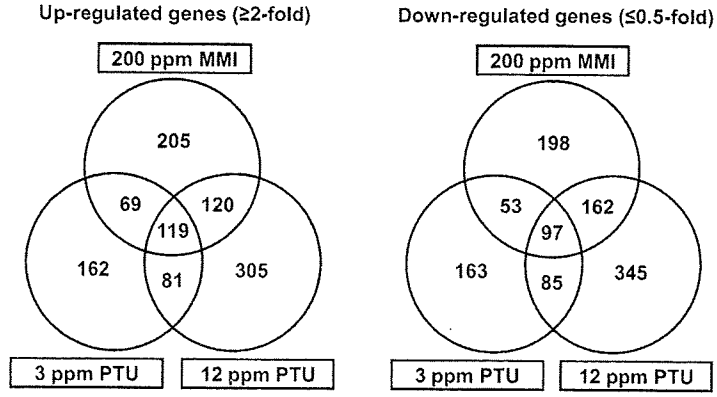

Fig. 2. Venn diagram of gene populations showing altered expression in the hippocampal CA1 pyramidal cell layer at postnatal day 20 in response to maternal exposure to propylthiouracil and/or 2-mercapto-1-methylimidazole compared with the untreated controls. (Left) Up-regulated genes (≥2-fold). (Right) Down-regulated genes (≤0.5-fold). Abbreviations: MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil.

Table 2. Validation of microarray data by real-time RT-PCR

Gene	200 ppm MMI			3 ppm PTU			12 ppm PTU		
	Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to	
		Hprt[a)]	Gapdh[b)]		Hprt	Gapdh		Hprt	Gapdh
Tacr3[c)]	7.32 ± 2.21**	4.29 ± 1.27	4.08 ± 1.15*	6.19 ± 2.19**	3.46 ± 1.42	3.76 ± 1.51*	12.49 ± 1.56**	9.23 ± 3.00**	8.81 ± 1.60**
Calb1[d)]	4.48 ± 0.66*	3.96 ± 0.74	3.67 ± 0.16	4.85 ± 2.53*	4.74 ± 2.48	4.93 ± 3.79	9.00 ± 1.85**	11.13 ± 2.13**	10.53 ± 3.26**
Slit2[e)]	3.04 ± 0.79	2.83 ± 0.90	4.08 ± 1.15*	2.62 ± 1.16	1.33 ± 0.67	3.67 ± 1.51*	7.08 ± 2.15**	4.72 ± 2.57**	8.81 ± 1.60**
Plgl1[f)]	3.10 ± 1.57	12.67 ± 5.00	11.5 ± 7.50	4.28 ± 2.88	18.33 ± 6.00	19.00 ± 9.00*	6.86 ± 2.85**	30.67 ± 5.33**	27.00 ± 8.00**
Mobp[g)]	0.35 ± 0.15**	0.6 ± 0.22*	0.52 ± 0.16**	0.18 ± 0.07**	0.24 ± 0.07**	0.24 ± 0.05**	0.12 ± 0.02**	0.18 ± 0.04**	0.16 ± 0.04**
Edg8[h)]	0.40 ± 0.11*	0.49 ± 0.16*	0.43 ± 0.13*	0.06 ± 0.05**	0.29 ± 0.10**	0.28 ± 0.08**	0.08 ± 0.07**	0.21 ± 0.07**	0.18 ± 0.03**
Cebpb[i)]	0.31 ± 0.06**	0.43 ± 0.04**	0.38 ± 0.06**	0.43 ± 0.18**	0.77 ± 0.07	0.76 ± 0.10	0.26 ± 0.04**	0.39 ± 0.16**	0.35 ± 0.22**

a) Hprt, Hypoxanthine-guanine phosphoribosyltransferase; b) Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; c) Tacr3, Tachykinin receptor 3; d) Calb1, Calbindin 1; e) Slit2, Slit homolog 2 (Drosophila); f) Plgl1, Pleomorphic adenoma gene-like 1; g) Mobp, Myelin-associated oligodendrocytic basic protein; h) Edg8, Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8; i) Cebpb, CCAAT/enhancer binding protein (C/EBP), beta.

Values are mean ± SD (n=4) relative to the expression level in the untreated controls. Real-time RT-PCR analysis of Hprt and Gapdh was performed in the analysis of each target gene.

*, **: Significantly different from the untreated controls at $P<0.05$ and $P<0.01$, respectively (Dunnett's multiple comparison test).

tion in response to 12 ppm PTU was approximately 2-fold higher than that with 3 ppm PTU. The number of genes showing up- or down-regulation in response to 200 ppm MMI was in between that elicited by 3 or 12 ppm PTU. One-hundred nineteen genes were up-regulated in common by MMI and PTU, with PTU showing up-regulation from 3 ppm. On the other hand, 97 genes showed down-regulation in all MMI and PTU groups. Representative genes showing up- or down-regulation in all three groups are shown in the Table 1. Among the genes listed, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were found to be up-regulated and those for myelination were often down-regulated.

Real-time RT-PCR analysis: For confirmation of the microarray data, four genes that were up-regulated and three that were down-regulated in response to anti-thyroid agents were selected for mRNA expression analysis by real-time RT-PCR and the results are summarized in Table 2.

In all exposure groups, many of the expression changes were similar in the two analysis systems, except for much higher expression of *Plagl1* in all exposure groups by real-time RT-PCR as compared with findings from the microarray system.

Although we performed expression analysis of *Efna5* by real-time RT-PCR, expression values were rather low with great variability between samples, and therefore, reliable quantitative data could not be obtained (data not shown).

Immunolocalization of EphA5 and Tacr3 in the hippoc-

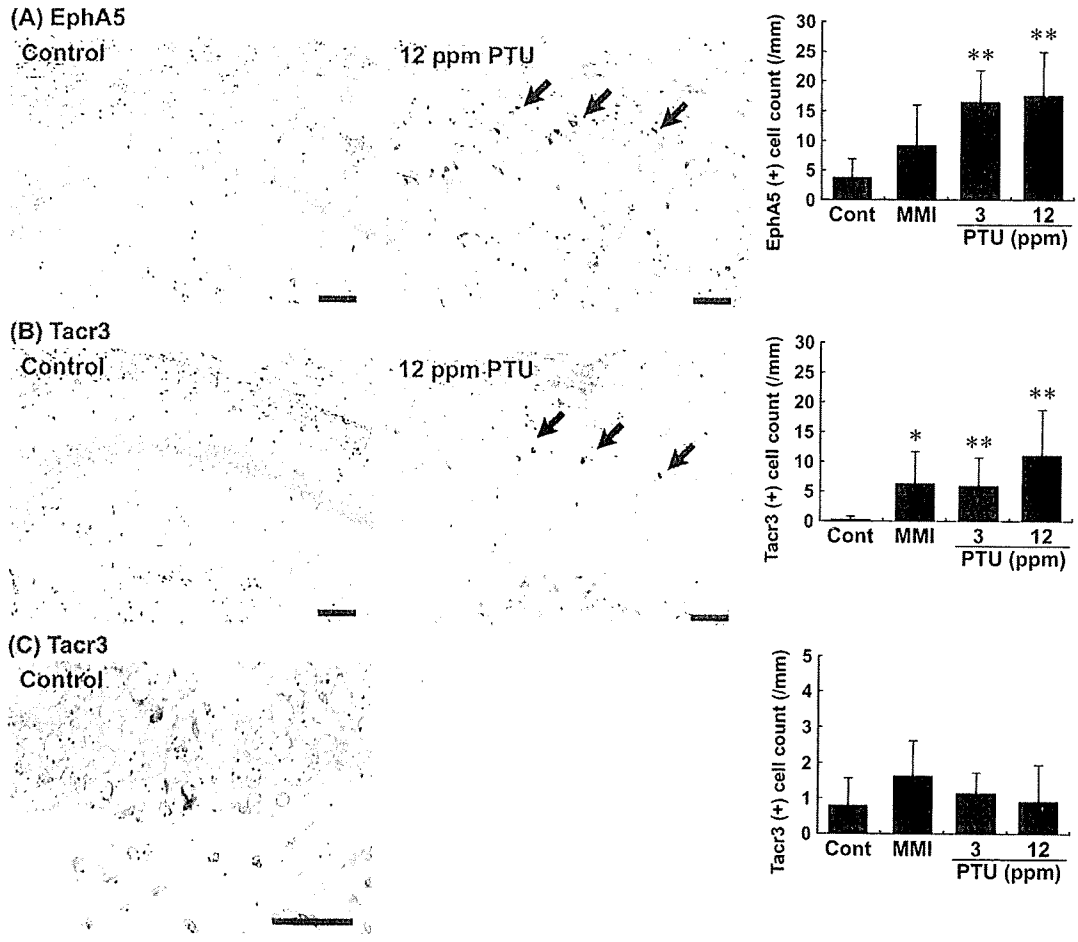

Fig. 3. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation in rats at PND 20 after maternal exposure to anti-thyroid agents. (A) EphA5-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of EphA5-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar =100 μm. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. ** $P<0.01$ versus untreated controls (Student's *t*-test). (B) Tacr3-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of Tacr3-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar =100 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P<0.05$, ** $P<0.01$ versus untreated controls (Student's *t*-test). (C) Tacr3-immunoreactive cells located in the subgranular zone of the dentate gyrus. Bar =50 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the subgranular zone of bilateral hemispheres. Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil, Tacr3, Tachykinin receptor 3.

ampal formation: Immunohistochemical localization of EphA5 and Tacr3 in the hippocampal formation was examined at PND 20 and PNW 11.

On PND 20, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. On the other hand, very sparse distribution of strongly immunoreactive cells for EphA5 was observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control animals, but immunoreactive cells were significantly increased showing scattered distribution by PTU at both 3 and 12 ppm (Fig. 3A). MMI-exposed animals also showed a small increase in the number of strongly positive cells with EphA5. Increased intensity in immunoreactivity of EphA5 was also observed in the gray matter consisting of neuropil at the stratum oriens of the CA1 region (Fig. 3A), and also in the molecular layer of the dentate gyrus at PND 20 after exposure to anti-thyroid agents, especially in PTU-exposed groups (data not shown).

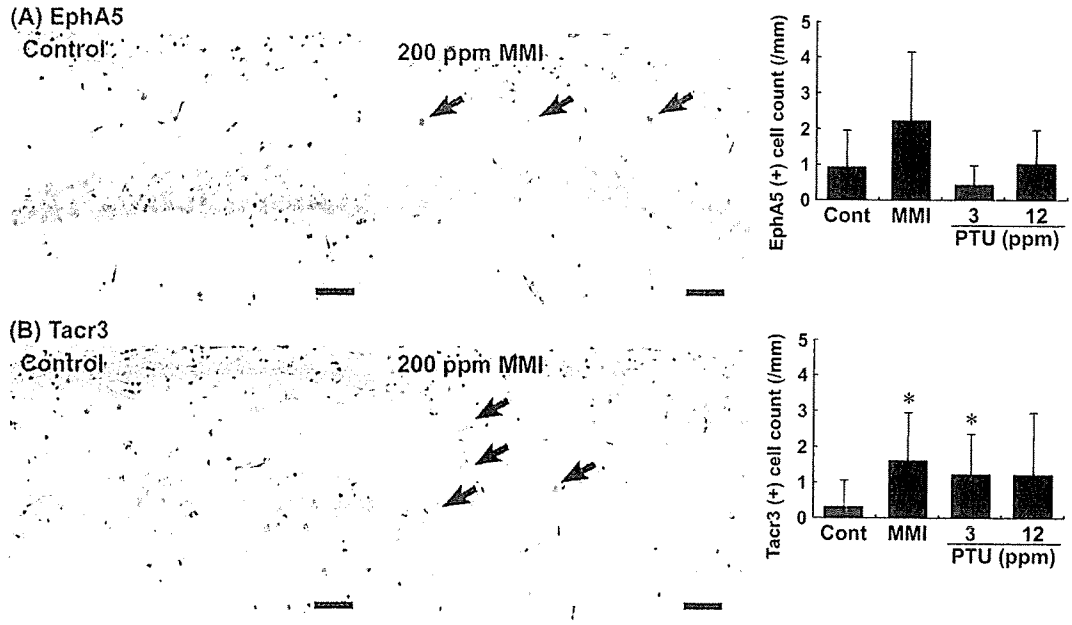

Fig. 4. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation at PNW 11 of rats exposed maternally to anti-thyroid agents. (A) EphA5-immunoreactive cells with moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region. EphA5-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). The arrows show positive cells. Bar =50 μm. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. (B) Tacr3-immunoreactive cells with weak to moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Immunoreactivity is rather faint as compared with that observed at PND 20. Note the higher number of Tacr3-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). Bar =50 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P<0.05$ versus untreated controls (Student's t-test). Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil; Tacr3, Tachykinin receptor 3.

With regards to Tacr3, the number of positive cells was increased with a scattered distribution showing strong intensity in the CA1 region similarly to that of EphA5 in the animals exposed to MMI or PTU on PND 20, but they were mostly absent in the untreated controls (Fig. 3B). Similarly, Tacr3-immunoreactive cells were sparse in the subgranular zone of the dentate gyrus in the MMI and PTU-exposed animals and in the untreated controls, but there were no differences in the number of positive cells as compared with the untreated controls (Fig. 3C). In addition, increased intensity in neuropil-immunoreactivity of Tacr3 was also observed in the strata oriens and radiatum of the CA1 region in all exposure groups of anti-thyroid agents (Fig. 3B).

On PNW 11, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. EphA5-immunoreactive cells with moderate staining intensity were very sparsely observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control animals. There was no statistically significant increase in the number of these immunoreactive cells after exposure to PTU, while animals exposed to MMI showed a tendency for an increased number of immunoreactive cells (Fig. 4A). Increased neuropil-immunoreactivity of EphA5 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).

As well as at PND 20, Tacr3-immunoreactive cells were mostly absent in the untreated controls at PNW 11; however, a few immunoreactive cells with weak to moderate intensity were observed in the stratum oriens of the CA1 region in the animals exposed to anti-thyroid agents. There was a statistically significant difference in the animals treated with MMI or 3 ppm PTU compared with the untreated controls (Fig. 4B). Although the change was non-significant and lacked dose-dependence, 12 ppm PTU also showed an increasing tendency in the number of Tacr3-immunoreactive cells. In addition, increased neuropil-immunoreactivity of Tacr3 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).

DISCUSSION

In our recent study using rats [24], after maternal exposure to MMI or PTU, we detected typical hypothyroidism-related changes in the thyroid-related hormone levels, and hippocampal CA1 pyramidal neurons due to neuronal mismigration, as previously reported [8]. We also observed white matter changes, which seem to be due to impaired oligodendroglial development [6, 21]. To visualize molecules related to impaired neuronal development, microdissected CA1 region-specific global gene expression profiling was performed in the present study using the same animals that were used in our previous study. Two recently published studies have used microarrays to examine the expression profiles in the cerebral cortex and hippocampus of genes linked to developmental hypothyroidism caused by maternal PTU-exposure [7, 19]. In accordance with these studies, the genes that were significantly down-regulated in the present study included those that play roles in myelination, such as *Mobp* and myelin-associated glycoprotein, suggestive of the reflection of suppressed myelination by developmental hypothyroidism [21]. However, the genes that were found to be up-regulated on microdissected CA1 pyramidal cell layer, including *Efna5* and *Tacr3*, in the present study, have not been identified in previous studies. This difference may be related to the target tissues collected and the methods used, including microdissection of CA1 pyramidal cell layer from paraffin-embedded sections in the present study versus manual dissection of the cortical tissues from unfixed tissues in the previous studies.

EphA5 is a tyrosine kinase receptor that is almost exclusively expressed in the nervous system [15]. EphA5 and its ligand are important in mediating axon guidance, topographic projection, development, cell migration and the plasticity of limbic structures [15]. In addition, the transient expression of EphA5 during development is correlated with early neurogenesis and the migration of differentiated cells in the midbrain [3]. Thus, although expression of EphA5 was mostly weak in the euthyroid CA1 pyramidal neurons at PND 20, the increased number of EphA5-expressing cells with strong intensity in the CA1 region during developmental hypothyroidism in the present study reflects the neuronal mismigration caused by anti-thyroid agents. However, this increase was recovered after cessation of developmental hypothyroidism. Ephrins and their receptors are recently identified molecules and functional relationship between subfamily proteins is largely unknown; however, we, in the present study, found down-regulation of Epha7, another subfamily ephrin receptor, in all exposure groups of anti-thyroid agents (Table 1).

Tacr3, a member of the mammalian tachykinin peptide neurotransmitter/neuromodulator receptor family, is predominantly expressed in neurons in both the peripheral and central nervous systems, including the hippocampus [25]. There is increasing evidence of the role of Tacr3 on the survival and function of dopaminergic neurons. The survival of mesencephalic dopaminergic neurons during development largely depends on excitatory inputs, and tachykinins, through their receptors, are reported to play role in excitation [20]. On the other hand, senktide, a Tacr3 agonist, activates dopaminergic neurons to stimulate the release of dopamine and serotonin, and hyperlocomotion in gerbils [14]. Abnormal excitatory action of D_2-like receptor, one of the major subtypes of dopaminergic receptors, was observed on glutamatergic transmission in the CA1 synapses in the adult stage of rats after developmental hypothyroidism, suggesting a permanent disruption of synaptic integration in the CA1 neural networks [16]. While the role of Tacr3 in the hippocampal CA1 region during development is not clear, the increase in Tacr3-positive cells with strong intensity in this region during developmental hypothyroidism suggests a cell survival effect of tachykinin-3. Although the magnitude of the change was decreased, as compared with that at the end of the developmental hypothyroidism, the increased number of Tacr3-positive cells in the CA1 region of MMI and 3 ppm PTU-exposed animals may be an outcome of permanent disruption of synaptic integration, as described by Oh-Nishi *et al.* [16]. However, sparse distribution of Tacr3-positive cells may reflect that impairment sustained in a small population of aberrantly migrated neurons.

In conclusion, in this study, we have shown gene expression profiles showing altered expression in response to developmental hypothyroidism by analysis on microdissected hippocampal CA1 pyramidal cell layer in rats. Immunohistochemical analysis of the two candidate molecules revealed that developmental hypothyroidism until weaning is associated with the persistence of Tacr3-expressing neurons until the adult stage in the CA1 region, suggestive of the reflection of permanent disruption of synaptic integration. These findings probably reflect a mechanism to facilitate cell survival of aberrantly developed neurons due to mismigration.

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Sustained production of Reelin-expressing interneurons in the hippocampal dentate hilus after developmental exposure to anti-thyroid agents in rats

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ABSTRACT

To detect molecular evidence reflecting a permanent disruption of neuronal development due to hypothyroidism, distribution of Reelin-producing cells that function in neuronal migration and positioning was analyzed in the hippocampal dentate hilus using rats. From gestation day 10, maternal rats were administered either 6-propyl-2-thiouracil (PTU) at 3 or 12 ppm (0.57 or 1.97 mg/kg body weight/day) or methimazole (MMI) at 200 ppm (27.2 mg/kg body weight/day) in the drinking water and male offspring were immunohistochemically examined at the end of exposure on weaning (postnatal day 20) and at the adult stage (11-week-old). Offspring with MMI and 12 ppm PTU displayed evidence of growth retardation lasting into the adult stage. On the other hand, all exposure groups showed a sustained increase in Reelin-expressing cells in the dentate hilus until the adult stage in parallel with Calbindin-D-28K-expressing cells at weaning and with glutamic acid decarboxylase 67-positive cells in the adult stage, confirming an increase in γ-aminobutyric acid (GABA)ergic interneurons. At the adult stage, NeuN-positive postmitotic mature neurons were also increased in the hilus in all exposure groups, however, the increased population of Reelin-producing cells at this stage was either weakly positive or negative for NeuN, indicative of immature neurons. At weaning, neuroblast-producing subgranular zone of the dentate gyrus showed increased apoptosis and decreased cell proliferation suggestive of impaired neurogenesis. The results suggest that sustained increases of immature GABAergic interneurons synthesizing Reelin in the hilus could be a signature of compensatory regulation for impaired neurogenesis and mismigration during the neuronal development as a hypothyroidism-related brain effect rather than that secondary to systemic growth retardation.

1. Introduction

Thyroid hormones are essential for normal fetal and neonatal brain development. They control neuronal and glial proliferation in definitive brain regions and regulate neural migration and differentiation [1–3]. In humans, maternal hypothyroxinemia, early in pregnancy, may have adverse effects on fetal brain development and importantly, even mild–moderate hypothyroxinemia may result in suboptimal neurodevelopment [4]. These results may increase the concern of impaired brain development by exposure to thyroid hormone-disrupting chemicals in the environment. Particularly, groups of persistent organic pollutants, such as organochlorine pesticides and polychlorinated biphenyls, have been shown to be ubiquitous environmental pollutants because of their great chemical stability and lipid solubility [5]. In addition to the variety of effects including immunologic, teratogenic, reproductive, carcinogenic, and neurological effects [6], many of these compounds are known to induce hypothyroidism [7].

Experimentally, developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning actions [8,9]. Rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) show impaired brain development, with impaired neuronal migration and white matter hypoplasia involving limited axonal myelination and oligodendrocytic

Abbreviations: CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; Calb-D-28K, Calbindin-D-28K; DH, dentate hilus; GABA, γ-aminobutyric acid; GAD67, glutamic acid decarboxylase 67; GD, gestation day; MMI, methimazole; NeuN, neuron-specific nuclear protein; PCNA, proliferating cell nuclear antigen; PND, postnatal day; PNW, postnatal week; PTU, 6-propyl-2-thiouracil; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone.

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Table 1
Serum levels of thyroid-related hormones of the male offspring exposed to anti-thyroid agents during the period from the mid-gestation and lactation periods.

	Untreated controls	Anti-thyroid agent in the drinking water		
		200 ppm MMI	3 ppm PTU	12 ppm PTU
PND 20				
No. of offspring examined	10	9	10	9[a]
T_3 (ng/ml)	1.22 ± 0.10[b]	0.43 ± 0.19**	0.97 ± 0.31*	0.25 ± 0.03**
T_4 (μg/ml)	4.72 ± 0.84	1.06 ± 0.44**	1.86 ± 0.41**	1.06 ± 0.32**
TSH (ng/ml)	6.80 ± 2.11	35.33 ± 12.69**	27.38 ± 13.66**	27.69 ± 5.74*
PNW 11				
No. of offspring examined	10	10	10	6
T_3 (ng/ml)	1.02 ± 0.08	0.88 ± 0.09**	0.93 ± 0.11	0.84 ± 0.10**
T_4 (μg/ml)	5.11 ± 0.70	4.57 ± 1.04	5.12 ± 0.73	4.05 ± 0.71
TSH (ng/ml)	9.81 ± 3.16	9.41 ± 4.40	9.10 ± 3.25	7.75 ± 2.23

[a] $N = 7$ for measurement of T_3 and T_4 levels.
[b] Mean ± SD.
* Significantly different from the untreated controls ($^*P < 0.05$).
** Significantly different from the untreated controls ($^{**}P < 0.01$).

accumulation [2,10,11]. The outcome of this type of impaired brain development is permanent and is accompanied by apparent structural and functional abnormalities. However, it is still unclear whether the molecular aberrations remain in the retarded brain after maturation.

In the hippocampal formation, neuronal subpopulations are known to produce Reelin from embryonic period throughout adult life [12–16]. Reelin is a secreted extracellular matrix glycoprotein that plays a critical role in neuronal migration and positioning during brain development in the process regulated by thyroid hormone [13,17]. Also, in adults, it is suggested that Reelin released by γ-aminobutyric acid (GABA)ergic interneurons could regulate the migration and maturation of newborn granular cells in the dentate granular cell layer [18]. Altered Reelin signaling has been reported in the dentate gyrus of some neurological disease conditions, such as depression and epilepsy [18,19]. Within hippocampal formation, dentate gyrus is the unique structure that can continue neurogenesis during postnatal life and is a well-known target of developmental hypothyroidism [20].

In the present study, to detect a key molecular event reflecting permanent disruption of neuronal development due to exposure to xenobiotic chemicals that can interfere with thyroid hormone signaling, we examined temporal distribution change of Reelin-expressing cells in the dentate gyrus of rat offspring after developmental exposure to anti-thyroid agents. To distinguish chemical-specific expression changes from hypothyroidism-linked ones, two different anti-thyroid agents, methimazole (MMI) and PTU, were used, and dose-related responses were also examined with PTU.

Because of the similarities in the DNA binding domain of estrogen response element and thyroid hormone response element, crosstalk between the estrogen receptors and thyroid hormone receptors has been reported in previous studies [21,22]. Therefore, male offspring were selected for immunohistochemical analysis as well as measurement of serum thyroid-related hormones to avoid possible influence of estrogen in the present study.

2. Materials and methods

2.1. Chemicals and animals

Methimazole (2-mercapto-1-methylimidazole: MMI; CAS No. 60-56-0) and 6-propyl-2-thiouracil (PTU; CAS No. 51-52-5) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pregnant Crj:CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestation day (GD) 3 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature: 24 ± 1 °C; relative humidity: 55 ± 5%) with a 12-h light/dark cycle and allowed *ad libitum* access to food and tap water. A soy-free diet (Oriental Yeast Co. Ltd., Tokyo, Japan) was chosen as the basal diet for the maternal animals to eliminate possible phytoestrogen effects [23], and water was given *ad libitum* throughout the experimental period including the 1-week acclimation period. On the other hand, all offspring consumed a regular CRF-1 basal diet (Oriental Yeast Co. Ltd.) and water *ad libitum* from PND 20 onwards (PND 0: the day of delivery). Although the formula is not open, CRF-1 contains soybean/alfalfa-derived proteins and oil including daidzin and genistin at concentrations of 87 and 102 ppm in diet according to the supplier's analysis, and coumestrol of less than 3 ppm based on the content of lucerne meal in the diet (supplier's comment). Soy-free diet was prepared based on the formulation of the NIH-07 open formula rodent diet, in which soybean meal and soy oil were replaced with ground corn, ground wheat, wheat middlings and corn oil. Values for phytoestrogens in this diet were below the detection limit (0.5 ppm), except for coumestrol with 3 ppm. Estrogen equivalents of phytoestrogens included in each CRF-1 and soy-free diet were roughly calculated as 0.91 and 0.06 ppm of β-estradiol, respectively, based on the relative binding affinities in a rat endometrial-derived experimental model [24]. Nutritional standards did not differ between soy-free diet and CRF-1 (supplier's analysis).

2.2. Experimental design

The animal experiments were identical to those in a previous study [25]. In brief, maternal animals were randomly divided into four groups including untreated controls. Eight dams per group were treated with 200 ppm of MMI or 3 ppm or 12 ppm of PTU in the drinking water from GD 10 to PND 20. Dose finding study on PTU and MMI was preliminarily performed based on the dose range to show changes in neuronal or oligodendroglial parameters in previous reports [2,26–28]. With the dose setting at the level of 9 ppm or 12 ppm for PTU and 200 ppm or 250 ppm for MMI in the drinking water, dams ($n = 2$/dose) were treated from GD 10 to PND 20, apart from the untreated control dams ($n = 2$). As a result, PTU at 12 ppm and MMI at 200 ppm exhibited clear hypothyroidism-linked effects to dams, i.e., increased relative thyroid weights and thyroid follicular cell hypertrophy, but did not affect pregnancy, implantation, delivery, or nursing until PND 20 (data not shown).

On PND 2, the litters were culled randomly, leaving four male and four female offspring. On PND 20, 20 male and 20 female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy [25,29].

The remaining animals were maintained until postnatal week (PNW) 11. All offspring consumed the CRF-1 basal diet and tap water *ad libitum* from PND 20 onwards. At PNW 11, all pups were subjected to adult stage necropsy [25,29].

All animals used in the present study were weighed and sacrificed by exsanguination from the abdominal aorta under deep anesthesia with ether. These protocols were reviewed in terms of animal welfare and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.3. Thyroid-related hormone measurement

At the necropsies of animals sacrificed on PND 20 and PNW 11, blood samples of male offspring were collected from the abdominal aorta under anesthesia. Serum was prepared and stored at −30 °C to measure thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) concentrations at SRL, Inc. (Tokyo, Japan). Number of animals examined was described in Table 1.

2.4. Immunohistochemistry and Cresyl Violet staining

To evaluate the immunohistochemical distribution of the molecules, brains in the subgroups of male offspring killed at PND 20 and PNW 11 were fixed in Bouin's solution at room temperature overnight. Six animals were used as untreated controls, six for 200 ppm MMI, eight for 3 ppm PTU, and nine for 12 ppm PTU on PND 20.

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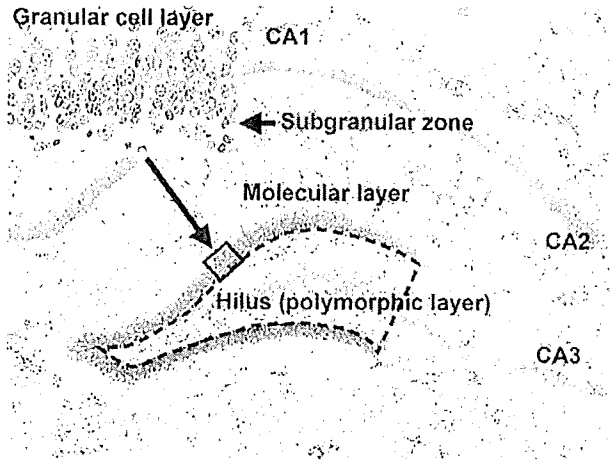

Fig. 1. Overview of the hippocampal formation of a male rat at postnatal day 20 stained with hematoxylin and eosin. Inset shows higher magnification of the granular cell layer and subgranular zone. Magnification, 40× (Inset: 200×). Number of immunoreactive cells for Reelin, Calb-D-28K, and GAD67 in the hilus of the dentate gyrus, as enclosed by the dotted line, was counted and normalized for the unit area. Small-sized neurons in this area showed positive immunoreactivity with these antigens, but large-sized CA3 neurons distributed in this area were not immunoreactive with these antigens. Number of NeuN-immunoreactive cells was similarly counted and normalized for the unit area, but CA3 neurons distributed in this area and also positive for NeuN were excluded from counting.

On PNW 11, 10 animals were used as untreated controls and 10 for 200 ppm MMI, nine for 3 ppm PTU, and six for 12 ppm PTU. Coronal slices at the positions of −3.0 and −3.5 mm from the bregma were prepared from brains of PND 20 and PNW 11, respectively.

Immunohistochemistry was performed on the brain sections (3 μm in thickness) of PND 20 and PNW 11 animals with antibodies against Reelin (clone G10, mouse $IgG_{1\kappa}$, 1:1000; Novus Biologicals, Inc., Littleton, CO, USA) and Calbindin-D-28K (Calb-D-28K; clone CB-955, mouse IgG_1, 1:500; Sigma Chemical Co.), which were incubated with the tissue sections overnight at 4 °C. On the brain sections at PNW 11, immunohistochemistry of neuron-specific nuclear protein (NeuN; clone A60, mouse IgG_1, 1:1000, Chemicon, Billerica, MA, USA), which specifically detects postmitotic neurons, was also performed. In addition, immunohistochemistry of glutamic acid decarboxylase 67 (GAD67; clone 1G10.2, mouse IgG, 1:50, Chemicon) and proliferating cell nuclear antigen (PCNA; clone PC10, mouse IgG_{2a}, 1:200, Dako, Glostrup, Denmark) was performed on PND 20 and PNW 11 ($n=5$ in each group) in untreated controls and rats treated with 12 ppm PTU. Antigen retrieval treatment was not performed for these antigens. Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen, as previously described [30]. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

For double staining of NeuN and Reelin, 3,3′,5,5′-tetramethylbenzidine (Vector Laboratories) was used to visualize Reelin and DAB was used to visualize NeuN.

For evaluation of apoptosis in the subgranular zone of the dentate gyrus, apoptotic bodies were detected by Cresyl Violet staining as described by others [31].

2.5. Morphometry of immunolocalized cells and apoptotic cells

Reelin-, NeuN-, Calb-D-28K- or GAD67-positive cells distributed in the hilus of the dentate gyrus were bilaterally counted and normalized for the number per unit area of the hilar area (polymorphic layer) as enclosed by the dotted line in Fig. 1. In the subgranular zone of the dentate gyrus (Inset of Fig. 1), apoptotic bodies as detected by Cresyl Violet staining and proliferating cells as detected by nuclear immunoreactivity of PCNA were bilaterally counted and normalized the number with the length of the granular cell layer measured. For quantitative measurement of each immunoreactive cellular component, digital photomicrographs at 100-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co.), and quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

2.6. Statistical analysis

Numerical data of the thyroid-related hormone levels and the number of immunoreactive cells were assessed using Student's *t*-test to compare the untreated controls with each of the anti-thyroid agent-exposed groups when the variance was homogenous among the groups using a test for equal variance. If a significant difference in variance was observed, Aspin–Welch's *t*-test was used instead.

3. Results

3.1. Effects on dams

During the gestation period, slight but statistically significant decrease of water consumption during GD 10–GD 15 and food intake during GD 15–GD 20 were observed with 200 ppm MMI compared with the untreated dams [25]. During the lactation period, both water consumption and food intake of dams decreased with 12 ppm PTU and MMI with statistical significance. However, treatment did not affect the body weight gain during the exposure period and the body weight of dams at weaning [25]. With regard to the maternal clinical signs, all dams in the groups of 12 ppm PTU and MMI exhibited somewhat higher sensitivity against handling stimuli as compared with untreated controls and 3 ppm PTU after delivery. However, no dams abandoned rearing offspring.

By monitoring water consumption, chemical intake of dams treated with 3 ppm PTU was calculated to be 0.57 mg/kg body weight/day during the whole exposure period (0.39 mg/kg body weight/day during GD 10–GD 20 and 0.67 mg/kg body weight/day during PND 1–PND 20) [25]. In case of dams treated with 12 ppm PTU, intake value was 1.97 mg/kg body weight/day during the whole exposure period (1.54 mg/kg body weight/day during GD 10–GD 20 and 2.20 mg/kg body weight/day during PND 1–PND 20). In case of dams treated with 200 ppm MMI, intake value was 27.2 mg/kg body weight/day during the whole exposure period (19.7 mg/kg body weight/day during GD 10–GD 20 and 31.2 mg/kg body weight/day during PND 1–PND 20).

3.2. Effects on offspring growth and survival

With regard to the reproductive parameters, no significant alterations in the number of implantation sites, number of live offspring, and sex ratio were observed by the exposure to anti-thyroid agents. At PND 1, a slight and non-significant decrease of the body weight was observed in all exposure groups of both sexes [25]. All animals survived during the lactation period. At PND 20, a decrease of body weight was observed after exposure to anti-thyroid agents in both sexes, which was statistically significant in the males of the 12 ppm PTU and MMI groups and in females of all exposure groups. After weaning, four out of ten males and six out of ten females receiving 12 ppm PTU were found dead or subjected to moribund sacrifice. During observation, many of these animals were hyperactive and aggressive in nature and sometimes raced around to bump into a cage wall. During necropsy, most of these animals showed evidence of acute hemorrhage of the brain surface.

At the necropsy of 11-week rats, only six males and four females remained in the 12 ppm PTU group, whereas 10 animals/sex remained in other groups. Offspring of dams receiving 12 ppm PTU and MMI showed a statistically significant decrease in body weight in both sexes [25].

3.3. Serum levels of thyroid-related hormones

At PND 20, decreases of serum levels of T_3 and T_4 were evident in animals that were administered anti-thyroid agents with statistical significance in all groups of animals exposed to anti-thyroid agents for both T_3 and T_4 (Table 1). Reductions of T_3 and T_4 with PTU occurred in a dose-dependent fashion. Significantly elevated TSH levels were observed with MMI and PTU at both doses. At PNW 11, a slight but statistically significant decrease of T_3 levels was observed with MMI and 12 ppm PTU groups.

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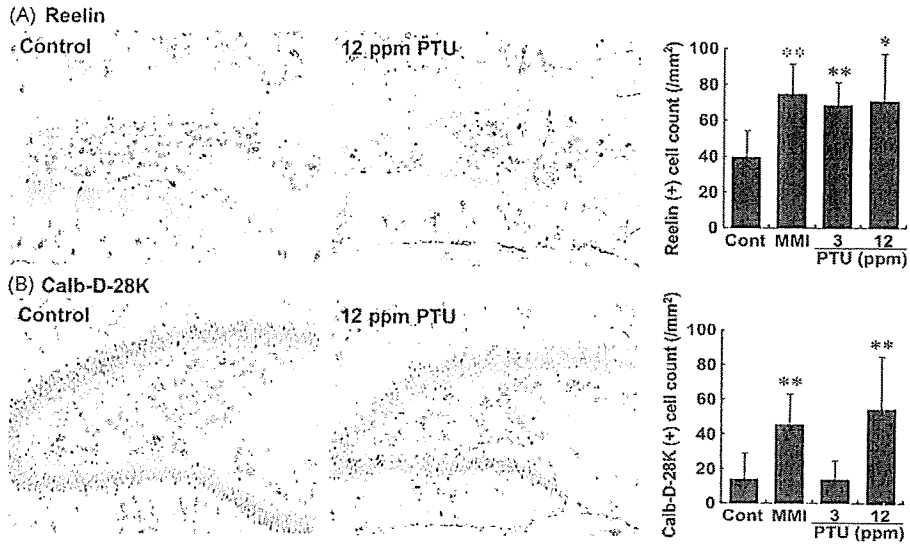

Fig. 2. Distribution of immunoreactive cells for Reelin and Calb-D-28K in the hippocampal formation in male rats at PND 20 after maternal exposure to anti-thyroid agents. (A) Reelin-immunoreactive cells in the hilus of the dentate gyrus. Reelin-positive cells with abundant cytoplasm show scattered distribution within the hilar region of the dentate gyrus. Note the higher number of Reelin-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Magnification, 100×. The graph shows the number of Reelin-positive cells/unit area (mm^2) of the hilus of the bilateral hemispheres. $^*P<0.05$, $^{**}P<0.01$ *versus* untreated controls. (B) Calb-D-28K-immunoreactive cells in the hilus of the dentate gyrus. Calb-D-28K-positive cells are mainly distributed in the area medial to the subgranular zone. Note the higher number of Calb-D-28K-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Magnification, 100×. The graph shows the number of Calb-D-28K-positive cells/unit area (mm^2) of the hilus of the bilateral hemispheres. $^{**}P<0.01$ *versus* untreated controls.

3.4. Immunolocalization of Reelin and Calb-D-28K in the hippocampal formation at PND 20

The distribution of Reelin-immunoreactive cells in the hippocampal formation that included the CA1–3 regions was similar to that described in the literature [14]. In the dentate gyrus, Reelin was expressed predominantly in the interneurons located in the hilus (polymorphic layer), whereas Reelin-containing neurons were sparse in the molecular layer (Fig. 2A). Morphometrically, the number of Reelin-positive cells in the dentate hilus was normalized in terms of unit area, and all of the animals exposed to MMI or PTU showed an increased number of Reelin-positive cells with a rather diffuse distribution within the hilus.

With regard to Calb-D-28K, the CA1 pyramidal neurons expressed this molecule with intense immunoreactivity in the inner pyramidal cells. Within the dentate gyrus, neurons in the granular cell layer showed strong immunoreactivity, while cells in the subgranular zone showed no expression (Fig. 2B). In addition to Reelin, Calb-D-28K-immunoreactive cells were frequently observed in the dentate hilus at the position medial to the subgranular zone. As compared with the untreated controls, animals exposed to MMI or 12 ppm PTU exhibited an increased number of Calb-D-28K-positive cells in the dentate hilus, while 3 ppm PTU did not show any change.

3.5. Immunolocalization of Reelin and Calb-D-28K in the hippocampal formation at PNW 11

On PNW 11, Reelin-immunoreactive cells showed similar distributions to those at PND 20 within the hippocampal formation, although the total number was reduced in the CA1–3 regions, by contrast to the comparable numbers in the dentate gyrus at PND 20. In the hilus of the dentate gyrus, the immunoreactive cells were increased in the MMI- and both doses of PTU-exposed animals (Fig. 3).

There were no cells immunoreactive for Calb-D-28K in the hippocampal formation in both untreated controls and MMI or PTU-exposed animals.

3.6. Characterization of the neuronal cell population in the dentate hilus

Within the hilus of the dentate gyrus, the number of NeuN-positive cells was apparently increased in the MMI- and both doses of PTU-exposed animals at PNW 11 as indicated by solid line in Fig. 4A.

The number of GAD67-positive cells in the animals treated with 12 ppm PTU tended to increase at PNW 11 compared with untreated controls, but was unchanged at PND 20 (Fig. 4B, C).

Evaluation of the co-localization of Reelin and NeuN in the dentate hilus at PNW 11 in untreated controls and 12 ppm PTU-exposed animals revealed that more than half of the Reelin-positive cells were weakly positive (±) or negative (−) for NeuN in the untreated controls, and 12 ppm PTU apparently increased this cell population (Fig. 4D).

3.7. Apoptotic and proliferating cell indices in the dentate subgranular zone

Apoptotic bodies were not found in the subgranular zone of the untreated controls at PND 20 (Fig. 5A). Also, no apoptotic bodies were detected after exposure to 3 ppm PTU at this time point. Although the number was very few, apoptotic bodies were increased after exposure to 12 ppm PTU. MMI-exposed animals also showed an increasing tendency in the number of apoptotic bodies. At PNW 11, no apoptotic bodies were detected in all cases including untreated controls, except for one apoptotic body detected in one case out of six untreated control animals.

With regard to the PCNA-immunoreactivity in the subgranular zone in untreated control animals, positive nuclei were sparsely observed at PND 20, and the number was decreased at PNW 11 (Fig. 5B). When the number of PCNA-immunoreactive nuclei was compared between the untreated controls and 12 ppm PTU, the latter decreased the number at PND 20. On the other hand, at PNW 11, no statistical difference was observed in the number of immunoreactive cells between the two groups.

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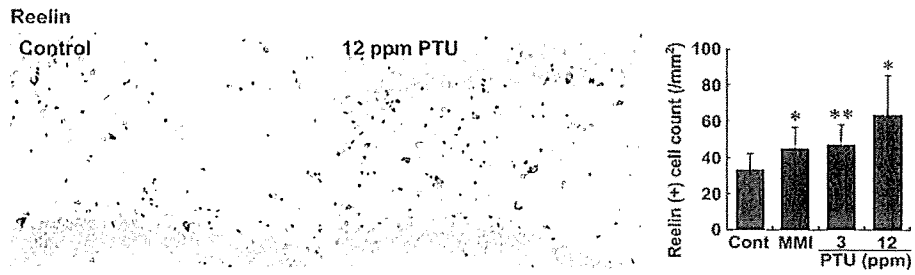

Fig. 3. Distribution of immunoreactive cells for Reelin in the hilus of the hippocampal dentate gyrus at PNW 11 of male rats exposed maternally to anti-thyroid agents. Similar to PND 20, the Reelin-positive cells with abundant cytoplasm show a scattered distribution within the hilar region of the dentate gyrus. Note the higher number of Reelin-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Magnification, 100×. The graph shows the number of Reelin-positive cells/unit area (mm²) of the hilus of the bilateral hemispheres. $^{*}P<0.05$, $^{**}P<0.01$ *versus* untreated controls.

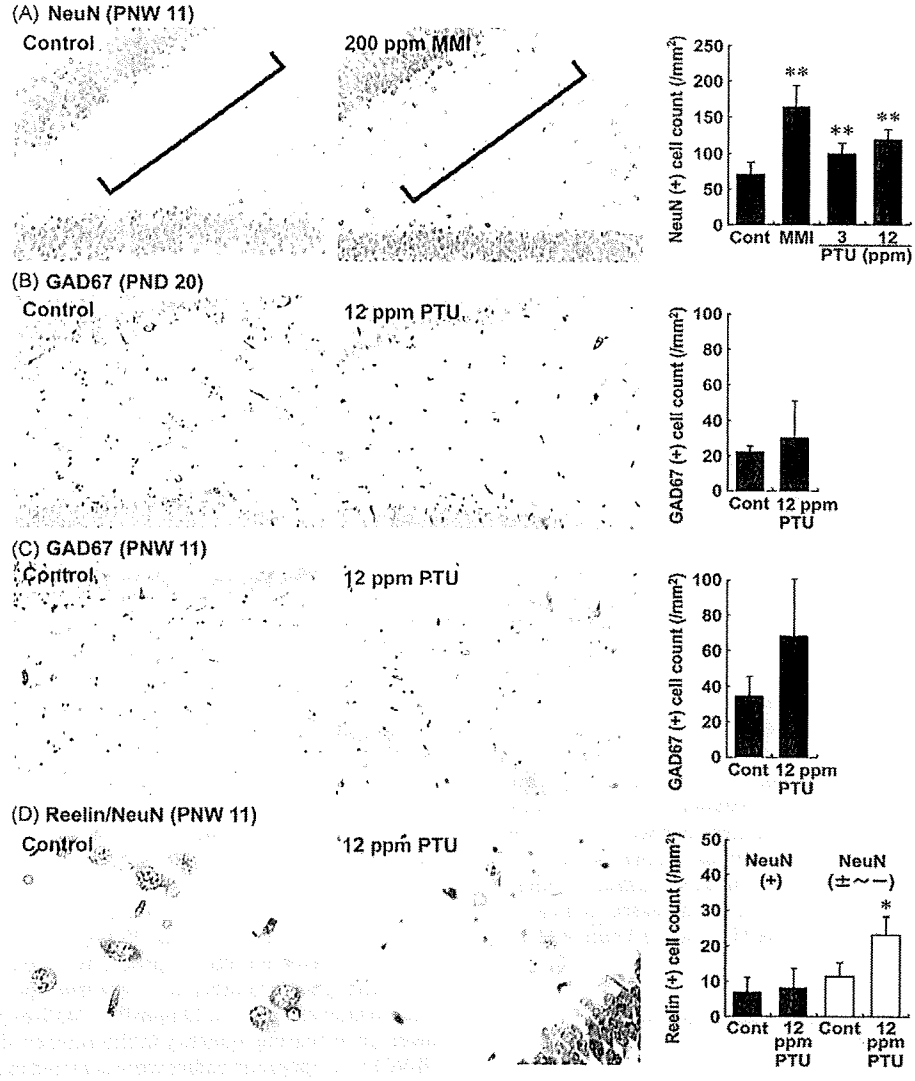

Fig. 4. Distribution of immunoreactive cells for NeuN and GAD67, and co-localization of Reelin and NeuN in the dentate hilus of male rats exposed maternally to anti-thyroid agents. (A) NeuN-immunoreactive cells at PNW 11. NeuN-positive cells are mainly distributed in the hilar area medial to the subgranular zone. Note the higher number of NeuN-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). Magnification, 200×. The graph shows the number of NeuN-positive cells/unit area (mm²) of the hilus of the bilateral hemispheres. $^{**}P<0.01$ *versus* untreated controls. (B) GAD67-immunoreactive cells at PND 20. GAD67-positive cells with abundant cytoplasm show a scattered distribution within the hilar region. Magnification, 200×. Note there is no change in the number of GAD67-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). The graph shows the number of GAD67-positive cells/unit area (mm²) of the hilus of bilateral hemispheres. (C) GAD67-immunoreactive cells at PNW 11. Similar to the PND 20 cases, the GAD67-positive cells with abundant cytoplasm show a scattered distribution within the hilar region. Magnification, 200×. Note the higher number of GAD67-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). The graph shows the number of GAD67-positive cells/unit area (mm²) of the hilus of the bilateral hemispheres. (D) Double staining of Reelin and NeuN at PNW 11. Magnification, 600× (Left); 400× (Right). Note the increased number of Reelin-positive cells showing weak or no immunoreactivity for NeuN in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). The graph shows the number of Reelin-positive cells with apparent (+) or weak to negative (±~−) NeuN-immunoreactivity/unit area (mm²) of the hilus of the bilateral hemispheres. $^{*}P<0.01$ *versus* untreated controls.

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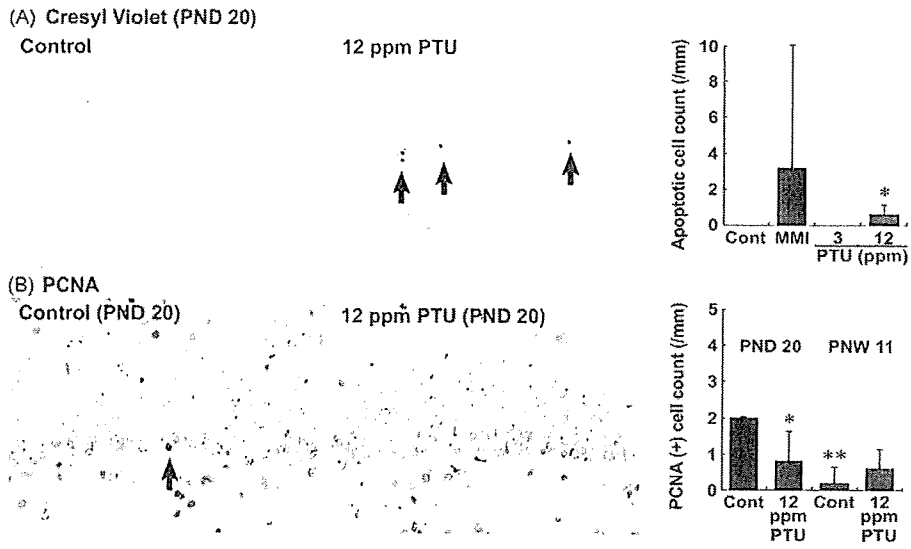

Fig. 5. Distribution of apoptotic cells and proliferating cells in the dentate subgranular zone of male rats exposed maternally to anti-thyroid agents. (A) Apoptotic cells detected as apoptotic bodies with Cresyl Violet staining at PND 20. Note sparse distribution of apoptotic bodies (arrows) in a case exposed to 12 ppm PTU (Right) as compared with the control animal without apoptotic bodies (Left). Magnification, 400×. The graph shows the number of apoptotic bodies/unit length (mm) of the subgranular zone of bilateral hemispheres at PND 20. $^{*}P<0.05$ *versus* untreated controls. (B) Proliferating cells as detected by PCNA-positive nuclei at PND 20. Note one PCNA-positive nucleus (arrow) in the micrographic view of the control animal (Left), while no positive nuclei were found in a case of 12 ppm PTU (Right). Magnification, 400×. The graph shows the number of PCNA-positive nuclei/unit length (mm) of the subgranular zone of bilateral hemispheres at both PND 20 and PNW 11. $^{*}P<0.05$, $^{**}P<0.01$ *versus* untreated controls at PND 20.

4. Discussion

In our recent study using animals of the identical experiment of the present study, we detected typical hypothyroidism-related changes, such as thyroid follicular cell hypertrophy accompanied with increased thyroid weight, and fluctuations in the thyroid-related hormone levels at the end of maternal exposure to MMI or PTU [25]. Morphometrical analysis at the adult stage also revealed hypothyroidism-related brain changes reflecting neuronal mismigration [10] and impaired oligodendroglial development [2], in both chemicals, with PTU showing dose-dependence. Offspring also displayed evidence of growth retardation lasting into the adult stage with MMI and PTU at 12 ppm. Dams in these groups exhibited reductions in food intake and water consumption during the lactation period suggestive of the relation to the growth suppression of offspring. On the other hand, offspring exposed to 3 ppm PTU also exhibited reduced body weights at weaning, with a statistically significant difference in females, without a concurrent reduction of food intake and water consumption of dams, suggesting that the reduced body weight was due to the development of hypothyroidism [32].

With regard to male offspring exposed to 3 ppm PTU, a clear increase in Reelin-expressing cells was evident at the end of developmental exposure with the magnitude similar to other treatment groups, while body weight reduction with 3 ppm PTU was weak and non-significant. Increase of Reelin-expressing cells continued until adult stage in this group, irrespective of the no reduction in the terminal body weight. These results may suggest that the increase in Reelin-expressing cells was the reflection of hypothyroidism-related brain effect rather than systemic growth retardation. We recently found that experimental undernutrition of offspring during GD 10–PND 21 utilizing a rat intrauterine growth restriction model did not change the number of Reelin-expressing cells until adult stage (Ohishi and Shibutani, unpublished data).

During early postnatal life, Reelin expression becomes established in a subpopulation of GABAergic interneurons in the dentate gyrus, with a high density in the hilus and along the base of the granule cell layer [16], where Reelin is maintained throughout adult life [14,15]. In the present study, we also found an increase in Calb-D-28K-immunoeactive cells, as with Reelin-positive cells, in the dentate hilus after exposure to anti-thyroid agents at PND 20. Calcium binding protein Calb-D-28K and parvalbumin are known to form distinct subpopulations of GABAergic interneurons in the rodent hippocampal formation [33]. During early postnatal development in human, subpopulations of Reelin-positive interneurons express Calb-D-28K in the dentate hilus [34]. These results suggest that the increased Reelin-expressing cells in the dentate hilus at the end of developmental hypothyroidism in the present study are GABAergic interneurons. However, the Calb-D-28K-expressing cells disappeared at the adult stage, regardless of the presence of Reelin-expressing cells in the present study. Because aged rats lack Calb-D-28K expression in the hippocampal interneurons [35], Calb-D-28K may play a role in functional maturation of these cells [36]. Interestingly, experimental induction of epileptic seizures in rats facilitates neurogenesis of cells expressing Calb-D-28K in the dentate hilus [37,38].

In the adult stage, we also found increased numbers of Reelin-positive cells and NeuN-positive cells in the dentate hilus after developmental exposure to anti-thyroid agents. In parallel with the increase in NeuN-positive cells, the increased GAD67-positive population was confirmed in the 12 ppm PTU-exposed animals. On the other hand, double staining of Reelin and NeuN revealed that developmental hypothyroidism resulted in an increase in Reelin-positive cells showing weakly positive or negative NeuN-immunoreactivity. Faint expression of mature neuronal markers, such as NeuN and microtubule-associated protein-2, was reported to reflect immature nature of neurons [39]. These results suggest that developmental hypothyroidism resulted in a sustained increase in GABAergic interneurons in the dentate hilus until the adult stage, a subpopulation of these cells produced Reelin with immature neuronal nature.

In line with its role in neuronal migration during development, Reelin was reported to be involved in the rostral migratory stream

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in adult rats [40]. Hippocampal heterotopias seen after exposure to methylazoxymethanol during uterine life [41] are not observed at birth but become progressively more evident between P5 and P21. Similarly, developmental hypothyroidism induced subcortical band heterotopia at weaning (PND 20), and this becomes more evident at the adult stage [25]. Because Reelin acts as a stop signal [42], postnatal overexpression of Reelin in the dentate hilus in the present study could be responsible for heterotopias by maintaining the migrating granular cells in an incorrect position.

On the other hand, the transient prenatal disturbance of neurogenesis by treatment with methylazoxymethanol induces a long-term increase in the number of neurons expressing Reelin in the hippocampus [43]. In the dentate gyrus, the neuronal stem/progenitor cells are located within the subgranular zone, and neurogenesis occurs constitutively throughout postnatal life in adult mammals [44]. During postnatal hypothyroidism, neural progenitor proliferation and differentiation have been shown to be impaired [3]. In the present study, we found a slight increase or increasing tendency in apoptotic cells as well as a slight suppression of cell proliferation activity in the dentate subgranular zone at PND 20 at the end of exposure to anti-thyroid agents, while these changes were not continued until adult stage. Similar supportive data have recently been reported showing gene expression changes suggestive of facilitation of apoptosis and suppression of cell proliferation in the hippocampal formation during the postnatal hypothyroidism and down-regulation of anti-apoptotic genes at the adult stage [20]. Thus, although the reason and consequence of overexpression of Reelin during adulthood is unclear, Reelin production can be sustained in later life in response to impaired neurogenesis as with mismigration in the dentate granular cells during development, and interneurons may play a role in sustained Reelin production. It is suggested that GABAergic inputs to dentate progenitor cells in adult stage promote activity-dependent neuronal differentiation [45].

5. Conclusions

In this study, we have shown persistent increases in GABAergic interneurons with Reelin production in an immature population until the adult stage in the dentate hilus after developmental hypothyroidism. These findings probably reflect a compensatory regulation for impaired neurogenesis and mismigration. There are many xenobiotic chemicals having a potential to interfere with thyroid hormone signaling in the developing brain. Considering an increasing demand to develop efficient screening method of developmental neurotoxicants, monitoring of Reelin-expressing interneurons in the hippocampal dentate hilus may provide a valuable tool for detection of chemicals that can affect neurogenesis and migration.

Conflict of interest

All of the authors disclose that there are no conflicts of interest that could inappropriately influence the outcomes of the present study.

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RAPID COMMUNICATION

Hippocampal Epigenetic Modification at the Brain-Derived Neurotrophic Factor Gene Induced by an Enriched Environment

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ABSTRACT: Environmental enrichment is an experimental paradigm that increases brain-derived neurotrophic factor (BDNF) gene expression accompanied by neurogenesis in the hippocampus of rodents. In the present study, we investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice. Exposure to an enriched environment for 3–4 weeks caused a dramatic increase in the mRNA expression of BDNF, but not platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), epidermal growth factor (EGF), or glial fibrillary acidic protein (GFAP), in the hippocampus of mice. Under these conditions, exposure to an enriched environment induced a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters, in contrast to significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. The expression levels of several microRNAs in the hippocampus were not changed by an enriched environment. These results suggest that an enriched environment increases BDNF mRNA expression via sustained epigenetic modification in the mouse hippocampus. © 2010 Wiley-Liss, Inc.

KEY WORDS: mouse hippocampus; epigenome; BDNF gene; neurogenesis; environmental enrichment

INTRODUCTION

Over the past few decades, exposure to an enriched environment, which consists of housing groups of animals together in a complex environment with various toys to provide more opportunity for learning and social interaction than standard laboratory living conditions, has been shown to enhance behavioral performance in various learning tasks. Consistent with these behavioral tests, exposure to an enriched environment has been shown to induce biochemical and structural changes in the hippocampal dentate gyrus (DG) and CA1 region, such as an increased number of dendritic branches and spines, enlargement of synapses, and an increased number of glial cells. Moreover, exposure of adult rodents to increased environmental complexity induces hippocampal progenitor proliferation and neurogenesis (Nilsson et al., 1999; van Praag et al., 1999). However, the detailed mechanisms that control neurogenesis in the hippocampus of animals housed in an enriched environment are still unclear.

It has been reported that brain-derived neurotrophic factor (BDNF) promotes neuronal differentiation from endogenous progenitor cells in the ventricular wall of the adult forebrain (Ahmed et al., 1995; Kirschenbaum and Goldman, 1995), and the increased expression of BDNF is required for the environmental induction of hippocampal neurogenesis in rodents (Rossi et al., 2006). The BDNF gene and the regulation of its expression are highly complex, and have been examined in both human and rodent brains (Timmusk et al., 1993; Liu et al., 2006; Aid et al., 2007; Pruunsild et al., 2007). The mouse BDNF gene, which shows a high degree of sequence homology to its human congener, contains multiple 5′ noncoding exons and a single 3′ coding exon for the mature BDNF protein (Aid et al., 2007). These noncoding exons undergo alternative splicing with the common coding exon to produce multiple exon-specific BDNF transcripts. Nine BDNF promoters have been previously identified in the mouse (Aid et al., 2007), and each drives the transcription of BDNF mRNAs containing one of the four 5′ noncoding exons (I, II, III, IV, V, VI, VII, or VIII) spliced to the common 3′ coding exon.

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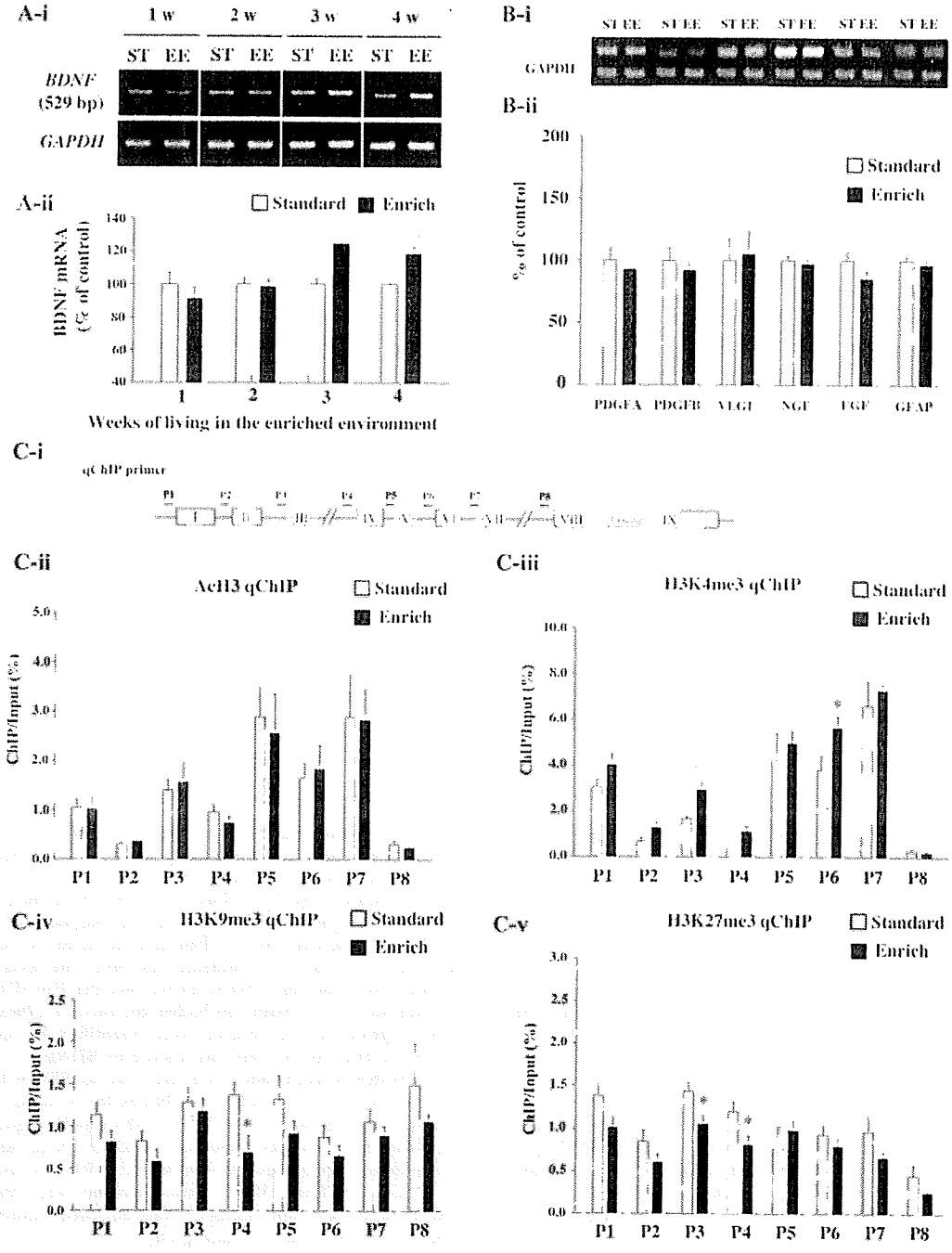
Chromatin remodeling at gene promoter regions is becoming increasingly recognized as a key control point of gene expression. Histone modification represents one prominent form of chromatin remodeling. According to the "histone code theory," different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state that encodes gene activation vs. gene silencing (Jenuwein and Allis, 2001). Intriguing correlations have been found between cellular plasticity, including transformation and such epigenetic modification at a specific gene (Kouzarides, 2007; Borrelli et al., 2008), indicating that possible epigenetic modification at BDNF gene promoters may partly contribute to adult neurogenesis. Therefore, in the present study, we evaluated whether an enriched environment could induce histone modification at several BDNF gene promoters in mice.

Male C57BL/6J mice (Jackson Laboratory), weighing 18–23 g, were used in the present study. Control mice were housed four per standard (16.5 × 26.5 × 13.5 cm^3) plexiglass cage. Mice



in the enriched environment group were kept eight per large (25.5 × 42.5 × 39 cm^3) wire-mesh, two-storied cage, which contained tunnels and running wheels, for 4 weeks (Supporting Information Fig. 1A).

In the DG of mice housed in the enriched environment for 4 weeks, immunoreactivity (IR) for doublecortin, which is a microtubule-associated protein that is expressed specifically in virtually all migrating neuronal precursors of the CNS and which has been used as a candidate marker for neural migration and differentiation, was increased compared to that in mice housed in the standard cage (Supporting Information Fig. 1B). Furthermore, IR for NeuroD, which is another marker for the differentiation of granule cells in the hippocampus (Miyata et al., 1999), was clearly increased in the DG of mice housed in an enriched environment (Supporting Information Fig. 1C). Additionally, the number of BrdU-positive cells in the DG that were classified as newly dividing cells was markedly increased in mice housed in an enriched environment (Supporting Information Fig. 2A), and theses were clearly colocalized with the neuronal marker NeuN (Supporting Information Fig. 2B). In paralleled with adult neurogenesis, the expression of BDNF mRNA in the hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks (Fig. 1A). In contrast, mRNA levels of glial fibrillary acidic protein (GFAP), platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and epidermal growth factor (EGF) in the hippocampus were not altered by exposure to an enriched environment for 4 weeks (Fig. 1B).

Under these conditions, a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters was observed upon exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter, and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters were seen in the hippocampus of mice under an enriched environment. In contrast, an enriched environment did not produce the hyperacetylation of H3 in the hippocampus of enriched mice (Fig. 1C).

In terms of changes in mRNA levels of several histone methylases and demethylases in the hippocampus, an enriched environment failed to change the mRNA expression of MLL1, LSD1, Jarid1a, Jarid1b, jmjd2B, jmjd2C, jmjd2D, EZH2, UTX, or jmjd3 (Fig. 2A).

As with histone methylases and demethylases, no significant changes in microRNA9 (miR9), miR124a, miR132, miR133b, or miR145 were observed in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in a standard cage (Fig. 2B).

In the present study, we demonstrated hippocampal neurogenesis in mice that were exposed to an enriched environment. This notion is supported by previous reports that exposure of adult rodents to an enriched environment increased neurogenesis in the hippocampus (Kempermann et al., 1997; Nilsson et al., 1999).

During development, growth factors provide important extracellular signals that regulate the proliferation and differentiation of neural stem cells in the CNS (Calof, 1995). Several investigations have examined the role of these factors in the adult brain (Calof, 1995; Kuhn et al., 1997). Furthermore, it has been shown that exposure to an enriched environment increased the expression of BDNF genes (Falkenberg et al., 1992). In support of these findings, the present study showed that the expression of BDNF mRNA in the hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks. In contrast, mRNA levels of GFAP, PDGF-A, PDGF-B, VEGF, NGF, and EGF in the hippocampus were not altered under the present conditions. In our *in vitro* study using neural stem cells cultured from the mouse embryonic forebrain, neuronal differentiation was clearly observed following exposure to recombinant BDNF (Supporting Information Fig. 3). These findings raise the possibility that an enriched environment may stimulate expression of the BDNF gene in the hippocampus and, in turn, the enhanced

FIGURE 1. (A) Time course of changes in the expression of BDNF mRNA in the hippocampus. (A-i) Representative RT-PCR for BDNF mRNA in the hippocampus obtained from standard or enriched mice. (A-ii) The intensity of the bands was semiquantified using NIH Image software. The value for BDNF mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for enriched mice is expressed as a percentage of the increase in standard mice. Each column represents the mean ± S.E.M. of six samples. $^{**}P < 0.01$ vs. the standard group. (B) Upper: Representative RT-PCR for PDGFA, PDGFB, VEGF, NGF, EGF, and GFAP mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The values for mRNAs were normalized by that for GAPDH mRNA. Each column represents the mean ± S.E.M. of six samples. (C-i) Schematic of the BDNF gene: The BDNF gene contains eight noncoding exons I–VIII upstream of the coding exon IX in mouse. Exons I–VIII can each be alternatively spliced next to exon IX, from the 5′ UTR region of different mRNA splice variants, BDNF I-VIII, which can promote the expression of their corresponding transcript variants. For an mRNA analysis of total BDNF, primers were used to amplify exon IX. For ChIP analysis, primers were designed around the putative promoters, P1-P8, which are located upstream of exons I–VIII. (C-ii, iii, iv, v) Stable changes in histone modifications in the hippocampus in mice housed under standard or enriched conditions for 4 weeks. ChIP assays were performed to measure the levels of several histone modifications at the eight BDNF promoters Ex1-Ex8 (P1–8) in the hippocampus using specific antibodies for each modification state. Levels of promoter enrichment were quantified by quantitative PCR. (C-ii) Histone H3 was not altered at BDNF P1–P8. (C-iii) Histone H3K4 trimethylation was increased at BDNF P3 and P6 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group, $^{**}P < 0.01$ vs. the standard group. (C-iv) H3K9 trimethylation was decreased at BDNF P4 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group. (C-v) Histone H3K27 trimethylation was decreased at BDNF P3 and P4 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group.

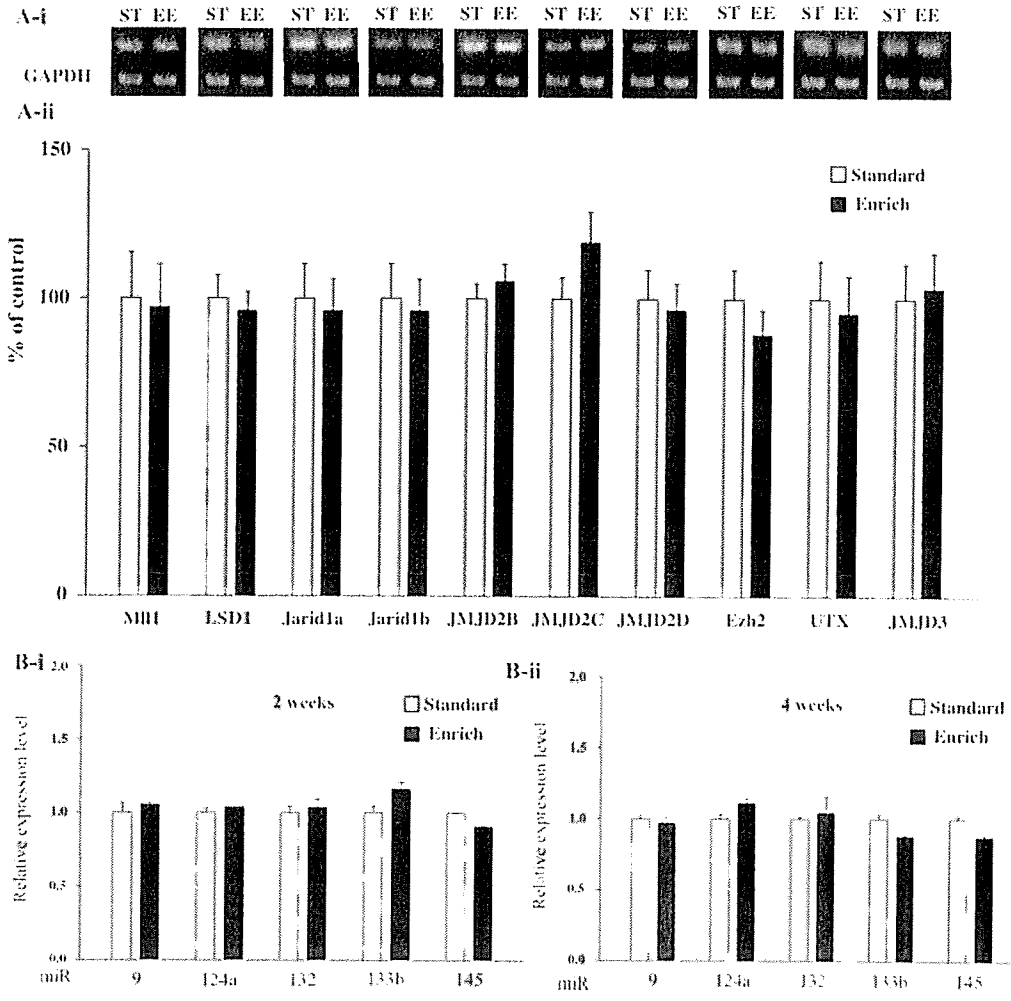


FIGURE 2. (A) Upper: Representative RT-PCR for MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a, and Jarid1b (H3K4 demethylases), JMJD2B, JMJD2C and JMJD2D (H3K9 demethylases), Ezh2 (H3K27 methyltransferase), and UTX and JMJD3 (H3K27 demethylases). mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The value for mRNA was normalized by that for GAPDH mRNA. Each column represents the mean ± SEM of six samples. (B) Expression levels of miRNAs were measured in the hippocampus of mice housed in an enriched environment for 2 weeks (B-i) and 4 weeks (B-ii). The value for miRNA was normalized by that for the internal standard snoRNA202.

BDNF protein may lead to neural differentiation from its precursors in the hippocampal DG.

We next evaluated whether an enriched environment increases BDNF gene expression through chromatin-specific events that promote the expression of distinct transcript variants. In this study we analyzed two active histone modifications (acetylation of histone H3, AcH3, and trimethylation of H3K4) and two repressive histone modifications (trimethylation of H3K9 and trimethylation of H3K27) at different BDNF promoter regions in the hippocampus. As a result, we detected a significant increase in H3K4 trimethylation, an activated histone modification marker, at the BDNF P3 and P6 promoters after exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in H3K9 trimethylation, a repressive histone modification marker, at the BDNF P4 promoter, and H3K27 trimethylation, another repressive histone modification marker, at the BDNF P3 and P4 promoters were seen after exposure to an enriched environment. Under these conditions, we observed that an enriched environment did not produce the hyperacetylation of H3 in enriched mice.

The methylation of H3K9 and H3K27 can be directly modulated by histone methylases and demethylases that target specific lysine residues and methylation states (Jenuwein and Allis, 2001; Kouzarides, 2007). Thus, we investigated whether an enriched environment could alter the mRNA level of several histone methylases and demethylases in the hippocampus. We found that an enriched environment did not change the

mRNA expression of MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a or Jarid1b (H3K4 demethylases), jmjd2B, jmjd2C or jmjd2D (H3K9 demethylases), EZH2 (an H3K27 methyltransferase), or UTX or jmjd3 (H3K27 demethylases).

Recently, microRNAs (miRNAs), a class of small, noncoding RNAs, have been identified as important regulators of many biological processes, including organogenesis and disease development (Kim et al., 2007; Chen et al., 2008; Hutchison et al., 2009). Indeed, it has been shown that epigenetic factors such as DNA methylation, histone modification, and regulatory noncoding RNAs affect the fate of neural stem cells (Chi and Bernstein, 2009). miRNAs have the potential to specifically regulate a large set of target molecules, which may affect the cell's fate in a programmatic way, and the role of miRNAs in stem cell gene networks is being actively explored. Their ability to potentially regulate large numbers of target genes simultaneously suggests that they may be important sculptors of transcriptional networks. In this study, we found that miR9, miR124a, miR132, miR133b, and miR145 are expressed in the hippocampus of adult mice. It has been reported that miR145 regulates Oct4, Sox2, and Klf4 and suppresses the potential of human embryonic stem cells to generate any differentiated cell type (pluripotency) (Xu et al., 2009). miR124, one of these signature miRNAs that is enriched in the brain, regulates adult neurogenesis in the subventricular zone stem cell niche (Cheng et al., 2009). miR132 is localized and synthesized, in part, at synaptic sites in dendrites to regulate synaptic formation and plasticity (Vo et al., 2005). miR9 is expressed specifically in the hippocampus and may be involved in neural stem cell self-renewal and differentiation (Krichevsky et al., 2006; Bak et al., 2008). In the present study, there were no significant changes in miR9, miR124a, miR132, miR133b, or miR145 in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in a standard cage. Although further studies are required to investigate the molecular mechanism of hippocampal neurogenesis induced by an enriched environment, we propose that an enriched environment may increase BDNF expression accompanied by histone modification without directly changing the expression of histone H3 methylases and demethylases, and miRs in the hippocampus.

In conclusion, the present study demonstrated that an enriched environment stimulates neuronal differentiation from precursors in the hippocampal DG. Furthermore, the increased expression of BDNF was observed in the hippocampus of mice that had been exposed to an enriched environment. This enrichment induced a significant increase in H3K4 trimethylation at the BDNF P3 and P6 promoters and a significant decrease in H3K9 trimethylation at the BDNF P4 promoter and H3K27 trimethylation at the BDNF P3 and P4 promoters in the hippocampus of mice. These results suggest that an enriched environment may increase BDNF expression with notably sustained chromatin regulation in the mouse hippocampus. This phenomenon could partly explain the hippocampal neurogenesis induced by an enriched environment in mice.

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Hippocampal Epigenetic Modification at the Doublecortin Gene Is Involved in the Impairment of Neurogenesis With Aging

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ABSTRACT Recent research has suggested that epigenetic mechanisms, which exert lasting control over gene expression without altering the genetic code, could mediate stable changes in brain function. A growing body of evidence supports the idea that epigenetic changes play a role in the etiology of aging and its associated brain dysfunction. The present study was undertaken to evaluate the age-related changes in the expression of doublecortin, which is a marker for neuronal precursors, along with epigenetic modification in the hippocampus of aged mice. In the present study, the doublecortin-positive cells were almost completely absent from the dentate gyrus of the hippocampus of 28-month-old mice. Furthermore, the expression level of doublecortin mRNA was significantly decreased in the hippocampus of aged mice. Under these conditions, a significant decrease in H3K4 trimethylation and a significant increase in H3K27 trimethylation at doublecortin promoters were observed with aging without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. These findings suggest that aging produces a dramatic decrease in the expression of doublecortin along with epigenetic modifications in the hippocampus. **Synapse 00:000–000, 2010.**

INTRODUCTION

Since the average human life span has increased dramatically over the last century, there are growing concerns about malfunctions associated with aging. The dysfunction of neurotransmission in normal aging and neuropsychiatric diseases late in life may contribute to the behavioral changes commonly observed in the elderly.

Neurogenesis occurs in specific areas in the adult brain throughout life, e.g., in the subventricular zone at the telencephalic level and in the dentate gyrus of the hippocampus (Eriksson et al., 1998; Lois and Alvarez-Buylla, 1993). The dentate gyrus, the hippocampus proper, and the subiculum constitute the hippocampal formation, which is critical for certain forms of learning and memory (Bliss and Collingridge, 1993). A positive correlation has been established between neurogenesis in the dentate gyrus and an animal's performance in behavioral tasks (Kempermann et al., 1997; van Praag et al., 1999). Aging is associated with a progressive accumulation of damaged molecules and impaired energy metabolism in brain cells. Neurons and glial cells may adapt to the adversities of aging by compensating for lost or damaged cells by producing new neurons and glia, and remodeling neuronal circuits.

The influence of age on rates of neurogenesis has been studied by several groups (Kuhn et al., 1996; Lichtenwalner et al., 2001). Changes in the relative proportion of young dentate gyrus neurons may have important consequences for hippocampal function and could possibly contribute to age-dependent structural

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