Developmental Hypothyroidism Abolishes Bilateral Differences in Sonic Hedgehog Gene Control in the Rat Hippocampal Dentate Gyrus

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ABSTRACT

Both developmental and adult-stage hypothyroidism disrupt rat hippocampal neurogenesis. We previously showed that exposing mouse offspring to manganese permanently disrupts hippocampal neurogenesis and abolishes the asymmetric distribution of cells expressing Mid1, a molecule regulated by sonic hedgehog (Shh) signaling. The present study examined the involvement of Shh signaling on the disruption of hippocampal neurogenesis in rats with hypothyroidism. Pregnant rats were treated with methimazole (MMI) at 0 or 200 ppm in the drinking water from gestation day 10–21 days after delivery (developmental hypothyroidism). Adult male rats were treated with MMI in the same manner from postnatal day (PND) 46 to PND 77 (adult-stage hypothyroidism). Developmental hypothyroidism reduced the number of Mid1+ cells within the subgranular zone of the dentate gyrus of offspring on PND 21, and consequently abolished the normal asymmetric predominance of Mid1+ cells on the right side through the adult stage. In control animals, Shh was expressed in a subpopulation of hilar neurons, showing asymmetric distribution with left side predominance on PND 21; however, this asymmetry did not continue through the adult stage. Developmental hypothyroidism increased Shh+ neurons bilaterally and abolished the asymmetric distribution pattern on PND 21. Adult hypothyroidism also disrupted the asymmetric distribution of Mid1+ cells but did not affect the distribution of Shh+ hilar neurons. The results suggest that the hippocampal neurogenesis disruption seen in hypothyroidism involves changes in asymmetric Shh+ neuron distribution in developmental hypothyroidism and altered Mid1 expression in both developmental and adult-stage hypothyroidism.

Key words: hypothyroidism; hippocampus; midline 1 (Mid1); sonic hedgehog (Shh); neurogenesis; rat

It is well known that thyroid hormones are indispensable for brain development throughout the fetal and postnatal stages as they play many important roles in neural proliferation and neuronal differentiation (Bernal, 2002). Additionally, thyroid hormone deficiencies seriously affect neurological functions involving learning and memory and suppress physical growth (Sawano et al., 2013; Zoeller and Rovet, 2004). Rat offspring exposed to anti-thyroid agents, such as 6-propyl-2-thiouracil (PTU) or methimazole (MMI), demonstrated significant impairments in hippocampal learning (Axelstad et al., 2008), neurochemistry (Gong et al., 2010), synaptic transmission (Gilbert, 2011), and neuronal migration (Lavado-Autric et al., 2003). These neurophysiological impairments involve changes in the hippocampal cornu ammonis 1 and dentate gyrus, specifically (Gilbert and Sui, 2006). Moreover, hypothyroidism in adult rats caused by these anti-thyroid agents results in impaired neural...
cell morphology and brain function (Cortés et al., 2012; Koromilas et al., 2010) and behavioral changes characterized as a depressive-like state (Montero-Pedraza et al., 2006).

The hippocampus is known as a brain region responsible for learning, memory, and motivation. In the granule cell layer (GCL) of the hippocampal dentate gyrus, the subgranular zone (SGZ) retains the capacity to produce new neurons throughout adult life (Kempermann et al., 2004). Adult neurogenesis involves self-renewal of neuronal stem cells, proliferation of progenitor cells, and differentiation of progenitor cells into immature granule cells and then finally into post-mitotic mature granule cells (Hodge et al., 2008). These stages are considered to be susceptible to neurotoxins, especially during development.

We previously reported that developmental hypothyroidism caused by maternal exposure to MMI permanently affects neurogenesis by targeting stem cells and earlier progenitor cells in the rat hippocampal dentate gyrus (Shiraki et al., 2012). We also found that both adult-stage and developmental hypothyroidism caused fluctuations in the distribution of γ-aminobutyric acid (GABA)-ergic interneuron subpopulations in the hilus of the dentate gyrus (Sægusa et al., 2010; Shiraki et al., 2012). In another study, we observed a sustained disruption of hippocampal neurogenesis accompanied with population changes of GABAergic interneurons of offspring by maternal exposure to manganese (Mn) in mice (Wang et al., 2012). By global methylation analysis using CpG island microarrays, we found hypermethylation of the Mid1 gene in the dentate gyrus of these mouse offspring (Wang et al., 2013). Using immunohistochemistry, we found asymmetrical Mid1 expression with right side predominance in the late-stage granule cell progenitors or post-mitotic granule cells and hilar interneurons in untreated control animals. Maternal Mn exposure reduced the Mid1 expression on the right side and disrupted the asymmetric distribution through the adult stage (Wang et al., 2013). We considered that Mn exposure induces Mid1 hypermethylation in neuronal stem cells that could be heritable in the SGZ, causing a permanent reduction in mid1 † late-stage progenitors or postmitotic granule cells.

Mid1 is known to encode a ubiquitin ligase (Trockenbacher et al., 2001) and this ligase controls brain development by regulating expression of GLI-Kruppel family member GLI3 (Gli3) (Krauss et al., 2008). Gli3 mediates sonic hedgehog (Shh) signaling as either repressor or activator (Litingtung and Chiang, 2000). Shh is reported to repress the expression of Mi1, whereas Mi1 represses Shh expression through the induction of bone morphogenetic protein 4 (Bmp4) in the developmental period (Granata and Quaderi, 2003). The Shh pathway is involved in both the oligodendroglial and neuronal differentiation of neural progenitor cells (Mellough et al., 2005). Because Shh is known to be expressed in GABAAergic interneurons in embryonic mice (Favaro et al., 2009), regulatory interactions among these proteins are thought to control neuronal development and population by GABAAergic interneurons.

As above-mentioned, disruptive epigenetic regulation of Mid1 gene involving stem cells may be responsible for permanent disruption of neurogenesis. Because developmental hypothyroidism also caused sustained disruption of neurogenesis in rats (Shiraki et al., 2012), the present study was performed to clarify whether hypothyroidism affects Mid1 signaling in the SGZ. For this purpose, we examined the effect of developmental or adult-stage hypothyroidism on Shh signaling and Mid1 expression in the rat dentate gyrus using MMI as an anti-thyroid agent.

**MATERIALS AND METHODS**

**Chemicals and animals.** MMI (2-mercapto-1-methylimidazole; CAS No. 60-56-0) was purchased from Sigma Chemical Co (St Louis, Missouri). Pregnant Sprague Dawley (SD) rats and adult male SD rats were obtained from Japan SLC, Inc (Hamamatsu, Japan) at gestational day (GD) 1 (observation of vaginal plug was designated as GD 0) and on postnatal day (PND) 35 (where PND 0 is the day of delivery), respectively.

Animals were individually housed in polycarbonate cages with wood chip bedding in an air-conditioned animal room (temperature: 23 ± 2°C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Animals were provided with a pelleted basal diet ad libitum (MF; Oriental Yeast Co, Ltd, Tokyo, Japan) and tap water before the experimental period. From PND 21 onwards, offspring were reared with 3 or 4 animals per cage and provided with the MF diet and tap water ad libitum.

**Experimental design.** Animals and experimental design of the MMI-mediated induction of developmental and adult-stage hypothyroidism were identical to those previously reported (Shiraki et al., 2012). In Experiment 1 (the developmental hypothyroidism experiment), MMI was administered to dams. Twenty-four dams were randomly divided into 3 groups of 8 dams each and treated with MMI (0, 50, or 200 ppm) in the drinking water from GD 10 to PND 21. The high dose level and the duration of exposure period were determined according to previous reports that showed apparent aberrations in neuronal development in the hippocampus involving neurogenesis in the dentate gyrus of offspring (Sægusa et al., 2010; Shibutani et al., 2009). On PND 2, dams were subjected to litter culling, which left randomly 8 male offspring per dam. Where male offspring were less than 8, additional offspring were cross-fostered from other dams in each group. On PND 21, all dams and 32 male offspring per group were euthanized by exsanguination from the abdominal aorta under deep anesthesia and subjected to prepubertal necropsy. The remaining male offspring were maintained until PND 77 without administration of MMI and subjected to adult-stage necropsy.

In Experiment 2 (the adult-stage hypothyroidism experiment), male SD rats were randomly divided into 3 groups of 16 animals each and treated with 0, 50, or 200 ppm MMI in the drinking water from PND 46 to PND 77. All animals were euthanized on PND 77 in the same way as described for Experiment 1 and subjected to necropsy.

In the present study, animals from the 0 ppm (control) and 200 ppm MMI groups were used for immunohistochemical and real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses in both of Experiments 1 and 2.

All animal experiments were conducted in accordance with the “Guidelines for Proper Conduct of Animal Experiments” (Science Council of Japan, June 1, 2006) and the protocol was approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

**Tissue sample preparation.** For RNA analysis, brains of 22 male PND 21 and PND 77 pups per group (Experiment 1) and 6 animals per group (Experiment 2) were removed under anesthesia and then fixed in methacarn solution for 5h at 4°C. From each brain, a 2-mm-thick coronal brain slice was prepared at the position of –2.8 mm from bregma. Portions of the hippocampal dentate gyrus were collected using a biopsy punch (Φ1.0 mm, Kai Industries Co, Ltd, Seki, Japan) and stored in ethanol at –80°C until extraction.
For immunohistochemistry, brains of 10 male PND 21 and PND 77 pups per group (Experiment 1) and 10 animals per group (Experiment 2) were fixed in Bouin’s solution overnight at room temperature. Paraaffin-embedded coronal slices from −3.0 mm from bregma for the PND 21 samples and −3.5 mm from bregma for the PND 77 samples were prepared.

Real-time RT-PCR analysis. Total RNA samples (N = 6/group in each of Experiments 1 and 2) were extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) in combination with QIAzol (Qiagen) according to the manufacturer’s instruction. First-strand cDNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, California).

Real-time PCR analysis was performed using the Power SYBR Green PCR Master Mix (Life Technologies) and StepOnePlus Real-Time PCR System (Life Technologies). The PCR primers for the target genes (Table 1) were designed using Primer Express software (version 3.0; Life Technologies). Threshold cycle (C_T) values were first normalized to a housekeeping gene, either hypoxanthine guanine phosphoribosyltransferase 1 (Hprt1) or glyceraldehyde 3-phosphate dehydrogenase (Gapdh), as an endogenous control in the same sample. The relative differences in gene expression compared with control C_T values were calculated by the 2^−ΔΔC_T method (Livak and Schmittgen, 2001).

Immunohistochemistry. Primary antibodies against SRY (sex determining region Y)-box 2 (Sry2; mouse IgG, clone 9-9-3, 1:8000, Abcam Inc, Cambridge, UK; positive control: fetal rat brain), midline 1 (Mid1; rabbit IgG, 1:150, Sigma-Aldrich Chemical Co, St Louis, Missouri; positive control: fetal rat brain), sonic hedgehog (Shh; rabbit IgG, 1:400, Biorbyt Ltd, Cambridge, UK; positive control: fetal rat brain), and Gli family zinc finger 3 (Gli3; rabbit IgG, 1:150, Abcam Inc; positive control: rat testis) were used in immunohistochemistry experiments on brain sections. To quench endogenous peroxidase, deparaffinized sections were incubated in 0.3% (vol/vol) hydrogen peroxide in absolute methanol for 30 min. Heat-induced antigen retrieval was performed for Mid1 and Shh by autoclaving at 121°C for 10 min, and for Gli3 by microwaving at 90°C for 10 min in 10 mM citrate buffer (pH 6.0). No antigen retrieval was performed for Sox2. Immunodetection was performed using a Vectastain Elite ABC kit (Vector Laboratories Inc, Burlingame, California) with 3,3′-diaminobenzidine/H2O2 as the chromogen. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination. For negative control, the primary antibody was omitted and absence of immunoreactivity was confirmed.

Morphometry of immunolocalized cells. Shh+ cells distributed in the hilus of both sides of the dentate gyrus were counted and normalized for the number per unit area of the hilar area (polymorphic layer). In the SGZ or GCL of the dentate gyrus, all Sox2+, Gli3+, and Mid1+ cells were counted bilaterally and the numbers were normalized to the entire length of the GCL measured as previously described (Wang et al., 2012). For quantitative measurement of each immunoreactive cellular component, digital photomicrographs at 100-, 200-, or 400-fold magnification were taken using a BX53 microscope (Olympus Optical Co, Ltd, Tokyo, Japan) attached to a DP72 Digital Camera System (Olympus Optical Co, Ltd). Quantitative measurements were performed using the WinROOF image analysis software package (version 5.7; Mitani Corp, Fukui, Japan).

Statistical analysis. Numerical data are presented as mean ± SD. For all data, the individual animal was examined as the experimental unit. Differences between the control and MMI groups were evaluated as follows. The F test was used to determine the homogeneity of variance. If the variance was homologous, the Student’s t test was applied. If variance was heterogeneous, Aspin-Welch’s t test was applied. All analyses were performed using the Excel Statistics 2010 software package (Social Survey Research Information Co, Ltd, Tokyo, Japan).

RESULTS

Transcript Expression Changes
In the developmental hypothyroidism experiment, Shh and Gli3 mRNA expression levels were statistically significantly higher in MMI-exposed PND 21 offspring when compared with untreated controls after normalization to either Hprt1 or Gapdh. In contrast, Mid1 mRNA expression levels were significantly lower in the PND 21 MMI-exposed offspring when compared with untreated controls after normalization to either Hprt1 or Gapdh. MMI exposure did not alter Sox2 or Bmp4 mRNA levels. Gli3 continued to show significantly higher expression levels in MMI-exposed offspring when compared with untreated controls on PND 77, whereas other genes did not show any significant differences at this point (Table 2).

In the adult-stage hypothyroidism experiment, Gli3 mRNA expression levels were significantly higher in MMI-exposed animals when compared with untreated controls after normalization with either Hprt1 or Gapdh. The other genes did not show significant mRNA expression changes (Table 2).

Immunohistochemical Cellular Distribution
On PND 21, Mid1 immunoreactivity was sparsely observed in both the nucleus and cytoplasm of progenitor cells distributed limited at the SGZ of the untreated control offspring (Fig. 1A). The number of Mid1+ cells increased accompanying extended distribution to GCL on PND 77, especially at the center of the

<table>
<thead>
<tr>
<th>Table 1. Primer Sequence for Real-Time RT-PCR Analysis</th>
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<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>Sox2</td>
</tr>
<tr>
<td>Bmp4</td>
</tr>
<tr>
<td>Shh</td>
</tr>
<tr>
<td>Gli3</td>
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<tr>
<td>Mid1</td>
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<tr>
<td>Hprt1</td>
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<td>Gapdh</td>
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Notes: Bmp4, bone morphogenetic protein 4; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gli3, GLI-Kruppel family member GLI3; Hprt1, hypoxanthine phosphoribosyltransferase 1; Mid1, midline 1; Shh, sonic hedgehog; Sox2, SRY (sex determining region Y)-box 2.
TABLE 2. Transcript Expression in the Hippocampal Dentate Gyrus after Developmental and Adult-Stage Hypothyroidism

<table>
<thead>
<tr>
<th>MMI (ppm)</th>
<th>0 (Control)</th>
<th>200</th>
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<tbody>
<tr>
<td></td>
<td>Relative Transcript Level Normalized to Hprt1</td>
<td>Gapdh</td>
</tr>
<tr>
<td>Developmental hypothyroidism (PND 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals examined</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Sox2</td>
<td>1.09 ± 0.05</td>
<td>1.06 ± 0.31</td>
</tr>
<tr>
<td>Bmp4</td>
<td>1.08 ± 0.39</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>Shh</td>
<td>1.03 ± 0.05</td>
<td>1.03 ± 0.16</td>
</tr>
<tr>
<td>Gli3</td>
<td>0.97 ± 0.10</td>
<td>1.01 ± 0.18</td>
</tr>
<tr>
<td>Mid1</td>
<td>1.04 ± 0.92</td>
<td>1.02 ± 0.36</td>
</tr>
<tr>
<td>Developmental hypothyroidism (PND 77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals examined</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Sox2</td>
<td>1.01 ± 0.14</td>
<td>1.05 ± 0.21</td>
</tr>
<tr>
<td>Bmp4</td>
<td>1.04 ± 0.29</td>
<td>1.02 ± 0.22</td>
</tr>
<tr>
<td>Shh</td>
<td>1.01 ± 0.15</td>
<td>1.03 ± 0.10</td>
</tr>
<tr>
<td>Gli3</td>
<td>1.07 ± 0.10</td>
<td>1.02 ± 0.08</td>
</tr>
<tr>
<td>Mid1</td>
<td>1.02 ± 0.83</td>
<td>1.07 ± 0.76</td>
</tr>
<tr>
<td>Adult-stage hypothyroidism (PND 77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals examined</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Sox2</td>
<td>1.05 ± 0.04</td>
<td>1.07 ± 0.05</td>
</tr>
<tr>
<td>Bmp4</td>
<td>1.03 ± 0.19</td>
<td>1.02 ± 0.12</td>
</tr>
<tr>
<td>Shh</td>
<td>1.05 ± 0.05</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>Gli3</td>
<td>1.01 ± 0.12</td>
<td>1.02 ± 0.18</td>
</tr>
<tr>
<td>Mid1</td>
<td>1.02 ± 0.73</td>
<td>1.06 ± 0.86</td>
</tr>
</tbody>
</table>

Notes: Bmp4, bone morphogenetic protein 4; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gli3, GLI-Kruppel family member GLI3; Hprt1, hypoxanthine phosphoribosyltransferase 1; Mid1, midline 1; MMI, methimazole; PND, postnatal day; Shh, sonic hedgehog; Sox2, SRY (sex determining region Y)-box 2.

*P < 0.05, **P < 0.01, significantly different from 0 ppm (control) group by Student’s t test or Aspin-Welch’s t test.

suprapyramidal blade and lateral side of the infrapyramidal blade in the coronal section (Fig. 1B). The number of SGZ Mid1− cells was significantly greater in the right side compared with the left side on both PND 21 and PND 77, but the magnitude of difference was greater on PND 21 than PND 77 (Figs. 1A and 1B). Developmental hypothyroidism by MMI exposure decreased the number of Mid1− cells on the right side of the offspring’s SGZ and abolished the bilateral difference between the left and right sides that was seen in the untreated control offspring at both PND 21 and PND 77. The total number of bilateral Mid1− cells was also significantly lower in the MMI-exposed offspring.

The untreated control animals in the adult-stage hypothyroidism experiment showed similar distribution of Mid1− cells as the untreated pups with right side predominance on PND 77. Adult-stage hypothyroidism reduced the number of Mid1− cells in the right side so that it was equal level to the left side. The total number of Mid1− cells in the treated animals was smaller when compared with the total number from the untreated controls, and there was no statistically significant difference in the number of Mid1− cells between the left and right sides (Fig. 1C).

With regard to Shh, cytoplasmic immunoreactivity was observed in the hilar neurons of the untreated control offspring on PND 21, and the number of Shh− cell was significantly greater on the left side compared with the right side (Fig. 2A). On PND 77, the number of Shh− cells decreased when compared with the number at PND 21, and the left side predominance disappeared at this time point. Developmental hypothyroidism significantly increased the number of bilateral Shh− cells on PND 21, showing a statistically significant difference in the sum of the left and right sides when compared with the sum in the untreated control offspring. Additionally, a bilateral difference between the left and right sides was not observed, unlike in the control offspring. On PND 77, there was no difference between the untreated controls and MMI-exposed offspring in the total number of Shh− cells or in the difference between the number of cells in the left and right sides (Fig. 2B).

In the rats with adult-stage hypothyroidism, there was no difference in the number of cells found between the left and right sides or in the total number of Shh− cells between the untreated controls and MMI-exposed animals (Fig. 2C).

In Experiment 1, Gli3 immunoreactivity was observed in both the nucleus and cytoplasm of neurons within the SGZ and GCL in the untreated PND 21 control offspring. The number of Gli3− cells was the same between the left and right sides (Supplementary Fig. 1). On PND 77, the total number of Gli3− cells in the untreated controls was similar to that at PND 21 (Figs. 3A and 3B). MMI exposure significantly increased the total number of bilateral Gli3− cells on both PND 21 and PND 77. In Experiment 2, the MMI-exposed group showed a significant increase in the total number of bilateral Gli3− cells compared with untreated control animals (Fig. 3C).

In Experiment 1, Sox2 nuclear immunoreactivity was observed in the SGZ of the untreated control offspring on PND 21, and the number of Sox2− cells showed no difference between the left and right sides (Fig. 4A and Supplementary Fig. 1). On PND 77, there was no difference in the distribution of Sox2− cells between the left and right sides, and the total number of bilateral Sox2− cells decreased when compared with
the number at PND 21 (Fig. 4B). MMI exposure did not alter the number of Sox2⁺ cells on either PND 21 or PND 77 when compared with the untreated controls. In Experiment 2, no change was observed in the number of Sox2⁺ cells in the MMI-exposed animals compared with the untreated control animals (Fig. 4C).

**DISCUSSION**

We have previously reported that the study samples used in the present study revealed apparent decreases of serum triiodothyronine and thyroxine concentrations as well as decreases of the body weight at the end of both developmental and adult-stage hypothyroidism by MMI exposure; however, there were no obvious clinical signs in MMI-treated maternal and offspring animals and adult animals (Shiraki et al., 2012). With regard to the toxic potential of MMI, cytotoxicity is reported only with isolated rat hepatocytes (Heidari et al., 2013). Although ingested MMI has been shown to specifically accumulate in the thyroid, distribution of MMI to the brain was scarce in rats (Marchant and Alexander, 1972), suggesting a low possibility of direct MMI toxicity to the brain. Moreover, exposure effects of MMI on brain development were the same with those of PTU (Fujimoto et al., 2012; Saegusa et al., 2010; Shibutani et al., 2009), another representative anti-thyroid agent as with MMI (Axelstad et al., 2008). Therefore, effects of MMI on brain development in rats may essentially be through induction of developmental hypothyroidism.

We have previously found affection of both granule cell lineage and GABAergic interneuron subpopulations in the dentate gyrus by developmental MMI exposure, whereas adult-stage MMI exposure only revealed affection of GABAergic interneuron subpopulations in the study samples used in the present study (Shiraki et al., 2012). Considering the higher activity of neurogenesis during development than adult stage (Lemaire et al., 2000), impact of hypothyroidism on neurogenesis may be higher in infants than adults. However, effect on Mid1 expression was observed with both developmental and adult-stage hypothyroidism, suggesting an operation of Mid1-linked target mechanism on neurogenesis by hypothyroidism even at the adult stage.

In the present study, developmental hypothyroidism reduced the number of Mid1⁺ cells distributed within the SGZ on PND 21. Interestingly, we saw an asymmetric distribution of Mid1⁺ cells with right-side predominance and its abolishment by developmental hypothyroidism, which dramatically decreased the total number of bilateral Mid1⁺ cells. This is similar to the disruption of hippocampal neurogenesis we saw in mouse offspring following maternal Mn exposure (Wang et al., 2013). In the present study, the disruption of Mid1 asymmetry continued through the adult stage, even after...
cessation of the developmental hypothyroidism. Considering that the effect of developmental Mn exposure on hippocampal neurogenesis was permanent in nature similarly to developmental hypothyroidism (Shiraki et al., 2012; Wang et al., 2013), disruption of Mid1 expression may be the signature of permanent effect on neurogenesis at least by developmental exposure. On the other hand, Shh⁺ hilar neurons showed left-side predominance in normal development on PND 21, but this asymmetry disappeared in adulthood. The present study is the first to show asymmetric Shh expression in the brain in early postnatal life. Developmental hypothyroidism increased bilaterally Shh⁺ cells and abolished the left-side Shh predominance on PND 21.

The present study also revealed a disruption of the asymmetric Mid1⁺ cell distribution with adult-stage hypothyroidism; however, any change was lacking in Shh⁺ hilar neuron distribution. Different localization of Mid1⁻ cells and Shh⁺ cells may suggest limited molecular interaction between the 2 proteins. This is different from what has been found in the Hensen’s node of chick embryos (Granata and Quaderi, 2003). The abolishment of the asymmetric distribution of Shh⁺ cells in the developmental hypothyroidism coupled with the lack of Shh⁺ expression change in adult-stage hypothyroidism may suggest a temporal regulation of Shh in the hippocampus that is limited to early postnatal life. In contrast, the persistence of the asymmetric Mid1⁺ cell distribution pattern through the adult stage and its sustained disruption by developmental hypothyroidism suggests that developmental hypothyroidism induces a permanent alteration of Mid1 gene regulation. Molecular asymmetries among nerve cells in the brain are known to be involved in learning and memory processes, especially in the hippocampus (Kohl et al., 2011). Considering that Mid1 knockout mice exhibit learning impairments (Lancioni et al., 2010), our finding that hypothyroidism abolishes asymmetric Mid1 expression accompanied with reduction of total number of bilateral Mid1⁺ cells may suggest that hypothyroidism disrupts higher brain functions specialized in the left or right side of the brain. Imbalance of symmetric neurochemical distribution is found in some brain disorders such as schizophrenia, depression, and Alzheimer’s disease (Ramírez et al., 2004).

Shh is known to play important roles as a morphogen in embryogenesis and also as a neural stem cell regulator in both developmental and postnatal neurogenesis (Charytoniuk et al., 2002; Han et al., 2008; Lai et al., 2003). Granata and Quaderi (2003) reported that cMid1, a MID1 ortholog, and Shh regulate the expression of one another to produce molecular asymmetry for determination of left-right identity in the Hensen’s node that appears during the gastrulation phase of chick embryogenesis. cMid1 is expressed in the gastrula cells with right-side predominance and represses the Shh expression on the right side of the node. In contrast, Shh expression is restricted to the left side and is thought to repress cMid1 expression on this side. In the present study, we observed increased Shh mRNA expression at the end of developmental hypothyroidism; however, we did not detect any difference in mRNA expression on PND 77 after developmental hypothyroidism or during adult-stage hypothyroidism. Immunohistochemically, the number of Shh⁺ neurons distributed within the dentate hilus decreased from PND 21 to PND 77, in accordance with the previous studies (Fang et al., 2011; Machold et al., 2003). We also found that hypothyroidism...
abolished the left-side predominance of Shh⁺ neurons on PND 21 by increasing bilateral Shh⁺ cell population.

Shh is reported to facilitate proliferation and to shift the balance between self-renewal and differentiation of neural stem cells in neurogenesis (Palma et al., 2005). Shh is a secreted protein and its receptors patched and smoothened are highly expressed in granule cells (Traiffort et al., 1999). We previously found that Mid1⁺ population was mostly late-stage granule cell progenitors or post-mitotic granule cells in the mouse hippocampal dentate gyrus on PND 77 (Wang et al., 2013). In rats, we here also observed Mid1⁺ population within SGZ and GCL on PND 77, suggestive of Mid1⁺ population in granule cell progenitors or post-mitotic granule cells. Because hilar interneurons innervate axons to type-2 progenitor cells (Tozuka et al., 2005), it is likely that Shh secreted from hilar interneurons could repress Mid1 expression in granule cell progenitors or post-mitotic granule cells. On the other hand, Mid1 represses expression of Shh through induction of Bmp4 during development, and Bmp4 is known to be expressed in the dentate hilus (Tang et al., 2009). However, we did not observe mRNA expression change of Bmp4 in the dentate gyrus by developmental hypothyroidism. Therefore, it is unlikely that Mid1 in granule cell lineages regulates Shh in the hilar neurons. Although the detailed regulatory mechanism between Mid1 and Shh is not clear, Granata and Quaderi (2003) demonstrated that ectopically implanted Shh protein could prevent the induction of cMid1 expression in the chick Hensen’s node. Therefore, developmental hypothyroidism that causes a bilateral increase in the number of Shh⁺ neurons and abolishes the left-side Shh⁺ predominance at PND 21 may in turn abolish the right-side predominance of Mid1⁺ cells seen in the present study. Because the left-side predominance of Shh⁺ neurons is limited during postnatal development, Shh probably does not control Mid1 expression after maturation.

Similar to what we saw in mouse offspring following maternal exposure to Mn (Wang et al., 2012), we observed a sustained reduction in the Mid1⁺ cells by developmental hypothyroidism in the current study. Our previous study revealed that the methylation level of the CpG islands within the Mid1 promoter region was lower on the right side than on the left side in the untreated control mice at both PND 21 and PND 77 (Wang et al., 2013); however, maternal Mn exposure decreased Mid1⁺ cells in the left side without affecting Mid1 promoter methylation levels at both PND 21 and PND 77. These results suggest that Mn treatment changes Mid1 expression through gene regulatory mechanisms other than promoter region hypermethylation. In contrast, we found in the present study that developmental and
adult-stage hypothyroidism in rats did not decrease the number of Mid1$^+$ cells in the left side but instead decreased the number of them in the right side. Although we are not aware of changes in methylation status of Mid1 caused by hypothyroidism because rat Mid1 promoter sequence information is unavailable, the maintenance of Mid1 expression in the left side suggests methylation is unaffected by hypothyroidism at this side. On the other hand, the inherent nature of methylation status after cell division suggested that the CpG island hypermethylation might have occurred in type-1 stem cells. Considering that developmental hypothyroidism targets broad populations of granule cell lineages to decrease the number from type-1 stem cells (Shiraki et al., 2012), it may be possible that developmental hypothyroidism induces Mid1 hypermethylation in right side neuronal stem cells to cause sustained effect on postnatal neurogenesis.

Gli3, a transcription factor that mediates activation or suppression of Shh signaling, is usually processed to repressor form and attenuates Shh signaling (Cambray et al., 2012). However, Mid1 expression mediates maturation of Gli3 into its activator form and thus dysregulation of Mid1 may cause the repressor form to dominate in inappropriate situations (Krauss et al., 2008). In the present study, we found that both developmental and adult-stage hypothyroidism increased Gli3 mRNA levels and Gli3$^+$ cells. Because there was no asymmetrical distribution of Gli3$^+$ cells in untreated animals, hypothyroidism may simply enhance Gli3 function.

Sox2 is a transcription factor and Shh requires Sox2-dependent regulation for hippocampal development and neural stem cell maintenance (Favaro et al., 2009). The present study did not reveal asymmetric Sox2$^+$ cell distribution in untreated controls and also fluctuation in Sox2 mRNA levels or Sox2$^+$ cells by hypothyroidism. Considering that Sox2$^+$ cells are symmetrically distributed type-1 stem cells and type-2a and type-2b progenitor cells within the SGZ (Hodge et al., 2008), it is unlikely that Sox2 differentially regulates Shh expressed in hilar neurons between the left and right sides. It is known that Shh can be transcribed by forkhead family transcription factors, such as Foxa1 and Foxa2, in embryonic midbrains (Mavromatakis et al., 2011). However, as far as we examined, we could not find Foxa1 and Foxa2 in the postnatal hippocampal tissues immunohistochemically (data not shown). Some unknown mechanism may be regulating Shh expression in postnatal hilar neurons.

In conclusion, developmental hypothyroidism reduced the number of Mid1$^+$ cells, abolished the asymmetric Mid1$^+$ cell distribution through the adult stage, increased the number of Shh$^+$ neurons, and abolished the asymmetric Shh$^+$ cell distribution on PND 21; however, this change did not continue through...
the adult stage. In contrast, adult-stage hypothyroidism disrupted the asymmetric Mid1+ cell distribution without changing Shh+ hilar neuron distribution. These results suggest that disruption of postnatal neurogenesis by hypothyroidism involves disrupted regulation of Shh in developmental hypothyroidism and Mid1 in both of developmental and adult-stage hypothyroidism. Our results suggest that disruption of postnatal neurogenesis by hypothyroidism may involve asymmetric regulation of neuronal architecture and may impair neuronal functions in the hippocampus.

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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