



# Temporal effects of thyroid hormone (TH) and decabrominated diphenyl ether (BDE209) on Purkinje cell dendrite arborization

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**Summary:** Thyroid hormones (TH) 3,3',4-tri-iodothyronine (T3) and 3,3',4,4'-tetra-iodothyronine (T4) plays crucial role in cerebellar development. Deficiency of TH consistently results in aberrant growth and development of the cerebellum including reduced growth and branching of the Purkinje cells. In rodents, the critical period of thyroid hormone action on cerebellum development is within the first two to three weeks, after which thyroid hormone replacement cannot fully reverse abnormal cerebellar development induced by thyroid hormone insult. Decabrominated diphenyl ether (BDE209) is an industrial reagent used as an additive flame retardant to reduce flammability of various commercial and household produce. BDE209 has bio-accumulative potential and is neurotoxic. Previously, we have shown that T4 ( $10^{-8}$  M) induced extensive dendrite arborization of Purkinje cells and low dose BDE209 ( $10^{-10}$  M) remarkably suppressed TH-induced Purkinje cell dendrite arborization. In the present study, we show that the critical period for TH-induced Purkinje cell growth and dendrite arborization in culture is much earlier than reported in animal models. Also, we show for the first time that low dose BDE209 suppressed TH-induced dendrite arborization in a time-dependent manner. Taken together, our study indicates that hypothyroidism and exposure to BDE209 during critical stage of cerebellar development can lead to impaired Purkinje cell growth and dendrite arborization and may consequently disrupt normal cerebellar functions.

**Keywords:** BDE209, Cerebellum, Purkinje cells, Thyroid hormone.

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## INTRODUCTION

The thyroid hormones (TH) (3,3',4-tri-iodothyronine, T3: and 3,3',4,4'-tetra-iodothyronine, T4) are essential for normal neuronal development including the cerebellum (Koibuchi and Chin 2000). TH mediates a number of physio-biochemical processes that converts the fetal brain to that of an adult (Porterfield and Hendrich 1993). The critical period of brain growth spurt in human extends from the third trimester of pregnancy through the first two years of neonatal life, and in rodents extends through the first three weeks after birth (Dobbing and Sands 1979). TH deficiency during the critical perinatal period results in numerous neuronal abnormalities.

Specifically, perinatal hypothyroidism resulted in remarkable reduction in the growth and branching of the Purkinje cell dendrite *in vivo* (Nicholson and Altman 1972). Synaptic contact between the Purkinje cells dendrites and granule cells axons were dramatically reduced (Nicholson and Altman 1972).

The disappearance of the external granule cell layer was delayed and granule cells migrated at much slower pace from the external granule cell layer to the internal granule cell layer (Nicholson and Altman 1972). Also, myelination and synaptic connections among cerebellar cells were impaired (Bala'zs et al, 1971; Haj'os et al, 1973).

The predominant mode of TH action includes its signaling via thyroid hormone receptors (TRs) which bind as homodimers or as heterodimers with retinoic acid receptors to thyroid hormone responsive-elements (TRE) in the promoter regions of target genes. TRs are widely expressed in the developing cerebellum (Bradley et al, 1992).

Although the dose-dependent effect of THs on Purkinje cell dendrite arborization in culture have been studied (Ibhazehiebo et al, 2011; Kimura-Kuroda et al, 2002), there are yet no studies to our knowledge detailing the temporal effect of TH on cerebellar Purkinje cell development and identification of the critical period for Purkinje cell dendrite arborization *in vitro*.

Polybrominated diphenyl ethers (PBDEs) are group of industrial halogenated chemicals extensively used for decades as additive flame retardants to reduce combustibility of several everyday appliances such as electronics, textiles and furniture upholstery. PBDE are commercially classified as pentaBDE, octaBDE or decaBDE (BDE209). Production of penta and octa BDEs have been banned in Europe and voluntarily withdrawn in the United States because of their health hazards and ability to bioaccumulate (Lind et al, 2003; Lober 2008; Meironyte et al, 1999). Production of decaBDE has continued in Europe and North America (Hale et al, 2003; Sjodin et al, 2003), though the two US producers of BDE209 and the largest US importer have agreed to phase out BDE209 by the end of 2013 (USEPA, 2010).

Although PBDE are useful to prevent fire accidents, they however disrupt normal endocrine functions and interfere with thyroid hormone homeostasis (Danerud 2008). In animal studies, PBDE have been shown to alter the levels of thyroid hormones in offspring after maternal exposure to low dose PBDE in rodents and sheep (Kuriyama et al, 2007; Abdelouahab et al, 2009). In humans, elevated PBDE levels in door dust have been attributed to abnormal hormonal levels in men (Meeker et al, 2009) and abnormally high levels of PBDE have been detected in breast milk of mothers who gave birth to newborn males with cryptorchidism (Main et al, 2007). BDE209 have been found at very high concentrations in indoor environment, eg, household dust (Harrad et al, 2008; Vorkamp et al, in press), as well as in humans and wildlife (Gomara et al, 2007; Vorkamp et al, 2005).

We previously reported that low dose PBDE disrupted TR-mediated transcription via DNA-binding domain of TR and impaired Purkinje cell dendrite arborization (Ibhazehiebo et al 2011). In the present study, we showed that BDE209 (deca-BDE) impairs Purkinje cell dendrite arborization in a time-dependent manner. Also, we established a critical period for BDE209 action *in vitro*.

## MATERIALS AND METHODS

**Chemicals:** T4 and dexamethasone (Dex) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2',3,3',4,4',5,5',6,6'-decaBDE (BDE209) was purchased from AccuStandard Chemicals (New Haven, CT). BDE209 congener was >99% pure.

**Culture medium:** The serum-free culture medium was composed of Dulbecco's Modified Eagles Medium (DMEM)/F12 (GIBCO) supplemented with 10 µg/ml

bovine insulin (SIGMA), 100 µg/ml transferrin (SIGMA), 30 nM sodium selenite and 100 µg/ml penicillin-streptomycin (GIBCO) (Kimura-Kuroda et al, 2002). Stock solution of thyroxine (T4, SIGMA) was prepared with DMSO as vehicle. Reagents were dissolved immediately before use and repeated freeze-thawing was avoided.

**Primary cerebellar Culture:** Pregnant Wistar rats were purchased from Japan SLC, Inc (Hamamatsu, Japan). Newborn rats were euthanised under diethylether anaesthesia 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 number of animals used and their sufferings. Detailed protocol of the culture is described elsewhere (Kimura-kuroda et al, 2002). Briefly, the cerebella were digested with 0.2 units/ml of papain (Worthington, Lakewood, NJ) in phosphate-buffered saline (PBS) containing 0.2 mg/ml DL-cysteine, 0.2 mg/ml bovine serum Albumin (Intergen, Purchase, NY), 5 mg/ml glucose and 0.02 mg/ml DNase I (Sigma, 400-600 units/mg) for 25 minutes at 37°C. Dissociated cells were suspended on a serum-free medium without TH and plated at a density of  $2.5 \times 10^5$  cells/0.2 ml in wells of chamber slides (8-mm-diameter wells, NUNC Lab-Tek, IL, USA), pre-coated with 0.1mg/ml poly-L-lysine (Sigma). Next day after plating, T<sub>4</sub> and/or BDE209 were added to the culture medium and one-half of the medium was replaced with fresh medium every 3-4 days and mixed cerebella cells were cultivated in a 5% CO<sub>2</sub> incubator for 17 days. Effects of dimethyl sulfoxide (DMSO) were excluded by using control and experimental media at final concentration of 0.05% and avoiding repeated freezing and thawing.

## Immunocytochemistry to analyze Purkinje cell development

Immunocytochemistry of the cultured cells were previously described (Ibhazehiebo et al, 2011; Kimura-Kuroda et al, 2002). Briefly, Purkinje cells were immuno-stained with mouse- monoclonal anti-calbindin-28 K antibody (1:1000; McAB 300, Swant, Bellinzona, Switzerland) and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse antibody (1:200; Molecular Probes,

Oregon, USA) and observed under a laser confocal scanning microscope (FV1000D spectral type inverted microscope IX81, Olympus, Tokyo, Japan). To quantify dendrite arborization, the total area covered by the dendritic tree on randomly selected Purkinje cells in each experiment was determined by tracing the outline of the cells and dendritic branches and computing the area using NIH image software. Ten randomly selected Purkinje cells were used for each treatment group because of the limitations of photo-bleaching associated with the use of the laser confocal microscope. Data shown represent mean  $\pm$  S.E.M., and results from one experiment are shown graphically. More than two independent experiments were performed and results were confirmed for each experiment. The relative dendritic area of Purkinje cells was shown.

### Statistical Analysis:

Data was analyzed using ANOVA, post-hoc comparison was made using Bonferroni's test. The  $p$ -values  $<0.05$  were considered significant and marked with asterisk in the figures.

## RESULTS

### T4 induce Purkinje cell dendrite arborization in a time-dependent manner:

To examine the temporal effect of TH on Purkinje cell development and thus establish the critical period of TH-induced Purkinje cell development in culture, we performed primary cerebellar culture. Seventeen days after onset of culture, cells were fixed and immunostained with anti-calbindin antibody to visualize Purkinje cells. Thyroid hormone (10 nM T4) greatly promoted the dendritic arborization of

cerebellar Purkinje cells in contrast with control culture without T4 treatment (Figure 2). The Purkinje cells in the control medium showed poor growth, while those in the medium with T4 showed elaborate dendritic arborization (Figure 2).

Specifically, addition of 10 nM T4 to the medium on second day of culture resulted in Purkinje cells with well arborized dendrites. Culture wells to which T4 was added on the third day also showed well arborizing dendrite whose dendritic area was not significantly different from those in which T4 was added on the second day of culture (Figure 3A). However, when T<sub>4</sub> was added on the fifth day to the thirteenth day of culture, Purkinje cell dendritic development was significantly impaired (Figure 2).

Also, the area of Purkinje cell dendrite correlated positively with days of treatment by T4 (Figure 3A). On the other hand, there was no significant difference in the areas of Purkinje cell bodies after treatment with T4 on different days (Figure 3B). Taken together, our study indicates that the critical period for Purkinje cell dendritogenesis in vitro may be between the second and third day after onset of culture.

### BDE209 impairs Purkinje cell dendrite development in a time-dependent manner.

To further examine the temporal effect of BDE209 action on TH-induced Purkinje cell development, we studied the effect of BDE209 on T4-induced dendrite arborization of cerebellar Purkinje cells in primary culture. While Purkinje cells developed well arborizing dendrites in the presence of T4 (Figure 4), the addition of 10<sup>-10</sup> M of BDE209 (deca-BDE) (the concentration at which effective suppression of TR-mediated transcription in vitro was seen in our previous study, Ibhazehiebo et al 2011) to the cerebellar culture together with T4 inhibited dendritic development of Purkinje cells (Figure 4). Purkinje cells cultures with BDE209 developed abnormally

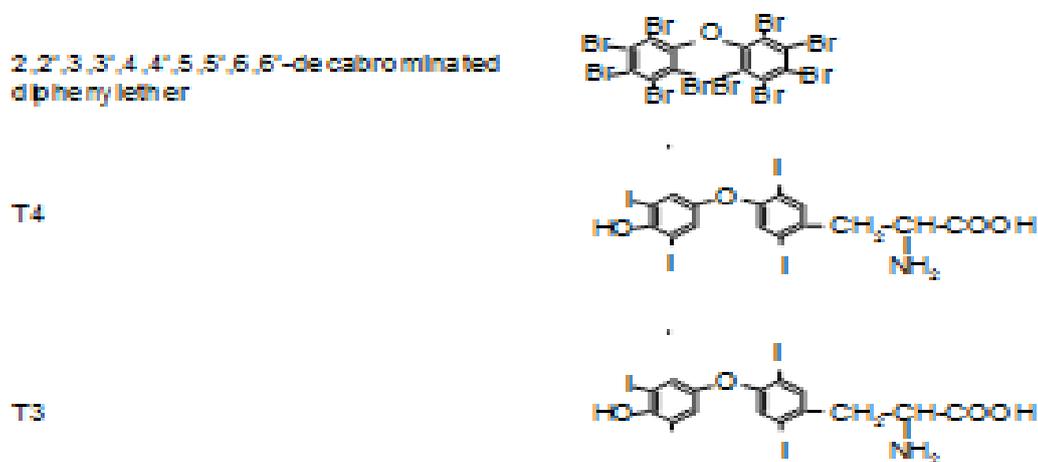
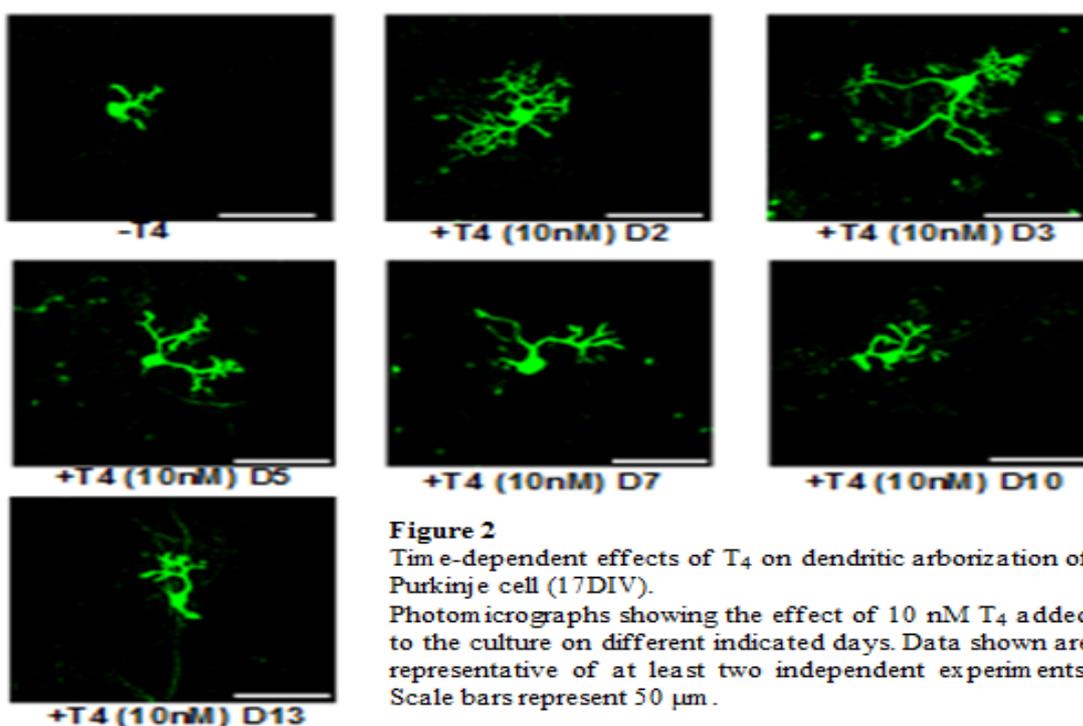
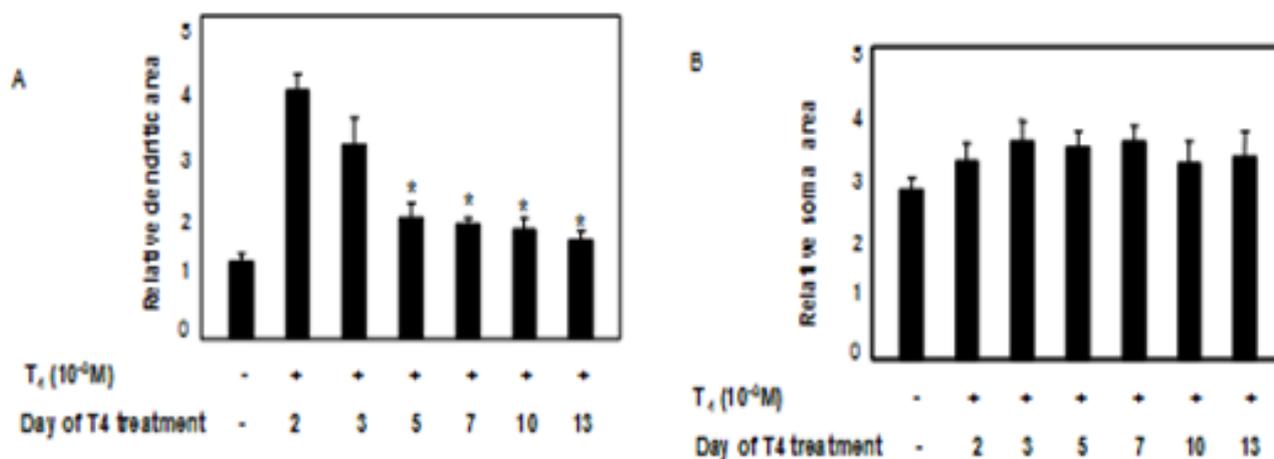


Figure 1:  
Structure of BDE209 and T4.



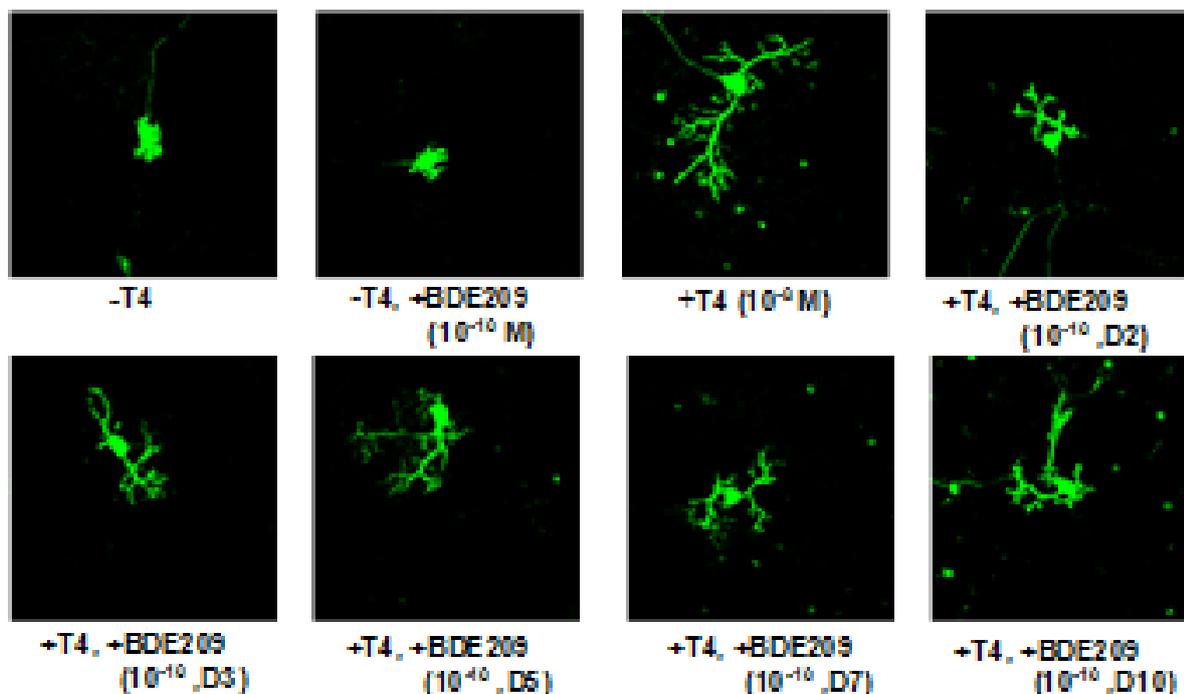
**Figure 2**  
Time-dependent effects of T<sub>4</sub> on dendritic arborization of Purkinje cell (17DIV). Photomicrographs showing the effect of 10 nM T<sub>4</sub> added to the culture on different indicated days. Data shown are representative of at least two independent experiments. Scale bars represent 50 μm.



**Figure 3.** Time-dependent effect of T<sub>4</sub> on Purkinje cell dendritic area (17DIV). **A**, 10 nM T<sub>4</sub> was added to the culture on different indicated days. Data are expressed as mean ± S.E.M. (n = 10 determinations). \*, statistically significant p < 0.01 by ANOVA) for T<sub>4</sub> (+) Day 2 vs. T<sub>4</sub> (-) and Day 5 to Day 13. Data shown are representative of at least two independent experiments. **B**, 10 nM T<sub>4</sub> was added to the culture on different indicated days. Data are expressed as mean ± S.E.M. (n = 10 determinations). Data shown are representative of at least two independent experiments. No significance was uncovered by ANOVA.

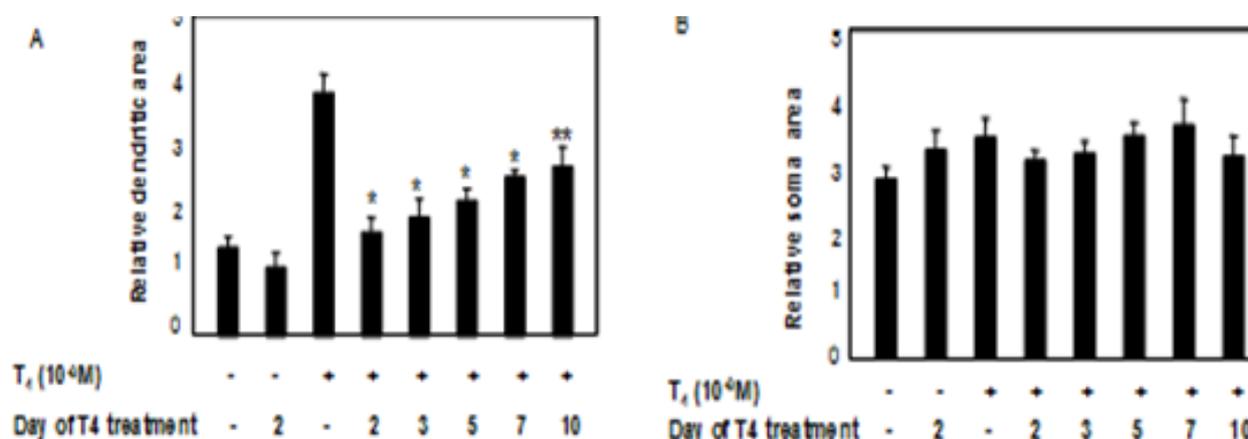
shaped dendrite, with very poor growth and the secondary branches particularly shrank (Figure 4). Also the area of dendrite arborization of Purkinje cells was significantly reduced (Figure 5A). Purkinje cell culture T<sub>4</sub> (-) and BDE209 (+) showed almost complete absence of dendrite (Figure 4). Specifically, the ability of BDE209 to inhibit dendritogenesis of Purkinje cell was time-dependent. The degree of impairment of Purkinje cell dendrite arborization was greatest when culture was incubated with BDE209 and T<sub>4</sub> on second day of culture (Figure 4). When culture was treated with BDE209

and T<sub>4</sub> on tenth day of culture, Purkinje cell developed better arborizing dendrite but was still significantly less than those treated in the presence of T<sub>4</sub> only (Figure 4). Purkinje cell dendrite development and growth becomes impaired on addition of BDE209 (Figure 4 and 5A). However, the area of Purkinje cell soma was not significantly altered in the presence of BDE209 treatment on different days (Figure 5B). Taken together, these data indicate that PBDE suppress Purkinje cell dendrite arborization in a time-dependent manner, and that



**Figure 4.**

Temporal effect of BDE209 on Purkinje cell dendrite arborization (17DIV). Photomicrographs showing the effect of  $10^{-10}$  M BDE209 added to the culture in the presence of T4 (10 nM) on different indicated days. Data shown are representative of at least two independent experiments. Scale bars represent 50  $\mu$ m.



**Figure 5.**

Figure 5 Temporal effect of BDE209 on Purkinje cell dendritic area (17DIV). **A.**  $10^{-10}$  M BDE209 was added to the culture in the presence of T4 (10 nM) on different indicated days. Data are expressed as mean  $\pm$  S.E.M. (n = 10 determinations). \*, statistically significant p <0.01 by ANOVA) for T4 (+), BDE209 (-) vs. T4 (-),BDE209 and. T4 (+), BDE 209 (+). \*\*, statistically significant p <0.05 by ANOVA) for T4 (+), BDE209 (-) vs. T4 (-), BDE209 and. T4 (+), BDE 209 (+).Data shown are representative of at least two independent experiments. **B.**  $10^{-10}$  M BDE209 was added to the culture in the presence of T4 (10 nM) on different indicated days. Data are expressed as mean  $\pm$  S.E.M. (n = 10 determinations). Data shown are representative of at least two independent experiments. No significance was uncovered by ANOVA.

BDE209 largely inhibits further development of Purkinje cell dendritogenesis immediately after its addition.

## DISCUSSION

In the present study, we showed temporal effect of TH on Purkinje cell dendritogenesis, proposed a critical period of TH-induced Purkinje cell dendrite

development, and also showed temporal effects of BDE209 on Purkinje cell dendrite morphogenesis. TH regulatory control of cerebellar growth and development has been well established (Nicholson and Altman 1972), many complex physiological and biochemical processes under TH regulations ensures normal neuronal development in the cerebellum (Porterfield and Hendrich 1993). Also, the critical period of brain development in humans and rodents in vivo have been well defined (Dobbing and Sands

1979), However, less well defined is the temporal effect of TH and BDE209 on cerebellar neuronal development, particularly on the Purkinje cells.

Our study shows that dendritic development of the Purkinje cells in culture depends greatly on the time of T4. Purkinje cells treated with T4 on second and third day of culture developed normal well arborizing dendrites characterized by numerous branchings and bifurcations (Figure 2). On the other hand, Purkinje cells treated with T4 on fifth to thirteenth day of culture or those cultured in the absence of TH developed abnormally shaped dendrites with less branching and bifurcations (Figure 2), particularly, those cultured in the absence of TH showed poorly developed dendrites (Figure 2). Also, the dendritic area of Purkinje cells treated with T4 on second and third day of culture was significantly greater than those with T4 treatment on day 5 to day 13 (Figure 3A). However, the area of Purkinje cell soma was not significantly different amongst the different treatment groups (Figure 3B).

BDE209 though useful as flame retardant, have been implicated in various neurobehavioral abnormalities (Eriksson et al, 2001). We have previously shown that BDE209 suppresses TH-mediated Purkinje cell dendrite arborization at low dose after seventeen days in culture (Ibhazehiebo et al, 2011). In this present study, we show that BDE209 suppresses T4 –induced Purkinje cell dendrite development in a temporal manner. Specifically, the extent to which Purkinje cells arborize dendrites is clearly dependent on time of BDE209 treatment (Figure 4). Purkinje cells treated with BDE209 on day 2 of culture show less dendritic arborization compared to those treated with BDE209 on day 7 or day 10 in the presence of T4 (Figure 4). Also, the dendritic areas of the Purkinje cells correlated positively with day of BDE209 treatment (Figure 5A). On the other hand, the area of Purkinje cell soma was not significantly affected by treatment with BDE209 on different days (Figure 5B).

TRs are expressed in most cerebellar neuronal cells including the Purkinje and granule cells during development (Bradley et al, 1989, 1992) and previous studies have shown that TH induces the dendritic development of Purkinje cells via TR (Strait et al, 1991). Thus the inhibitory effects by BDE209 observed in our temporal study could be due to its action on TR-mediated gene expression in cerebellar neurons especially on the Purkinje cells given that PBDEs can pass through the blood brain barrier and accumulate in the brain (Costa and Giordano, 2007; Naerts et al, 2007) and that PBDEs has been implicated in various neurobehavioral, neurochemical and neuroanatomical changes (Eriksson et al, 2001; Haddow et al, 1999; Viberg et al, 2003a).

Our present study shows that TH and BDE209 affect Purkinje cell dendrite growth and development in a temporal manner. We hope that our study will further help to clarify the temporal effects of TH and BDE209 on Purkinje cell dendritogenesis.

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