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LIGAND-INDUCED CHANGES IN OESTROGEN AND THYROID HORMONE RECEPTOR EXPRESSION IN THE DEVELOPING RAT CEREBELLUM: A COMPARATIVE QUANTITATIVE PCR AND WESTERN BLOT STUDY

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Oestrogen (E2) and thyroid hormones (THs) are key regulators of cerebellar development. Recent reports implicate a complex mechanism through which E2 and THs influence the expression levels of each other's receptors (ERs and TRs) to precisely mediate developmental signals and modulate signal strength. We examined the modulating effects of E2 and THs on the expression levels of their receptor mRNAs and proteins in cultured cerebellar cells obtained from 7-day-old rat pups. Cerebellar granule cell cultures were treated with either E2, THs or a combination of these hormones, and resulting receptor expression levels were determined by quantitative PCR and Western blot techniques. The results were compared to non-treated controls and to samples obtained from 14-day-old *in situ* cerebella. Additionally, we determined the glial effects on the regulation of ER-TR expression levels. The results show that (*i*) ER and TR expression depends on the combined presence of E2 and THs; (*ii*) glial cells mediate the hormonal regulation of neuronal ER-TR expression and (*iii*) loss of tissue integrity results in characteristic changes in ER-TR expression levels. These observations suggest that both E2 and THs, in adequate amounts, are required for the precise orchestration of cerebellar development and that alterations in the ratio of E2/THs may influence signalling mechanisms involved in neurodevelopment. Comparison of data from *in vitro* and *in situ* samples revealed a shift in receptor expression levels after loss of tissue integrity, suggesting that such adjusting/regenerative mechanisms may function after cerebellar tissue injury as well.

Key words: Oestrogen, thyroid, receptors, interaction, developing, cerebellum

Numerous studies provided evidence for the role of 17β-oestradiol (oestrogen or E2) (Ikeda, 2008; Fan et al., 2010) and thyroid hormones, i.e. triiodothyronine, thyroxine (THs, T3 and T4, respectively) (Koibuchi, 2008; Horn and

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Heuer, 2010) in the regulation of normal cerebellar development. Oestrogen, as a traditionally known reproductive hormone and 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. Such E2-TH regulated processes are neuronal/glial maturation and migration (Kirby et al., 2004; Belcher et al., 2009), and the regulation of intracellular metabolism, the latter of 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 receptors (mostly nuclear, ERs and TRs) that function as transcription factors when activated by bound hormone ligands (generally considered as genomic effects) (Jakab et al., 2001; Belcher and Zsarnovszky, 2001; Ikeda, 2008; Fan et al., 2010); (2) putative plasma membrane-bound/incorporated ligand-receptor complexes that activate rapid, non-genomic intracellular signalling cascades (e.g. Pekary et al., 2006; Belcher, 2008; Leonard, 2008); and (3) crosstalk on multiple levels of genomic and/or non-genomic E2- and TH-activated intracellular signalling pathways (Vasudevan et al., 2001*a,b*; Zhao et al., 2005), where hormone effects are evident but the exact role of the ligand alone or the ligand-receptor complex is not yet clarified. Thus, the numerous trophic effects of E2 and THs that are mediated by ERα,β and TRα,β, are the result of the two hormones' interactive effects on the expression level of each other's receptors, thereby modulating intracellular mechanisms that are dependent on the receptor's signal mediating functions. ERs (Shughrue et al., 1997) and TRs (Murray et al., 1988; Hodin et al., 1989) are widespread in the brain; however, their expression level depends on the brain region, age (Bernal, 2007; Al-Bader et al., 2008) and the 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 with 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 *in vitro* experimental model that is suitable to investigate isolated granule cell responses to various single or combined hormone treatments, where cell–cell interactions due to physical cellular contact could be discounted and/or glial effects could be experimentally manipulated. Glia-containing or glia-destroyed cerebellar granule cell cultures were treated with either E2, T3, T4 or a combination of these hormones, and the resulting receptor expression levels were determined by quantitative PCR (qPCR) and Western blot techniques. The results were compared to non-treated controls and to samples obtained from 14-day-old (age-matched, non-treated) *in situ* cerebella. Since our experiments on ERα mRNA and protein expression levels resulted in highly variable values (unpublished observations), those studies will only be regarded in a general context when appropriate.

Materials and methods

Animals

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; vendor: Charles River Laboratories, Inc., Hungary) 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. To make our *in vitro* results comparable to physiological ER-TR expression values, cerebella of P14 pups without any pre-treatments were used to obtain the *in situ* cerebellar samples as reference controls. Following the guidelines established by the NIH, 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).

Preparation of primary granule cell cultures

Primary cerebellar cultures were prepared as described earlier (Wong et al., 2001), with the following modifications. 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 described previously (Wong et al., 2001). 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 neurones that localised to a distance from each other. Thus, cell–cell interactions (please see the role of glia below) under various experimental conditions could be accounted for as the results of paracrine intercellular signalling.

Treatments

For analysis of mature primary cerebellar granule cells in a glia-destroyed environment, a final concentration of 10 μM cytosine β-D-arabinofuranoside (AraC; Sigma Aldrich Ltd., Hungary, Cat. # C1768) was added 24 h after seeding to inhibit the proliferation of non-neuronal cells (AraC+ experimental groups). In contrast, no AraC was added to the media for analysis of neurones grown in a glia-containing environment (AraC– experimental groups). Cultures

were treated with either of the following hormones (at physiologically relevant concentrations) 7 days after seeding and 6 h (for qPCR) or 18 h (for Western blot) before harvesting: 17β-oestradiol (E2, 1.16×10^{-10} M, Sigma Aldrich Ltd., Hungary, water soluble, Cat. # E4389); 3,3',5-triiodo-L-thyronine (T3, 0.92 nM, Sigma Aldrich Ltd., Hungary, Cat. #T2877); L-thyroxine (T4, 65 nM, Sigma Aldrich Ltd., Hungary, Cat. #T1775); E2+T3 or E2+T4 (at concentrations described above). Reference cultures without any hormone treatments were included in both the AraC+ and AraC– groups (ntC[AraC+/–]).

Western blot studies

Cell harvesting was performed as described by Wong et al. (2001). Samples were then homogenised in (in mM) 20 Tris-HCl, pH 7.5, 150 NaCl, 1 PMSF, 1 EGTA, 1 EDTA, 2.5 sodium pyrophosphate, 1 beta-glycerol phosphate, and 1 Na_3VO_4 plus 1 mg/ml Pefabloc, 10 µg/ml leupeptin 10 µg/ml pepstatin, 1 µg/ml aprotinin, and 1% Triton X-100, 0.05% sodium deoxycholate. 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). Membranes were blocked with 5% nonfat dry milk for 1 h in TBS-T and incubated with appropriate antisera (Primary antibodies: anti-oestrogen receptor beta, Sigma Aldrich, Hungary, Cat. # E-1276, dilution: 1:1,000; anti-thyroid hormone receptor alpha1, AbD Serotec, UK, Cat. # 0100-0486, dilution: 1:1,000; anti-thyroid hormone receptor beta1, AbD Serotec, UK, Cat. # 0100-0484, dilution: 1:555. 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. Multiple exposures of each blot were collected, and those in the linear range of the film were used for densitometric analysis. Optical densities were calculated as arbitrary units, normalised to the protein concentrations of samples, and to a reference sample (cerebellum obtained from a 14-day-old rat pup) that has been added to each gel. Results are expressed as fold changes relative to ntC[AraC–] as appears on graphs. 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 TRIzol Reagent (Invitrogen, Carlsbad, CA, USA, Cat. # 15596-026) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction was carried out as described by Sayed-Ahmed et al. (2004). Primer sequences were as published by

Billon et al. (2002) for TRα, Kariv et al. (2003) for TRβ, and Vaillant et al. (2002) for ERβ. Cellular gene expression was quantified by quantitative PCR reactions (qPCR; LightCycler 2.0, F. Hoffmann-La Roche Ltd., Basle, Switzerland) using LightCycler DNA Master SYBRGReen I fluorescent dye (Hoffmann-La Roche Ltd., Basle, Switzerland, Cat. # 15015099001). 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 Center, Gödöllő, Hungary). Real-time PCR threshold cycle (Ct) data were analysed using the REST-XL software version 2.0 (Pfaffl et al., 2002). The target Ct of each sample was normalised to the Ct of the reference gene (rat cytoplasmic beta actin) in the same sample. Ct values in the treated groups were compared to the control group. Differences in the Ct values were converted into relative amounts of mRNA based on the assumption that the amplification efficiency was 2.00. Control mRNA value (ntC[AraC–] group) was arbitrarily set to 1 and results from other groups were expressed as fold changes relative to the ntC[AraC–] control group.

Immunohistochemical labelling of glial fibrillary acidic protein (GFAP)

Astroglia in the cultures was identified by immunohistochemical labelling for the specific astroglia marker GFAP (Fig. 1). Standard immunohistochemistry protocol was followed as described earlier (Wong et al., 2003), with the exception of the visualisation method. Specifically, binding of the antibodies (primary antibody: polyclonal rabbit anti-GFAP, dilution: 1:200, Cat. number G4546, Sigma-Aldrich Hungary; secondary antibody: biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA) was visualised with diamino-benzidine by the avidin-biotin peroxidase complex method following standard protocols (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). In negative control experiments, the primary antibody was omitted. Omission of the primary antibody resulted in no immunostaining. Stained cultures (kept in washing solution after staining) were examined and photographed in a Zeiss Axiovert 135 inverted microscope. Images were processed for publication by Adobe Photoshop v. 7.0 software.

Statistical analyses

Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test as appropriate. Data were analysed with Excel (Microsoft, Microsoft Co., Redmond, WA, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

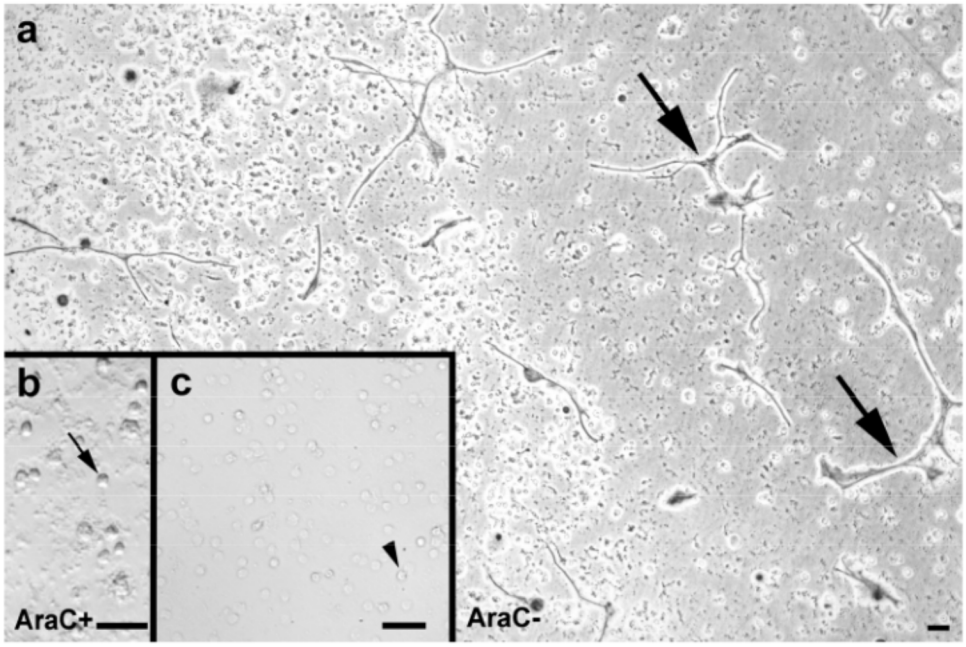

Fig. 1. Identification of glial fibrillary acidic protein (GFAP) labelled glia in primary cerebellar cell cultures. **a:** Cultures not treated with cytosine β-D-arabinofuranoside (AraC–) contained numerous GFAP-labelled glial cells that possessed long cell processes (arrows). **b:** Negative control experiment, where the primary antibody for GFAP was omitted during the process of immunostaining (AraC+). Small arrow points to a granule neurone. **c:** Cytosine β-D-arabinofuranoside treated cultures (AraC+) contained very few, scattered GFAP positive large cell bodies that have not developed any cell processes; their glial origin could only be determined by their GFAP content. Bars represent 50 μm

Results

General observations

In general, all experimental conditions applied resulted in increases in mRNA expression levels compared to respective *in situ* controls. Increased mRNA levels, however, were accompanied by an increase in ERβ protein levels, while TR[AraC–] protein levels were generally comparable to, and TR[AraC+] protein levels were lower than, those measured in *in situ* controls. The variance in mRNA[AraC+] values was remarkably higher than under all other experimental conditions. In general, protein expression levels were higher, while mRNA levels were lower in AraC– groups compared to respective AraC+ cultures. (The results of experiments on ERα mRNA and protein expression were inconsistent; therefore, those results are not published here.)

Thyroid hormone receptor alpha

Western blot results. In general, in AraC– (glia present) cultures the levels of TRα protein expression did not differ significantly from that detected in *in situ* cerebellar samples. E2 treatment, however, resulted in an exception by inducing a significant fall in TRα protein levels compared to *in situ* results. It is worthy of note that in these cultures, where glial cells were present, levels of variances were considerably higher than in the AraC+ (glia destroyed) group. In the latter experimental group (AraC+), where the growth of glia was blocked, all samples, including the non-treated control (ntC), displayed significantly lower TRα protein expression than that seen in the *in situ* cerebellum. Additionally, T3, T4 and E2+T4 treatments led to a significant reduction in TRα protein expression compared to their comparably treated AraC– counterparts.

PCR results. The overall pattern of TRα mRNA and TRα protein expressions markedly differed from each other (Fig. 2). All AraC+/– samples had significantly higher TRα mRNA levels than the *in situ* control. Variances within AraC- groups were markedly lower compared to those in ntC[AraC+] and T3-T4[AraC+] samples. The relative amount of TRα mRNA in the AraC– subgroup was less dependent on the hormone ligand used than the highly variable TRα mRNA expression levels in the different AraC+ samples. The mean values of ntC[AraC+] and T3[AraC+] groups were particularly higher than their AraC– counterparts. When comparing all TRα results (including qPCR and Western blots), the most heterogeneity in values occurred within the TRα mRNA[AraC+] subgroup. Interestingly, all TRα mRNA[AraC+] expression levels were higher than TRα protein[AraC+] values relative to their own *in situ* controls. Figure 2 illustrates relative TRα mRNA and protein expression levels in those experimental conditions.

Thyroid hormone receptor beta

Western blot results. As with TRα, overall TRβ protein expression levels in the AraC– group were comparable to those detected in *in situ* samples, nevertheless, in non-treated AraC– controls significantly lower TRβ protein levels were observed (Fig. 3). The presence of physiological amounts of T3 or T4 maintained TRβ protein expression near *in situ* values and were significantly higher than in the AraC– non-treated controls. Although mean TRβ protein values were higher in all AraC– treatment groups than in their non-treated control, only T3 or T4 differed significantly from ntC[AraC–]. In contrast, all hormone treatments in the AraC– group resulted in significantly higher TRβ protein levels than that found in non-treated AraC+ controls (with the exception of E2+T4[AraC–], where this difference did not reach significance).

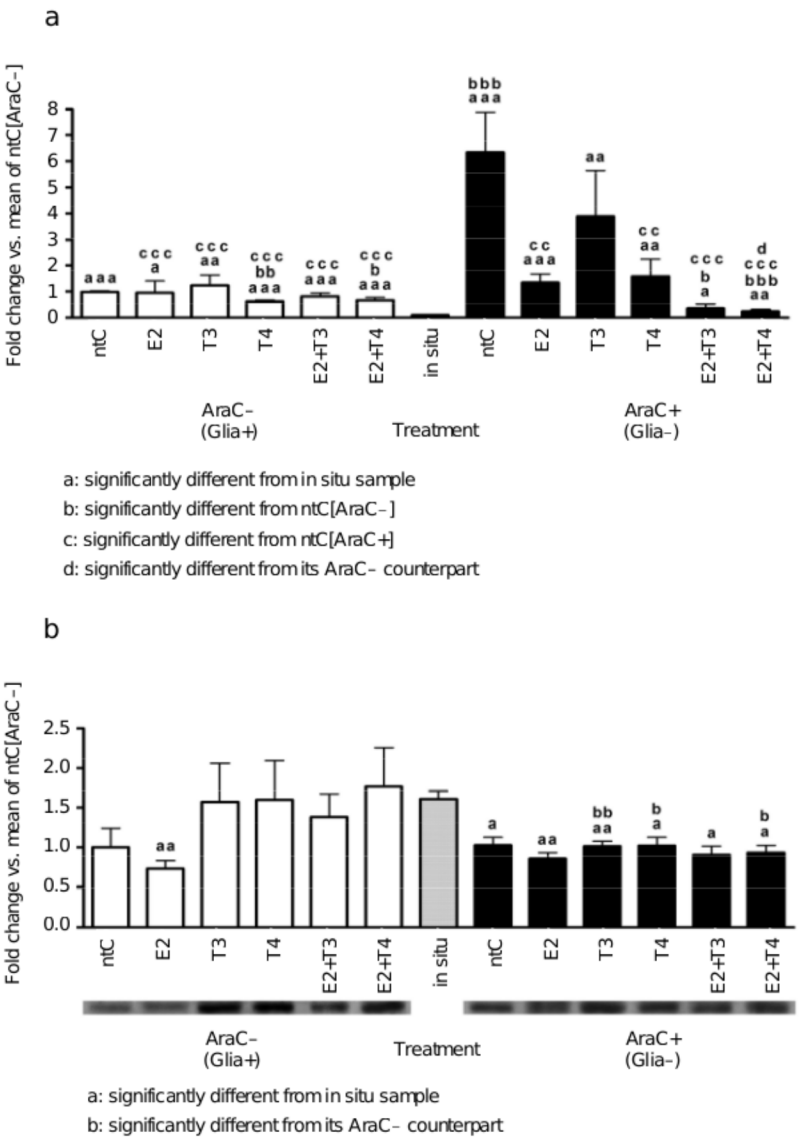

Fig. 2. TRα mRNA and protein expression levels after hormone treatments in the presence (AraC–) or absence (AraC+) of glia. **a:** TRα mRNA expression. The overall pattern of TRα mRNA and TRα protein expressions markedly differed from each other. All AraC+/– samples had significantly higher TRα mRNA levels than the *in situ* control. **b:** TRα protein expression. In general, in AraC– cultures, the levels TRα protein expression did not differ from that in *in situ* cerebellar samples. E2 treatment, however, resulted in an exception by inducing a significant fall in TR levels compared to *in situ* results. In AraC+, where the growth of glia was blocked, samples displayed significantly lower TRα expression than that seen in the *in situ* cerebellum. Abbreviations: ntC: non-treated control; E2: oestrogen; T3: triiodothyronine; T4: thyroxine; AraC+: treated with cytosine β-D-arabinofuranoside; AraC–: not treated with cytosine β-D-arabinofuranoside. Levels of significance: single letter if $P < 0.05$; double letter if $P < 0.01$; triple letter if $P < 0.001$

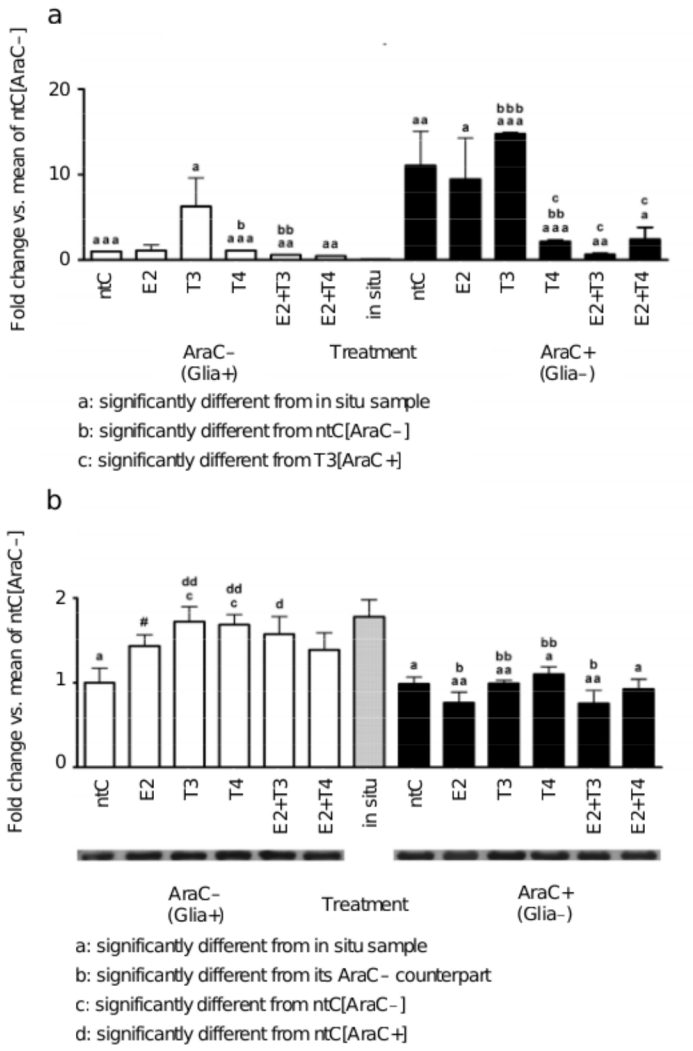

Fig. 3. TRβ mRNA and protein expression levels after hormone treatments in the presence (AraC–) or absence (AraC+) of glia. **a:** TRβ mRNA expression. As with TRα mRNAs, TRβ mRNA expression levels were higher in all cultures than in *in situ* controls. Comparison of qPCR and Western blot results revealed that, as in the case of TRα, TRβ mRNA[AraC+] values were higher, while TRβ protein[AraC+] values were lower than their respective *in situ* control. **b:** TRβ protein expression. As with TRα, overall TRβ expression levels in the AraC– group were comparable to those in *in situ* samples, nevertheless, in non-treated AraC– controls significantly lower TRβ protein levels were observed. All hormone treatments in the AraC– group resulted in significantly higher TRβ levels than that found in AraC+ non-treated controls (with the exception of E2+T4[AraC–], where this difference did not reach significance). All AraC+ groups displayed significantly lower TRβ expression levels than that seen in *in situ* samples. Abbreviations: ntC: non-treated control; E2: oestrogen; T3: triiodothyronine; T4: thyroxine; AraC+: treated with cytosine β-D-arabinofuranoside; AraC–: not treated with cytosine β-D-arabinofuranoside. Levels of significance: single letter if $P < 0.05$; double letter if $P < 0.01$; triple letter if $P < 0.001$

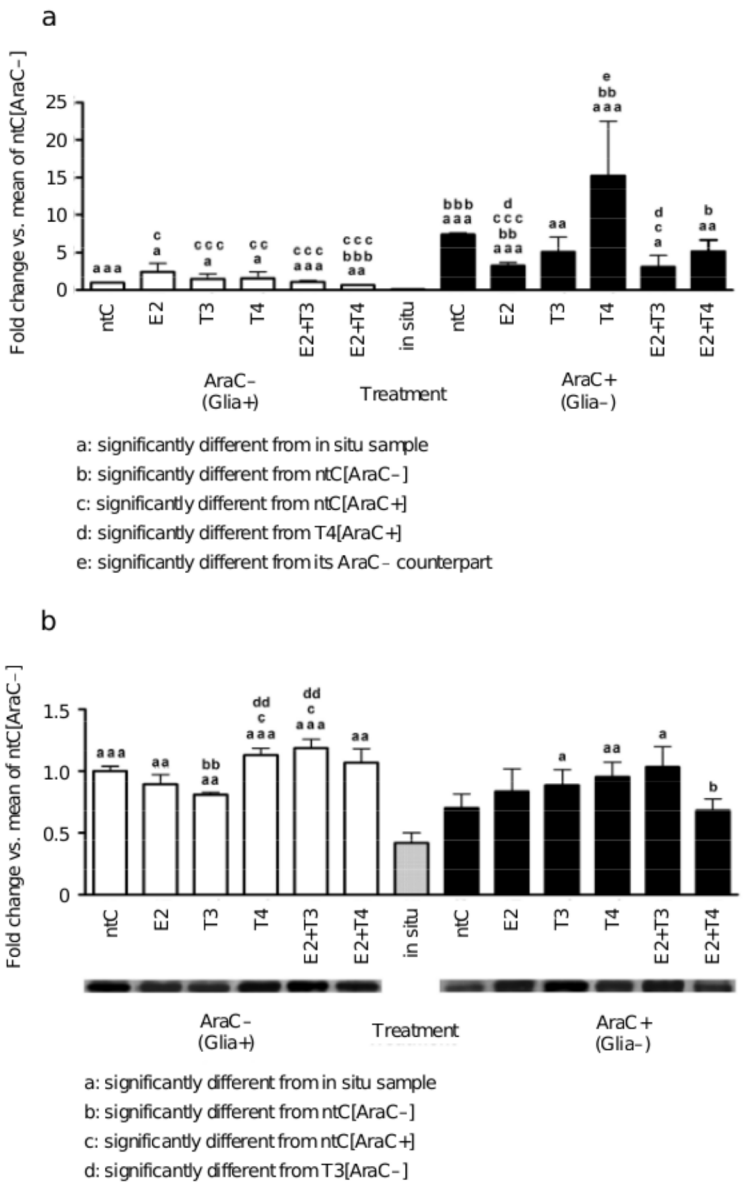

Fig. 4. ERβ mRNA and protein expression levels after hormone treatments in the presence (AraC–) or absence (AraC+) of glia. **a:** ERβ mRNA expression. Comparison of PCR and Western results revealed that in their adjustment to the applied experimental conditions, cultured cells reacted by increasing both their ERβ mRNA and protein expression levels relative to their respective *in situ* controls. **b:** ERβ protein expression. ERβ protein levels were higher in cultures than in *in situ* samples. Abbreviations: ntC: non-treated control; E2: oestrogen; T3: triiodothyronine; T4: thyroxine; AraC+: treated with cytosine β-D-arabinofuranoside; AraC–: not treated with cytosine β-D-arabinofuranoside. Levels of significance: single letter if $P < 0.05$; double letter if $P < 0.01$; triple letter if $P < 0.001$

All AraC+ groups displayed significantly lower TRβ protein expression levels than that seen in *in situ* samples. At the same time, AraC+ group members did not significantly differ from each other, however, E2-, T3-, T4- and E2+T3-treated cultures had significantly lower TRβ protein levels than their AraC– counterparts.

PCR results. As with TRα mRNAs, TRβ mRNA expression levels were higher in all cultures (with the exception of E2[AraC–]) than in *in situ* controls. AraC– samples displayed particularly low variances with the exception of T3[AraC–], where the relatively high mean value was accompanied with high variance. Interestingly, in the AraC+ subgroup, T3 samples had the highest mean as well, with low variance. Comparison of qPCR and Western blot results revealed that, as in the case of TRα, TRβ mRNA[AraC+] values were higher, while TRβ protein[AraC+] values were lower than their respective *in situ* control. Figure 3 illustrates relative TRβ mRNA and protein expression levels in the experimental conditions applied.

Oestrogen receptor beta

Western blot results. In general, ERβ protein levels were remarkably higher in cultures than in *in situ* samples (Fig. 4); in this comparison, however, differences from the non-treated control only reached significance in T3[AraC–] and E2+T4[AraC+]. In the AraC– group, only T3 treatment resulted in a difference from the non-treated control of the group. In AraC– cultures, where T4 or E2+T3 was added to the culture medium, ERβ protein expression levels were significantly (and near significantly in E2+T4 treated) higher than the AraC+ non-treated control. Finally, in the AraC– group, we found a significant difference in ERβ protein expression levels between T3-treated (lower values) and T4- or E2+T3 treated (higher values; E2+T4: near significant) cultures.

PCR results. The overall pattern of ERβ mRNA expressions resembled that of TRs in that both AraC+/– subgroups had higher values than the *in situ* control, and in general, AraC+ values superseded those of the AraC– samples. However, a comparison of qPCR and Western results suggests that in their apparent adjustment to the applied experimental conditions, cultured cells presumably reacted by increasing both their ERβ mRNA and protein expression levels relative to their respective *in situ* controls. ERβ mRNA expression was significantly higher in ntC[AraC+] than in T3, T4, E2+T3, E2+T4 [AraC–] and E2, E2+T3[AraC+]. Within the glia-destroyed groups (AraC+) E2 and E2+T3 evoked significantly lower response in ERβ mRNA expression than did T4. Figure 4 illustrates relative ERβ mRNA and protein expression levels in the experimental conditions applied.

Discussion

In discussing our results we consider our *in situ* data as a base point of reference, where tissue integrity and the molecular environment, at the time of sampling, were intact. Compared to *in situ* samples, AraC+/– cultures lost their tissue integrity; in addition, in AraC+ cultures the glia is destroyed; E2 groups are deprived of THs, T3/T4 groups are deprived of E2, and the non-treated controls of AraC+/– groups are deprived of both E2 and THs (we are aware that such an approach to analyse our data is substantially simplistic, as there are countless biological differences between *in situ* and *in vitro* biological materials).

General observations

The applied experimental conditions lead to increases in mRNA expression levels compared to respective *in situ* controls. This observation suggests 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 (Duenas et al., 1996; Zhu et al., 2004; Lamirand et al., 2008; Spence et al., 2009; Shulga et al., 2009; Mirzatoni et al., 2010). In addition, it should be considered that the cultured cells were obtained from immature, developing cerebella; therefore, maintenance of gene activation necessary for the completion of genetically programmed developmental processes may also require higher transcriptional activity of ligand-bound TRs and ERs under the experimental conditions that we used. Interestingly, characteristic differences in the patterns of TRα,β mRNA[AraC–] vs TRα,β mRNA[AraC+] and protein[AraC–] vs. protein[AraC+] suggest that glial cells play key roles in the regulation of neuronal TRα,β protein biosynthesis, as higher than normal TRα,β mRNA expression levels accompanied lower than normal TRα,β protein levels in the absence of glia. One of the possible glial functions might be the mediation of molecular signals towards granule neurones that may be necessary links between the transcription and translation of TR genes. It should be noted, however, that the cell density in our cultures was set to prevent physical cellular contact. Therefore, all interactions in intercellular signalling observed could be accounted for by paracrine signalling, thereby supporting previous observations that paracrine signalling can activate gene expression in the brain of rodents or humans as well (Freitas et al., 2010). At this point, we do not know how and to what extent direct cellular contact would mask the apparent intercellular signalling between the glia and granule neurones; thus, further studies are needed to clarify the exact nature of *in vivo* glia-neurone interactions. End-products, or so-called key-proteins, of protein biosyntheses usually play feedback roles to down-regulate their own biosynthesis. In this respect, it is also possible that glial cells play a role in the aforementioned negative feedback signalling; therefore, the lack of glia may result in flawed feedback in receptor biosyntheses and thus, im-

balanced transcriptional and translational activities, leading to overt mRNA synthesis and insufficient protein production in glia-free neuronal populations.

With regard to ERβ, increased mRNA[AraC+/–] levels were also accompanied by increased ERβ protein[AraC+/–] 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.

TR÷

Results from the AraC– 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[AraC–] group. The maintenance of normal levels of TRα protein, however, seems to require higher than normal transcriptional activity, as relevant TRα mRNA[AraC–] expression levels were significantly increased compared to those of *in situ* controls. This is consistent with previous findings that hypothyroidism leads to an increase in TR expression levels (Chattopadhyay et al., 1995; Gereben et al., 1998), although in our experimental model this was only reflected in TRα,β mRNA expression, but not in receptor protein levels. The increase in TRα mRNA expression might also be the result of compensatory mechanisms to ameliorate the reduction of nutrient substrates in the culture compared to *in situ* conditions. Increased TRα levels in CNS cells after ischaemic conditions support this idea (Zhu et al., 2004). Additionally/alternatively, the observed increase in transcriptional activity may also reflect a regenerative action on the part of explanted cells (Panaite and Barakat-Walter, 2010).

When cultured cells were deprived of THs (E2 treatment only), not even increased transcriptional activity could maintain *in situ* levels of TRα protein production. Comparison of similarly treated subgroups of AraC–/AraC+ samples revealed that non-treated controls[AraC–/+] do not differ significantly from each other in their TRα protein levels. This phenomenon, however, was backed by significantly different respective ntC[AraC+] *vs.* ntC[AraC–] mRNA expression values. Therefore, it is reasonable to conclude that granule neurones possess a high degree of adaptability on (TRα) transcriptional level to compensate for the lack of glia and resulting lack of possible neurone–glia interactions.

These 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 (E2[AraC–]), as TRα protein expression values for both E2[AraC+/–] 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 turn, this phenomenon may also indicate that when the glia is present, cerebellar TRα protein expression depends on the presence of either of the used THs, but not on E2. Cerebellar glial cells are able to present T3

to granule neurones due to their T4 to T3 conversion by type 2 deiodinase activity (Guadano-Ferraz et al., 1997). Further, excess amounts or unnecessary T3 in neurones is deactivated by type 3 deiodinase (Escamez et al., 1999; Peeters et al., 2001). In order to prevent neuronal T3 deactivation before its physiological role is accomplished and to protect neurones, glial cells can inhibit neuronal type 3 deiodinase activity (Lamirand et al., 2008). This interactive mechanism between the glia and neurones might be the reason why TRα,β[AraC+/–] protein expressions tended to be at near similar levels. It is also possible, however, that the glial contribution to the maintenance of normal levels of TRα protein levels is not dependent on glial type 2 deiodinase activity, but rather, on some other glial signalling mechanism. Deprivation of cultured neurones from both E2 and THs or from E2 alone (ntC[AraC–]) does not lead to a significant change in TRα protein expression, although mean values were decreased. If glia growth was blocked (ntC[AraC+]), the latter decrease reached significance. This significant decrease in TRα protein levels observed in all AraC+ samples was accompanied by remarkably high mRNA expression levels (compared to respective *in situ* controls), where there was a significant heterogeneity between TRα mRNA[AraC+] groups. The aforementioned, treatment- (ligand-) dependent heterogeneity in mRNA levels might demonstrate how granule neurones attempt to maintain a subnormal but steady expression level of TRα proteins with the lack of glial contribution. Specifically, results from TRα mRNA[AraC+] samples suggest that the most TRα mRNA is required when no receptor ligand is present (ntC[AraC+]), while, considering the mean values, deprivation of neurones from either T4 or E2 (T3[AraC+] group) resulted in a state with the second highest TRα transcriptional activity. In contrast, mean values of E2/T4/E2+T3/E2+T4[AraC+] samples were closer to those of *in situ* controls, suggesting that the potency of T3 to induce TRα mRNA synthesis differs from that of the other hormones used, although, due to the high SEM values for the T3 treatment group, these differences did not reach significance. Altogether, mean values of TRα mRNA[AraC+] groups may indicate that in the absence of glia, TRα transcriptional activity may depend more on E2 than on THs. Additionally, the highly variable mean and SEM values also suggest that neurones that lack the glial contribution, possess a high degree of adaptability in TRα,β transcriptional levels to maintain a relatively steady (and lower than normal) level of TRα,β protein expression.

TR✤

In AraC+/– non-treated controls, TRβ protein expression levels fell significantly as compared to *in situ* values. Based on this observation, one may speculate that loss of tissue integrity leads to a decrease in TRβ protein expression. However, in the presence of glia (AraC–), loss of tissue integrity alone does not lead to a decrease in TRβ protein 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 (AraC+) displayed TRβ protein levels significantly lower than those detected in *in situ* controls, 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. This idea is supported by the finding that analogously treated counterparts (all AraC+ groups) displayed significantly lower levels of TRβ protein. Interestingly, in the presence of glia (AraC– groups), only THs maintained TRβ protein expression values at *in situ* control levels so as to significantly differ from the non-treated control of the AraC– group; nevertheless, all other treatment subgroups showed higher TRβ protein expression values than their AraC– non-treated control (albeit these differences did not reach significance). Comparison of E2[AraC+/–] subgroups indicated that the glia mediates both E2 and TH effects: deprivation of cultures from THs resulted in lower TRβ protein expression when the glia is absent, however, a glia-mediated E2 effect was also detected, as E2,T3,T4,E2+T3[AraC–] values were significantly different from those in the non-glia-containing samples.

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. Although there is no distinction between TRα and TRβ isoforms here, other researchers do make a distinction between TRα and TRβ isoforms ($TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$) since TRα and TRβ are derived from separate genes (Lazar, 1993; Oppenheimer et al., 1995; Williams, 2000); this may explain the relatively minor differences detected between TRα and TRβ expression patterns (on both transcriptional and translational levels).

It is perplexing that T3 in the TRα and TRβ and T4 in the ERβ mRNA expression graphs would show increased fold changes in AraC+ groups (without glia). However, previous work may offer some explanation (Zhao et al., 2005; Vasudevan and Pfaff, 2007), proposing that E2 and THs (T3, T4) may act synergistically and are subject to both genomic and nongenomic mechanisms characteristic of members of the nuclear family of steroid receptors (Evans, 1988).

ER✤

The most salient observation was the overall increase in ERβ mRNA and protein expression levels in cultures compared to *in situ* controls. 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 (Lee and McEwen, 2001; Wong et al., 2003). With regard to ERβ mRNA expression pattern, there are many similarities

between ligand-induced TR and ERβ transcriptional activities. For example, AraC– values (glia present) were closer to each other than those in the glia-destroyed samples. There were clear differences in both the magnitude and ligand-dependent variances between respective AraC– and AraC+ groups, which indicates the apparent effect of glia in the regulation of neuronal ERβ gene transcription. On the other hand, granule neurones seem to possess a great degree of adaptability in their ERβ transcriptional activity so as to set a final ERβ protein level adequate to the experimental conditions applied.

An interesting observation is that, while AraC– vs. AraC+ ERβ mRNA values displayed a considerably different pattern and therefore suggest obvious glial effects in the regulation of ERβ gene transcription, AraC– protein expression values were much less different from those found in AraC+ ERβ protein groups. This finding may suggest that the neuronal adaptability mentioned above could compensate for the absence of glial contribution. This idea raises the question of what the exact role of glia is in the regulation of neuronal ERβ (and TRα,β) expression, if ERβ (and TRα,β) protein expression values do not show (when comparing treatment groups within given AraC– and/or AraC+ groups) such an apparent regulatory role for the glia? ERβ has been identified in both neurones and glia of the developing cerebellum (Price and Handa, 2000; Jakab et al., 2001). Therefore, it is reasonable to assume that mechanisms that set ERβ expression levels that are adequate to a given environment are available in both cell types. This assumption does not rule out the possibility of glia–neurone interaction(s), when the glia is present, to synchronise their ERβ biosynthetic activity for optimal adjustment to conditions of experimentation.

Possible mechanisms of ligand-dependent ER–TR interactions

Although early reports are controversial about the presence of TRs in astrocytes of the rat brain (Carlson et al., 1994), the majority of the relevant studies reported that both ERs and TRs are expressed in cerebellar glia (Kolodny et al., 1985; Ortiz-Caro et al., 1986; Hubank et al., 1990; Lebel et al., 1993; Leonard et al., 1994; Carlson et al., 1996) and neurones (Wallis et al., 2010) as well. Thus, both cell types are direct targets of both E2 and THs. Therefore, hormonal and receptorial interactions are possibly intracellular, as well as intercellular via interactions between glial cells and neurones. ERs and TRs bind to hormone response elements, present in the promoter region of certain genes, as either homo- or heterodimers. Lee et al. (1998) suggested that ERβ can bind to TRs and form ER-TR heterodimers in yeast and mammalian two-hybrid tests. In addition, Zhu et al. (1996) pointed to 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, although both Lee's and Zhu's results were obtained from cultured and transfected cells, these findings altogether implicate the possibility of a high de-

gree of co-operation between certain functions of E2 and THs. Such a possibility is supported by a number of other studies as well (Vasudevan et al., 2001*a*,*b*; Vasudevan et al., 2002; Vasudevan and Pfaff, 2005).

As mentioned above, one of the likely mechanisms of hormonal-receptorial interactions involve hormonal signalling through specific receptors, i.e. ERs and TRs, that are transcription factors and, thus, are able to activate genes that possess oestrogen- and/or thyroid hormone responsive elements (EREs, THREs). Since TH actions through TRs can modulate E2-induced transcription from EREs in neuroblastoma cells (Zhao et al., 2005), it is possible that such mechanisms also function in neurones and glia of the developing cerebellum. Moreover, the findings of Zhao et al. (2005) also suggest a more complex interplay between the two hormones, as the aforementioned genomic interaction was mediated by MAPK activation, and other studies report that both E2 and THs can activate the MAPK pathway (Wong et al., 2003; Zsarnovszky et al., 2005; Ghosh et al., 2005; Lin et al., 2009). Interestingly, TH-dependent MAPK activation can also lead to ER phosphorylation (Tang et al., 2004). The MAPK pathway is but one of the rapid, non-genomic intracellular signalling mechanisms that represent potential crosstalk between E2 and TH signalling [a broad review of the molecular mechanisms of crosstalk between E2 and THS was provided by Vasudevan and Pfaff (2005)]. For example, brain-derived neurotrophic factor (BDNF) can also be activated by both hormones (Koibuchi et al., 1999; Sasahara et al., 2007). The cited literature also indicates that E2- and/or TH-induced MAPK- or BDNF activation mediates developmental signals. Since activation of genes involved in the regulation of developmental processes is sequential and follows a well-defined temporal pattern (Wechsler-Reya, 2003), the exact roles of the two hormones in the regulation of neurodevelopment seem to be even more interrelated, making distinctions more difficult to identify, as inadequate/imbalanced hormonal signalling is likely to affect a longer period of neurodevelopment. As part of this complexity of the discussed hormone interactions, it is worth mentioning that, especially in the cerebellum, the glia plays an important role in the mediation of developmental signals (maturational, migrational) towards neurones (Yamada and Watanabe, 2002; Morest and Silver, 2003).

Considering that we used a relatively diluted cell suspension to prevent physical cell–cell contact, all glial effects observed (a form of glia–neuron interaction) could only be mediated by a paracrine way of intercellular signalling. Since a previous study indicated that neurone–astroglia interactions in the cerebellum involve both cell contact and soluble factors (Martinez and Gomes, 2005), it is possible that one of the reasons for detecting differences between *in situ* and *in vitro* results is the lack of cell contact-based signalling mechanisms in our experimental model.

Conclusions

The 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 extreme complexity of interactions between E2 and TH signalling, both intracellularly and intercellularly, it is reasonable to hypothesise that the failure of the integrated ER-TR signalling at one or more points may account, at least in part, for the formation of neurone- or glia-based tumours.

Last but not least, our results also point out the importance of differences between *in situ* and *in vitro* experimental models.

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