Induction of the UDP-Glucuronosyltransferase 1A1 during the Perinatal Period Can Cause Neurodevelopmental Toxicity

Rika Hirashima, Hirofumi Michimae, Hiroaki Takemoto, Aya Sasaki, Yoshinori Kobayashi, Tomoo Itoh, Robert H. Tukey, and Ryoichi Fujiwara

Department of Pharmaceutics (R.H., A.S., T.I., R.F.), Division of Biostatistics (H.M.), and Department of Pharmacognosy (H.T., Y.K.), School of Pharmacy, Kitasato University, Tokyo, Japan; and Laboratory of Environmental Toxicology, Department of Pharmacology, University of California San Diego, La Jolla, California (R.H.T.)

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ABSTRACT

Anticonvulsants can increase the risk of developing neurotoxicity in infants; however, the underlying mechanism has not been elucidated to date. Thyroxine [3,5,3\(^{-\text{L}}\)-triiodothyronine (T\(_3\))] plays crucial roles in the development of the central nervous system. In this study, we hypothesized that induction of UDP-glucuronosyltransferase 1A1 (UGT1A1)—an enzyme involved in the metabolism of T\(_4\)—by anticonvulsants would reduce serum T\(_4\) levels and cause neurodevelopmental toxicity. Exposure of mice to phenytoin during both the prenatal and postnatal periods significantly induced UGT1A1 and decreased serum T\(_4\) levels on postnatal day 14. In the phenytoin-treated mice, the mRNA levels of synaptophysin and synapsin I in the hippocampus were lower than those in the control mice. The thickness of the external granule cell layer was greater in phenytoin-treated mice, indicating that induction of UGT1A1 during the perinatal period caused neurodevelopmental disorders. Exposure to phenytoin during only the postnatal period also caused these neurodevelopmental disorders. A T\(_4\) replacement attenuated the increase in thickness of the external granule cell layer, indicating that the reduced T\(_4\) was specifically associated with the phenytoin-induced neurodevelopmental disorder. In addition, these neurodevelopmental disorders were also found in the carbamazepine- and pregnenolone-16-a-carbonitrile-treated mice. Our study is the first to indicate that UGT1A1 can control neurodevelopment by regulating serum T\(_4\) levels.

Introduction

Hypothyroidism during the perinatal period results in irreversible damage and severe mental and physical retardation, which is known as cretinism in humans (Koibuchi and Chin, 2000). In rodents, perinatal hypothyroidism induced by propylthiouracil (PTU), methimazole, and thyroxin can lead to impaired performance on a variety of motor and behavioral learning tasks (Eayrs and Levine, 1963; Davenport and Dorcey, 1972; Hasebe et al., 2008). It has been reported that, in the cerebellum, perinatal hypothyroidism induced various anatomic alterations, including delayed migration of granule cells, reduction of growth and branching of dendritic arborization of Purkinje cells, reduction of synaptogenesis between Purkinje cells and granule cell axons, delayed myelination, and changes in the synaptic connection among cerebellar neurons and afferent neuronal fibers in the cerebellum in rodents (Nicholson and Altman, 1972a,b,c; Legrand, 1979, 1980). These neurotoxicities in the cerebellum can further cause impaired performance on motor tasks. In the hippocampus, hypothyroidism decreased the neurogenesis and synaptogenesis and changed a morphology of pyramidal cells in rats (Desouza et al., 2005; Koromilas et al., 2010). These neurotoxicities in the hippocampus have been regarded as the cause of impaired learning ability. These data indicate that thyroid hormones play crucial roles in the development of the central nervous system.

Thyroid hormones consist of 3,5,3\(^{-\text{L}}\)-triiodothyronine (T\(_3\)) and 3,5,3\(^{-\text{L}}\),5\(^{-\text{L}}\)-tetraiodothyronine (T\(_4\)). As T\(_4\) solely enters the developing brain more readily than T\(_3\), where thyroid hormone receptors (TRs) exist, the serum T\(_4\) level is tightly associated with brain development during the perinatal period (Calvo et al., 1990). The liver is a major organ of T\(_4\) metabolism (Ohnhaus and Studer, 1983; Malik and Hodgson, 2002). Glucuronidation by UDP-glucuronosyltransferase (UGT) 1A1 is one of the main pathways of T\(_4\) metabolism (Richardson et al., 2014). UGT1A1 can be induced by various drugs, foods, and environmental pollutants through nuclear receptors, such as aryl hydrogen carbon

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ABBIJECTIONS: CAR, constitutive androstane receptor; HPLC, high-performance liquid chromatography; hUGT1, humanized UGT1; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; PBS, phosphate-buffered saline; PC, polychlorinated biphenyl; PCN, pregnenolone-16-a-carbonitrile; PCR, polymerase chain reaction; PTU, propylthiouracil; PXR, pregnane X receptor; Sult, sulfotransferase; T\(_3\), 3,5,3\^-L-triiodothyronine; T\(_4\), 3,5,3\^-L,5\^-L-tetraiodothyronine; TR, thyroid hormone receptor; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.
receptor, constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator--activated receptor α (Jemmert et al., 2000; Xu et al., 2005). Polychlorinated biphenyl (PCB) is a potent activator of aryl hydrocarbon receptor (Sanderson et al., 1996), whereas phenytoin and carbamazepine are activators of CAR (Faucette et al., 2007; Kachaylo et al., 2011). The UGT enzyme is not directly involved in T4 function. However, induction of UGT1A1 has been shown to decrease serum T4, which does indicate that UGT1A1 plays a key role in modulating T4 levels (Franklyn et al., 1984; Koopman-esseboom et al., 1994).

A case-control study showed that children exposed to phenytoin during the fetal period had significantly lower intelligence quotient at 4–8 years of age (Vanoverloop et al., 1992). Another case-control study showed that children exposed to carbamazepine during the fetal period also had a significantly lower intelligence quotient at 6 months to 6 years of age (Ornony and Cohen, 1996). Several cohort studies reported that exposure to PCB during the fetal and neonatal periods impaired visual recognition memory and cognitive abilities (Jacobson and Jacobson, 1996). As phenytoin and carbamazepine can pharmacologically reduce the blood pressure of pregnant women, the lowered transfer of oxygen to fetus was originally hypothesized as a cause of mental retardation in neonates (Imosemi and Osinubi, 2011). However, it has been reported that children exposed to phenytoin during only the neonatal period exhibited cognitive and behavioral impairments (Bacon et al., 1981). Furthermore, PCB has been reported not to reduce blood pressure (Goncharov et al., 2011). These inconsistent findings indicate that the actual mechanism of neurodevelopmental disorder in children exposed to phenytoin, carbamazepine, and PCB remains unclear.

T4 is extremely essential for brain development during the perinatal period. As phenytoin, carbamazepine, and PCB can commonly induce UGT1A1 (Soars et al., 2004; Smith et al., 2005; Shelby and Klaassen, 2006), the lowered T4 level caused by UGT1A1 induction might be the cause of phenytoin-, carbamazepine-, and PCB-induced neurodevelopmental disorders. Therefore, in this study, we investigated the effect of UGT1A1 induction on neurodevelopment during the perinatal period in mice. We analyzed histologic development of the cerebellum and synaptogenesis in the hippocampus to understand the effect of phenytoin on neurodevelopment during the perinatal period. Synaptogenesis in the hippocampus was examined by determining the expression levels of synaptophysin and synapsin I, marker genes of synaptic density (Thiel, 1993). Development of the cerebellum was examined by measuring the migration of granule cell in the cerebellum (Rakic, 1971).

Materials and Methods

Chemicals and Reagents. UDP-glucuronic acid (UDPGA), L-thyroxine, estradiol, carbamazepine, pregnenolone-16β-carbonitrile (PCN), and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). FTU, phenytoin, and phenylhydrazine were purchased from Wako Pure Chemical (Osaka, Japan). Free-T4 AccuBind enzyme-linked immunosorbent assay kit was purchased from Monobind (Lake Forest, CA). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Animals, Treatments, and Tissue Collection. Tg (UGT1A1<sup>+/-</sup>)<sup>hUGT1</sup> (humanized UGT1 (hUGT1)) mice were developed previously in a C57BL/6 background (Fujiiwara et al., 2010). Wild-type mice (C57BL/6NcrSc) were obtained from SLC Japan (Shizuoka, Japan). All animals received food and water ad libitum, and mouse handling and experimental procedures were conducted in accordance with the animal care protocol approved by Kitasato University (Tokyo, Japan).

To investigate the neurodevelopment of mice exposed to phenytoin during the fetal and neonatal period, dams and their pups were treated with phenytoin daily from gestation day 12, 13, or 14 to postnatal day 0 (p.o. 80 mg/kg) and from postnatal day 1 to day 14 (s.c. 35 mg/kg). To investigate the neurodevelopment of mice exposed to phenytoin during the neonatal period only, phenytoin (s.c. 35 mg/kg), carbamazepine (s.c. 35 mg/kg), and PCN (s.c. 10 mg/kg) were administered to pups daily from postnatal day 1 or day 1 to day 14. To investigate the effect of T4 replacement on neurodevelopment in phenytoin-treated mice, phenytoin (s.c. 35 mg/kg) and T4 (s.c. 100 μg/kg) were administered to pups daily from postnatal day 1 to day 14. To develop hypothyroid models, mice were given PTU (50 ppm) in drinking water after the 14 days beginning of mating to postnatal day 14. To investigate the effect of bilirubin on serum T4 level, phenylhydrazine was administered to hUGT1 mice intraperitoneally on postnatal days 15 and 16 (20 mg/kg).

Mice were anesthetized by diethyl ether inhalation, and the liver was perfused with ice-cold 1.15% KCl on postnatal day 14. The liver was removed and rinsed in cold 1.15% KCl and stored at −80°C. Brains were removed and rinsed in cold phosphate-buffered saline (PBS). The hippocampus was dissected on ice immediately and stored at −80°C. For histologic analysis, mice were anesthetized by diethyl ether inhalation and perfused transcardially with 4% paraformaldehyde in PBS (pH 7.4) on postnatal day 14. Brains were removed, postfixed overnight in the same fixative, and dehydrated using gradual ethanol and embedded in paraffin.

Determination of Serum T4 and Bilirubin Levels. Blood was obtained from the submandibular vein on postnatal day 14 and incubated at 4°C for 60 minutes to clot. Then, the blood was centrifuged at 3000g for 5 minutes. The supernatant was used as a serum sample. Free thyroxine (T<sub>4</sub>) levels were quantified using a Free-T4 AccuBind enzyme-linked immunosorbent assay kit (Monobind). Procedures were performed according to the manufacturer’s instructions. Total serum bilirubin levels were quantified using a Bilirubinometer (B-105N; Erma, Tokyo, Japan).

Quantitative Reverse-Transcription Polymerase Chain Reaction. Total RNA of hUGT1 mouse liver and hippocampus was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA). The cDNA was synthesized from 1 μg of total RNA of hUGT1 mouse liver and hippocampus using ReverTra Ace (Toyobo, Osaka, Japan). After the reverse-transcription reaction at 37°C for 15 minutes and at 50°C for 5 minutes, the reaction mixture was incubated at 95°C for 5 minutes to deactivate the reverse transcriptase. Quantitative reverse-transcription polymerase chain reaction (PCR) was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo), and the reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primer pairs listed in Table 1 were used to detect cyclophilin B, UGT1A1, Sulfofransferei 1b (Sult1b1), type 1 iodothyronine deiodinase, type 2 iodothyronine deiodinase, type 3 iodothyronine deiodinase, synapsin I, and synaptophysin. After an initial denaturation at 95°C for 30 seconds, the amplification was performed by denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 45 cycles. Expression levels were normalized with mouse cyclophilin mRNA level.

Enzyme Assays. Perfluorinated liver with 1.15% KCl was homogenized in 3 volumes of homogenization buffer (1.15% KCl/10 mM potassium phosphate buffer, pH 7.4). The homogenate was centrifuged at 10,000g for 30 minutes at 4°C, and the supernatant was collected. The supernatant was centrifuged at 105,000g for 60 minutes at 4°C, and the pellet was suspended in the same buffer and used as the microsomal fraction. Protein concentrations of microsomal fractions were measured by the Bradford method using bovine serum albumin as a standard (Bradford, 1976). Thyroxine-glucuronide formation was determined according to the method of Kato et al. (2008) with slight...
The sample was subjected to high-performance liquid chromatography (HPLC). The mobile phase was 0.1% formic acid (A) and acetonitrile including 0.1% formic acid (B). The conditions for elution were as follows: 25% B (0–1 minute), 25–70% B (1–4 minutes), 70% B (4–10 minutes), 70–25% B (10–11 minutes). Linear gradients were used for all solvent changes. The flow rate was 0.5 mL/min. The eluent was introduced by electrospray ionization into the mass spectrometer (Xevo TQD; Waters) operating in positive ionization mode. Multiple reaction monitoring mode, using specific precursor/product ion transition, was used for quantification. The capillary and sampling cone voltages were set to 3500 and 35 V, respectively. Source and desolvation temperatures were set to 150 and 500°C, respectively, and the cone and desolvation gas flows were set to 50 and 600 L/h, respectively. The collision energy was set to 35 V. Two mass/charge (m/z) ion transitions were recorded in the multiple reaction monitoring mode: m/z 778 and 732 for thyroxine, and m/z 954 and 778 for thyroxine glucuronide. The retention times of thyroxine glucuronide and thyroxine were 7.1 and 8.9 minutes, respectively.

**HPLC Conditions.** Estradiol 3-O-glucuronidase was determined by the HPLC system with an LC-10AD pump (Shimadzu, Kyoto, Japan), an SLC-10A system controller (Shimadzu), an FP-2020 fluorescence detector (JASCO, Tokyo, Japan), an SIL-10A autosampler (Shimadzu), an SL-10A system controller (Shimadzu), and a Mightysil RP-18 GP column (4.6 × 150 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The mobile phases were 10 mM H3PO4 (10–8.9 minutes), 25% B (8.9–11 minutes). Linear gradients were used for all solvent changes. The flow rate was 1.0 mL/min. Estradiol 3-O-glucuronidase was detected with a fluorescence detector at 280-nm excitation and 310-nm emission. The retention time was 6.4 minutes.

**Western Blotting Analysis.** One hundred fifty micrograms of liver microsomes was subjected to neutral polyacrylamide gel electrophoresis (12% Bis-Tris Gel (Life Technologies) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) following the manufacturer's protocol. The membrane was blocked for 3 hours with 50 mg/mL skimmed milk in PBS and then incubated with anti-UGT1A1 antibody (sc-27415; Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted with PBS (1:200) as a primary antibody overnight. The membrane was washed with PBS three times and incubated with anti-goat secondary antibody (A5420; Sigma-Aldrich) diluted with PBS (1:10,000) for 1 hour. The bands were detected using Chemi-Lumi One Super western blotting detection reagents (Nacalai Tesque, Kyoto, Japan).

**Histologic Analysis.** The paraffin blocks of brains were sliced at a thickness of 8 μm. To maintain consistency of analysis, only the cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with H&E stain. External granule cell area and thickness of 8 μm cerebellar vermis region was used for histologic analysis.

**Rotarod Study.** Motor coordination and motor learning were assessed on an accelerating rota rod test. Mice treated with phenytoin during the perinatal period, control mice, and PTU-induced hypothyroidism mice were trained on a rota rod at a fixed speed of 6 rpm for 5 minutes. After 24 hours of training, mice were placed on a stationary rod, and acceleration was initiated. The speed of the rota rod accelerated from 6 to 20 over 5 minutes. The time to fall off the rod was measured.

**Statistical Analysis.** All data were presented as means (± S.D.). The differences in serum T4 concentration between wild-type mice and the effect of phenytoin through breast milk during the perinatal period, control mice, and PTU-induced hypothyroidism mice were trained on a rota rod at a fixed speed of 6 rpm for 5 minutes. After 24 hours of training, mice were placed on a stationary rod, and acceleration was initiated. The speed of the rota rod accelerated from 6 to 20 over 5 minutes. The time to fall off the rod was measured.
Results

UGT1A1 Activity Controls the Serum T4 Level. There is a species difference in UGT1A1 activity between wild-type mice and humans (Cai et al., 2010). To overcome the species difference in UGT1A1 activity, hUGT1 mice in which the original Ugt1 locus was disrupted and replaced with the human UGT1 locus have been developed (Fujiwara et al., 2010). Bilirubin is a specific substrate of UGT1A1. It was reported that serum bilirubin was 0.4 mg/dl in 2-day-old wild-type mice (Bortolussi et al., 2012), whereas serum bilirubin in 2-day-old hUGT1 mice was 2.5–3.0 mg/dl (Fujiwara et al., 2010). The bilirubin level in hUGT1 mice is more than 6-fold higher than the level in wild-type mice, suggesting that UGT1A1 activity in hUGT1 mice is lower than that in wild-type mice. In this study, serum free and total T4 levels in hUGT1 mice were statistically higher than those in wild-type mice (Table 2). Phenylhydrazine lyases erythrocytes, leading to a significant increase of serum bilirubin levels (Rice and Shapiro, 2008). Phenylhydrazine increased the serum bilirubin levels in hUGT1 mice from 3.8 to 20.1 mg/dl (Table 3). The serum T4 levels were similar between phenylhydrazine-treated and control mice, indicating that the difference in UGT1A1 activity, but not serum bilirubin, might be mainly controlling serum T4 level.

Effect of Phenytoin Treatments during Perinatal Period on Serum T4 Levels and T4 Metabolism-Related Gene Expression. In this study, phenytoin decreased serum T4 levels in 14-day-old hUGT1 mice (Table 4). Decreased protein binding of T4 can result in a reduced serum T4 level (Wang et al., 1998). Phenytoin significantly increased mRNA levels of UGT1A1, whereas mRNA levels of other T4-metabolizing enzymes, such as type 1 iodothyronine deiodinase, type 2 iodothyronine deiodinase, and sult1b1, did not statistically change by phenytoin (Fig. 1A). T4 glucuronidation activity toward T4 in liver microsomes of phenytoin-treated mice was higher than that in liver microsomes of control mice (Fig. 1B). Quantification of T4 glucuronidation activity in liver microsomes revealed that the exposure to phenytoin during the perinatal period increased T4 glucuronidation activity 38-fold. Salicylic acid, which is an inhibitor of T4 binding to serum proteins (Wang et al., 1999), increased the free T4 level in vitro (Fig. 1C). In contrast, phenytoin did not increase the free T4 level in vitro (Fig. 1C). These data indicated that phenytoin did not inhibit T4 binding to serum proteins but induced hepatic UGT1A1, causing the accelerated T4 metabolism.

mRNA Levels of Neurodevelopment-Related Genes in Brains of hUGT1 Mice Treated with Phenytoin during Perinatal Period. In humans and rodents, hypothyroidism impairs hippocampal function, leading to defective performance on a variety of behavioral learning tasks (Koromilas et al., 2010). Learning ability is strongly associated with hippocampus synaptogenesis (Shors, 2004). In the present study, we assessed the expression levels of synapsin I and synaptophysin, molecular markers of synaptic density (Thiel, 1993), to investigate the effect of phenytoin on synaptogenesis in the hippocampus. In PTU-induced hypothyroidism (Table 4), the mRNA expression levels of synapsin I and synaptophysin were significantly lower than those in control mice (Fig. 2A). In hUGT1 mice, phenytoin lowered mRNA expression levels of synapsin I and synaptophysin (Fig. 2B). These data indicated that the exposure to phenytoin during the perinatal period decreased the synaptogenesis in the hippocampus at the gene expression level in hUGT1 mice.

Effect of Exposure to Phenytoin during Perinatal Period on Migration of Granule Cells in hUGT1 Mice. Cerebellar lesions cause impairment of visual-motor coordination ability (Becker et al., 1990). Children exposed to phenytoin during pregnancy had a significantly lower score on a visual-motor integration test (Vanoverloop et al., 1992), suggesting that phenytoin led to impairments in the cerebellum. In the present study, to investigate the effect of phenytoin exposure during the perinatal period on cerebellum development, area of cerebellar granule cell migration was evaluated in control and phenytoin-treated mice. In the control mice, cerebellar granule cells completely migrated from the external granule layer to the internal granule layer (Fig. 3A). In contrast, cerebellar granule cells remained at the

TABLE 2
Serum T4 levels in neonatal wild-type (WT) and hUGT1 mice

<table>
<thead>
<tr>
<th></th>
<th>Free T4</th>
<th>Total T4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/dl</td>
<td>µg/dl</td>
</tr>
<tr>
<td>WT (n = 18)</td>
<td>2.89 ± 0.12</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>hUGT1 (n = 15)</td>
<td>3.04 ± 0.03*</td>
<td>8.1 ± 1.4*</td>
</tr>
</tbody>
</table>

*P < 0.05.

TABLE 3
Bilirubin and serum-free T4 levels in phenylhydrazine-treated hUGT1 mice

<table>
<thead>
<tr>
<th></th>
<th>Bilirubin</th>
<th>Free T4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td>µg/dl</td>
</tr>
<tr>
<td>Control</td>
<td>3.8 ± 4.4</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>20.1 ± 4.8*</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05.

TABLE 4
Effects of perinatal or postnatal chemical treatments on serum T4 levels

<table>
<thead>
<tr>
<th></th>
<th>Number of Animals</th>
<th>Free T4 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>8</td>
<td>1.4 ± 0.4**</td>
</tr>
<tr>
<td>PTU</td>
<td>4</td>
<td>0.6 ± 0.0**</td>
</tr>
<tr>
<td>Phenytoin (postnatal)</td>
<td>7</td>
<td>1.6 ± 0.8*</td>
</tr>
<tr>
<td>Phenytoin (postnatal) + T4</td>
<td>6</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Carbamazepine (postnatal)</td>
<td>4</td>
<td>2.4 ± 0.1**</td>
</tr>
<tr>
<td>PCN (postnatal)</td>
<td>4</td>
<td>1.5 ± 0.2**</td>
</tr>
<tr>
<td>PHT (through breast milk)</td>
<td>5</td>
<td>2.5 ± 0.2*</td>
</tr>
</tbody>
</table>

PHT, phenytoin.

*P < 0.05; **P < 0.01.
external granule layer in the phenytoin-treated mice (Fig. 3B). The phenytoin-induced delay in the migration of cerebellar granule cells was reproducible \((n = 6)\) (Fig. 3, C and D). The quantification of external granule cell area at the external granule layer showed that the external granule cell area in the phenytoin-treated mice increased 5.4-fold compared with that in the control mice (Fig. 3E). These results suggest that the exposure to phenytoin during the perinatal period delays cerebellum development at the morphology level.

**Effect of Exposure to Phenytoin during Perinatal Period on Rotarod Performance in hUGT1 Mice.** In humans and rodents, hypothyroidism led to impaired performance on motor coordination and behavioral learning tasks (Koibuchi and Chin, 2000; Koromilas et al., 2010). In the present study, the effect of exposure to phenytoin during the perinatal period on behavioral phenotypes was analyzed on a rotarod using Kaplan-Meier analysis (Fig. 3F). None of the control mice fell off the rod for the first 60 seconds. One of the 16 tested control mice fell off the rotarod during the next 60 seconds. In contrast, two of the seven phenytoin-treated mice fell off the rotarod in the first 60 seconds. Three of five PTU-treated mice fell off the rotarod in the first 60 seconds. A log-rank test for trend revealed that the rotarod performance of PTU-treated mice and phenytoin-treated mice was lower than that in control mice \((P < 0.001)\), indicating that exposure to phenytoin during the perinatal period impaired motor activity and motor learning ability, similar to hypothyroidism.

**Effects of Postnatal Phenytoin Treatment on UGT1A1 Activity and Rotarod Performance in Wild-Type Mice.** To investigate the effect of phenytoin treatment during the neonatal period in wild-type mice, we subcutaneously administered phenytoin to wild-type mice from postnatal day 1 to day 14. Microsomal UGT1A1 activity was increased 2-fold by phenytoin in wild-type mice (Supplemental Fig. 1A). All of the phenytoin-treated wild-type mice fell off the rotarod within 270 seconds (Supplemental Fig. 1B). Meanwhile, two out of 10 untreated wild-type mice did not fall off the rotarod for
migration of cerebellar granule cells in these mice. The 14 and assessed the synaptogenesis in the hippocampus and period on neurodevelopment, we subcutaneously adminis-
ment in mice (Fig. 3F). The expression levels of synapsin I and synaptophysin in the hippocampus were decreased by phenytoin (Fig. 4D). Furthermore, the delay in the migration of external granule cells was observed in hUGT1 mice exposed to phenytoin during the postnatal period (Fig. 4, E and F). The quantification of the external granule cell area showed that exposure to phenytoin during the postnatal period increased the external granule cell area 2.5-fold. These data showed that exposure to phenytoin, especially during the postnatal period, delayed neurodevelopment in hUGT1 mice. In terms of brain develop-
ment, the postnatal period in mice corresponds to the third trimester and postnatal period in humans (Zoeller and Rovet, 2004), suggesting that the administration of phenytoin during the third trimester and postnatal period can increase the risk for neurodevelopmental disorder in children of humans.

Effect of T4 Replacement on Phenytoin-Induced Neurodevelopmental Toxicity in hUGT1 Mice. As phenytoin functions in the brain as an anticonvulsant drug, phenytoin might have directly inhibited neurodevelopment without T4 reduction. We next investigated whether T4 replacement could attenuate neurodevelopmental disorder induced by phenytoin exposure during the postnatal period in hUGT1 mice. Phenytoin and T4 were simultaneously administered to hUGT1 mice daily from postnatal day 1 to day 14. The T4 replacement (100 μg/kg/day) increased the serum T4 level to the same level as control (Table 4). T4 replacement increased the mRNA expression levels of synapsin I and synaptophysin to the same levels as those in control mice (Fig. 4D). T4 replacement accelerated the migration of the external granule cells (Fig. 4, E and F). T4 replacement attenuated the neurodevelopmental disorder induced by phenytoin exposure during the postnatal period in hUGT1 mice, indicating that reduced T4 was tightly associated with the neurodevelopmental disorder induced by phenytoin.

Effect of Carbamazepine and PCN on Serum Free T4 Level and Neurodevelopmental Toxicity in hUGT1 Mice. Phenytoin induces UGT1A1 through CAR (Smith et al., 2005). Activation of CAR during the postnatal period by phenytoin might have induced the neurodevelopmental disorder. Treatments of hUGT1 mice with carbamazepine, another CAR activator, decreased serum T4 levels (Table 4). The mRNA level of UGT1A1 was induced 200-fold by the carbamazepine treatment (Fig. 5A). UGT1A1 glucuronidation activity was increased 2-fold by carbamazepine (Supplemental Fig. 2). Carbamazepine significantly decreased the expression levels of synapsin I and synaptophysin in the hippocampus (Fig. 5B). The delay in the migration of external granule cells was observed in hUGT1 mice exposed to carbamazepine (Fig. 5C). PCN, a PXR ligand, is an inducer

more than 300 seconds. Although phenytoin induced UGT1A1 activity and neurodevelopmental toxicity in both hUGT1 mice and wild-type mice, the effect was more significant in hUGT1 mice (Fig. 3F).

Effect of Exposure to Phenytoin during Postnatal Period on Serum Free T4 Levels and Neurodevelopment in hUGT1 Mice. To investigate the effect of the exposure of neonates to phenytoin during only the postnatal period on neurodevelopment, we subcutaneously adminis-
tered phenytoin to hUGT1 mice from postnatal day 1 to day 14 and assessed the synaptogenesis in the hippocampus and migration of cerebellar granule cells in these mice. The exposure of hUGT1 mice to phenytoin during the postnatal period decreased serum T4 levels (Table 4). hUGT1 mice exposed to phenytoin during only the postnatal period showed an induction of UGT1A1 mRNA (Fig. 4A) similar to the mice exposed to phenytoin during the perinatal period (Fig. 1A). It was reported that mRNA levels for UGT1A1 might not correlate with protein levels (Ohtsuki et al., 2012). However, western blot analysis of liver microsomes showed that phe-
nytoin treatment during the neonatal period induced UGT1A1 protein in the liver (Fig. 4B), which was in agreement with the finding that UGT1A1 mRNA level was increased by phenytoin treatment (Fig. 4A). It was further found that UGT1A1 activity was increased 3.5-fold by phenytoin treatment (Fig. 4C).

Fig. 3. Effect of exposure to phenytoin during the perinatal period on migration of granule cells and rotarod performance in hUGT1 mice. Dams and their pups were treated with phenytoin daily from gestation day 12, 13, or 14 to postnatal day 0 (p.o.) and from postnatal day 1 to day 14 (s.c.). On postnatal days 14, the midsagittal sections of the cerebellum vermis in control mice (A and C) and phenytoin (PHT)-treated mice (B and D) were stained with hematoxylin and eosin. (E) External granule cell area was measured within 50 μm in the horizontal direction of the external granule cell layer from randomly selected fields (n = 6). Prior to the rotarod study, control mice, phenytoin-treated mice, and PTU-induced hypothyroidism mice were trained on a rotarod at a fixed speed of 6 rpm for 5 minutes. In the rotarod study, the speed of the rotarod was accelerated from 6 to 20 over 5 minutes. The time to fall off the rod was measured. (F) The difference in rotarod performance between control mice (solid line, n = 15), phenytoin-treated mice (dashed line, n = 7), and PTU-treated mice (dotted line, n = 5) was analyzed by the Kaplan-Meier method and the log-rank test for trend. Error bars show the S.D. of biologic replicates. ***P < 0.001 compared with control. EG, external granule layer; IG, internal granule layer; ML, molecular layer.
of UGT1A1. Although Usui et al. (2006) reported that PCN did not induce UGT1A1 mRNA in HepG2 cells, Buckley and Klaassen (2009) and Wagner et al. (2005) showed that hepatic Ugt1a1 mRNA was induced 1.5-fold by PCN in mice. Treatments of hUGT1A1 mice with PCN decreased serum T4 levels (Table 4). In the present study, mRNA level of UGT1A1 was induced 10,000-fold by postnatal PCN treatment (Fig. 5A). UGT1A1 glucuronidation activity was increased 2.5-fold by PCN (Supplemental Fig. 2). PCN significantly decreased the expression levels of synapsin I and synaptophysin in the hippocampus (Fig. 5B). The delay in the migration of external granule cells was observed in hUGT1A1 mice exposed to PCN (Fig. 5C). The quantification of external granule cell area showed that carbamazepine and PCN increased the external granule cell area 5.1-fold and 16-fold, respectively (Fig. 5D). Not only CAR activation but also PXR activation led to neurodevelopmental disorder, indicating that UGT1A1 induction was the factor causing neurodevelopmental disorder.

Effect of Phenytoin Treatment during the Postnatal Period through Breast Milk on Serum T4 Levels and Neurodevelopment in hUGT1 Mice. Phenytoin treatment has no contraindication in breast-feeding women (Bar-Oz et al., 2000). As phenytoin can be slightly secreted to breast milk, children might be exposed to up to 5% of the therapeutic doses of phenytoin by breast-feeding (Steen et al., 1982). We investigated the effect of phenytoin exposure at low concentrations through breast milk on neurodevelopment in hUGT1A1 mice by administering a therapeutic dose of phenytoin (80 mg/kg) to lactating hUGT1A1 mice. Exposure to phenytoin through breast milk in hUGT1A1 mice decreased serum T4 levels (Table 4). In hUGT1 mice exposed to phenytoin via breast
milk, the expression levels of synapsin I and synaptophysin were lower than those in control mice (Fig. 6A). Furthermore, a delay in the migration of external granule cells was observed in *hUGT1* mice exposed to phenytoin via breast milk (Fig. 6B, upper panels). In the control mice, cerebellar granule cells completely migrated from the external granule layer to the internal granule layer, whereas cerebellar granule cells remained at the external granule layer in the phenytoin-treated mice (Fig. 6B, upper panels). The phenytoin-induced delay in the migration of cerebellar granule cells was reproducible (*n* = 6) (Fig. 6B, lower panels). The quantification of external granule cell area showed that the external granule cell area in the phenytoin-treated mice increased 1.7-fold compared with those in the control mice (Fig. 6C). These data suggest that the exposure to phenytoin at low concentrations through breast milk still caused the neurodevelopmental disorder in neonatal mice.

**Discussion**

In the present study, total and free serum thyroxine levels in *hUGT1* mice were higher than those in wild-type mice on postnatal day 14 (Table 2). The UGT1A1 activity in *hUGT1* mice was lower than that of wild-type mice (Fujiwara et al., 2010), indicating that the low activity of UGT1A1 was the cause for higher T4 level in *hUGT1* mice than in wild-type mice. The *Ugt1* knockout mice were, therefore, expected to have even higher T4 levels than wild-type mice. In contrast to our expectation, a previous study reported that total serum T4 levels were similar between *Ugt1* knockout mice and wild-type mice at 5 days after birth (Nguyen et al., 2008). As it has been reported that there are age differences in regulation of serum T4 levels (Ingbar, 1976), these inconsistent findings can be explained by the difference in age of mice. In fact, free T4 levels in 2-month-old *hUGT1* mice were lower than those in 2-month-old wild-type mice, whereas free T4 levels in 6-month-old *hUGT1* mice were similar to those in wild-type mice (Supplemental Fig. 3). As the serum T4 level is controlled by multiple factors, such as T4-metabolizing enzymes, T4-binding proteins, hypothalamic-pituitary-thyroid negative feedback regulation, and age, the process of controlling serum T4 levels is highly complex. Whereas lower UGT1A1 activities slightly affected the serum T4 levels (Table 2), increased UGT1A1 activities dramatically reduced the T4 levels (Fig. 1A; Table 4). Therefore, UGT1A1 can be the determining factor of serum T4 levels, especially when strongly induced.

Since exposure to phenytoin during the postnatal period caused a reduction of T4 and neurodevelopmental disorder in *hUGT1* mice (Figs. 2B and 3, E and F; Table 4), lowered transfer of oxygen to the fetus is not a primary cause of neurodevelopmental disorder in children exposed to phenytoin during the perinatal period. As phenytoin functions in the brain as an anticonvulsant drug, phenytoin might have...
directly induced neurodevelopmental toxicity without T₄ reduction. Due to the similarity of the chemical structure between PCBs and thyrroxine (Fig. 7), it has been proposed that PCBs inhibit the binding of thyrroxine to TRs (McKinney, 1989). As the structures of phenytoin and carbamazepine are also similar to that of thyrroxine, phenytoin- and carbamazepine-induced neurodevelopmental disorder might have been caused by disruption of T₄ binding to TRs. In addition, as phenytoin activates the nuclear receptor CAR (Smith et al., 2005), activation of CAR during the postnatal period might have induced neurodevelopmental disorder. The present study showed that T₄ replacement attenuated neurodevelopmental disorder induced by phenytoin exposure during the postnatal period in hUGT1 mice (Fig. 4, D–F), indicating that the reduced T₄ was specifically associated with neurodevelopmental disorder induced by phenytoin. Not only phenytoin but also the PXR activator PCN caused neurodevelopmental disorder (Fig. 5, B–D); therefore, CAR activation was not associated with phenytoin-induced neurodevelopmental disorder. In addition, PCN is not structurally similar to thyrroxine (Fig. 7), indicating that the disruption of T₄ binding to TRs might not be associated with neurodevelopmental disorder. The present study showed that phenytoin neither inhibited binding of T₄ to T₄-binding proteins nor induced T₂-metabolizing enzymes except for UGT1A1 (Fig. 1A), supporting the fact that induction of UGT1A1 caused neurodevelopmental disorder in hUGT1 mice. Further studies demonstrated that phenytoin-induced neurodevelopmental disorder resulted from the reduced T₄ caused by the induction of UGT1A1.

An important question was whether UGT1A1 induction by phenytoin could actually induce neurodevelopmental disorder in humans. First, it was unclear whether a therapeutic dose of phenytoin could reach a concentration sufficient to induce UGT1A1 and reduce serum T₄ levels in humans. In this study, the maximum plasma concentration of phenytoin in 13-day-old hUGT1 mice treated with phenytoin (s.c. 35 mg/kg) was about 100 μM (data not shown). The effective therapeutic range of phenytoin is from 50 to 100 μM in humans (Bochner et al., 1972), indicating that the plasma concentration of phenytoin could reach a concentration sufficient to induce UGT1A1 and reduce the serum T₄ level by the administration of a therapeutic dose of phenytoin. In fact, a clinical study reported that exposure to phenytoin resulted in about 20% decrease of serum free and total T₄ levels in humans (Franklyn et al., 1984). Second, it remained unclear whether the reduced serum T₄ level could induce neurodevelopmental disorder in humans. In human infants, a normal level of serum free T₄ level ranges from 0.9 to 2.6 ng/dl (Singer et al., 2015). A case report demonstrated that an approximately 30% decrease of serum free T₄ level in infants caused neurodevelopmental disorder (Namba et al., 2008). These data suggest that the present results in hUGT1 mice are translatable to humans.

In the present study, exposure to phenytoin through breast milk at therapeutic doses caused neurodevelopmental disorder in neonatal mice (Fig. 6, A and B). These data suggest that pregnant women who take UGT1A1 inducers such as phenytoin should avoid breast-feeding after delivery so that the children do not develop neurodevelopmental disorder. Furthermore, to avoid the potential development of neurodevelopmental disorder in children, UGT1A1 inducers should not be administered to pregnant women during the third trimester. However, as withdrawal of anticonvulsant drugs and a switch to other anticonvulsant drugs can cause recurrences (Lownstein and Alldredge, 1993; Wang et al., 2013), certain pregnant women still require phenytoin and carbamazepine. In our study, T₄ replacement attenuated neurodevelopmental disorder in hUGT1 mice exposed to phenytoin (Fig. 4, D and E). Therefore, T₄ replacement therapy would attenuate neurodevelopmental disorder induced by UGT1A1 inducers during pregnancy in humans. Withdrawal of UGT1A1 inducers, T₄ replacement, and bottle-feeding can decrease the risk for neurodevelopmental disorder in humans.

References


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Authorship Contributions

Participated in research design: Kobayashi, Itou, Tukey, Fujiwara.
Conducted experiments: Hirashima, Takemoto, Sasaki, Fujiwara.
Contributed new reagents or analytic tools: Takemoto.
Performed data analysis: Hirashima, Michimae, Fujiwara.
Wrote or contributed to the writing of the manuscript: Hirashima, Tukey, Fujiwara.


