The Rat Cerebellar Development in Hypothyroidism Induced by the Anti-thyroid Drug

（薬剤誘発低甲状腺ホルモンラット産子の小脳発達）

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Contents

General Introduction

Thyroid hormone (TH) exerts most of its effects on the maturation of the developing mammalian brain. Late brain development is characterized by maturation of the organ. The processes of axonal and dendritic growth, synapse formation, myelination, cell migration, proliferation of specific population of cells, such as the glial cells and ceratin late arising neurons, all occur late in brain development and are regulated by TH (Anderson, 2001). TH regulates gene transcription by binding to the TH receptor which in turn binds to specific DNA sequences known to as TH response elements in a TH-responsive gene promoter (Anderson, 2008).

The developing cerebellum is a well-recognized target of TH. In rat cerebellum, the deep nuclear neurons, Purkinje and Golgi cells are originated from the ventricular zone during prenatal development, whereas the internal granule cells are from the external granular layer (EGL) during postnatal development (Sotelo, 2004). Following the extensive mitotic activity, the external granule cells migrate toward the molecular layer (ML) and then descend further to the internal granular layer (IGL), leaving their axons in the ML to establish connection with the

Purkinje cells (PCs). In more detail, the EGL is thin on Postnatal Day (P) 1, and is composed mostly of the densely packed outer proliferative zone. The EGL reaches its maximal thickness between P 5 to P 10, with major contribution made to this change by the expansion of inner, loosely packed cell zone. By P 20, the EGL has virtually disappeared. The ML is quite thin on P 1, and progressively increases in thickness on the succeeding days and weeks. The PC layer is reduced in thickness between P 1 and 10 and this is attributable to its transformation from a multicellular to a monocellular sheet of PCs. The soma of PCs has increased in size during this period but thereafter the major change is the reduction in its packing density. The IGL is first recognized by P 5 and the thickness has increased greatly by P 10. Various anatomical alterations induced by perinatal HT have been well documented. These include: reduction of growth and branching of dendritic arborization of PCs; reduction of synaptogenesis between PCs and granule cell axons; delayed proliferation and migration granule cells; delayed myelination; and changes in synaptic connection among cerebellar neurons and afferent neuronal fibers.

From perspective of cerebellar lobulation, the five cardinal lobes are well defined in all parts of the cerebellar cortex by P 1. The central lobe, one of the five cardinal lobes, splits into lobules VI, VII, and VIII, and lobule VI segregates into sublobules VIa, VIb, and VIc. Already by P 5, most of the lobes and some of the sublobes are already delineated in both vermis and the hemisphere. By P 20, lobulation of the cerebellar cortex reached the mature pattern. The adult rat vermis consists of ten lobes and contains more sublobes (Altman and Bayer, 1997). Although a number of theories have been proposed, it remains unknown how the conserved position of the lobes is determined, and what genetic mechanism regulate the size and complexity of the lobes. It is also not known whether postioning of the lobe and regulation of the number of lobe/sublobe are independent or inter-related events (Corrales et al., 2006). The cerebellum is highly organized and the set of lobes is largely generated postnatally during the expansion of the granule cells precursor (GCP) pool. Sonic hedgehog (Shh) is one of candidates regulating the cerebellar lobulation pattern. Since the secreted factor Shh is expressed in PCs and functions as a GCP mitogen in vitro, it is possible that Shh influences lobulation

during cerebellum development by regulating the position and/or size of lobes (Dahmane and Ruiz-i-Altaba, 1999; Lewis et al., 2004). Although the mechanisms underlying cerebellar lobulation are thought to influence the mechanical forces created by the expanding granule cell population, which are controlled by TH-regulated genes. It is not clear whether TH regulates Shh or how the Shh expression is in the HT.

There are several genes known to be regulated by TH in the cerebellum at transcriptional level. For example, *reelin* which plays a crucial role in neuronal migration and lamination, has been shown to be under TH control. The level of *reelin* mRNA is decreased by hypothyroidism at an earlier stage of cerebellar development. During the migration period of the granule cell, *reelin* expression is still under control of TH. These results indicate that the abnormal neuronal migration seen in the HT animal might be mediated in part by the change in *reelin* expression. Whether *reelin* is under direct control of TH is not known. Interestingly, brain derived neurotropic factor (BDNF) regulates *reelin* expression. Therefore, changes in *reelin* expression seen in the HT cerebellum may be exerted, in part, through change in BDNF gene

expression (Hirotsune et al., 1995; Koibuchi and Chin, 2000; Takahashi et al., 2008; Anderson, 2008). As described earlier, the role of TH in cell proliferation, migration, differentiation, and migration has been investigated in detail. Programmed cell death (PCD), an important process of development, has received less attention in TH and brain development. Bax, a member of the pro-apoptotic Bcl-2 family gene is an important regulator of apoptosis. As shown in a previous report (Singh et al., 2003), the relative amount of Bax in cytosol varied with age in the euthyroid condition. Initially, low levels of Bax expression were observed from P 0 to P 20, thereafter they increased at P 24 and adult stages. On the other hand, in experimental rat hypothyroidism induced by methimazole (MMI), Bax expression was high from P 0 to 12, before decreasing at P 20, increasing again at P 24 and further decreasing at adult stages. It was demonstrated that normal levels of TH substantially prevent cerebellar apoptosis. It is not clear whether TH acts as a physiological signal to trigger PCD to adjust cell number or to prevent lethal differentiation of brain cells, or whether the lack of TH in the postnatal period affects the expression of apoptotic genes to modulate cell death during the cerebellar

neurogenesis (Singh et al., 2003).

Hyperthyroidism including Graves' disease is the result of excess thyroid hormone production. The drugs for hyperthyroidism commonly used are MMI, carbimazole, and propylthiouracil (PTU). There is a risk that treatment of pregnant patients or breast-feeder of hyperthyroidism with antithyroid drugs could result in fetus or postnatal baby getting HT. In human and also in rats, MMI passages across the placenta and breast epithelium to a far higher extent than do PTU. PTU is recommended as the antithyroid drug for pregnant woman and breastfeeder. MMI are used worldwide to treat pregnant woman with hyperthyroidism (Mandel and Cooper, 2001). The effects of experimental hypothyroidism on the morphogenesis of rat brain have has been largely investigated using PTU not MMI.

In this investigation, MMI was used to induce the experimental congenital hypothyroidism in rats and examined the effects of HT on the rat cerebellar development from behavioral, morphological, and genetic aspects. The gene expression assay about 3 genes correlated with the morphological development of the cerebellum (Shh, *reelin* and Bax) was

carried out using quantitative real-time PCR.

Chapter I

Effects of an anti-thyroid drug, methimazole, administration to rat dams on the cerebellar cortex development in their pups

Summary

In the present study, the effect of methimazole (a major anti-thyroid drug) administration to rat dams on the development of cerebellum of their pups was investigated with morphological, morphometrical and functional procedures. A motor performance in the pups was evaluated by a rota-rod test. Brains removed on 6, 9, 12, 15, 25, and 30 postnatal days were analyzed using the serial sagittal sections of the cerebellum. Results showed that orally administered methimazole to dams produced a congenital hypothyroid model accompanied with an impaired motor coordination assured by the reduced thyroid hormones. The prominent anomaly was found in the internal granular layer in that there were excess bulges or branching and formation of excess sublobules although the normal lobulation pattern was kept. Three dimensional reconstruction imaging revealed the complex morphological pattern of internal granular layer of the cerebellar hemispheres as well as of the vermis, in which bulges and branches were viewed stereoscopically as the smooth ridges rather than irregular or nodal. In addition, the external granular layer in hypothyroidism survived another several days than that in controls. It is

suggested that the complex internal granular layer resulted from the overproduced internal granule cells, which originate in the prolonged external granular layer.

Introduction

The role of thyroid hormone (3,5,3'-L-triiodithyronine, T_3; 3,5,3',5'-L-tetraiodothyronine, T_4; TH) in mammalian brain development is extensively studied (Porterfield and Hendrich, 1993). Because rat pups are born with a relatively undeveloped brain, especially the immature cerebellum, perinatal hypothyroidism (HT) dramatically affects cerebellar development. Therefore, the neonatal rat cerebellum would be an excellent model to investigate the roles of TH in development.

The lack of TH in early life has a marked effect on the development of the rat cerebellum. In the murine cerebellum, the deep nuclear neurons, Purkinje and Golgi cells are originated from the ventricular zone during prenatal development, whereas the internal granule cells are from the external granular layer (EGL) during postnatal development (Sotelo, 2004). Following the extensive mitotic activity, the external granule cells migrate toward the molecular layer (ML) and then descend further to the

internal granular layer (IGL), leaving their axons in the ML to establish connection with the Purkinje cells. In the development of rat cerebellum, HT prolongs cell proliferation in EGL resulting in retarded disappearance of EGL and shows retarded growth of the cerebellar cortex (Nicholson and Altman, 1972; Lauder et al., 1974; Legrand, 1979; Oppenheimer and Schwartz, 1997; Xiao and Nikodem, 1998; Thompson and Potter, 2000).

In human, cretinism, a congenital HT, is characterized by stunted body growth and mental retardation (Franklyn et al., 2005). Meanwhile, hyperthyroidism including Graves' disease is the result of excess thyroid hormone production. The drugs for hyperthyroidism commonly used are methimazole (MMI), carbimazole, and propylthiouracil (PTU). Carbimazole is converted to MMI in the body. There is a risk that treatment of pregnant patients or breast-feeder of hyperthyroidism with antithyroid drugs could result in fetus or postnatal baby getting HT. In human and also in rats, MMI passages across the placenta and breast epithelium to a far higher extent than do PTU. PTU is recommended as the antithyroid drug for pregnant woman and breastfeeder. However, MMI are used worldwide to treat pregnant woman (Mandel and Cooper, 2001).

The effects of experimental hypothyroidism on the morphogenesis of rat brain have has been largely investigated using PTU not MMI. Although if hyperthyroid mothers keep an adequate dosage of antithyroid drug, the risk to babies is low (Momotani et al., 1997; Wing et al, 1994), it is required to elucidate a risky impact on pups of MMI administered to dam.

In this study, we examined the effects of orally administered MMI, in which induced HT in rat dams, on the cerebellar development in their pups. In the previous studies on the cerebellar development in the HT rats, histological observations have been exclusively carried out in parasagittal planes. We also covered the cerebellar hemispheres as well as the vermis by three dimensional reconstruction imaging. Furthermore, we assessed the cerebellar functions of HT pups using a rota-rod test.

Materials and Methods

Animals

Mated female Crl(CD)SD rats (11 weeks old) were delivered from Charles River Inc. on the 7th of gestational days. All rats were kept in controlled dark-light cycles (Light on: 7 a.m. to 7 p.m.) and temperature

(22 ± 3°C). All animal studies were conducted in accordance with principle and procedures approved by Banyu Institutional Animal Care and Use Committee.

Induction of experimental HT

Maternal animals were administered 20 mg/kg/day MMI (2-Mercapto-1-methylimidazole, Sigma) orally from the 17th gestational days onwards. The maternal animals were allowed to deliver pups naturally. The administration of MMI was continued even during the lactation. The fetuses had access to the drug by placental transfer and pups through milk secretion. Mothers in the control group received distilled water. The thyroid status was verified by measuring plasma thyroid hormones, T_3 and T_4 in dams and their pups using an electrospray Ionization LC-MS/MS with on-line column extraction method.

Rota-rod test

On Postnatal Day (P) 30, the accelerating rota-rod (Rota-Rod Tredmill for Rats, Ugo Basile, Italy) test was performed on pups (5 males/group) to

assess motor ability and motor coordination. With the minimum speed of 2 rpm, each rat was placed in its section in order to familiarize it with the revolving drum. After 2 training run for 2 minutes at intervals of 2 to 3 hours, the rat was tested. The speed was slowly increased from 2 rpm to 20 rpm for 5 minutes. Rats were tested for three trials, and the latency on the device was recorded.

Morphology and morphometry of cerebellums

The male pups were sacrificed by CO_2 and their brains were removed on P 6, 9, 12, 15, 25, and 30 (6 to 8 males/group/postnatal day). The brain was immersed in 10% neutral formalin prior to embedding paraffin. Brains were sectioned at 3μm, taking only midsagittal sections of the vermis, and stained with Cresyl Violet. Area of midsagittal section of the cerebellum, area of the EGL and number of the Purkinje cells of Lobule **V** and **VI** (the both sides of primal fissure) from the brain chronologically collected were measured using Digital life science imaging system (.slide, Soft Imaging System GmbH, Germany). In addition, area of the IGL and the ML in the midsagittal section of cerebellum on P 30 were measured

using the same software.

Three dimensional reconstruction of IGL

For three dimensional analysis, we used P 15, 20 and 30 HT pups and P 20 and 30 normal pups (1 male/group/postnatal day). The cerebella were removed and fixed in a mixture of 10% formalin and 1 % glutaraldehyde. Tissues were embedded in paraffin and sectioned serially at 5 μm in sagittal plane, and one in each five serial sections was stained with hematoxylin and eosin. We digitized images of the unilateral cerebellum using scanner and then stored only the IGL using Adobe Photoshop Limited 5.0 (Adobe Systems, Tokyo, Japan). Three dimensional reconstructions were performed using DeltaViewer 2.1.1, three dimensional image reconstruction software, a freely distributed PC-based program (http://vivaldi.ics.nara-wu.ac.jp/~wada/DeltaViewer/index-j.html).

Statistical analysis

The statistical significance of drug effects was evaluated using

Student's t-test. The statistical analysis was carried out with StatView for Windows (SAS institute Inc, NC, USA). $P \leq 0.05$ was considered statistically significant.

Results

Concentrations of plasma T_3 and T_4

Maternal and pup plasma T_3 and T_4 concentrations, summarized in Table 1, were measured on P 15. The plasma T_3 and T_4 concentration in HT dams and their pups were less than the lower limit of quantitation (0.122 ng/mL for T_3 and 10.7 ng/mL for T_4). As expected, MMI treatment effectively reduced plasma T_3 and T_4 levels in the pups in addition to their dams.

Table 1. Plasma T_3 and T_4 concentrations

	T_3 (ng/mL)[a]		T_4 (ng/mL)[a]	
	Control	HT	Control	HT
Dams	0.464 ± 0.0707	< 0.122 [b]	40.5 ± 3.00	< 10.7 [b]
Pups (P 15)	0.915 ± 0.118	< 0.122 [b]	57.7 ± 2.65	< 10.7 [b]

a Mean ± SEM (N=5/group)

b Lower limit of quantitation

Rota-rod test

The accelerating rota-rod was used to assess motor coordination in each five MMI-treated and control pups. Duration of stay on accelerating rota-rod in control pups was 203.8 ± 18.3 s and in the HT pups was 122.2 ± 25.4 s (Fig. 1). The duration in the HT pups was significantly ($p \leq 0.05$) shorter compared with controls. The fore and hind limb grip strength per body weights in the HT pups were comparable to controls (data not shown).

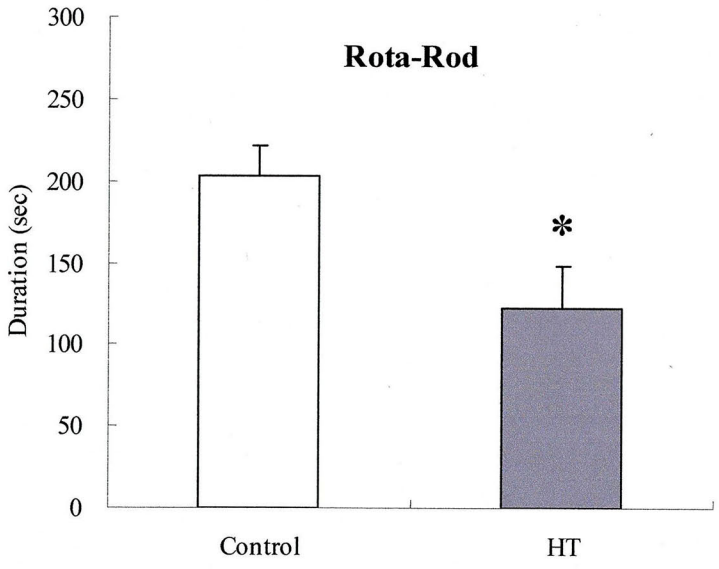

Fig. 1. The mean (±SEM) rota-rod performance. The HT male pups exhibited poorer performance on this task than the control pups. * $p \leq 0.05$

Morphology and morphometry of cerebellums

The measurement of area of the cerebellum in the midsagittal section was not clearly distinguished HT pups from control pups (Fig. 2) but there was trend of decreases in this area on P 30 without statistical significance (P=0.0747). Normal pattern of the lobulation in the cerebellum of HT pups was essentially kept (Fig. 3). In other words, ten lobules were easily distinguished (Figs. 4A and B). However, the IGL was partially irregular with excess bulges or complex branching pattern compared with the age matched controls from P 15 and more. Furthermore, some lobules were subdivided to form extra sublobules (Figs. 3J - L and 4B).

The three dimensional images showed (1) irregularity of the IGL was found in the hemisphere as well as the vermis, (2) the excess bulges and branches of the IGL extended laterally to form ridges, (3) these ridges were relatively smooth rather than irregular or nodal, (4) although the appearance of the overproduced ridges in the IGL was not constant in location and complexity, these ridges were frequently found in lobules Ⅵ, Ⅶ, and Ⅷ (Fig. 5).

Contrary, reduced foliation was also observed at a lower frequency

(9.5 % of HT pups on P 15, 25 and 30; Fig. 4C).

In the control pups, the area of the EGL increased until about P 9, then declines and finally disappeared by P 25, due to the progressive increase in the rates of differentiation and migration over cell proliferation. In the HT pups, the peak of the increment of EGL was delayed 3 days (Fig. 6A). The EGL of HT cerebellum consisted of three to four layers of cells at P 25 (Fig. 7B) and disappeared by P 30. In HT pups on P 30, the area of IGL/cerebellum was statistically significantly (P=0.0044) greater than that of controls, although number of internal granule cell assessed by the area of IGL was not affected (Table 2).

Number of Purkinje cells (Fig. 6B) was not affected by HT. However, the area of ML and the area ML/cerebellum statistical significantly (P=0.0141 and 0.0069, respectively) reduced on P 30 (Table 2).

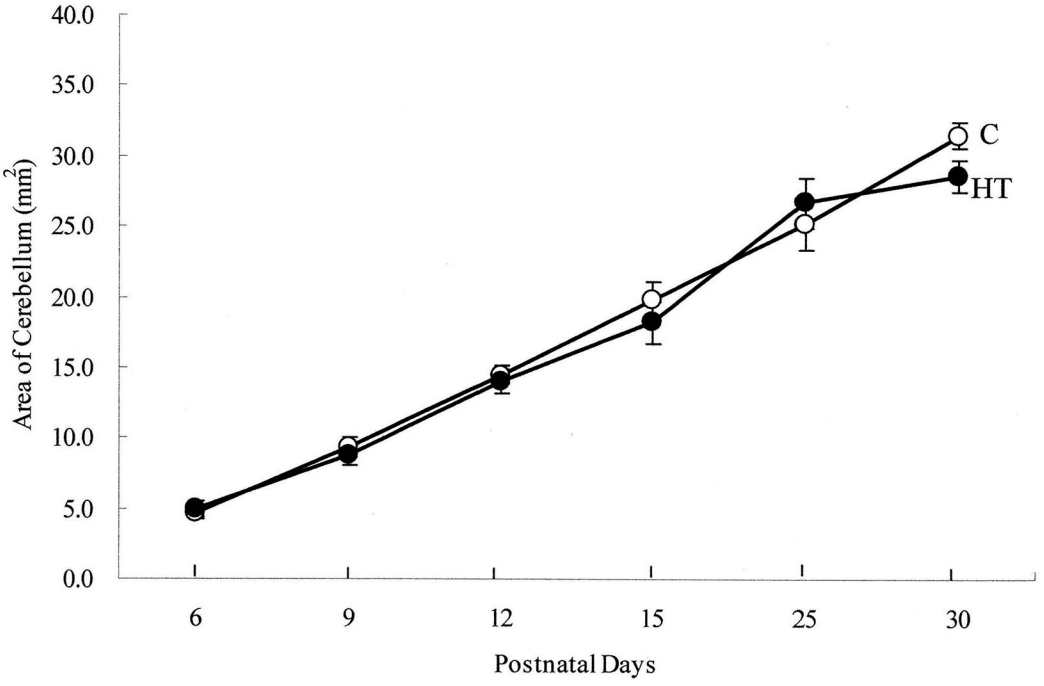

Fig. 2. Area of cerebellum in the midsagittal section (Data from HT pups with the reduced lobulation was excluded from these data analyses). Open circle, Control; Closed circle, HT; The values are means (±SEM).

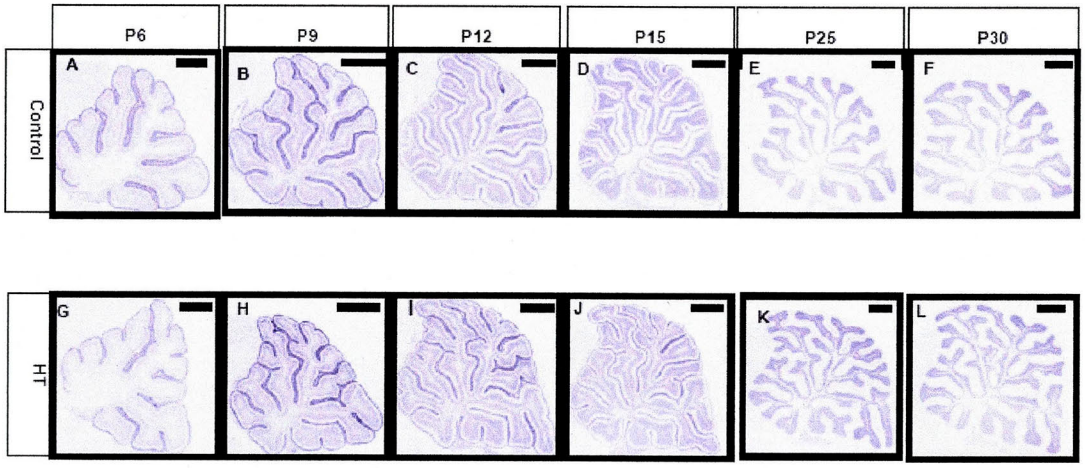

Fig. 3. Midsagittal sections of the cerebellum (vermis) on P6, 9, 12, 15, 25, and 30 in control and HT pups. The normal structure of the lobulation in the cerebellum was kept in HT pups but HT pups show further subdivided lobes compared with the age matched controls. Scale bar: 500 μm in A, G; 1 mm in B-F, H-K Anterior is to the left in a section.

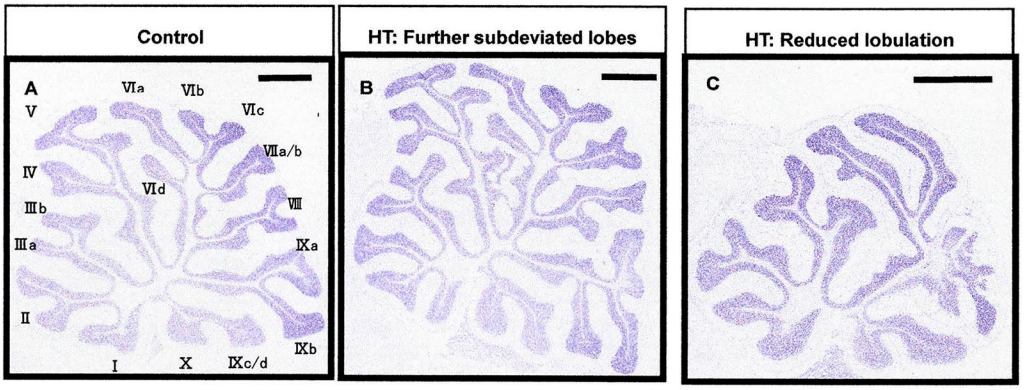

Fig. 4. Midsagittal sections of the cerebellum (vermis) on P 30. HT pups showed further subdivided lobules (B) or reduced lobulation (C) compared to controls (A). I to X: Larsell lobules Scale bar: 1 mm

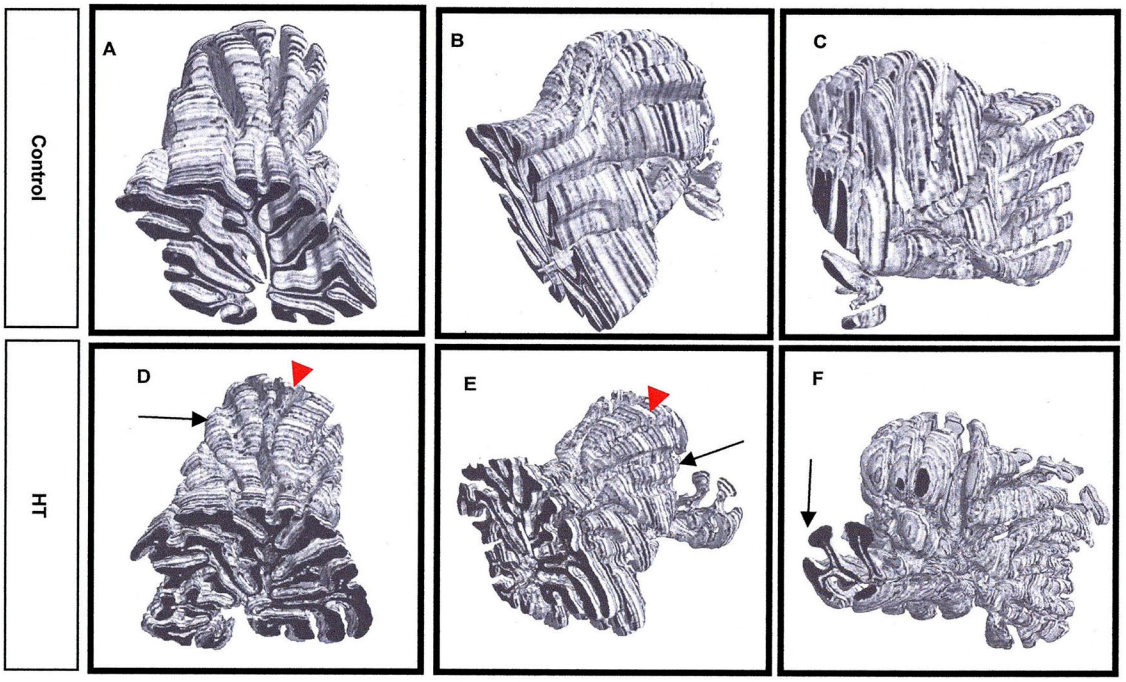

Fig. 5. Three dimensional reconstruction imaging of IGL on P30. Irregularity of the IGL in HT pups (D, E, and F) is found both in the vermis and cerebellar hemisphere. The excess branches of IGL at D; anisiform lobule 1, sublobule a (arrow) and anisiform lobule 2, sublobule a (arrow head): E; copula pyramidis, sublobule a: (arrow) F; paraflocculus (arrow).

The deeper fissure of IGL was also recognized at anisiform lobule 2, sublobule b (E, arrow head)

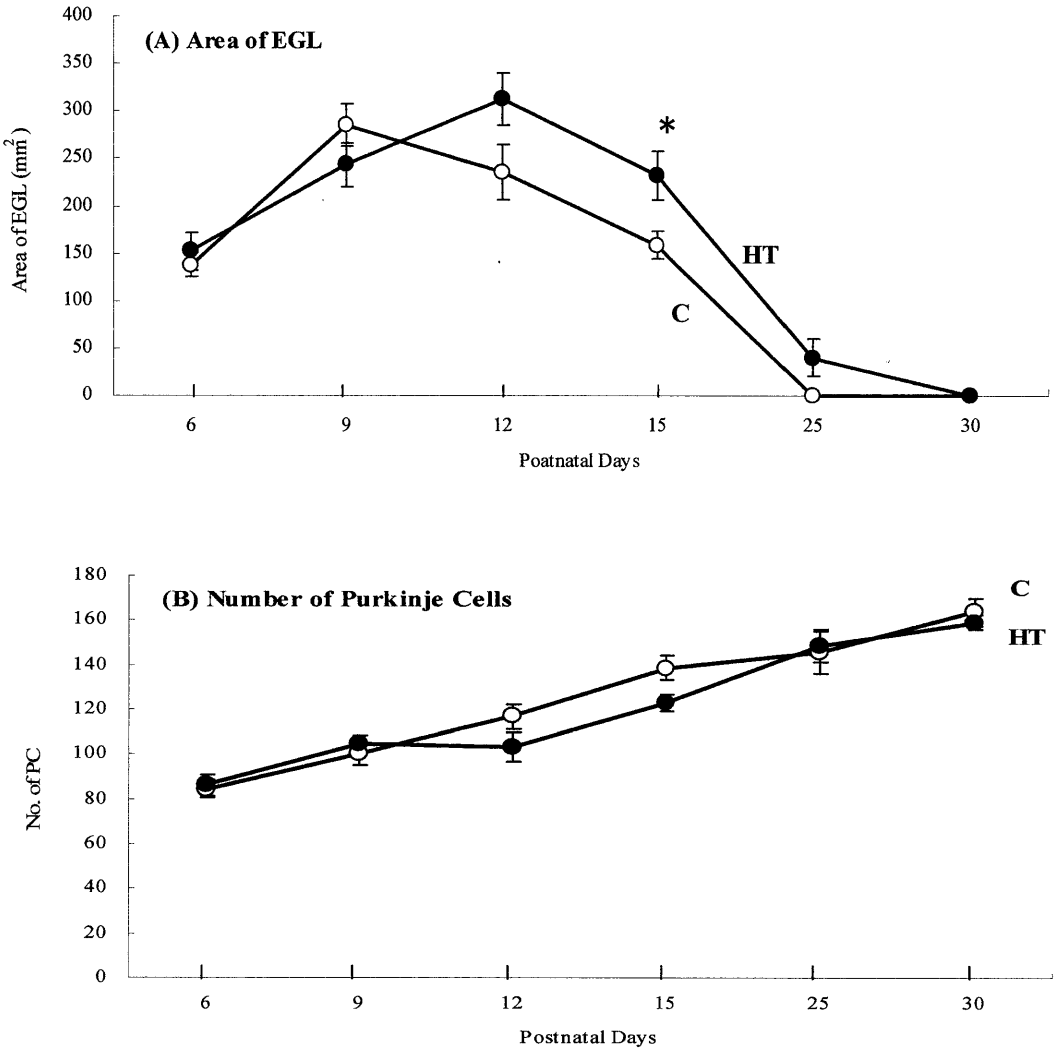

Fig. 6. Developmental changes in the area of EGL (A) and number of the Purkinje cells (B) along the primary fissure. The values are means (±SEM). * $p \leq 0.05$

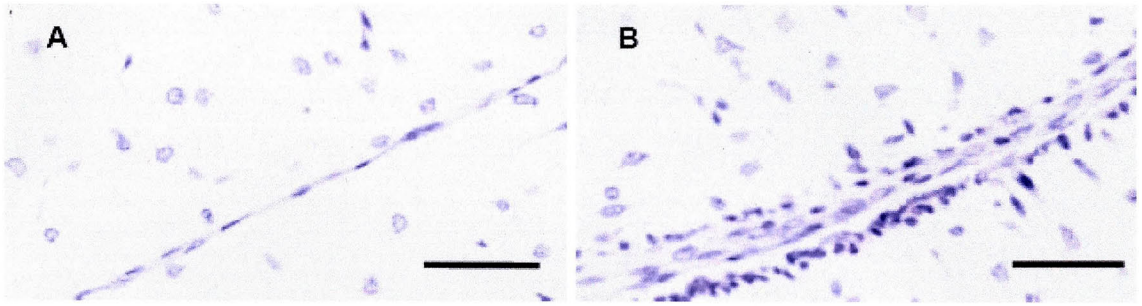

Fig. 7. EGL along the primary fissure on P 25. The EGL is disappeared by P 25 in the control pups (A), but the layer is still present on P 25 in HT pups (B). Scale bar: 50 μm

Table 2. Measurements of area of cerebellum on P 30

	Total Area of Cerebellum (mm^2)	IGL (mm^2) (%)	ML (mm^2) (%)
Control	31.4 ± 0.9	12.0 ± 0.5 (38.4)	14.4 ± 0.3 (45.9)
HT	28.5 ± 1.1	11.9 ± 0.4 (41.9*)	12.1 ± 0.6* (42.5*)

a Mean ± SEM (N=5-6/group; except for the cerebellum with reduced lobulation)

* $p \leq 0.05$

( ) percent IGL/ML of total area of cerebellum is indicated in parentheses.

Discussion

Although it is not arguable that TH is crucial for the cerebellum development, it has not been clearly established whether HT of pup induced by MMI treatment to pregnant and lactating dam impairs its cerebellar development. Direct injection of an anti-thyroid drug to rat pups has been used to induce rat HT in most experiments, and PTU is a most common drug to produce the HT model. Orally administered MMI transmits easily to breast milk because MMI is minimally bound to serum proteins (Mandel and Cooper, 2001; Zatón et al., 1988) and is not ionized in serum (Johansen et al., 1982; Zatón et al., 1988). In this study, plasma T_3 and T_4 levels reduced markedly in pups after oral administration of MMI to their dam. HT pups showed morphological cerebellar abnormalities including a complex pattern of IGL, an increased number of fissures and a thinning of ML. Use of the rota-rod is very common to assess the motor performance (Bogo et al., 1981; Rustay et al., 2003). Moreover, the accelerating rota-rod is recommended to eliminate the need for extensive training or the introduction of a maximal time limit for performance (Jones and Roberts, 1968). The HT pups in this study

showed an impairment of motor coordination evaluated by the rota-rod test, indicating the functional defect of the cerebellum. For all of these reasons, we can conclude that MMI orally administered to dam can effortlessly induce HT in postnatal rats.

The development of the cerebellum in HT animals has been extensively examined in murines. Previous studies on HT rats have revealed that (1) the number of Purkinje cells is not decreased, (2) their maturation is permanently affected, as reflected by abnormal organization of the dendritic tree, persistent hypoplasia of the dendritic field and decrease in number of dendritic spines, (3) proliferation, migration, and differentiation of external granule cells are retarded, (4) parallel fibers are shorter and have fewer synaptic contact with Purkinje cells, and (5) synaptic density degrade throughout the cerebellar cortex (Nicholson and Altman, 1972; Clos et al., 1974; Lauder, 1974; Legrand, 1979; Rabié et al, 1980; Dussault and Ruel, 1987; Oppenheimer and Schwartz, 1997; Xiao and Nikodem, 1998; Koibuchi and Chin, 2000; Thompson and Potter, 2000; Anderson, 2001; Mussa et al., 2001). In this study, most prominent alteration emerged as a formation of excess bulges and branching in the

IGL and the increase in number of sublobules with preserving the normal cerebellar lobulation pattern. Three dimensional reconstruction of IGL clearly showed these above findings bulges and branches to form continuous ridges and a high ridge to induce a new sublobule in the vermis as well as the hemisphere. It should be put emphasis on HT to get complex morphological pattern of the IGL. According to Lauder et al. (1974), who observed the lobulation process of the cerebellum in hypo- and hyperthyroidism, hypothyroidism leads ultimately to the formation of an increased number of lobules with fissures of decreased depth and hyperthyroidism leads a reduction number of fissures of normal depth. Their report has been minimized and not referred in almost all reviews on the cerebellar development in HT. However, the description about the cerebellar lobulation of Lauder et al. (1974) is not correct. More accurately, HT or hyperthyroidism do not play a role in the lobulation and are merely involved in the formation of sublobule. Their figures show that the cerebellar lobulation is not abnormal and the basic lobulation pattern is preserved both in HT and hyperthyroidism.

In the cerebellum of HT pups, the external granule cells are retarded

both in cell migration and proliferation but present a longer time than in normal, resulting in acquisition of a greater number of the internal granule cells (Nicholson and Altman, 1972; Lewis et al., 1976; Patel et al., 1976; Lauder, 1979). Thus, HT prolongs the expansion of the granule cells and eventually leads the formation of excess number of fissures. Our results in this study basically supported their findings. Furthermore, HT reduces the thickness of ML for the stunted dendrite of Purkinje cells and the undeveloped parallel fibers, resulting in the formation of shallow fissures (Lauder et al., 1974). In fact, the mechanism underlying cerebellar lobulation has been considered the likely influence of mechanical forces created by the expanding granule cell population, which are controlled by the patterning genes including the sonic hedgehog gene (Corrales et al., 2006).

In a few cases, the HT cerebellum showed a reduction of lobulation and the morphologically simplified IGL. There are two phases in cerebellar lobulation, the granule cell dependent and independent phases (Doughty et al., 1998). In normal rats, the five principle lobules are established by the arrangement of five distinct cluster of Purkinje cells

beneath the EGL between embryonic day 17 (Altman and Bayer, 1997) and birth. In our cases, since a reduction of lobulation was not found prior to P 25 and body weights at birth in HT rats with a reduction of lobulation were comparable to those of their litter mates, the five principle lobes were established and lobulation of granule cell independent mechanism may be normal. In the cerebellar development of rats, apoptosis is limited to the internal granule cells in normal but found mainly in IGL and also in EGL and ML in the HT rats. Apoptosis in HT rats is higher in rate and longer in time than in control animals (Xiao and Nikodem, 1998). The HT cerebellum with abnormal lobulation may be caused by a disturbance of the granule cell dependent mechanism induced by reduction of proliferation in the EGL and/or abnormally increased apoptosis in the cerebellar cortex.

Chapter Ⅱ

Expression of sonic hedgehog regulates morphological changes of rat developing cerebellum in hypothyroidism

Summary

Although thyroid hormones are crucial for cerebellar development, and several thyroid hormone-dependent genes are known to be correlated with morphological development of the cerebellum, the precise mechanisms of morphological cerebellar changes in hypothyroidism (HT) remain unknown. To investigate these mechanisms in experimental rat HT induced by the anti-thyroid drug methimazole (MMI-HT rat), we carried out gene expression analysis (sonic hedgehog [Shh], *reelin*, and Bax) using quantitative real-time PCR. Histological examination revealed cerebellar abnormalities, including reductions in the thickness of the molecular layer and delayed disappearance of the external granular layer (EGL), as well as excess bulges or sublobules in the internal granular layer (IGL). At Postnatal Day (P) 6, Shh expression in MMI-HT rat was comparable to that in controls, thus suggesting that Shh expression was sufficient to form the lobes in the initial phase. However, Shh expression decreased in the later phases, as compared with age-matched controls. This demonstrated that stronger and sustained signaling is necessary for partitioning of the cardinal lobes into lobes and sublobes. Although *reelin* expression was not clearly different from that in controls, Bax expression

decreased at P 15. The attrition of Bax at P 15 as well as Shh in the later phase may be related to irregularities in the IGL and the relatively large numbers of internal granular cells. Taken together, these results suggest that Shh expression is related to the morphological cerebellar changes in experimental hypothyroidism and that sustained signaling by Shh may play a key role in normal development, particularly lobulation, in the cerebellum.

Introduction

The role of thyroid hormones (3,5,3'-L-triiodithyronine, T_3; 3,5,3',5'-L-tetraiodothyronine, T_4; TH) in mammalian brain development has been extensively studied (Porterfield and Hendrich, 1993), and they are known to be essential for normal development of various organs, including the brain (Oppenheimer and Schwartz, 1997). For example, a lack of TH in early life has a marked effect on development of the rat cerebellum. Hypothyroidism (HT) in rats prolongs cell proliferation in the external granular layer (EGL) resulting in retarded disappearance of EGL and retarded growth of the cerebellar cortex (Nicholson and Altman,

1972; Lauder et al., 1979; Legrand, 1979; Oppenheimer and Schwartz, 1997; Xiao and Nikodem, 1998; Thompson and Potter, 2000). Furthermore, HT reduces the thickness of the molecular layer (ML) in stunted dendrites of Purkinje cells (PCs) and undeveloped parallel fibers, resulting in the formation of shallow fissures (Lauder et al., 1974). In fact, the mechanisms underlying cerebellar lobulation are thought to influence the mechanical forces created by the expanding granule cell population, which are controlled by TH-regulated genes.

DNA microarrays are an efficient tool for comprehensive analyses of gene expression and have been used in recent studies to identify TH-regulated genes in the cerebellum during experimental HT. Numerous candidate genes involved in TH-regulated process have thus been identified. Numerous lines of evidence have suggested that the mitogenic effects of PC on granule cell precursors (GCPs) is mediated by sonic hedgehog (Shh), a secreted factor expressed in PC from Embryonic Day (E) 17.5 onwards in mice. Indeed, a reduction in Shh signaling results in less lobulation with a corresponding reduction in the temporal length of GCPs proliferation, whereas increased Shh signaling produces a more

complex lobulation pattern (Corrales et al., 2006).

In the present study, we investigated whether Shh expression is related to morphological cerebellar changes in experimental hypothyroidism induced by an anti-thyroid drug. We sampled brains chronologically and carried out gene expression analysis using quantitative real-time PCR. In addition, 2 other genes correlated with the morphological development of the cerebellum (*reelin* and Bax) were examined in the same manner.

Materials and Methods

Experimental animals

Mated female Crl(CD)SD rats (age, 11 weeks) were delivered from Charles River Inc. (Ibaraki, Japan) on gestational day (GD) 7 (GD 0 = day of the copulatory plug positive). All rats were kept under a controlled dark-light cycle (lights on: 7 a.m. to 7 p.m.) and temperature (22 ± 3°C). Pregnant rats were divided into two groups (n=5 in each group). Experimental hypothyroidism was induced by MMI (2-Mercapto-1-methylimidazole, Sigma, St. Louis, MO, USA) in rats (MMI-HT rats) as described previously (Hasebe et al., 2008). Briefly,

maternal animals were administered 20 mg/kg/day MMI orally from GD 17 to Postnatal Day (P) 29. The dose level of 20 mg/kg/day was produced impaired motor coordination in the pups without maternal toxicities (Hasebe et al., 2008). Maternal animals were weighed on GD 17 and 21, and once a week during the postnatal period (P 0, 7, 14, 21, and P28) to calculate dose volumes. The maternal animals were allowed to deliver pups naturally and housed with their pups until termination. The fetuses had access to the drug by placental transfer and pups through milk secretion. For mRNA detection, pups were sacrificed using CO_2 and their brains were removed on P 6, 15, 21 and 30 (4 males/group/postnatal day; birthday= P 0). All animal studies were conducted in accordance with the principles and procedures of the Banyu Institutional Animal Care and Use Committee.

Histological Analysis

Pups were sacrificed using CO_2 and their brains were removed on P 6, 15, and 30 (6 to 8 males/group/postnatal day). Brain tissue was immersed in 10% neutral formalin prior to paraffin embedding. Brains were

sectioned at 3 μm, taking only midsagittal sections of the vermis, followed by staining with Cresyl Violet.

Reverse transcription and quantitative real-time PCR

Total RNA was isolated from the cerebella of age-matched controls and MMI-treated pups with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For each sample, first-strand cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) from 1 μg of total RNA. cDNA was synthesized using a PCR Thermal Cycler TP400 (Takara Shuzo Co., Ltd., Shiga, Japan) under the following conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5 s. Quantitative real-time PCR analysis was performed with an Applied Biosystems Prism 7900HT Sequence Detection System using TaqMan® gene expression master mix according to the manufacturer's specifications (Applied Biosystems) for Shh, *reelin*, and Bax, for which validated TaqMan® Gene Expression Assays are available. The TaqMan® probes and primers for Shh (assay identification number Rn00568129_m1), *reelin* (assay identification number Rn00589609_m1)

and Bax (assay identification number Rn02532082_g1) were inventoried gene expression products (Applied Biosystems). The rat Actb gene was used as an endogenous control (Applied Biosystems, catalog number 4352340E). Gene-specific probes were labeled using the reporter dye FAM, and the Actb internal control probe was labeled with a different reporter dye, VIC, at the 5'-end. A nonfluorescent quencher and the minor groove binder were linked at the 3'-end of the probe as quenchers. Thermal cycler conditions were as follows: hold for 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 s and by 60 °C for 1 min. All procedures were performed in triplicate. Amplification data were analyzed using Applied Biosystems Prism Sequence Detection Software version 2.2 (Applied Biosystems). To normalize the relative expression of the genes, standard curves were prepared for each gene, as well as Actb, in each experiment. Relative expression levels were obtained by normalizing the amount of mRNA against that of Actb RNA in each sample (Heid et al., 1995; Gibson et al., 1996).

Statistical analysis

The statistical significance of drug effects on each postnatal day was evaluated using Student's t-test (Fig. 2. Statistical analysis was carried out using StatView for Windows (SAS Institute Inc., Cary, NC, USA). $P \leq 0.05$ was considered to indicate statistical significance.

Results

Disturbed cerebellar development in MMI-HT rats

In a previous study, we found that the midsagittal section of the cerebellum could not be clearly distinguished between MMI-HT and control pups from P 6 to P 25, but there were decreases in this area on P 30 (data not shown). Midsagittal sections of MMI-HT cerebella revealed irregularities, with excess bulges or complex branching patterns in the internal granular cell (IGL), as compared with age-matched controls (Fig. 1A), although the normal pattern of lobulation in MMI-HT pups was essentially maintained (Fig. 1B). This phenotype can be observed beginning on P 15. At P 15, the EGL of MMI-HT rats was thicker, and the ML was thinner than those of controls (Fig. 1C and D), and although

the EGL in controls completely disappeared at P 30 (Fig. 1E), a very thin EGL remained present in MMI-HT rats (Fig. 1F). These irregularities in the IGL, EGL and ML suggest that the development of granule cells and PCs was affected by MMI-HT. Furthermore, we previously demonstrated that the relative area of IGL to total area of cerebellum was increased, while the thickness of ML was reduced in MMI-HT rats at P 30.

Gene expression in the developing cerebellum in the MMI-HT rats

In order to determine whether the expression of Shh mRNA was disrupted in MMI-HT rats, real-time PCR analysis was performed. As shown in Fig. 2A, MMI-HT negatively affected the expression of Shh and the levels relative to age-matched controls were significantly lower ($p \leq 0.05$) at P 15 and 21. Reduced expression of Bax was observed at P 15 in MMI-HT rats, but no apparent effects were noted in the expression of *reelin* throughout development or in Bax in subsequent postnatal days (Fig. 2B and C).

Fig. 1

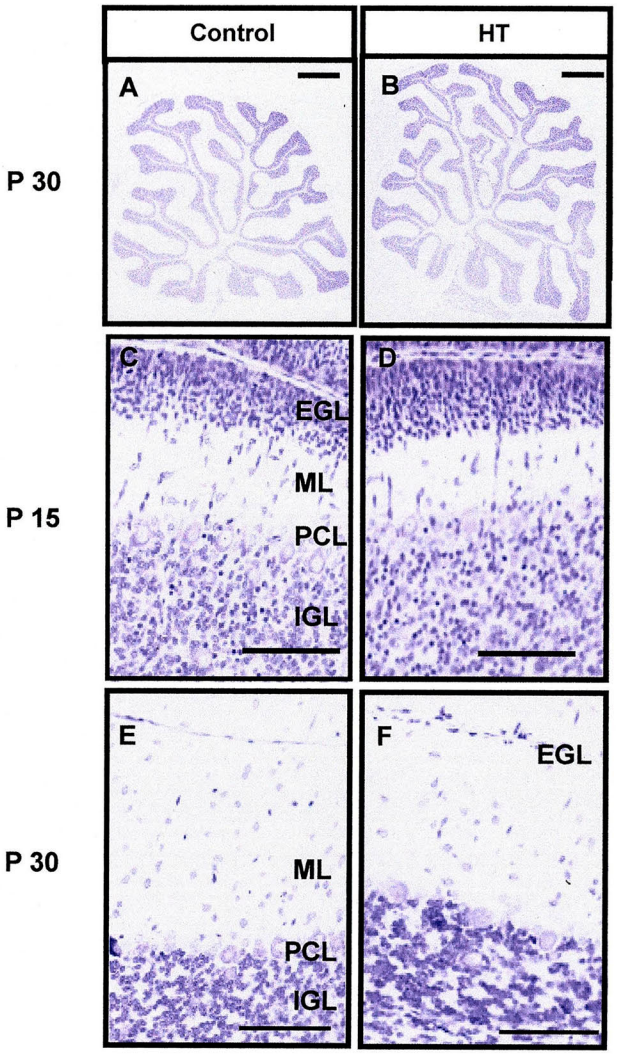

Fig. 2.

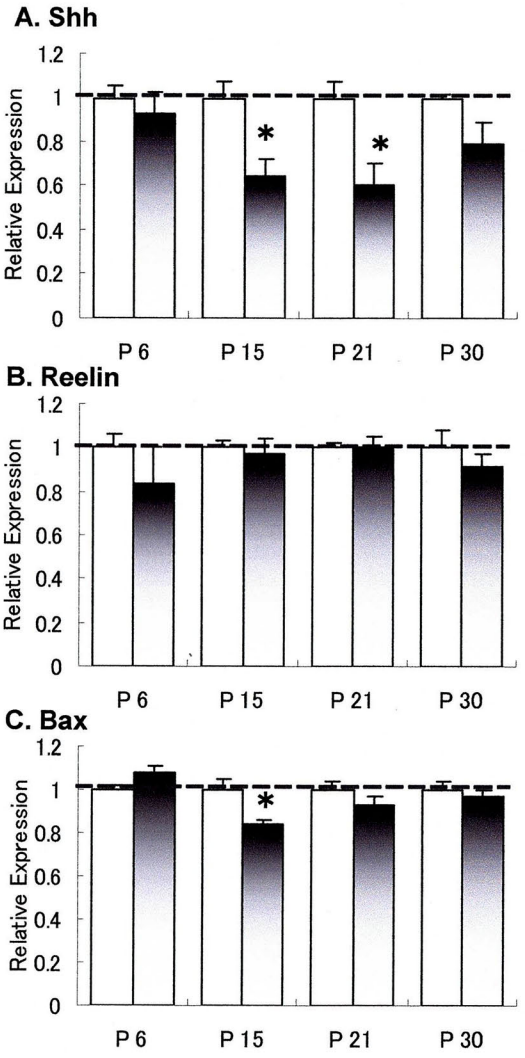

Fig. 1. A and B: Midsagittal sections of the cerebellum (vermis) on P 30. MMI-HT pups showed further subdivided lobules. Bars: 1 mm
C to F: Differences in cortical layer thickness in developing control and MMI-HT rats (P 15 and 30). Bars: 100 μm

Fig. 2. mRNA expression of Shh (A), *reelin* (B) and Bax (C). mRNA levels are expressed relative to those in age-matched controls (mean ± SEM, n=4). * $p \leq 0.05$
Light bar; Controls; Dark bar; MMI-HT
Relative expression = MMI-HT group mean / control group mean on each postnatal day

Discussion

Rat pups are born with a relatively undeveloped brain, and a particularly immature cerebellum. Thus, perinatal HT markedly affects cerebellar development (Porterfield and Hendrich, 1993). We used the developing rat cerebellum in an effort to better understand TH-modulated gene expression. Lobulation of the rodent cerebellum is generated in distinct phases (Altman and Bayer, 1997). First, the smooth cerebellar surface is divided into five cardinal lobes by four principle fissures in the vermis. Next, the process of lobulation divides the cardinal lobes into the individual lobules present in the adult cerebellum. Some of these lobules are then further subdivided into sublobules, and each lobule then grows to a specific size. In the mouse and rat, the emergence of the five cardinal lobes is observed at birth.

Recently, several lines of evidence have suggested that the mitogenic effect of PCs on granule cells is mediated by Shh, a secreted factor expressed in PCs from E17.5 onwards in mice. Corrales et al. (2004) demonstrated that Shh signaling is correlated spatially and temporally with fissure formation. Progressive deletion or inhibition of Shh reduces

proliferation of granule cell precursors and disorganizes lobulation (Dahmane and Ruiz-i-Altaba, 1999; Lewis et al., 2004).

In the present study, the cerebellar cortex in MMI-HT rats was normal with regard to basic lobulation but showed irregular sublobules. The morphological abnormalities in the cerebellum became apparent at P 15. We also observed that ML in MMI-HT rats reduced in thickness, suggesting the stunted dendrites of PCs and undeveloped parallel fibers. These finding likely resulted in the formation of shallow fissures (Lauder et al., 1974). At P 6, the expression of Shh in MMI-HT was comparable to controls. As Corrales et al. (2006) reported using the transgenic mice, the initial phase of lobulation requires very minimal levels of Shh signaling. In our experiments, the Shh expression in MMI-HT rats was likely sufficient to form the lobes in the initial phase. However, Shh expression in the later phase decreased by 64.2%, 60.1% and 79.5% of control values at P 15, 21 and 30, respectively (statistical significance was not noted at P 30; $p=0.0756$), as compared with age-matched controls. In the investigation by Corrales et al. (2006), higher and sustained signaling is necessary for partitioning of the cardinal lobes into lobes and sublobes.

They also demonstrated that Shh signaling is not required for the generation of the cardinal lobes, but instead is required to maintain the proliferative pool of GCPs such that a sufficient number of internal granule cells are generated to achieve full lobe growth, and to complete the lobulation and sublobulation processes. The transition of Shh signaling in the experimental hypothyroidism induced by MMI was similar to that in the transgenic HT model. As mentioned above, the mitogenic effect of PCs on granule cells is mediated by Shh (Corrales et al., 2004), the reduction of Shh in MMI-HT rats may related to irregularities such as the excess bulges or complex branching pattern in IGL, and the relatively large number of internal granule cells observed in this study.

The *reelin* gene is known be TH-responsive, and plays a role in cerebellar neuron migration (Porcionatto, 2006) and PC development (Beffert, et al., 2004). During early central nervous system development in mice, *reelin* mRNA is expressed by Cajal-Retzius cells in the cerebral cortex (E10-12), by Cajal-Retzius-like cells in the marginal zone of the developing hippocampus (E13-14), and by rhombic lip cells, external

neuroepithelium, differentiating PCs, and deep nuclear neurons, as well as by elements of the cerebellar peduncles (E13-14). During the postnatal cerebellar development, *reelin* mRNA is expressed by the internal granule cells and by cells present in the inner layer of the EGL (Hirotsune et al., 1995). TH regulates *reelin* expression in postnatal cerebellar granule cells. Mutation of this gene is associated with severe cerebellar abnormalities that resemble the abnormalities observed in MMI-HT (Takahashi et al., 2008; Anderson, 2008). However, it is unlikely that *reelin* is involved in the abnormalities seen in the present study, as its expression was not significantly inhibited by the present experiment.

Bax, a member of the pro-apoptotic Bcl-2 family gene is an important regulator of apoptosis. As shown in a previous report (Singh et al., 2003), the relative amount of protein was determined quantitatively using Western blotting and the relative amount of Bax in cytosol varied with age in the euthyroid condition. Initially, low levels of Bax expression were observed from P 0 to P 20, thereafter they increased at P 24 and adult stages. On the other hand, in the MMI-HT group, Bax expression was high from P 0 to 12, before decreasing at P 20, increasing again at P 24

and further decreasing at adult stages. It was demonstrated that normal levels of TH substantially prevent cerebellar apoptosis. Although statistical significance was noted only at P 15 in our study, the profile of changes in Bax expression was confirmed by quantitative real-time PCR and similar to their findings. In cerebellar development in rats, apoptosis is normally limited to the internal granule cells, but is primarily seen in the IGL, EGL and ML in HT rats. Apoptosis in HT rats occurs at a much higher rate and for longer periods than in control animals (Xiao and Nikodem, 1998). In addition to reduction of Shh, the attrition of Bax at P 15 may be also related to irregularities such as the excess bulges or complex branching pattern in IGL, and the relatively large number of internal granule cells.

General Conclusion

In these experiments, methimazole was orally administered to dams to produce a congenital hypothyroid model (MMI-HT). Morphologically, although the normal lobulation pattern was kept, there were excess bulges or branching and formation of excess sublobules in the internal granular layer, increment of internal granular cells and thinness of molecular layer in MMI-HT rats. Three dimensional reconstruction imaging revealed the complex morphological pattern of internal granular layer of the cerebellar hemispheres as well as of the vermis. The above-mentioned anomalies became apparent at Postnatal Day (P) 15.

To investigate how the conserved position of the lobes is determined, and what genetic mechanism regulate the size and complexity of the lobes, the gene expression which was correlated with morphological development of the cerebellum (Shh, *reeln* and BAX) was examined using quantitative real-time PCR. The results were as follows; 1) at P 6, Shh expression in MMI-HT rat was comparable to that in controls, thus suggesting that Shh expression was sufficient to form the lobes in the initial phase. However, Shh expression decreased in the later phases, as

compared with age-matched controls. This demonstrated that stronger and sustained signaling was necessary for partitioning of the cardinal lobes into lobes and sublobes; 2) the reduction of Shh (the mitogen of granule cells precursor), in MMI-HT rats may related to irregularities such as the excess bulges or complex branching pattern in IGL, and the relatively large number of internal granule cells observed in this study; 3) although *reelin* expression was not clearly different from that in controls, Bax (the regulator of apoptosis) expression decreased at P 15. In addition to changes in Shh expression, the attrition of Bax may be related to irregularities in the IGL and the relatively large numbers of internal granular cells, which become apparent at P15.

In conclusion, congenital hypothyroidism resulted in abnormal cellebellar development accompanied with an impaired motor coordination and the sustained signaling by Shh may play a key role in normal development, particularly lobulation, in the cerebellum.

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