DE71 suppresses thyroid hormone-mediated dendritogenesis and neuritogenesis in the developing cerebellum

[*1,2]Ibhazehiebo Kingsley, [1]Koibuchi Noriyuki

[1]*Department of Integrative Physiology, Gunma University Graduate School of Medicine, Maebashi, Japan.*
[2]*Department of Physiology, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin-City, Nigeria*

Summary: Polybrominated diphenylethers (PBDEs) are synthesized chemicals essential to minimize accidents and deaths resulting from fire-outbreaks. Despite their usefulness, public health concern is on the increase over their use. PBDE is global in use, persistent in the environment, and possess the ability to bio-accumulate. Previous studies have suggested that they may interfere with thyroid hormone homeostasis, and are neurotoxic. We therefore investigated the effects of DE71 (a PBDE mixture) on thyroid hormone (TH)-mediated developments in the cerebellum. Employing primary cerebellar culture from new born rats, our study revealed that low dose DE71 significantly suppressed TH-mediated Purkinje cell dendrite arborization. Also, low dose DE71 remarkably impaired neurite extension of granule cells obtained from reaggregate culture of new born rat cerebella. Taken together, our study clearly reveals that DE71 can impair TH-mediated neuronal development in the cerebellum and may therefore interfere with normal TH-induced brain growth and function.

**Keywords*:* Brain development, DE71, granule cell, Purkinje cell, Thyroid hormone.

Address for correspondence: dockings22@yahoo.com

INTRODUCTION

Thyroid hormone (TH) homeostasis is regulated by a sensitive feedback loop within the hypothalamic-pituitary-thyroid (HPT) axis (Capen, 1997). Thyroid stimulating hormone (TSH) induces the thyroid to synthesise thyroxine (T_4), which can rapidly cross the blood brain barrier (BBB) and enter into the brain where it is then de-iodinated by 5'-deiodinase enzyme contained in astrocytes to tri-iodothyronine (T_3). T_3 which is the more biologically active form is taken up by the neuronal cells and bind to TH receptor (Koibuchi et al, 2008). Disruption of thyroid homeostasis especially during perinatal period of rapid brain growth and development may lead to hypothyroidism and morphological alterations in the brain (Koibuchi and Chin, 1999).

Polybrominated diphenylethers (PBDEs) are synthetic chemicals essential to minimize accidents and deaths resulting from fire-outbreaks. PBDE are incorporated into house-hold and commercial products including textiles and other everyday appliances (IPCS, 1994). Currently, PBDE is a global environmental contaminant. It has been detected in both human samples and wide-life species (Ohta et al,

2002). The human body burden of PBDE is on the increase (She et al, 2002) due to their persistence and bio-accumulations (de Wit, 2002).There is currently, great health concern over PBDE usage (Hooper and McDonald, 2000).

DE71 is one of most abundant commercial PBDE mixture, and contain mostly tetra and penta congeners (Sjodin, 2000) and nearly 10,000 tons are produced annually in the United States alone. Previous studies have shown that exposure to DE71 induced hypothyroxineamia in rodents (Darnerud and Sinjar, 1996; Fowles et al, 1994; Zhou et al, 2001). Also, reduction in plasma thyroxine levels have been reported in rodents following exposure to DE71 (Hallgren and Darnerud, 2002; Zhou et al, 2001). DE71 have been shown to induce both phase 1 ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD) and phase 2 uridinediphosphate-glucoronosyltransferase (UDPGT) metabolic enzyme activities (Carlson, 1980a, b; Fowles et al, 1994; Zhou et al, 2001). It is also suggested that DE71 increases T_4-glucoronidation by phase 11 UDPGT enzymes in the liver with subsequent increased biliary excretion (Brouwer et al, 1998; Hallgren and Darnerud, 2002).

However, the effect of DE71 on cerebellar neuronal morphology and its implication for normal brain development and function is not fully clarified in the literatures.

The present study therefore seeks to examine the effects of DE71 on TH-mediated cerebellar Purkinje cell dendritogenesis and on TH-induced granule cell neurite extension.

MATERIALS AND METHODS

Chemicals: Thyroxine (T4) and tri-iodothyronine (T3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DE71 was purchased from AccuStandard Chemicals (New Haven, CT, USA) and was >98% pure.

Primary cerebellar cultures
Pregnant wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Newborn rats were sacrificed under diethylether aneasthesia on the first day of birth. The animal experimentation protocol in the present study was approved by the Animal Care and Experimentation Committee, Gunma University and all efforts were made to minimize numbers of animals used and alleviate their sufferings. Details of the culture methods are described elsewhere (Kimura-Kuroda et al, 2007; Ibhazehiebo et al, 2011a). Briefly, the cerebella were digested with papain and dissociated cells were suspended in a serum-free medium without TH and plated in wells of chamber slides (8-mm-diameter wells, Nunc Lab-Tek, IL) at a density of 2.5×10^5 cells /0.2 ml. The slides were pre-coated with 0.1 mg/ml poly-L-lysine (SIGMA). Next day after cell plating, T4 and/ DE71 10^{-10} M was added to the culture medium, while DE71 10^{-8} M was added on culture day 5. Half of the culture medium was replaced with fresh medium every 2-3 days for 17 days. To exclude the effects of dimethy sulfoxide (DMSO), all experimental media used had a final concentration of 0.01% DMSO. Repeated freezing and thawing of reagents were avoided.

Immunohistochemistry to analyze Purkinje cell dendritc development
Immunocytochemistry of the cultured cells are described elsewhere (Kimura-Kuroda et al. 2002, 2007; Ibhazehiebo et al, 2011a). Briefly, Purkinje cells were immunostained with mouse-monoclonal (1:1000; McAB 300, Swant, Bellinzona, Switzerland) anti-calbindin-28 K antibody and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse antibody (1:200; Molecular probes,

Oregon, USA) and observed under a laser conforcal scanning microscope (FV1000D spectral type inverted microscope IX81, Olympus, Tokyo, Japan). To quantify dendritic arborization, the total area covered by the dendritic tree on ten randomly selected Purkinje cells in each experiment was determined by tracing the outline of the cell and dendritic branches and computing the area using NIH image software. Data shown represent mean ± S.E.M. and results from one set of experiments are shown graphically. Three independent experiments were performed and consistent results were obtained for each experiment. The relative dendritic area is shown.

Preparation of granule cell precursors (GCPs)
Wistar rats with postnatal day 4 (P4) pups were purchased from Japan SLC, Inc. (Hamamatsu, Japan) .All efforts were made to minimize numbers of animals used and their sufferings. Detailed protocol of the culture with modifications is described elsewhere (Okano-Uchida et al, 2003). Briefly, cerebella from P7 pups were taken under diethylether aneasthesia, cut into small piece and incubated at 37°C for 30 min in papain solution (16.5 units/ml papain/200μg/ml L-cysteine/0.008% DNase).Tissue was rinsed in Dulbecco's phosphate buffered solution (PBS) containing 1.5 mg/ml ovomucoid, 1.5 mg/ml bovine serum albumin (BSA), and 0.008%DNase and triturated in same solution to obtain a single cell suspension. Cells were centrifuged at 1,000rpm for 10 min at room temperature and suspended in Dulbecco's PBS containing 10 mg/ml ovomucoid and 10 mg/ml BSA and centrifuged again. Cells were resuspended in panning buffer (Dulbecco's PBS containing 0.002% BSA and 5μg/ml insulin) and passed through a cell strainer (Falcon). To obtain a fraction enriched in GCPs, the cell suspension was loaded onto a step gradient of 35% and 60% Percoll (Amersham Biosciences) and centrifuged at 3,000 rpm for 25 min at room temperature. GCPs were recovered from the 35%/60% interface, washed twice in panning buffer and suspended in neurobasal medium (GIBCO) containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 2% B-27 (all obtained from Invitrogen), 5

µg/ml insulin, 100 µg/ml apotransferrin, 100 µg/ml BSA, 16 µg/ml putrescine, 40 ng/ml sodium selenite, and 30 µM N-acetly cysteine (all obtained from Sigma) and plated in a 100 mm tissue culture dish precoated with 25 µg/ml poly-L-lysine and incubated for 20 min at room temperature. The dish was shaken vigorously and the non adherent cells were seeded onto 12-well plates (Falcon) at a density of $1x10^6$ cells/ml to form aggregates. Next day, GCP aggregates were plated on 8-well slide glasses (Matsunami Osaka) precoated with 100 µg/ml poly-L-lysine and treated with T_3 and or DE71 and incubated for 48 hours at 37°C, 5% CO_2.

Analysis of Granule cell neurite extension

Granule cell neurite extension was observed using CCD video camera and area of neurite extension was analysed using ImageJ software. Data represent mean ± S.E.M., and results from one experiment are shown graphically. More than two independent experiments were performed and results were consistent for each experiment. The relative neurite area of granule cells is shown.

Statistical analysis

Statistical significance was determined using ANOVA and *p*-values <0.05 were considered significant and marked with asterisk in the figures.

RESULTS

DE71 suppressed thyroid hormone-dependent arborization of Purkinje cell dendrite

We examined the effect of DE71 on T_4 –induced dendrite arborization of cerebellar Purkinje cell using primary cerebellar culture. Seventeen days after onset of culture, cells were fixed and immunostained with anti-calbindin antibody to visualize Purkinje cells. Thyroid hormone (T_4) greatly promoted the dendritic arborization of cerebellar Purkinje cells in contrast with control culture without T_4 (Figure 1A). The Purkinje cells in the control medium show little dendritic growth, while those in the medium with T_4 showed elaborate dendrites characterized by the presence of main thick primary shaft and several other secondary shafts with bifurcating branches (Figure 1A). The addition of (10^{-8} M) T_4 and (10^{-10} M) DE71 to the cerebellar culture did not significantly impair Purkinje cell dendrite arborization (Figure 1A). This was also confirmed by quantitative analysis (Figure 1B). However, addition of (10^{-8} M) T_4 and (10^{-8} M) DE71 (concentration at which DE71 suppressed

TR-mediated transcription *in vitro* (Ibhazehiebo et al, 2011a) remarkably inhibited dendritic development of Purkinje cells (Figure 2A). Purkinje cells cultured in the presence of DE7 (10^{-8} M) and T_4 (10^{-8} M) developed abnormally shaped dendrite, with very poor growth and the secondary branches particularly shrank (Figure 2A), Also the area of dendrite arborization of Purkinje cells was significantly reduced (Figure 2B) Taken together, these data indicate that DE71 suppress TH-induced cerebellar Purkinje cell dendrite arborization and consequently may disrupt normal neuronal development.

DE71 suppressed TH-induced neurite extension of granule cells

Further, we investigated the effects of DE71 on TH-mediated neuritogenesis using purified granule cell reaggregate culture. Forty-eight hours after onset of
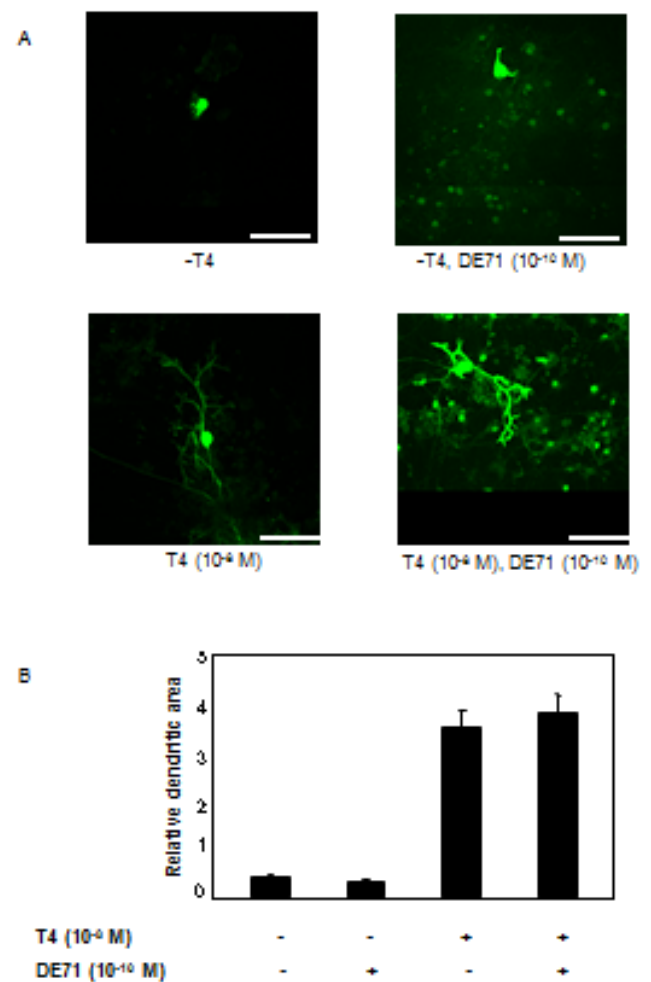

Figure 1 **10^{-10} M DE71 did not suppress TH-induced dendrite arborization of Purkinje cell (17 DIV)** A, Photomicrographs showing the effect of 10^{-10} M DE71 on Purkinje cell dendrite arborization in the absence or presence of T_4 (10 nM). Scale bars indicate 50 uM. B, Effect of DE71 on Purkinje cell dendrite development (17DIV) Data are expressed as mean ± S.E.M. (n = 10 determinations). No significance was uncovered by ANOVA.
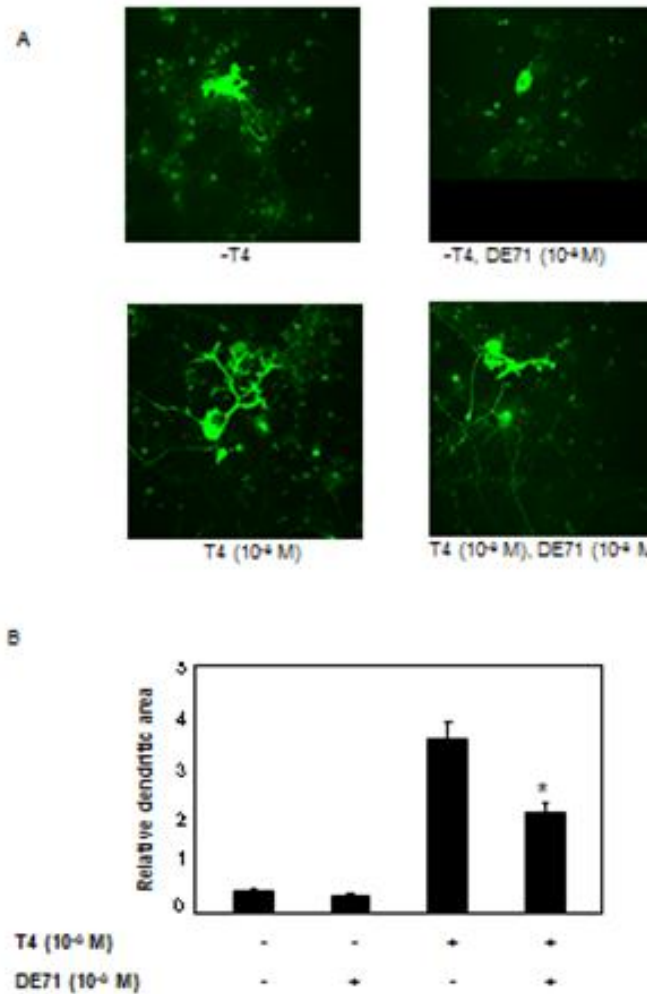
Figure 2. **10^{-8} M DE71 suppressed TH-induced dendrite arborization of Purkinje cell (17 DIV)**A, Photomicrographs showing the effect of 10^{-8} M DE71 added on day 5 on Purkinje cell dendrite arborization in the absence or presence of T_4 (10 nM). Bar indicates 50 uM. B, Effect of DE71 on Purkinje cell dendrite development (17DIV) Data are expressed as mean ± S.E.M (n=10 determinations). *statistically significant $p<0.01$ by ANOVA) for T_4 (+), DE71(-) vs. T_4 (+), DE71 (+). Data shown are representative of at least three independent experiments.

culture, granule cell neurite growth was observed real time using CCD camera. TH (T_3) remarkably enhanced neurite growth and extension of granule cells compared to control without T_3 (Figure 3A). Granule cell aggregate in the presence of T_3 form elaborate tree like neurites with several secondary shafts and bifurcating branches while those in the control medium showed fewer neurites that were thinner and had less bifurcating branches (Figure 3A). Addition of 10^{-8} M DE71 to the aggregate culture together with T_3, greatly suppressed neurite growth and extension of the granule cell aggregate (Figure 3A). Such granule cell aggregate developed fewer neurite with poor growth, markedly reduced length and the secondary branches and bifurcations were less (Figure 3A). The area of neurite extension of granule cells was also reduced as confirmed by quantitative analysis (Figure 3B). These data clearly
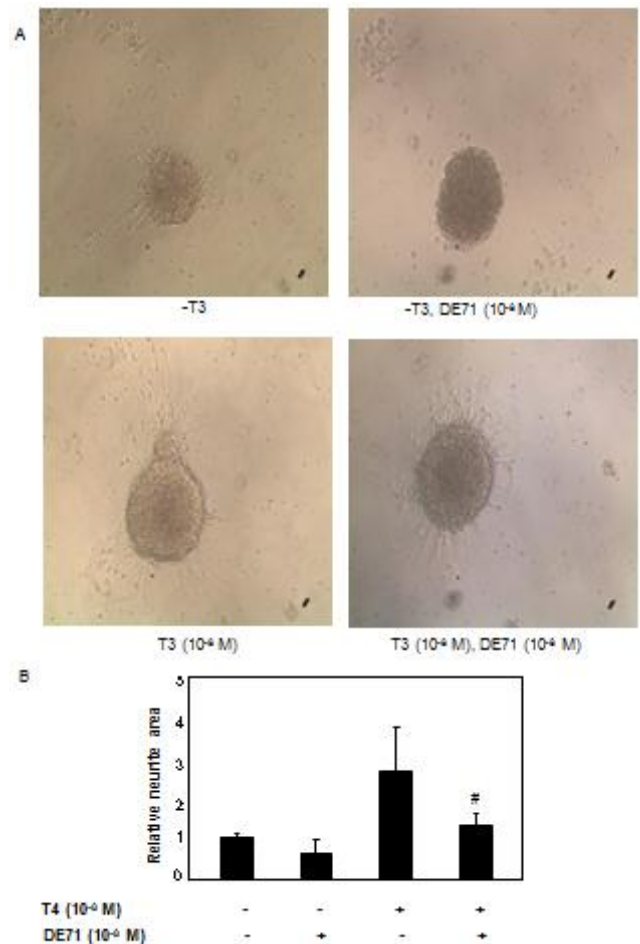

Figure 3 **Low dose DE71 suppress TH-induced neurite extension of granule cells** A, photomicrographs showing the effect of 10^{-8} M DE71 on granule cell neurite extensions in the absence or presence of T_3 (10 nM). Scale bars indicates 50 uM. B, Effect of DE71 on granule cell aggregate neurite extension Data are expressed as mean ± S.E.M. (n = 3 determinations). #, statistically significant $p<0.05$ by ANOVA) for T_3 (+), DE71 (-) vs. T_3 (+), DE71 (+). Data shown are representative of at least two independent experiments.

indicate that DE71 effectively suppressed TH-dependent growth and extension of granule cell neurite at low dose.

DISCUSSION

In the present study, we show that DE71 significantly suppressed TH-mediated dendritogenesis and neuritogenesis of neuronal cells in the developing cerebellum, and may consequently be inimical to normal brain development. The ability of TH to regulate neuronal development is well established (Potterfield, 2000). Also, morphogenetic disruptions arising from perturbations of TH homeostasis have been well characterized (Nicholson and Altman, 1972). Less well defined however, is the effect of DE71 on neuronal morphogenesis in the developing cerebellum.

The present study lend credence to our previous findings that TH remarkably enhanced Purkinje cell dendritic development and growth (Figure 1A; Ibhazehiebo et al, 2011a). Purkinje cells cultured in the presence of TH showed remarkable growth of dendrite (Figure 1A) compared to those cultured in the absence of TH (Figure 1A). Treatment of culture with TH and DE71 (10^{-10} M) on day 2 of culture did not significantly alter Purkinje cell dendritogenesis (Figure 1A). neither did it alter the area of dendrites (Figure 1B). This is consistent with our previous study that showed that 10^{-10} M DE71 did not suppress thyroid hormone receptor (TR)-mediated transcription in CV-1 cells (Ibhazehiebo et al, 2011a).

On the other hand, addition of 10^{-8} M DE71 together with TH to the culture on day 5 lead to significant suppression of Purkinje cell dendrites. Such Purkinje cells exhibited poorly formed dendrites, with reduced primary shafts, secondary branches and birfurcations (Figure 2A). Also, the dendritic areas of such Purkinje cells were dramatically reduced (Figure 2B). This data is in consonance with our previous study that showed that 10^{-8} M DE71 significantly impaired TR-mediated transcription (Ibhazehiebo et al, 2011a).

Moreover, we showed in the present study that low dose DE71 impaired the ability of granule cell to extend neurites (Figure 3A). The ability of granule cells to extend neurites was remarkably enhanced in the presence of T_3 (Figure 3A). Such neurites had more robust diameters, extended longer distances and had more branches compared to those cultured in the absence of T_3 (Figure 3A). Addition of 10^{-8} M DE71 to the aggregate culture together with T_3, greatly suppressed neurite growth and extension of the granule cell aggregate (Figure 3A). Such granule cell aggregate developed fewer neurite with poor growth, markedly reduced length and diameter, and the secondary branches and bifurcations were less (Figure 3A). The area of neurite extension of granule cells was also reduced as confirmed by quantitative analysis (Figure 3B). This also is in line with our recently published findings that hexabromo-cyclododecane (HBCD) and BP-6 which are also endocrine disruptors can suppress T3-mediated granule cell neurite extension (Ibhazehiebo et al, 2011b : 2011c)

TRs which mediates TH actions are abundantly expressed in the developing cerebellar cells including Purkinje and granule cells (Bradley et al, 1992), and because previous study have shown that growth and development of cerebellar neuronal cells is via TR (Strait et al, 1991), The morphological aberrations in Purkinje and granule cells observed with DE71 may be due to its interaction with TRs. Further investigations are required to clearly unravel DE71 interactions with TRs in Purkinje and granule cells.

Our study clearly shows that DE71 (a PBDE mixture) impaired dendritogenesis and neuritogenesis in developing cerebellar neurones, and may consequently affect TH-mediated brain development.

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