^{-10} M, Sigma Aldrich Ltd., Hungary; Cat # A239658); BPA + E_2; BPA + T3; BPA + E_2 + T3; BPA + T4; BPA + E_2 + T4; the latter BPA-containing combined treatments were carried out in both Glia– and Glia+ groups. Cultures without hormone treatments were included in both Glia+ and Glia– groups (non-treated control, ntC). The applied concentration of BPA (10^{-10} M) was chosen based on our previous experiments (Wong et al., 2003) as follows: The biphasic effects of E_2 and BPA at the low concentration range (10^{-12} to 10^{-9} M) were assumed to act at two binding sites, one stimulatory with a high affinity and one with a lower affinity that inhibits the effect of the first site. Based on the observed similarity in effect at each concentration it was also assumed that neither of the two sites distinguishes between E_2 and BPA. Thus, data points were fit with the Hill-type equation:

$$\text{Effect} = \text{maximal stimulation} \frac{1}{1+\left(\frac{EC50_{Stim}}{[C]}\right)^{H1}} \frac{1}{1+\left(\frac{[C]}{EC50_{Inhib}}\right)^{H2}}$$

where [C] is the concentration of the ligand. A best fit of the equation to the data resulted from setting the maximum effect of the stimulatory site (maximal stimulation) at 110%, with resulting values of 0.4 and 2 obtained for the stimulatory and the inhibitory Hill coefficients (H1 and H2), respectively. Under these conditions, EC_{50Stim} and $EC_{50Inhib}$ were determined as 8×10^{-12} M and 4×10^{-9} M, respectively.

Western blot studies

Cell harvesting was performed as described by Wong et al. (2001). Samples were homogenized in (in mM) 20 Tris [(Tris-(hydroxymethyl)aminomethane, Reanal Cat. # 34780-1-99-38) pH 7.6, 150 NaCl (Sigma Aldrich Ltd., Hungary, Cat. # S5886), 1 PMSF (Sigma Aldrich Ltd., Hungary, Cat. # P7626), 1 EGTA (Sigma Aldrich Ltd., Hungary, Cat. # E3889), 1 EDTA (Reanal-Ker Ltd., Hungary, Cat. # 19012-1-38), 2.5 sodium pyrophosphate (Sigma Aldrich Ltd., Hun-

gary, Cat. # P8010), 1-β-glycerol phosphate (Sigma Aldrich Ltd., Hungary, Cat, # G9891), and 1 Na_3VO_4 (Sigma Aldrich Ltd., Hungary, Cat. # S6508) plus 1 mg/ml Pefabloc (Fluka, Cat. # 76307), 10 μg/ml leupeptin (Sigma Aldrich Ltd., Hungary, Cat. # L2884), 10 μg/ml pepstatin (Sigma Aldrich Ltd., Hungary, Cat. # P5318), 1 μg/ml aprotinin (Sigma Aldrich Ltd., Hungary, Cat. # A3428), 1% Triton X-100 (Sigma Aldrich Ltd., Hungary, Cat. # T8787), and 0.05% sodium deoxycholate (Sigma Aldrich Ltd., Hungary, Cat. # D6750). Homogenates were sonicated for 5 sec a total of 5 times and cleared by centrifugation at 14,000 × *g* for 1 min at 2 °C. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Western blotting and densitometric analysis were performed by standard protocols (Wong et al., 2001); primary antibodies: anti-thyroid hormone receptor alpha, Abnova PAB-11276, dilution: 1:1,000; anti-thyroid hormone receptor beta, Abnova PAB-11277, dilution: 1:1000. Secondary antibodies: peroxidase labelled goat-anti rabbit IgG, Vector Laboratories, UK, Cat. # PI-1,000, dilution: 1:2,000; peroxidase-labelled horse-anti mouse IgG, Vector Laboratories, UK, Cat. # PI-2,000, dilution: 1:2,000). Immunoreactive bands were visualised onto preflashed X-ray film by enhanced chemiluminescence and analyzed by densitometric analysis. Optical densities were calculated as arbitrary units, normalised to the protein concentrations of samples, and to the ntC of Glia+. Results are presented as fold changes relative to the ntC of Glia+ cultures. All data that have been presented are representative of at least three independent experiments (n = 6 per treatment).

Quantitative PCR measurements

Total RNA was isolated from the cell samples using TRI Reagent (Invitrogen, Carlsbad, CA, USA, Cat. # RT111) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction was carried out following standard protocols. Primer sequences were as published by Billon et al. (2002) for TRα, and Kariv et al. (2003) for TRβ. Cellular gene expression was quantified by quantitative PCR reactions (qPCR; LightCycler 2.0, F. Hoffmann-La Roche Ltd., Basel, Switzerland) using LightCycler DNA Master SYBRGReen I fluorescent dye (Hoffmann-La Roche Ltd., Basel, Switzerland, Cat. # 12015099001). Aliquots of cDNAs were dispensed according to the manufacturer's instructions. QPCR cycles and controls were planned according to the manufacturer's instructions and were optimised for each primer pair. Amplified products were identified by agarose gel electrophoresis, melting point and sequence analysis (Applied Biosystems ABI 3100 Genetic Analyzer, Agricultural Biotechnology Centre, Gödöllő, Hungary). Real-time PCR threshold cycle (Ct) data were analysed using the REST-XL software version 2.0. The target Ct of each sample was normalized to the Ct of the reference (housekeeping) gene (rat cytoplasmic beta actin) in the same sample. Differences in the Ct values were converted into relative amounts of mRNA based on the assumption of am-

plification efficiency = 2.0. Results are presented as fold changes relative to the ntC of Glia+ cultures (i.e., further normalised to the ntC of Glia+).

Data analysis

All data that have been presented are representative of at least three independent measurements. Statistical analyses were conducted using Excel (Microsoft, Microsoft Co., Redmond, WA, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA), by means of one-way ANOVA with Tukey's multiple comparison test. The level of statistical significance in differences between treatment groups as shown in the figures is $P < 0.05$.

Results

General observations

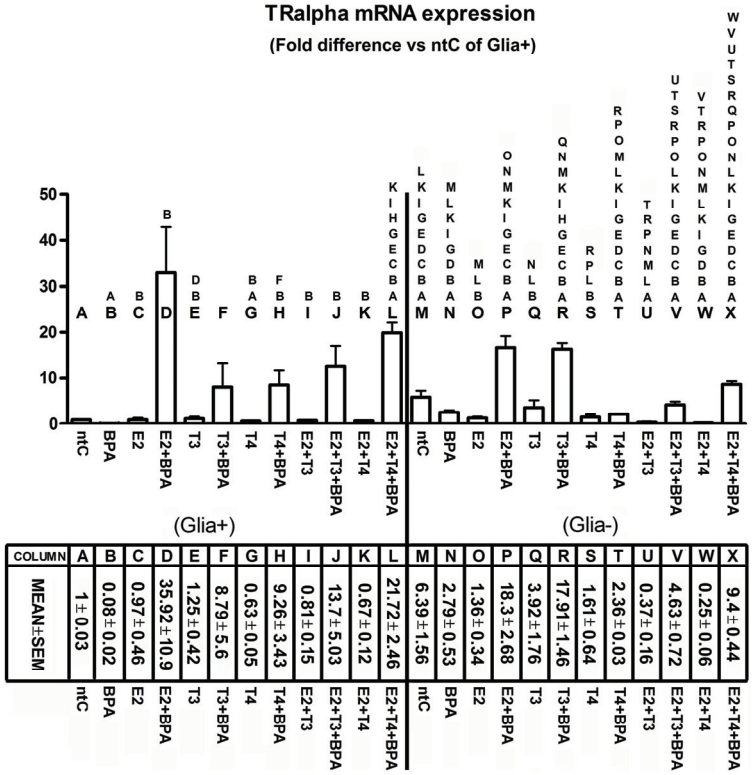
The results of hormone treatments were concordant with those we previously described (Scalise et al., 2012), so they will not be the major focus of this report; rather, these non-BPA-results are used as the reference base for the comparison between the effects of BPA alone and in combination with either E_2 and/or THs.

In general, transcriptional activity was higher in Glia– cultures compared to ntC [Glia+] (Figs 1 and 3). Regardless of the glia, in BPA-treated cultures receptor mRNA levels were lower than in ntCs (Figs 1 and 3; columns A *vs.* B and M *vs.* N), however, combined treatment with BPA and either E_2 or T3 resulted in increased receptor mRNA levels.

Thyroid hormone receptor alpha (TRα)

TRα mRNA: Glia+ vs. *Glia– (Fig. 1).* Non-treated controls of Glia+ and Glia– significantly differed from each other, with higher TRα mRNA values in Glia– cultures. Such a difference was found between BPA [Glia+] and BPA [Glia–] as well. With respect to combined treatment groups, with the exception of T3 + BPA, an opposite effect was evident: TRα mRNA expression was lower in E2 + BPA, T4 + BPA, E2 + T3 + BPA and E2 + T4 + BPA cultures of Glia– than of Glia+.

TRα protein: Glia+ vs. *Glia– (Fig. 2).* In Glia+ cultures (Fig. 2 columns A–L), TRα receptor protein expression was maintained at significantly higher level than the ntC in all treatment groups, with the exception of E2 treatment. In contrast, in Glia– cultures, differences were found between groups treated with hormones only and those exposed to BPA as well. In Glia–, BPA treatment alone resulted in the expression of just as much receptor protein as BPA in combination with any of the hormones used.

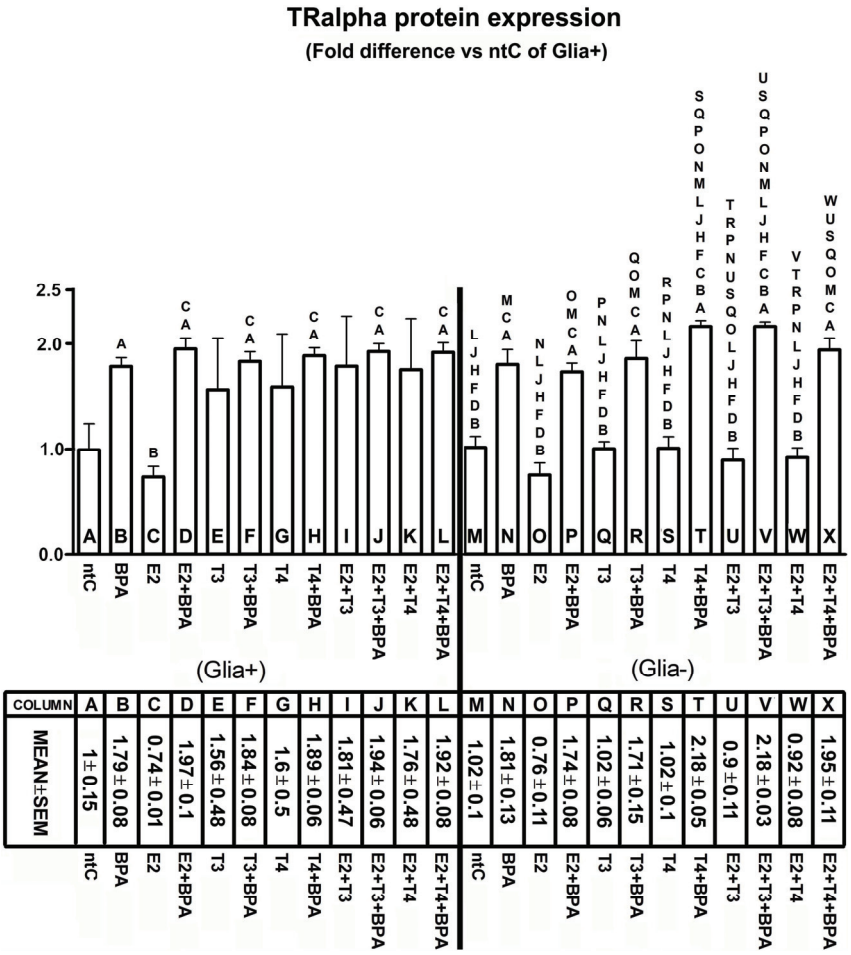


COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN±SEM	1±0.03	0.08±0.02	0.97±0.46	35.92±10.9	1.25±0.42	8.79±5.6	0.63±0.05	9.26±3.43	0.81±0.15	13.7±5.03	0.67±0.12	21.72±2.46	6.39±1.56	2.79±0.53	1.36±0.34	18.3±2.68	3.92±1.76	17.91±1.46	1.61±0.64	2.36±0.03	0.37±0.16	4.63±0.72	0.25±0.06	9.4±0.44
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Fig. 1. TRα mRNA expression in primary cerebellar cell cultures treated with BPA, E2, TH or a combination of these substances. X axis shows experimental groups; Y axis shows fold changes versus non-treated control (ntC) of Glia+. In Glia+ cultures, BPA decreased TRα mRNA expression compared to the ntC and resulted in mRNA levels lower than after E2 or TH treatment. In Glia– cultures, however, TRα mRNA expressions were less different between the BPA- and TH-treated groups. It is suggested that the glia may mediate BPA effects on transcription. All data that have been presented are representative of at least three independent measurements. Data show mean values ± SEM. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison test (n = 6). The level of statistical significance in differences between experimental groups is $P < 0.05$. Smaller size letters above the columns indicate significant differences from columns labelled with larger size letters

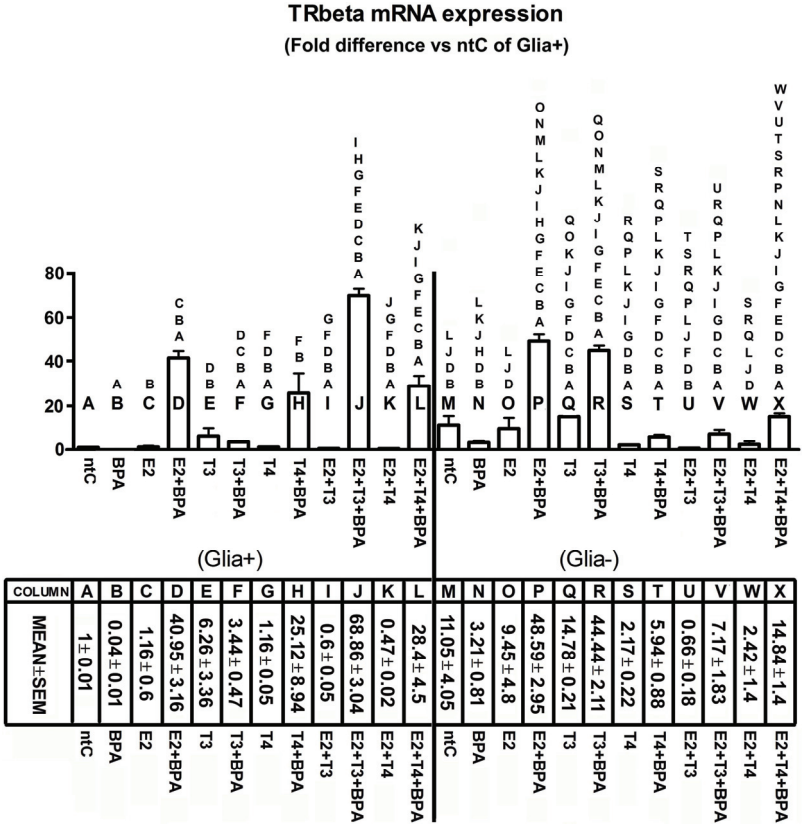
Thyroid hormone receptor beta (TRβ)

TRβ mRNA: Glia+ vs. *Glia– (Fig. 3).* Differences between the members of the Glia+ and Glia– showed comparable trends, although distinct differences between analogous treatment subgroups may be remarkable and resemble, in many respects, our TRα-related findings. Specifically, two exceptions were found from the above-stated phenomenon: (1) TRβ mRNA expression in T3 + BPA cultures was considerably higher in Glia– compared to Glia+ cultures; (2) in E_2 + T3 + BPA treatment groups, TRβ mRNA was significantly and prominently higher in Glia+ than in Glia–.



COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN±SEM	1±0.15	1.79±0.08	0.74±0.01	1.97±0.1	1.56±0.48	1.84±0.08	1.6±0.5	1.89±0.06	1.81±0.47	1.94±0.06	1.76±0.48	1.92±0.08	1.02±0.1	1.81±0.13	0.76±0.11	1.74±0.08	1.02±0.06	1.71±0.15	1.02±0.1	2.18±0.05	0.9±0.11	2.18±0.03	0.92±0.08	1.95±0.11
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

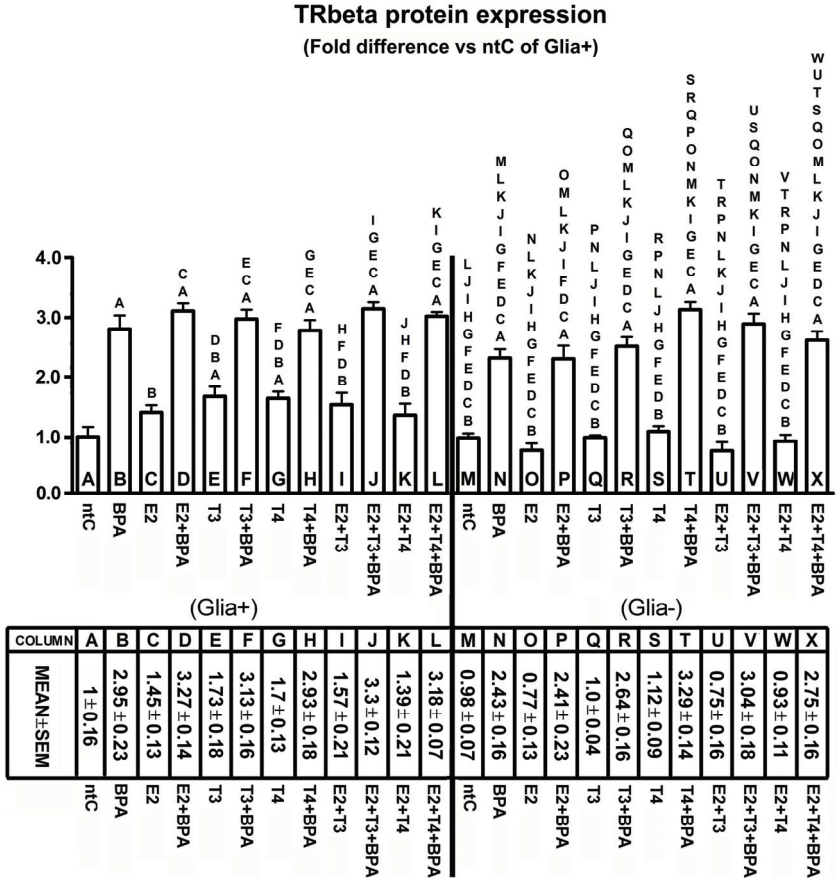
Fig. 2. TRα protein expression in primary cerebellar cell cultures treated with BPA, E2, TH or a combination of these substances. X axis shows experimental groups; Y axis shows fold changes versus non-treated control (ntC) of Glia+. In the presence of glia (Glia+), E2 alone did not cause a significant change compared to the ntC. In glia-destroyed environment (Glia–), significant differences were detected after BPA exposure compared to hormone-only treated cultures. All data that have been presented are representative of at least three independent measurements. Data show mean values ± SEM. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison test (n = 6). The level of statistical significance in differences between experimental groups is $P < 0.05$. Smaller size letters above the columns indicate significant differences from columns labelled with larger size letters



COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN±SEM	1±0.01	0.04±0.01	1.16±0.6	40.95±3.16	6.26±3.36	3.44±0.47	1.16±0.05	25.12±8.94	0.6±0.05	68.86±3.04	0.47±0.02	28.4±4.5	11.05±4.05	3.21±0.81	9.45±4.8	48.59±2.95	14.78±0.21	44.44±2.11	2.17±0.22	5.94±0.88	0.66±0.18	7.17±1.83	2.42±1.4	14.84±1.4
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Fig. 3. TRβ mRNA expression in primary cerebellar cell cultures treated with BPA, E2, TH or a combination of these substances. X axis shows experimental groups; Y axis shows fold changes versus non-treated control (ntC) of Glia+. E2 and E2 + BPA effects do not seem to be glia-dependent. However, when T3 was also added to the culture medium, significant differences were found between Glia+ and Glia– cultures. In Glia+ cultures, co-exposure of the cells to BPA and E2 or E2+T3 resulted in a nearly three-magnitude increase in TRβ transcription. All data that have been presented are representative of at least three independent measurements. Data show mean values ± SEM. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison test (n = 6). The level of statistical significance in differences between experimental groups is $P < 0.05$. Smaller size letters above the columns indicate significant differences from columns labelled with larger size letters

TRβ protein: Glia+ vs. *Glia– (Fig. 4).* TRβ protein expression measured in analogous subgroups of Glia+ and Glia– were relatively close to each other. TRβ protein expression levels were about twice as high in groups treated with BPA or BPA as well, than in cultures not treated with BPA, regardless of the presence or absence of glia. With respect to BPA exposure, clear glia effect on TRβ protein expression was only observed in analogous E_2 + BPA and E_2 + T4 + BPA groups.



COLUMN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
MEAN±SEM	1±0.16	2.95±0.23	1.45±0.13	3.27±0.14	1.73±0.18	3.13±0.16	1.7±0.13	2.93±0.18	1.57±0.21	3.3±0.12	1.39±0.21	3.18±0.07	0.98±0.07	2.43±0.16	0.77±0.13	2.41±0.23	1.0±0.04	2.64±0.16	1.12±0.09	3.29±0.14	0.75±0.16	3.04±0.18	0.93±0.11	2.75±0.16
	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA	ntC	BPA	E2	E2+BPA	T3	T3+BPA	T4	T4+BPA	E2+T3	E2+T3+BPA	E2+T4	E2+T4+BPA

Fig. 4. TRβ protein expression in primary cerebellar cell cultures treated with BPA, E2, TH or a combination of these substances. X axis shows experimental groups; Y axis shows fold changes versus non-treated control (ntC) of Glia+. Receptor protein expression levels were about twice as high in groups treated with BPA or BPA as well, than in cultures not treated with BPA, regardless of the presence or absence of glia. Clear glia effect was only observed in analogous E2 + BPA (columns D *vs.* P) and E2 + T4 + BPA groups (columns L *vs.* X). All data that have been presented are representative of at least three independent measurements. Data show mean values ± SEM. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison test (n = 6). The level of statistical significance in differences between experimental groups is $P < 0.05$. Smaller size letters above the columns indicate significant differences from columns labelled with larger size letters

Discussion

The primary cerebellar cell culture has been extensively used for the study of cellular responses to various experimental cues. A question that we should address is with regard to the effect of glia in the adjustment of TR expression. In

the present study, we compare endocrine effects in glia-containing versus glia-destroyed cultures. While differences between these groups is clearly the result of the presence or absence of glia, differences between treatment groups in Glia– cultures carry the enigma of the share of glial TR levels in the entire cultured cell population. Since, however, granule neurones extremely outnumber sporadic and rudimentary glial cells in these cultures, it seems to be safe to interpret the treatment effects as if they were only exerted by neurones.

Receptor mRNA levels

Under *in vitro* circumstances it is especially interesting that BPA alone suppresses TR mRNA expression. This is supported by the findings of Sheng et al. (2012); however, earlier data also suggest that the suppressing effect of BPA on TRs may only be temporary, and symptoms that emerge later in life resulting from perinatal exposure to BPA may develop on the grounds of BPA-linked mechanisms indirectly (Xu et al., 2007). 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 evident if the glia was present in the culture. While these observations are in concordance with previous results (Yamaguchi et al., 2006; Fauquier et al., 2014), our data also show that the effects of BPA on TR transcription are 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 (Zhang et al., 2013), yet, this finding should still be alarming. To our knowledge, currently there is no explanation for this massive upregulation, although it is likely that the ability of BPA to act on TRs plays a role in the potentiation of transcription (Zoeller, 2005).

Receptor protein levels

It was generally observed that the effects of BPA or BPA in combination with E_2 and/or THs on translation (receptor protein expression) were less prominent than those found with regard to mRNA expression. Since the 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 unrecognised for a long time in spite of the dramatically increased transcriptional activity. Several studies suggested that exposure to EDs early in life leads to altered CNS development and functional deficiencies later in adulthood (Mathisen et al., 2013). The present results suggest that the final, al-

tered outcome of hormonal signalling during ED exposure may only partly account for those anomalies, since the increased material and energy consumption by CNS cells in the process of enhanced transcription could also lead to energy-deficient intracellular conditions that could be, at least in part, responsible for developmental deficiencies. This idea is consonant with the report of Nakagawa and Tayama (2000) that BPA toxicity caused a decrease in cellular ATP levels in hepatocytes.

The unproportional ED effects in transcription *versus* translation also indicate that regulatory mechanisms, interposed between transcription and translation, such as microRNA regulation, may also be affected by EDs, as indicated by Avissar-Whiting et al. (2010) and Tilghman et al. (2012). These mechanisms apparently play a crucial role in 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.

Thyroid hormone receptor alpha (TRα)

TRα mRNA: Glia+ vs. *Glia– (Fig. 1)*. While differences between Glia+ and Glia– show comparable trends, it is noteworthy that in Glia+, BPA treatment alone resulted in lower TRα mRNA expression than E_2 treatment alone, in contrast to the opposite findings in Glia–. Whether or not differences between treatment groups compared to their respective ntC followed similar trends (changes relative to each other), results indicate that the differences are due to glial effects. One possible mechanism underlying the afore-mentioned idea is that the glia may mediate BPA effects on the level of transcription in a T3-dependent manner, which is likely due to the ability of astroglia to convert T4 to T3 through deiodination (Leonard, 1988).

TRα protein: Glia+ vs. *Glia– (Fig. 2)*. In Glia+, TRα receptor protein expression nearly doubled when cultures were exposed to any of the ligands used, with the exception of E_2. Thus, the potency of BPA, as a known oestrogenic chemical, to influence TRα expression more than E_2 underlines the importance of BPA to be considered as a general nuclear receptor modulator, rather than just a chemical with oestrogenic or thyroid effect.

In Glia–, significant 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 (compared to ntC [Glia+]), and that under such circumstances BPA does not further increase the TH-regulated TRα expression. The finding that in Glia– cultures these twofold ntC values were only detected if BPA was present alone or in combination with the hormones indicates that in neurones of Glia–, BPA (but not the hormones) determined the actual TRα protein expression values.

TRα mRNA vs. *TRα protein (Figs 1 and 2).* The seemingly ligand-independent receptor protein expression levels were based on highly ligand-dependent transcriptional activity, especially in cultures treated with BPA or BPA in combination with any of the hormones. In Glia+, with the exception of the ntC and the E_2 group, TRα protein expression levels were comparable (no significant differences), but were twofold compared to ntC. This phenomenon means that these cell populations could either not produce more TRα protein (because of their limited capacity or the activation of some down-regulating mechanism), or the increased TRα expression levels after treatments were necessary for the maintenance of survival under the applied experimental conditions. It is also possible that robust increases in transcription (e.g., in Glia+: E_2 + BPA, T3 + BPA, T4 + BPA and E_2 + T3/T4 +BPA) may potentially exhaust cellular energy resources, thereby influencing other energy-dependent cellular processes as well.

Based on the disproportionality between transcription and translation, it can be speculated that one of the cellular adaptation mechanisms may manifest in the form of a high degree of plasticity in transcription (and possibly linked mechanisms, such as microRNA regulation, etc.) in order to maintain a relatively steady receptor protein expression. In fact, this idea may apply to our TRβ results as well.

Thyroid hormone receptor beta (TRβ)

TRβ mRNA: Glia+ vs. *Glia– (Fig. 3).* Differences between the members of Glia+ and Glia– resemble, in many respects, to those found with regard to TRα mRNA. Differences between respective treatment groups in Glia+ *vs.* Glia– suggest that the mediating role of glia in the regulation of neuronal TRβ receptor expression is ligand dependent, and also indicate that this mediating activity, in addition to the mere glial presentation of T3 (after conversion of T4 to T3) to neurones, contains additional functional element(s) as well, whose identification warrants further experiments.

TRβ protein: Glia+ vs. *Glia– (Fig. 4), mRNA* vs. *TRβ protein (Figs 3 and 4).* 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 is also interesting that exposure to BPA, alone or in combination with any of the hormones used, leads to significantly elevated TRβ protein expression.

This finding suggests that BPA could influence TRβ protein in a hormone ligand-independent manner, as also indicated with regard to TRα. Again, this conclusion would be misleading regarding the effects of BPA on TRβ protein expression; therefore, we conclude that BPA effects on transcription and translation should be comparatively evaluated to understand the complexity of BPA and maybe other ED effects on the regulation of TR expression.

Further considerations

To the best of our knowledge, no clear definition of the differences between the functional roles of TRα and TRβ is available, most probably because of the usual co-activation of these receptors by their common ligands (Somogyi et al., 2011). There are, however, studies reporting on physiological functions partly regulated by either of these receptors. Based on knockout and knockin experiments, TRα was found to influence cardiac functions, thermogenesis, haematopoiesis, and the maturation of intestines and bones (O'Shea et al., 2005; Plateroti et al., 2006). TRβ is crucial for normal, physiological endocrine and sensory functions such as those regulated by the hypothalamic–pituitary–thyroid axis, hepatic reactions to T3, behaviour, audition, colour sensation, and tactile senses (Amma et al., 2001; Ng et al., 2001; Flores-Morales et al., 2002; Abel et al., 2003; Esaki et al., 2003; Siesser et al., 2005). TRα and TRβ, however, can be co-expressed in the same tissues and can substitute for each other's function to a certain extent (Gothe et al., 1999). Considering the latter potency of TRs allow us to reasonably suggest that one or more regulatory mechanisms interposed between transcription and translation is/are affected by BPA exposure. This idea is supported by reported alterations in microRNA expression in human placenta cells (Avissar-Whiting et al., 2010), MCF-7 breast cancer cells (Tilghman et al., 2012) and ovine fetal ovary (Veiga-Lopez et al., 2013) after BPA exposure.

We are aware that the *in vitro* conditions may 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 (Bradley et al., 1994). 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 (Pasquini and Adamo, 1994; Martinez-Galan et al., 1997; Simorangkir et al., 1997), 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 and generate oxidative stress conditions in mitochondria and endoplasmic reticulum (Huc et al., 2012; Babu et al., 2013).

The present results clearly indicate that BPA 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.

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