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Induction of the UDP-glucuronosyltransferase 1A1 during the perinatal period can cause neurodevelopmental toxicity

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Running title: Induction of the UGT1A1 during the perinatal period can cause neurodevelopmental toxicity

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Abbreviations: AhR, aryl hydrocarbon receptor; ANOVA, analysis of variance; CAR, *constitutive* androstane receptor; CPH, Cyclophilin B; D1, type 1 iodothyronine deiodinase; D2, type 2 iodothyronine deiodinase; D3, type 3 iodothyronine deiodinase; HE, hematoxylin and eosin; IQ, intelligent quotient; LC-MS/MS, liquid hromatography coupled with tandem mass spectrometry; PCB, Polychlorinated biphenyl; PCN, Pregnenolone-16-alpha-carbonitrile; PPARα, peroxisome proliferator-activated receptor *α*; PTU, propylthiouracil; PXR, Pregnane X receptor; Sult1b1, sulfotransferase1b1; T3, 3,5,3′-L-Triiodothyronine; T4, 3,5,3′5′-L-tetraiodothyronine; TR, thyroid hormone receptor; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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Abstract

Anticonvulsants can increase a risk of developing neurotoxicity in infants; however, the underlying mechanism has not been elucidated to date. Thyroxine (T4) plays crucial roles in the development of the central nervous system. In this study, we hypothesized that induction of UDP-glucuronosyltransferase1A1 (UGT1A1) – an enzyme involved in the metabolism of T4 – by anticonvulsants would reduce serum T4 levels and cause neurodevelopmental toxicity. An exposure of mice to phenytoin during both prenatal and postnatal period significantly induced UGT1A1 and decreased serum T4 levels on postnatal day 14. In the phenytoin-treated mice, the mRNA levels of synaptophysin and synapsin I in 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 perinatal period caused neurodevelopmental disorders. The exposure to phenytoin during only the postnatal period also caused these neurodevelopmental disorders. A T4 replacement attenuated the increase in thickness of external granule cell layer, indicting that the reduced T4 was specifically associated with the phenytoin-induced neurodevelopmental disorder. In addition, these neurodevelopmental disorders were also found in the carbamazepine- and pregnenolone-16-alpha-carbonitrile- (PCN-) treated mice. Our study indicates for the first time that UGT1A1 can control the neurodevelopment by regulating the serum T4 levels.

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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 thyroidectomy leads to impaired performance on a variety of motor and behavioral learning tasks (Hasebe et al., 2008; Davenport and Dorcey, 1972; Eayrs and Levine, 1963). In cerebellum, it has been reported that perinatal hypothyroidism induced various anatomical alterations, including delayed migration of granule cells, reduction of growth and branching of dendritic arborization of Purkinje cell, reduction of synaptogenesis between Purkinje cells and granule cell axons, delayed myelination, and changes in synaptic connection among cerebellar neurons and afferent neuronal fibers in the cerebellum in rodents (Legrand, 1979; Nicholson and Altman, 1972a; Nicholson and Altman, 1972b; Nicholson and Altman, 1972c; Legrand, 1980). These neurotoxicities in cerebellum can further cause the impaired performance on motor tasks. In 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 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′-L-Triiodothyronine (T_3) and 3,5,3′5′-L-tetraiodothyronine (T_4). As T_4 solely enters the developing brain more readily than T_3 , where thyroid hormone receptors (TRs) exist, serum T4 level is tightly associated with brain development during the perinatal period (Calvo et al., 1990). The liver is a major organ of T4 metabolism (Malik and Hodgson, 2002; Ohnhaus and Studer, 1983). Glucuronidation by UDP-glucuronosyltransferase (UGT) 1A1 is one of the main pathways of T4 metabolism (Richardson et al., 2013). UGT1A1 can be induced by various drugs, foods, and

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environmental pollutants through nuclear receptors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), Pregnane X receptor (PXR), and peroxisome proliferator-activated receptor α (PPARα) (Xu et al., 2005; Jemnitz et al., 2000). Polychlorinated biphenyl (PCB) is a potent activator of AhR (Sanderson et al., 1996), whereas phenytoin and carbamazepine are activators of CAR (Kachaylo et al., 2011; Faucette et al., 2007). 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 (Koopman-Esseboom et al., 1994; Franklyn et al., 1984).

A case control study showed that children exposed to phenytoin during fetal period had significantly lower intelligent quotient (IQ) at 4 to 8 years of age (Vanoverloop et al., 1992). Another case control study showed that children exposed to carbamazepine during fetal period also had a significantly lower IQ at 6 months to 6 years of age (Ornoy and Cohen, 1996). Several cohort studies reported that the exposure of PCB during fetal and neonatal period 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 Osinugi, 2011). However, it has been reported that the 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 the blood pressure (Goncharov et al., 2010). 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 perinatal period. As phenytoin, carbamazepine, and PCB can commonly induce UGT1A1 (Smith et al., 2005; Soars et al., 2004; Shelby and Klaassen, 2006), the lowered T4 level caused by UGT1A1 induction might

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be the cause of phenytoin-, carbamazepine-, and PCB-induced neurodevelopmental disorders. In this study, therefore, we investigated the effect of UGT1A1 induction on the neurodevelopment during the perinatal period in mice. We analyzed histological development of cerebellum and synaptogenesis in hippocampus to understand the effect of phenytoin on neurodevelopment during perinatal period. Synaptogenesis in hippocampus was examined by determinating the expression levels of synaptophysin and sinapsin 1, marker genes of synaptic density (Thiel, 1993). Development of cerebellum was examined by measuring the migration amount of granule cell in cerebellum (Rakic, 1971).

Materials and Methods

Chemicals and Reagents

UDPGA, L-thyroxine, estradiol, carbamazepine, Pregnenolone-16-alpha-carbonitrile (PCN), and alamethicin were purchased from Sigma–Aldrich (St Louis, MO, USA). PTU, phenytoin, and phenylhydrazine were purchased from Wako Pure Chemical (Osaka, Japan). Free-T4 AccuBind ELISA 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 (UGT1^{A1*28}) Ugt1$^{-/-}$ (humanized *UGT1*, *hUGT1*) mice were developed previously in a C57BL/6 background (Fujiwara et al., 2010). Wild type mice (C57BL/6NCrSlc) 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.

To investigate the neurodevelopment of mice exposed to phenytoin during 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

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mg/kg). To investigate the neurodevelopment of mice exposed to phenytoin during only neonatal period, 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 11 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) was administered to pups daily from postnatal day 1 to day 14. To develop hypothyroid models, mice were given PTU (50 ppm) in drinking water from 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 the postnatal day 15 and 16 days (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). Hippocampus were dissected on ice immediately and stored at -80°C. For histological 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 the postnatal day 14 and incubated at 4°C for 60 min to clot. Then, the blood was centrifuged at 3,000 g for 5 min. The supernatant was used as a serum sample. Free thyroxine (T4) levels were quantified using a Free-T4 AccuBind ELISA kit (Monobind, Lake Forest, CA, USA). Procedures were performed according to the manufacturer's instructions. Total serum bilirubin levels were quantified using a Bilirubinometer (B-105N, Erma, Tokyo, Japan).

Quantitative RT-PCR

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Total RNA of *hUGT1* mouse liver and hippocampus was extracted with TRIzol reagent (Life Technologies). The cDNA was synthesized from 1 μg total RNA of *hUGT1* mouse liver and hippocampus using ReverTra Ace (TOYOBO, Osaka, Japan). After the reverse transcription reaction at 37°C for 15 min and at 50°C for 5 min, the reaction mixture was incubated at 95 °C for 5 min to deactivate the reverse transcriptase. Quantitative RT-PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo), and the reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad). Primer pairs listed in Table 1 were used to detect Cyclophilin B (CPH), UGT1A1, Sulfotransferase 1b1 (Sult1b1), type 1 iodothyronine deiodinase (D1), type 2 iodothyronine deiodinase (D2), type 3 iodothyronine deiodinase (D3), Synapsin I, and Synaptophysin. After an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 95°C for 5 s, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 45 cycles. Expression levels were normalized with mouse CPH mRNA level.

Enzyme Assays

Perfused liver with 1.15% KCl was homogenized in three volumes of homogenization buffer (1.15% KCl/10 mM potassium phosphate buffer, pH 7.4). The homogenate was centrifuged at 10,000 g for 30 min at 4°C, and the supernatant was collected. The supernatant was centrifuged at 105,000 g for 60 min 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. (Kato et al., 2008) with slight modifications. Briefly, a typical incubation mixture (200 μl of total volume) contained 100 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 25 μg/ml alamethicin, 100 μM thyroxine, and 1 mg/mL liver microsomes of humanized *UGT1* mouse or 0.4 mg/mL human liver microsomes. The reaction was initiated

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by addition of 5 mM UDP-glucuronic acid (UDPGA) after a 3-min preincubation at 37°C. After incubation at 37°C for 240 min, the reaction was terminated by addition of 200 μl of cold acetonitrile. Although the incubation time (4 h) was slightly longer than the optimal condition, it was necessary to obtain a detectable peak area of T4-glucuronide in the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. After removal of the protein by centrifugation at 12,000 g for 5 min, a 10-μl portion of the sample was injected into the LC-MS/MS system. Quantification of T4 glucuronidation activity was performed by comparing the peak areas of T4 glucuronide in *hUGT1* mice liver microsomes to that in human liver microsomes and calculating by reference to the previous study (Yamanaka et al., 2007). Estradiol glucuronide formation was determined according to the previous method (Kutsuno et al., 2015), with slight modifications. In brief, a typical incubation mixture (200 μl of total volume) contained 100 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 25 μg/ml alamethicin, 50 μg/ml estradiol, and 0.4 mg/ml mouse liver microsomes or 0.2 mg/ml human liver microsomes. The reaction was initiated by addition of 2.5 mM UDPGA (final concentration) after a 3-min preincubation at 37°C. Incubation was performed for 30 min. The reaction was terminated by the addition of 200 μl of cold methanol. After removal of the protein by centrifugation at 12,000 g for 5 min, a 50-μl portion of the sample was subjected to high-performance liquid chromatography (HPLC).

T4-binding assay

A typical assay mixture (500 μl of total volume) contained pH7.4 0.15 M phosphate buffer, 2.5% serum from *hUGT1* mice, and 100 μM phenytoin or 100 μM salicylic acid. Phenytoin and salicylic acid were dissolved in ethanol, and the final concentration of ethanol in the assay tube was 0.5%. The assay mixture was incubated at 37°C for 1 h. The ultrafiltration device was rinsed with 0.15 M phosphate buffer and spun for at least 10 min at 1,500 g after rinsing to avoid dilution errors due to the presence of any remaining rinse

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solution in the device. The ultrafiltration experiments were carried out by placing a 500 μL aliquot of each test mixture into a Centrifree micropartition device (Millipore, Bedford, MA, USA) and spinning this mixture at 1,500 g for 10 min at 37°C. A 10-μl portion of filtrate was injected into the LC-MS/MS system for analysis of thyroxine content.

LC-MS/MS Analysis for Thyroxine Glucuronides

LC was performed using an Acquity UPLC H-Class system including a binary pump, an automatic sampler, and a column oven (Waters), which was equipped with a 50 mm × 2.1 mm Acquity 1.6 mm C18 column (Waters Corp, MA, USA). The column temperature was 35°C. 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 min); 25 to 70% B (1– 4 min); 70% B (4 –10 min); 70 to 25% B (10 –11 min). 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 (MRM) mode, using specific precursor/product ion transition, was employed 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 min and 8.9 min, respectively.

HPLC conditions

Estradiol 3-*O*-glucuronide was determined by the HPLC system with an LC-10AD pump (Shimadzu, Kyoto, Japan), an FP-2020 fluorescence detector (JASCO, Tokyo, Japan), a SIL-10A autosampler (Shimadzu), an SLC-10A system controller (Shimadzu), and a Mightysil

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RP-18 GP column (4.6 × 150 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The mobile phases were 10 mM H_3PO_4-methanol (45:55, v/v). The flow rate was 1.0 ml/min. Estradiol 3-*O*-glucuronide was detected with a fluorescence detector at 280-nm excitation and 310-nm emission. The retention time was 6.4 minutes.

Western blotting analysis

150 μg of liver microsomes were subjected to neutral polyacrylamide gel electrophoresis (NuPAGE) 4%–12% Bis-Tris Gel (Life Technologies, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) following the manufacurer's protocol. The membrane was blocked for 3 h with 50 mg/mL skimmed milk in phosphate-buffered saline (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) diluted with PBS (1:10,000) for 1 h. The bands were detected using Chemi-Lumi One Super Western-blotting detection reagents (Nacalai Tesque, Kyoto, Japan).

Histological analysis

The paraffin blocks of brains were sliced at a thickness of eight-micrometer. To maintain consistency of analysis, only the cerebellar vermis region was used for histological analysis. The brain sections were deparaffinized, hydrated with xylene and gradual ethanol, and then stained with hematoxylin and eosin (HE) stain. External granular cell area was measured within 50 μm in the horizontal direction of external granular cell layer from randomly selected fields using an image analysis software (Image J, Texelcraft, Tokyo, Japan).

Rotarod study

Motor coordination and motor learning were assessed on an accelerating rotarod test. Mice treated with phenytoin during perinatal period, control mice, and PTU-induced

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hypothyroidism mice were trained on rotarod at a fixed speed of 6 rpm for 5 minutes. After 24 h of training, mice were placed on a stationary rod and acceleration was initiated. The speed of the rotarod 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 (± SD). The differences in serum T4 concentration between *hUGT1* mice and wild-type mice and the effect of phenytoin during perinatal period and neonatal period through breast milk were analyzed by student's t test. The effect of T4 replacement and carbamazepine and PCN treatment during neonatal period and UGT1A1 inducibility of various UGT inducers were analyzed by analysis of variance (ANOVA) and Dunnett's procedure for multiple comparisons. Induction of UGT1A1 mRNA by carbamazepine and PCN was analyzed by student's t test with a Bonferroni correction for multiple comparisons. The difference in rotarod performance between control mice, phenytoin-treated mice, and PTU-treated mice was analyzed by the Kaplan-Meier method and the log-rank test for trend. $P < 0.05$ was considered significant.

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), while serum bilirubin in 2-day-old *hUGT1* mice was 2.5-3.0 mg/dL (Fujiwara et al.,

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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 lyses 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 not serum bilirubin, but the difference in UGT1A1 activity 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-days 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, while mRNA levels of other T4-metabolizing enzymes such as D1, D2, D3 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 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 free T4 level in vitro (Fig. 1C). In contrast, phenytoin did not increase 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

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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 sinapsin I and synaptophysin, molecular markers of synaptic density (Thiel, 1993), to investigate the effect of phenytoin on the synaptogenesis in hippocampus. In PTU-induced hypothyroidism (Table 4), the mRNA expression levels of synaptosin 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 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 caused the impairment of visual-motor coordination ability (Becker et al., 1990). Children exposed to phenytoin during the pregnancy had a significantly lower score in a visual motor integration test (Vanoverloop et al., 1992), suggesting that phenytoin lead to impairments in cerebellum. In the present study, to investigate the effect of phenytoin exposure during the perinatal period on the 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 external granular layer to internal granular layer (Fig. 3A). In contrast, cerebellar granule cells remained at external granular layer in the phenytoin-treated mice (Fig. 3B). The phenytoin-induced delay in the migration of cerebellar granular cells was reproducible (n = 6) (Fig. 3C and 3D). The quantification of external granule cell area at external granular 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.

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3E). These results suggest that the exposure to phenytoin during the perinatal period delays the cerebellum development at the morphology level.

Effect of exposure to phenytoin during perinatal period on rotarod performance in hUGT1 mice

In humans and rodents, the 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 the exposure to phenytoin during perinatal period on behavioral phenotypes was analyzed on rotarod using Kaplan-Meier analysis (Fig. 3F). None of the control mice fell off the rod for the first 60 sec. One of the 16 tested mice control mouse fell off the rotarod during the next 60 sec. In contrast, 2 of the 7 phenytoin-treated mice fell off the rotarod in the first 60 sec. Three of 5 PTU-treated mice fell off the rotarod in the first 60 sec. 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 ($P = 0.004$), indicating that the exposure to phenytoin during the perinatal period impaired motor activity and motor learning ability same as hypothyroidism.

Effects of postnatal phenytoin treatment on UGT1A1 activity and rotarod performance in wild-type mice

To investigate the effect of phenytoin treatment during 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 (Supplementary Figure 1A). All of the phenytoin-treated wild-type mice fell off the rotarod within 270 sec (Supplementary Figure 1B). Meanwhile, 2 out of 10 untreated wild-type mice did not fall off the rotarod for more than 300 sec. While 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)

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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 neonatal period on neurodevelopment, we subcutaneously administered phenytoin to *hUGT1* mice from postnatal day 1 to day 14 and assessed the synaptogenesis in hippocampus and migration of cerebellar granular cells in these mice. The exposure of *hUGT1* mice to phenytoin during postnatal period decreased serum T4 levels (Table 4). *hUGT1* mice exposed to phenytoin only during postnatal period showed a similar induction of UGT1A1 mRNA (Fig. 4A) 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 the phenytoin treatment during neonatal period induced UGT1A1 protein in the liver (Fig. 4B), which was in agreement with the result that UGT1A1 mRNA level was increased by the phenytoin treatment (Fig. 4A). It was further found that UGT1A1 activity was increased 3.5-fold by the phenytoin treatment (Fig. 4C). The expression levels of synapsin I and synaptophysin in 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. 4E and 4F). The quantification of external granule cell area showed that the exposure to phenytoin during postnatal period increased the external granule cell area 2.5-fold. These data showed that the exposure to phenytoin especially during the postnatal period delayed the neurodevelopment in *hUGT1* mice. In terms of brain development, the postnatal period in mice is corresponding to third trimester and postnatal period in humans (Zoeller and Rovet, 2004), suggesting that the administration of phenytoin during third trimester and postnatal period can increase the risk for neurodevelopmental disorder of children in humans.

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Effect of T4 replacement on phenytoin-induced neurodevelopmental toxicity in *hUGT1* mice

As phenytoin functions in brain as an anti-convulsant drug, phenytoin might have directly inhibited neurodevelopment without T4 reduction. We next investigated whether T4 replacement could attenuate the 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 serum T4 level to the same level as control (Table 4). The T4 replacement increased the mRNA expression levels of synapsin I and synaptophysin to the same level as those in control mice (Fig. 4D). The T4 replacement accelerated the migration of the external granule cells (Fig. 4E and 4F). 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). mRNA level of UGT1A1 was induced 200-fold by the carbamazepine treatment (Fig. 5A). UGT1A1 glucuronidation activity was increased 2-fold by carbamazepine (Supplementary Figure 2). Carbamazepine significantly decreased the expression levels of synapsin I and synaptophysin in 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 of UGT1A1. Although Usui et al. reported that

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PCN did not induce UGT1A1 mRNA in HepG2 cells (Usui et al., 2006), Buckley et al. and Wagner et al. showed that hepatic Ugt1a1 mRNA was induced 1.5-fold by PCN in mice (Buckley and Klaassen, 2009; Wagner et al., 2005). Treatments of *hUGT1* 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 (Supplementary Figure 2). PCN significantly decreased the expression levels of synapsin I and synaptophysin in hippocampus (Fig. 5B). The delay in the migration of external granule cells was observed in *hUGT1* 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 to 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 the phenytoin exposure at low concentration through breast milk on neurodevelopment in *hUGT1* mice by administering therapeutic dose of phenytoin (80 mg/kg) to lactating *hUGT1* mice. The exposure to phenytoin through breast milk in *hUGT1* 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, the 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 external granular layer to

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internal granular layer, while cerebellar granule cells remained at external granular layer in the phenytoin-treated mice (Fig. 6B upper panels). The phenytoin-induced delay in the migration of cerebellar granular cells was reproducible (n = 6) (Fig. 6B below 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 suggested that the exposure to phenytoin at low concentration 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 that in wild-type mice. The *Ugt1* knock-out 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* knock-out 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 months old *hUGT1* mice were lower than those in 2 months old wild-type mice, while free T4 levels in 6 months old *hUGT1* mice were similar to those in wild-type mice (Supplementary Figure 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 in controlling serum T4 levels is highly complex. While lowered UGT1A1 activities slightly affected the serum T4 levels (Table 2), increased UGT1A1 activities dramatically reduced the T4 levels (Table 4

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and Fig. 1A). Therefore, UGT1A1 can be the determining factor of serum T4 levels especially when strongly induced.

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

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induced neurodevelopmental disorder was resulted from the reduced T4 caused by the induction of UGT1A1.

An important question was whether UGT1A1 induction by phenytoin could actually induce the neurodevelopmental disorder in humans. First, it was unclear whether therapeutic dose of phenytoin could reach a concentration sufficient to induce UGT1A1 and reduce serum T4 level 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 μM to 100 μM in humans (Bochner et al., 1972), indicating that the plasma concentration of phenytoin can reach a concentration sufficient to induce UGT1A1 and reduce serum T4 level by the administration of 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). Secondly, it remained unclear whether the reduced serum T4 level could induce neurodevelopmental disorder in humans. In human infants, normal level of serum free T4 level is ranging from 0.9 – 2.6 ng/dL (Singer et al., 2012). A case report demonstrated that approximately 30% decrease of serum free T4 level in infants caused the neurodevelopmental disorder (Namba et al., 2008). These data suggest that the present results in *hUGT1* mice are translatable to humans.

In the present study, the exposure to phenytoin through breast milk at therapeutic doses caused the neurodevelopmental disorder in neonatal mice (Fig. 6A and 6B). These data suggested that pregnant women who take UGT1A1 inducers such as phenytoin should avoid breast-feed after delivery, so that the children would 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 switch to other anticonvulsant

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drugs can cause recurrences (Lowenstein and Alldredge, 1993; Wang et al., 2013), certain pregnant women still require phenytoin and carbamazepine. In our study, T4 replacement attenuated the neurodevelopmental disorder in *hUGT1* mice exposed to phenytoin (Fig. 4D and 4E). Therefore, T4 replacement therapy would attenuate the neurodevelopmental disorder induced by UGT1A1 inducers during pregnancy in humans. Withdrawal of UGT1A1 inducers, T4 replacement, and bottle-feeding can lead to a decrease in the prevalence rate for neurodevelopmental disorder in human children.

In the present study, we demonstrated that UGT1A1 induction decreased serum T4 levels, causing neurodevelopmental disorder in *hUGT1* mice. The neurodevelopmental disorder induced by the exposure to UGT1A1 inducers during the perinatal period was attenuated by a T4 replacement therapy. Not only anticonvulsants, but also other potential UGT1A1 inducers, through breast milk during the postnatal period might have a risk of developing neurodevelopmental disorder. Withdrawal of UGT1A1 inducers, T4 replacement, and bottle-feeding can decrease the risk for neurodevelopmental disorder in humans.

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Authorship contributions

Participated in research design: Y. Koboyashi, T. Itoh, R. H. Tukey, and R. Fujiwara.

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Wrote or contributed to the writing of the manuscript: R. Hirashima, R. H. Tukey, and R. Fujiwara.

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Footnotes

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Legends for Figures

Fig. 1. Effect of phenytoin treatments during perinatal period on serum T4 levels and T4 metabolism-related gene expression

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.). Total RNA was prepared from the livers and mRNA levels of T4-metabolizing enzymes were determined in control mice (closed columns, n = 3) and phenytoin (PHT)-treated mice (open columns, n = 3) (A). Liver microsomes were prepared and UGT1A1 activity toward T4 was measured. Tandem mass spectrometric chromatograms for T4 glucuronidation in control mice and phenytoin-treated mice were shown (B). The effect of salicylic acid (SA, n = 3) and phenytoin (n = 3) on T4 binding was determined in vitro (C). Each column is the mean ± S.D. of biological replicates. *, $P < 0.05$; **, $P < 0.01$ compared to control. PHT, phenytoin; SA, salicylic acid.

Fig. 2. mRNA levels of neurodevelopment-related genes in brains of *hUGT1* mice treated with phenytoin during perinatal period

PTU was given to dams from gestation day 14 to postnatal day 14. The mRNA levels of synapsin I and synaptophysin in hippocampus were measured in control (closed column, n = 3) and PTU-treated mice (shaded column, n = 3) (A). 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 day 14, mRNA levels of synapsin I and synaptophysin in hippocampus were determined in control (closed column, n = 3) and phenytoin (PHT)-treated mice (open column, n = 3) (B). Error bars show SD of biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to control. PHT, phenytoin.

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Fig. 3. Effect of exposure to phenytoin during 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 cerebellum vermis in control mice (A and C) and phenytoin (PHT)-treated mice (B and D) were stained with hematoxylin and eosin. External granular cell area was measured within 50 μm in the horizontal direction of external granular cell layer from randomly selected fields, n = 6 (E). Prior to the rotarod study, control mice, phenytoin-treated mice, and PTU-induced hypothyroidism mice were trained on 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. 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 (F). Error bars show SD of biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to control. EG, external granular layer; ML, molecular layer; IG, internal granular layer; PHT, phenytoin.

Fig. 4. Effect of exposure to phenytoin and T4 replacement during postnatal period on serum free T4 levels and neurodevelopment in *hUGT1* mice

Phenytoin (s.c. 35 mg/kg) was administered to pups daily from postnatal day 1 to day 14 (PHT group). Phenytoin (s.c. 35 mg/kg) and T4 (s.c. 100 μg/kg) was simultaneously administered to pups daily from postnatal day 1 to day 14 (PHT+T4 group). Total RNA was prepared from the livers of control (n = 3) and phenytoin-treated mice (n = 3) and mRNA levels of UGT1A1 were determined (A). Pooled liver microsomes were prepared from control (n = 3) and phenytoin-treated (n = 2) *hUGT1* mice and western blotting against

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UGT1A1 was performed (B). UGT1A1 activity toward estradiol was measured (C). Total RNA was prepared from hippocampus and mRNA levels of synapsin I and synaptophysin were determined in control group (closed columns, n = 3), PHT group (open columns, n = 3), and PHT+T4 group (gray columns, n = 3) (D). External granular cell area was measured within 50 μm in the horizontal direction of external granular cell layer from randomly selected fields, n =3 (E). The midsagittal sections of cerebellum vermis in control, PHT, and PHT+T4 group were stained with hematoxylin and eosin (F). Error bars show SD of biological replicates. *, $P < 0.05$ compared to control group. #, $P < 0.05$ compared to PHT group. PHT, phenytoin.

Fig. 5. Effect of exposure to carbamazepine and PCN during postnatal period on serum free T4 levels and neurodevelopment in *hUGT1* mice

Carbamazepine (CBZ, s.c. 35 mg/kg) and PCN (s.c. 10 mg/kg) were administered to pups daily from postnatal day 1 to day 14. Total RNA was prepared from livers of three *hUGT1* mice each and mRNA levels of UGT1A1 were determined (A). Total RNA was prepared from hippocampus and mRNA levels of synapsin I and synaptophysin in hippocampus were determined in control mice (closed columns, n = 3), CBZ-treated mice (white columns, n = 3), and PCN-treated mice (gray columns, n = 3) (B). The midsagittal sections of cerebellum vermis in control mice, CBZ-treated mice, and PCN-treated mice were stained with hematoxylin and eosin (C). External granular cell area was measured within 50 μm in the horizontal direction of external granular cell layer from randomly selected fields, n = 3 (D). Error bars show SD of biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to control. CBZ, carbamazepine.

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Fig. 6. Effect of phenytoin treatment during the postnatal period through breast milk on serum free T4 levels and neurodevelopment in *hUGT1* mice

Phenytoin (s.c. 35 mg/kg) was administered to dams daily from postnatal day 1 to day 14. Total RNA was prepared and mRNA levels of synapsin I and synaptophysin in hippocampus were determined in control (closed columns, n = 3) and phenytoin-treated mice (open columns, n = 3) (A). On postnatal days 14, the midsagittal sections of cerebellum vermis in control mice and phenytoin-treated mice were stained with hematoxylin and eosin (B). External granular cell area was measured within 50 μm in the horizontal direction of external granular cell layer from randomly selected fields, n = 6 (C). Error bars show SD of biological replicates. *, $P < 0.05$ compared to control. EG, external granular layer; ML, molecular layer; IG, internal granular layer.

Fig. 7. Chemical structures of thyroxine, phenytoin, PCBs, carbamazepine, and PCN

Chemical structures of phenytoin, PCBs, carbamazepine, and PCN were compared with that of thyroxine. Structural similarities among these compounds were indicated by spheres.

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Table 1. Sequence of primers used for quantitative RT-PCR.

Isoforms	Primers	Sequence
CPH	CPH-S	5'-CAG ACG CCA CTG TCG CTT T-3'
	CPH -AS	5'-TGT CTT TGG AAC TTT GTC TGC AA-3'
UGT1A1	UGT1A1-S	5'-CCT TGC CTC AGA ATT CCT TC-3'
	UGT1A1-AS	5'-ATT GAT CCC AAA GAG AAA ACC AC-3'
Sult1b1	Sult1b1-S	5'-TCC ATC TCA GGT CAC CAC CA-3'
	Sult1b1-AS	5'-TCT CCA AAC GTC TTC TGA GGC-3'
D1	D1-S	5′-GTT TAG CAC AAG CAA GAG GCA-3′
	D1-AS	5'-GCG CCT GCG ATT TGG TTT AG-3'
D2	D2-S	5'-GAA TCC CAT TGC CTC ACC GA-3'
	D2-AS	5'-AGG CTG CAA CAG GGT TTC TT-3'
D3	D3-S	5'-ATT GCT GTG GCT CGA ACT GA-3'
	D3-AS	5'-GAA ATG CTG GGG ACT TTC GC-3'
Synapsin I	Synapsin I-S	5'-TGC CAA CAA GAC GGA GAG TG-3'
	Synapsin I-AS	5'-TAG TGC CCC CTT TAA CGC AG -3'
Synaptophysin	Synaptophysin-S	5'-TTT GCC ATC TTC GCC TTT GC-3'
	Synaptophysin -AS	5'-GTG CAG CCT GAA TGG GTA CT-3'

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Table 2. Serum T4 levels in neonatal wild-type (WT) and *hUGT1* mice

	Free T4 (ng/dL)	Total T4 (μg/dL)
WT (n = 18)	2.89 ± 0.12	6.0 ± 0.4
hUGT1 (n = 15)	3.04 ± 0.03*	8.1 ± 1.4*

*, $P < 0.05$.

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Table 3. Bilirubin and serum free T4 levels in phenylhydrazine-treated *hUGT1* mice

	Bilirubin (mg/dL)	Free T4 (ng/dL)
Control (n = 3)	3.8 ± 4.4	1.9 ± 0.3
Phenylhydrazine (n = 3)	20.1 ± 4.8*	1.7 ± 0.1

*, $P < 0.05$.

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

	Number of animals	Free T4 (μM)
Control	15	3.0 ± 0.0
Phenytoin	8	1.4 ± 0.4**
PTU	4	0.6 ± 0.0**
Phenytoin (postnatal)	7	1.6 ± 0.8*
Phenytoin (postnatal) + T4	6	3.3 ± 0.3
Carbamazepine (postnatal)	4	2.4 ± 0.1*
PCN (postnatal)	4	1.5 ± 0.2**
PHT (through breast milk)	5	2.5 ± 0.2*

*, $P < 0.05$; **, $P < 0.01$.

Fig.1

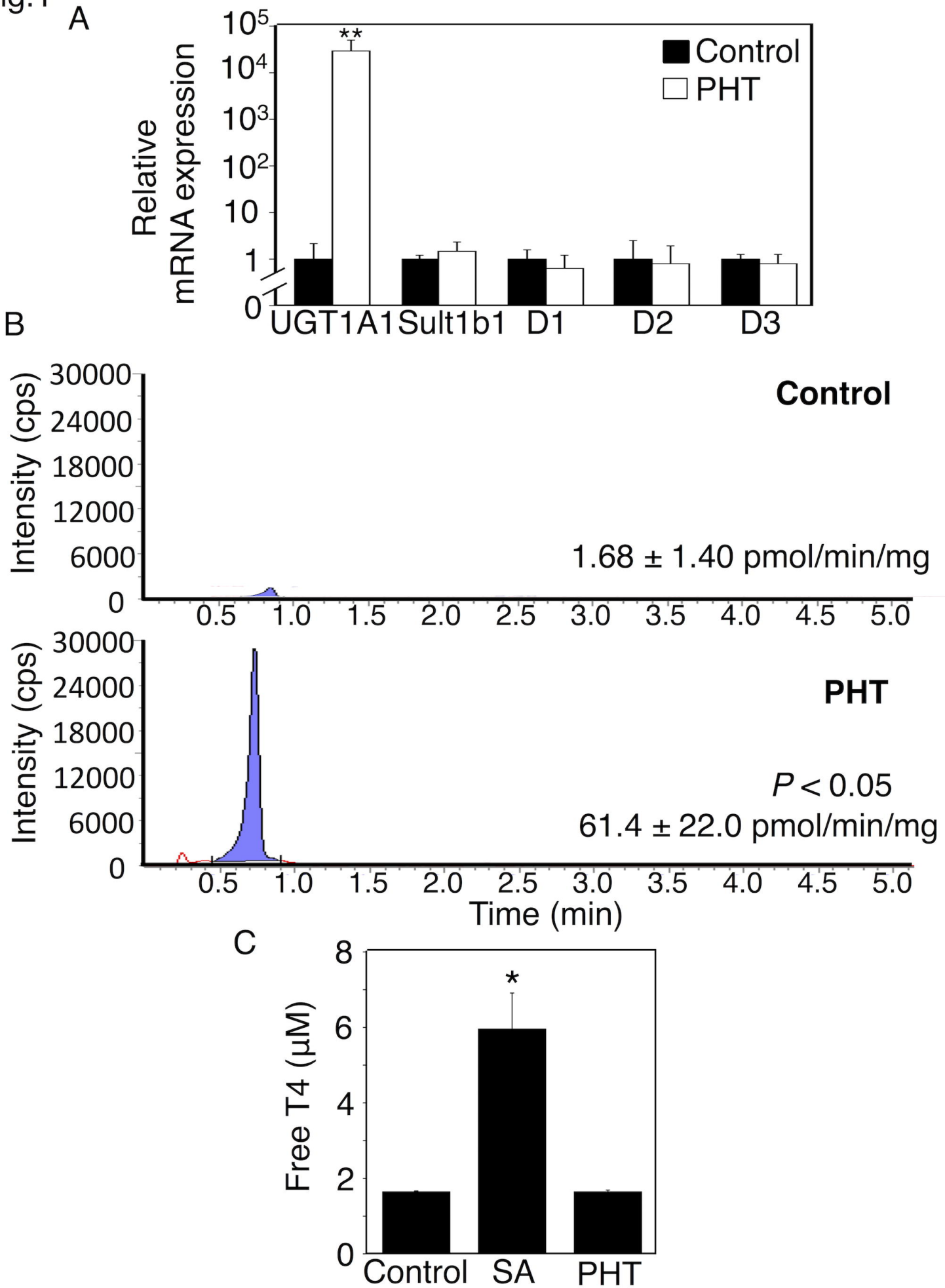
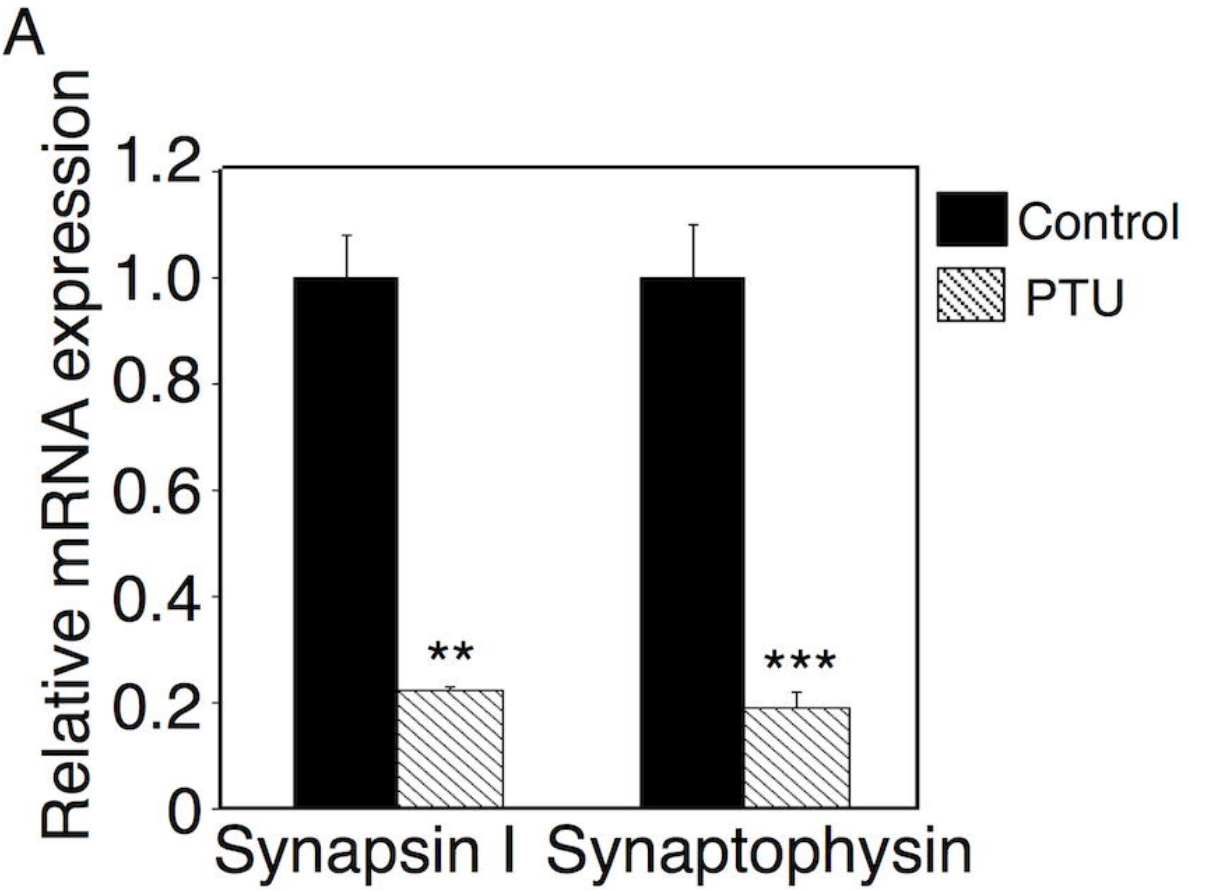

A
Relative mRNA expression
10^5
10^4
10^3
10^2
10
1
0
**
Control
PHT
UGT1A1
Sult1b1
D1
D2
D3
B
Intensity (cps)
30000
24000
18000
12000
6000
0
Control
1.68 ± 1.40 pmol/min/mg
PHT
P < 0.05
61.4 ± 22.0 pmol/min/mg
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Time (min)
C
Free T4 (μM)
8
6
4
2
0
*
Control
SA
PHT

Fig. 2


A
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
Control
PTU
Synapsin I
Synaptophysin

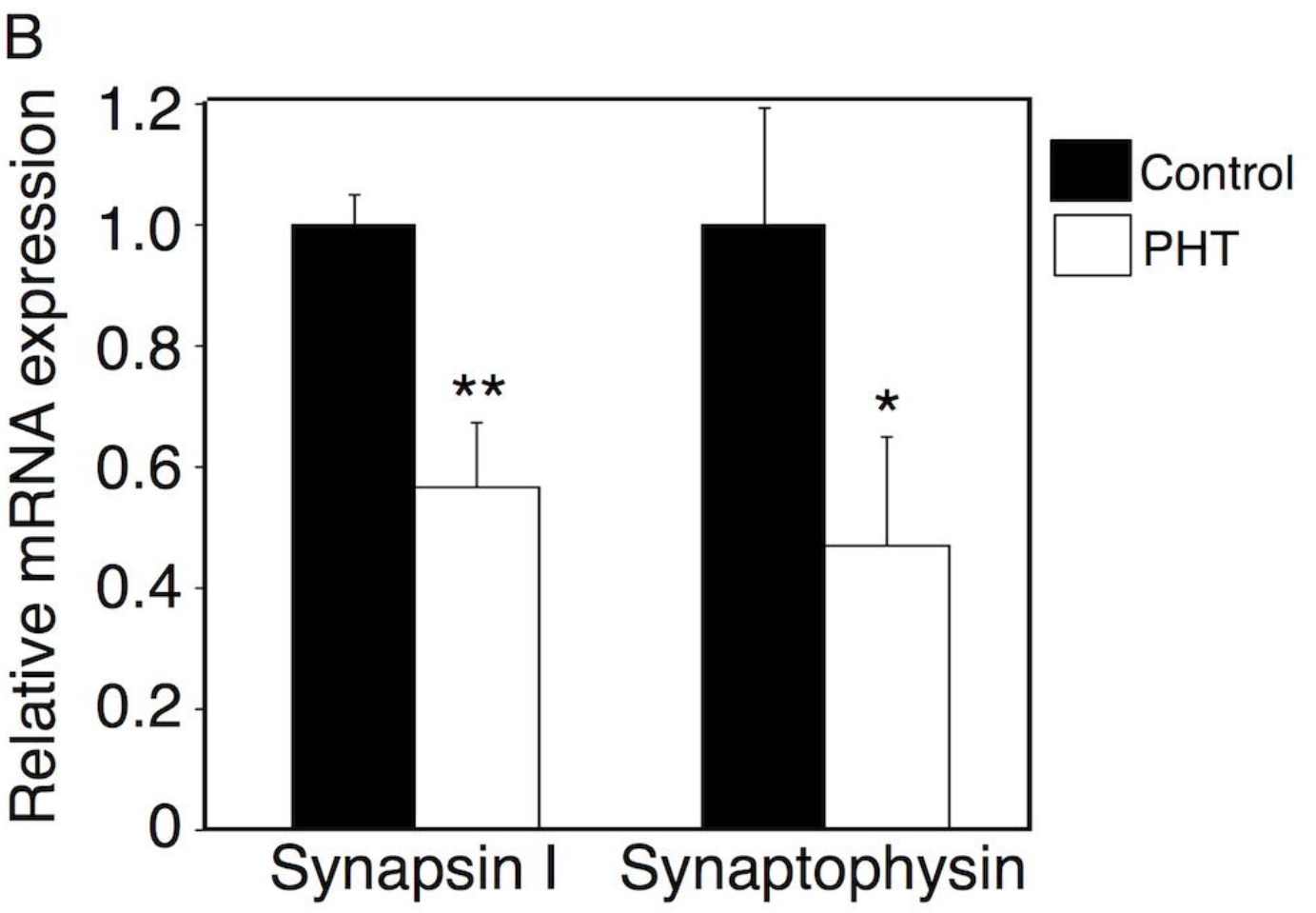
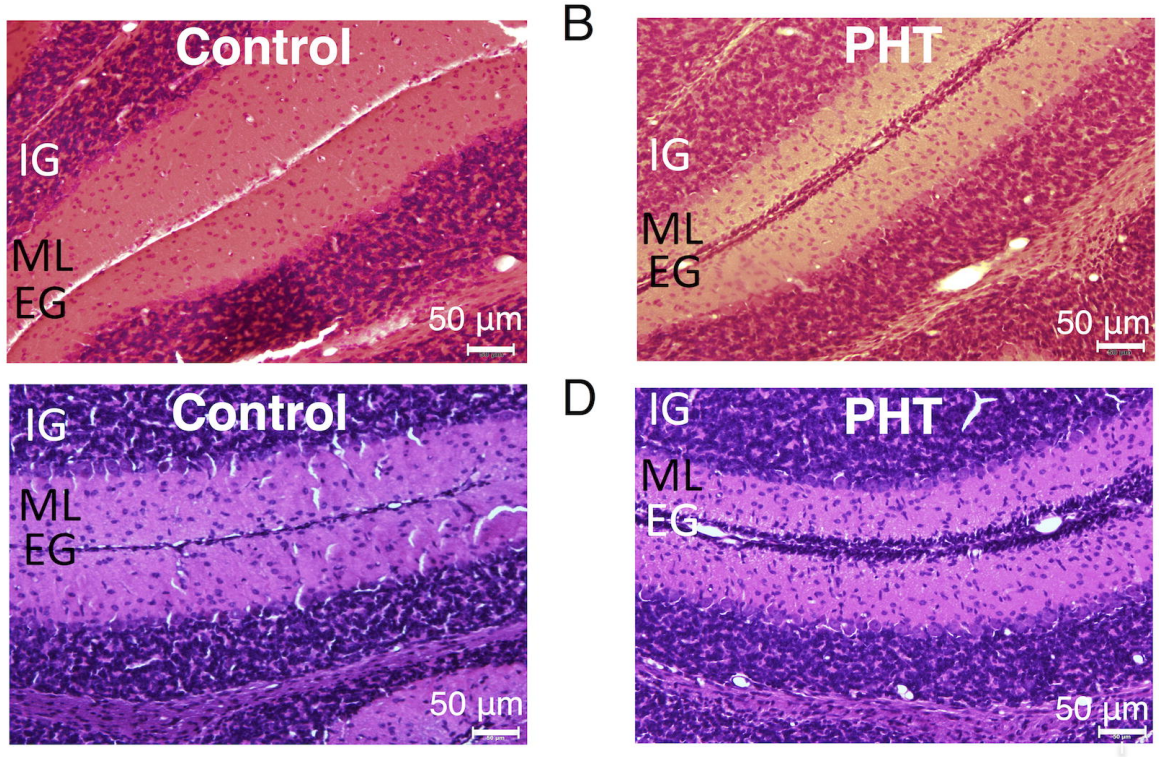
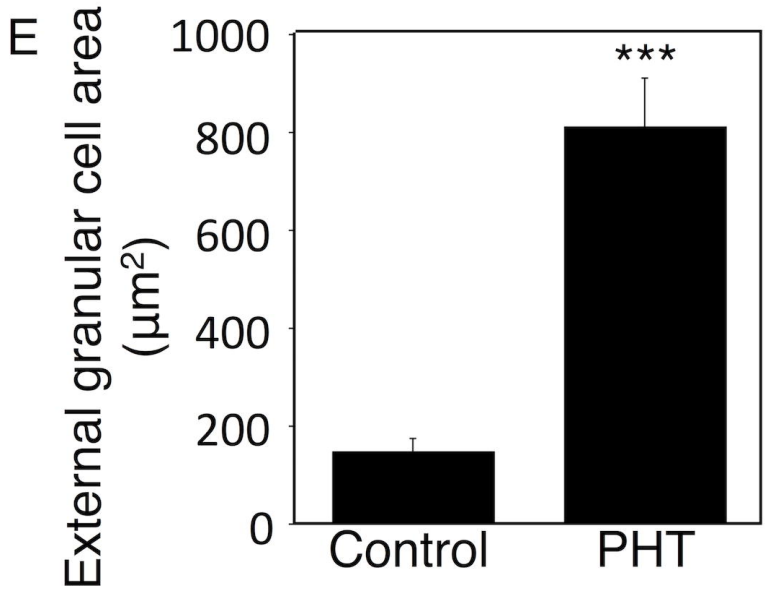

B
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
Control
PHT
Synapsin I
Synaptophysin

Fig.3


A
Control
IG
ML
EG
50 μm
B
PHT
IG
ML
EG
50 μm
C
Control
IG
ML
EG
50 μm
D
PHT
IG
ML
EG
50 μm


E
External granular cell area (μm²)
1000
800
600
400
200
0
***
Control
PHT

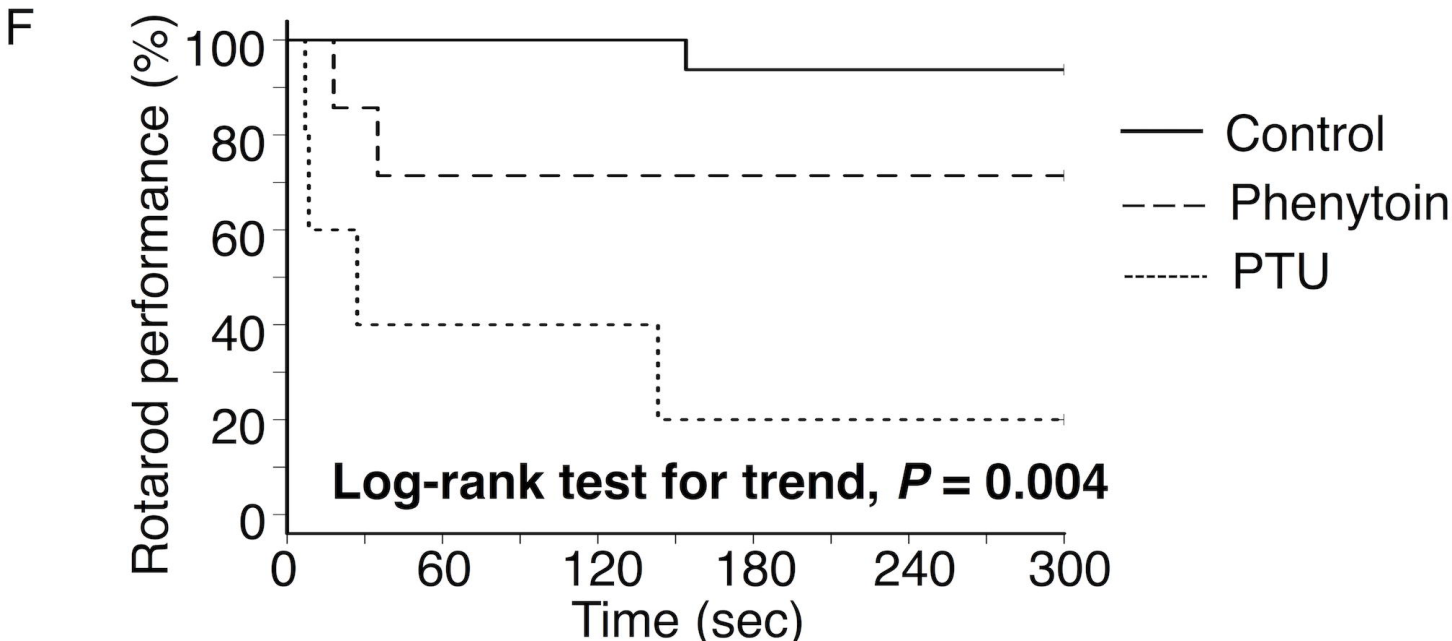

F
Rotarod performance (%)
100
80
60
40
20
0
0
60
120
180
240
300
Time (sec)
Log-rank test for trend, P = 0.004
Control
Phenytoin
PTU

Fig.4

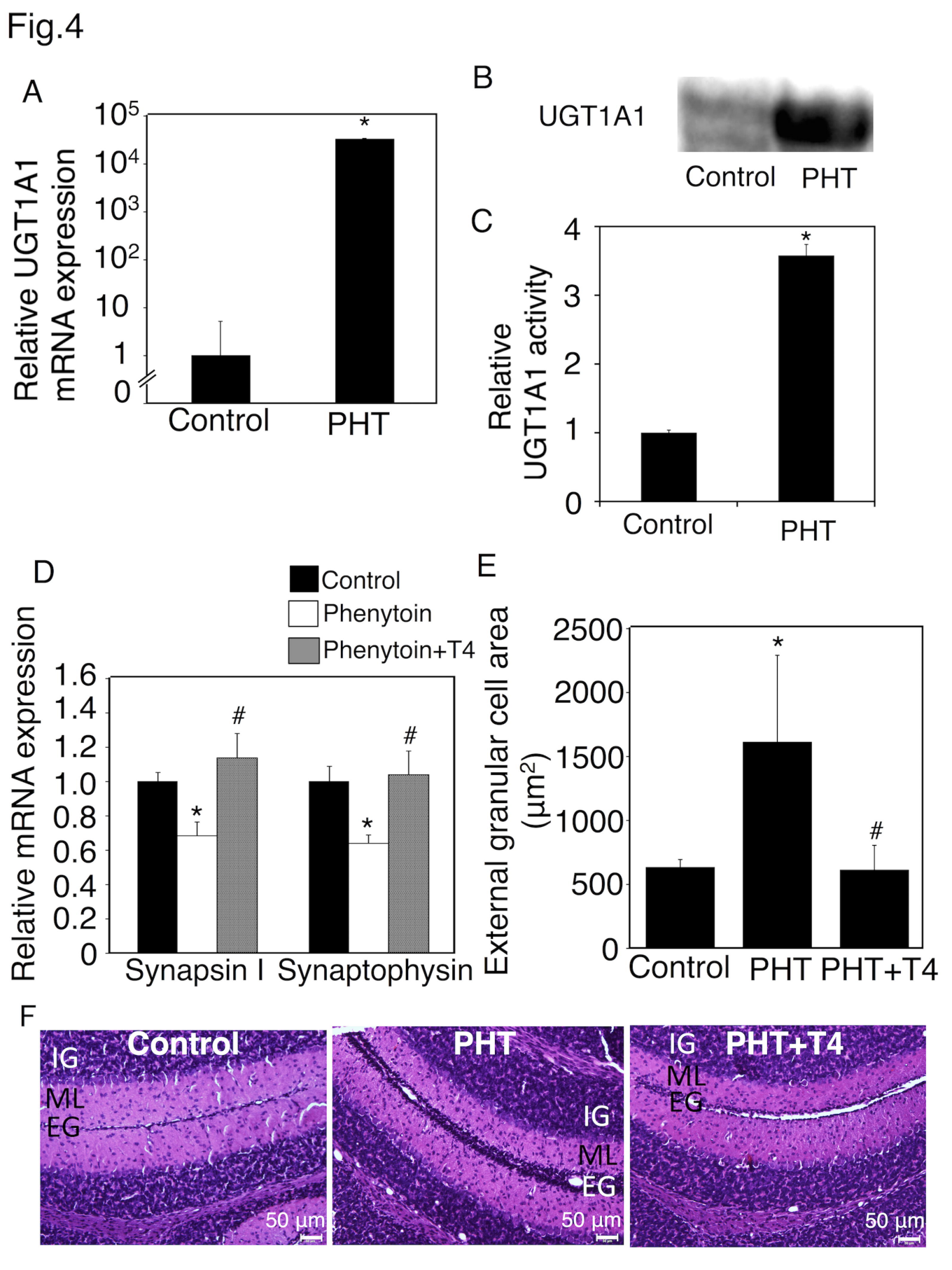

A
Relative UGT1A1 mRNA expression
10^5
10^4
10^3
10^2
10
1
0
*
Control
PHT
B
UGT1A1
Control
PHT
C
Relative UGT1A1 activity
4
3
2
1
0
*
Control
PHT
D
Control
Phenytoin
Phenytoin+T4
Relative mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
#
*
#
Synapsin I
Synaptophysin
E
External granular cell area (μm^2)
2500
2000
1500
1000
500
0
*
#
Control
PHT
PHT+T4
F
Control
IG
ML
EG
50 μm
PHT
IG
ML
EG
50 μm
PHT+T4
IG
ML
EG
50 μm

Fig.5

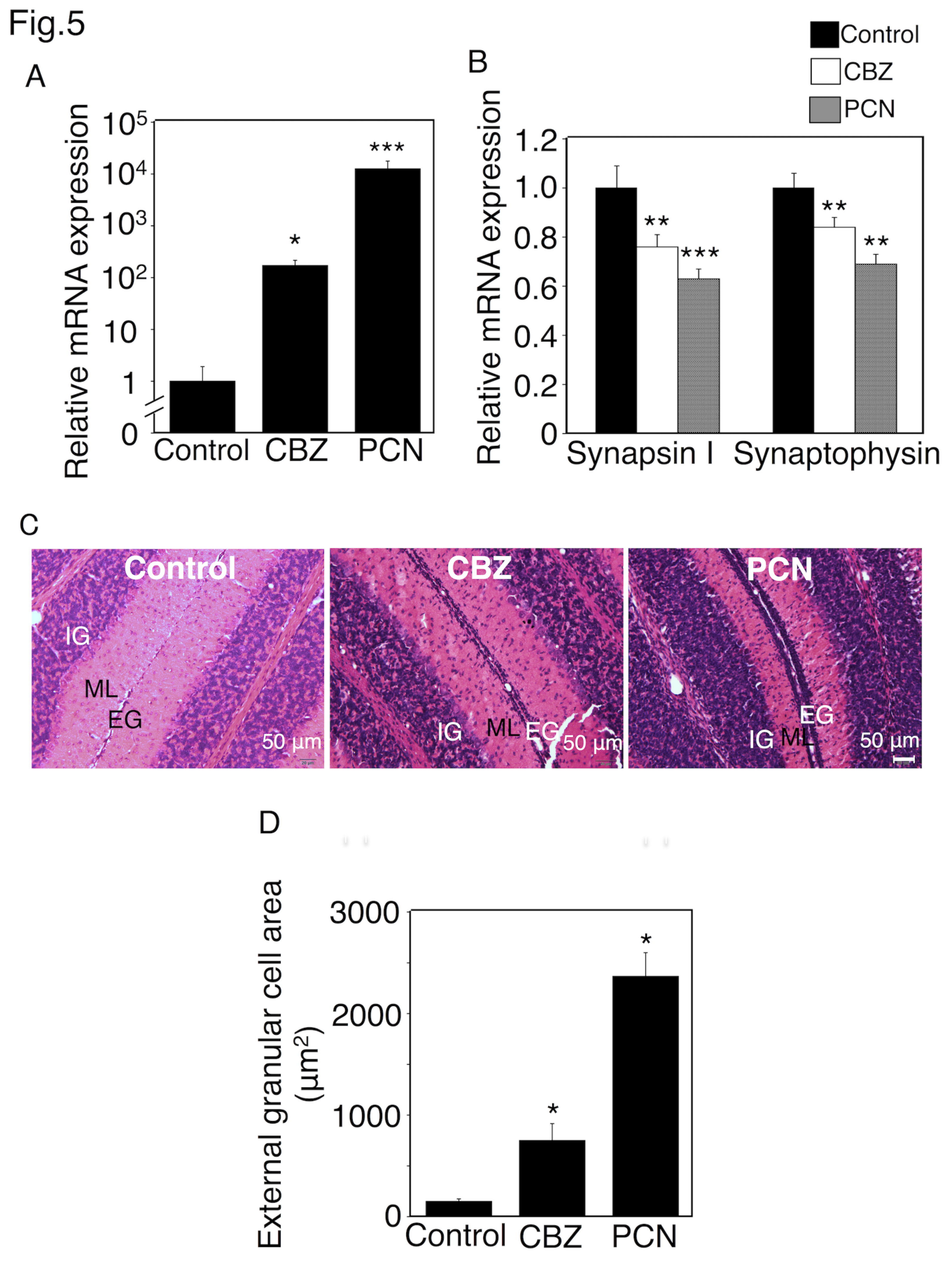

A
Relative mRNA expression
10^5
10^4
10^3
10^2
10
1
0
*
***
Control
CBZ
PCN
B
Control
CBZ
PCN
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
**
**
Synapsin I
Synaptophysin
C
Control
IG
ML
EG
50 μm
CBZ
IG
ML
EG
50 μm
PCN
EG
IG
ML
50 μm
D
External granular cell area
(μm²)
3000
2000
1000
0
*
*
Control
CBZ
PCN

Fig.6

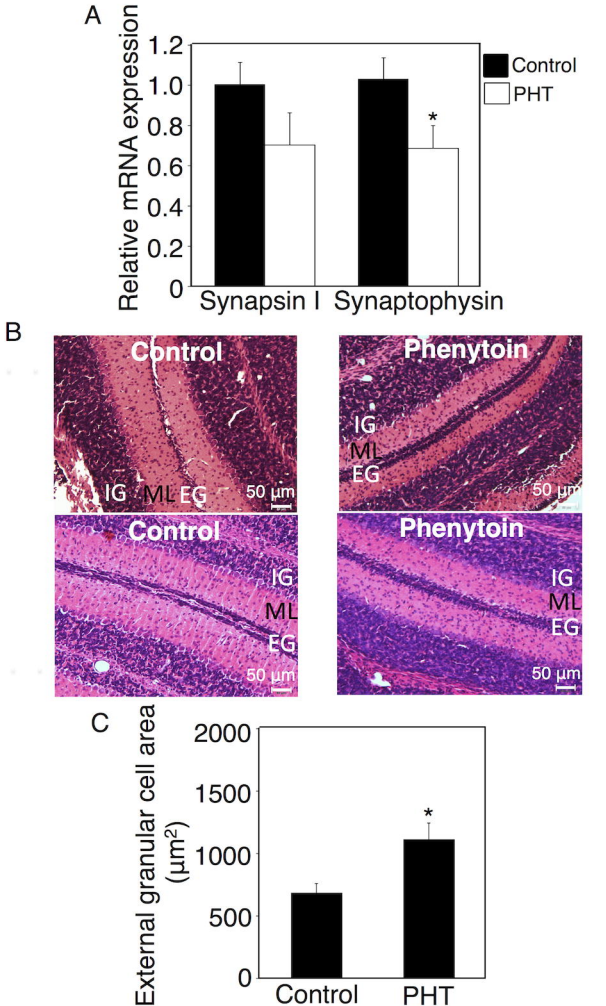
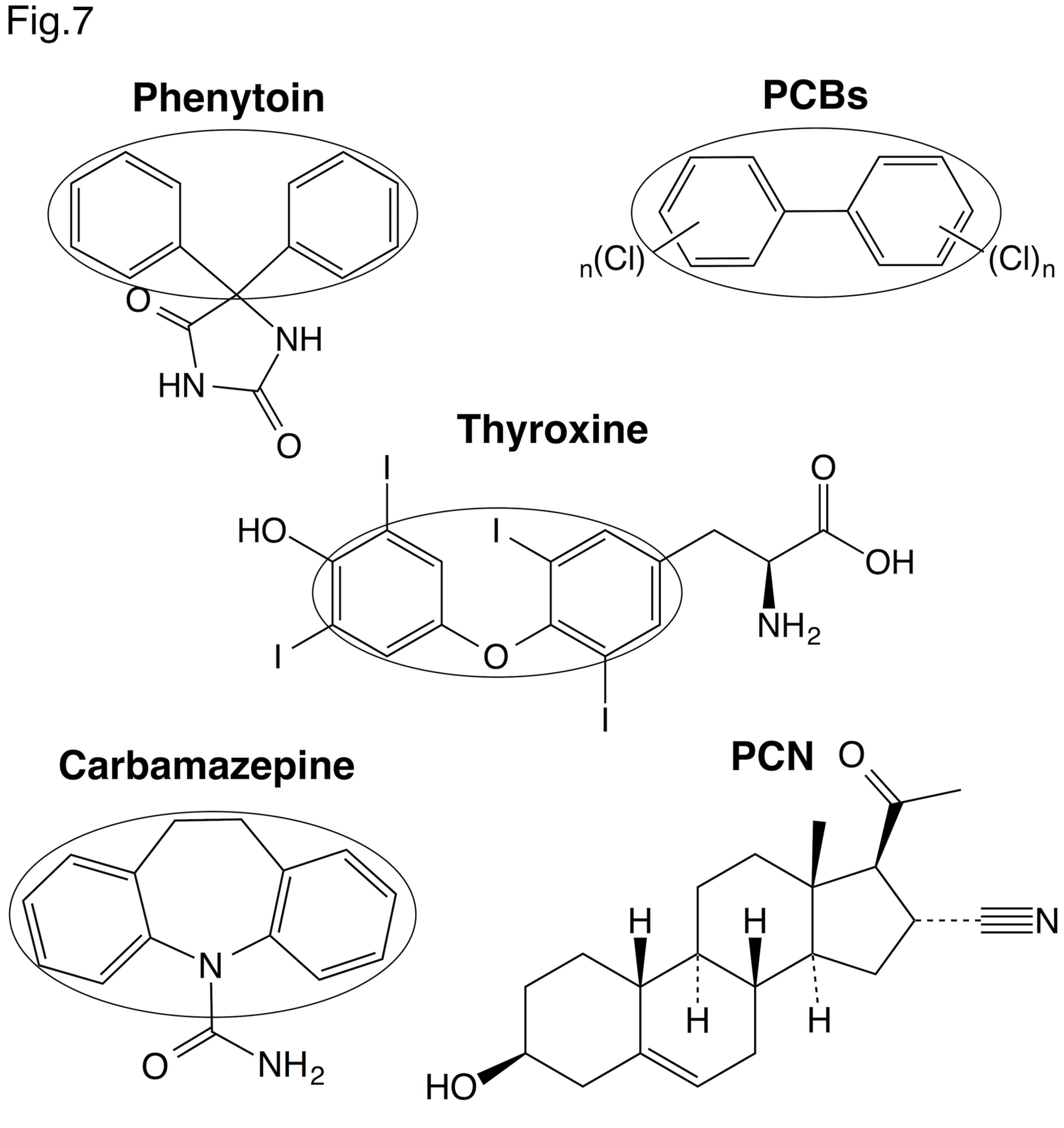

A
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
PHT
*
Synapsin I
Synaptophysin
B
Control
IG
ML
EG
50 μm
Phenytoin
IG
ML
EG
50 μm
Control
IG
ML
EG
50 μm
Phenytoin
IG
ML
EG
50 μm
C
External granular cell area (μm²)
2000
1500
1000
500
0
*
Control
PHT

Fig.7


Phenytoin
O
NH
HN
O
PCBs
n(Cl)
(Cl)n
Thyroxine
I
HO
I
O
I
I
O
OH
NH2
Carbamazepine
N
O
NH2
PCN
O
H
H
H
H
HO
N