

**Expression of *UDP-glucuronosyltransferase 1 (UGT1)* and glucuronidation activity toward  
endogenous substances in humanized *UGT1* mouse brain**

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**Running title:** Expression of brain UGT1A1 and UGT1A6 in *hUGT1* mice

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**Abbreviations:** UGT, UDP-glucuronosyltransferase; HPLC, high performance liquid chromatography; *hUGT1*, humanized *UGT1*; PBS, phosphate-buffered saline; CBZ, carbamazepine; TE, Tris-EDTA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CPH, cyclophilin; CYP, cytochrome P450; ANOVA, analysis of variance; Ct, threshold cycle; CAR, constitutive androstane receptor;

## Abstract

While UDP-glucuronosyltransferases (UGTs) are important phase II drug metabolizing enzymes, they are also involved in the metabolism of endogenous. Certain substrates of UGTs, such as serotonin and estradiol, play important roles in the brain. However, the expression of UGTs in human brain has not been fully clarified. Recently, humanized *UGT1* mice (*hUGT1* mice) in which the original *Ugt1* locus was disrupted and replaced with the human *UGT1* locus have been developed. In the present study, the expression pattern of UGT1As in brains from human and *hUGT1* mice was examined. We found that UGT1A1, 1A3, 1A6, and 1A10 were expressed in human brains. The expression pattern of UGT1As in *hUGT1* mouse brains was similar to that in human brains. In addition, we examined the expression of UGT1A1 and 1A6 in cerebellum, olfactory bulbs, midbrain, hippocampus, and cerebral cortex of *hUGT1* mice. UGT1A1 in all brain regions and UGT1A6 in cerebellum and cerebral cortex of 6 month-old *hUGT1* mice were expressed significantly higher than those of 2 week-old *hUGT1* mice. A difference in expression levels between brain regions was also observed. Brain microsomes exhibited glucuronidation activities toward estradiol and serotonin with the mean values of 0.13 pmol/min/mg and 5.17 pmol/min/mg, respectively. In conclusion, UGT1A1 and UGT1A6 might

play important role in function regulation of endogenous compounds in region- and age-dependent manner. Humanized *UGT1* mice might be useful to study the importance of brain UGTs *in vivo*.

## Introduction

UDP-glucuronosyltransferases (UGTs) catalyze glucuronidation of compounds by transferring glucuronic acid from a co-substrate, UDP-glucuronic acid (UDPGA), to substrates (Dutton et al., 1980). UGT isoforms have been identified to date belonging to 1A, 2A, 2B, 3A, and 8A subfamilies (Mackenzie et al., 2005). The human *UGT1* gene is located on chromosome 2q37 and encodes multiple unique exon 1s and common exons 2 to 5, producing nine functional UGT1A isoforms, UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (Ritter et al., 1992). Each UGT enzyme exhibits substrate specificity and is expressed in a tissue-specific manner (Tukey et al., 2000). While UGTs are important phase II drug metabolizing enzymes (Dutton et al., 1980), they are also involved in the metabolism of endogenous substances. UGT1As are involved in the metabolism of many endogenous substances such as bilirubin, estradiol, bile acids, thyroid hormone, and serotonin (de Leon, 2003).

Liver is the major organ of glucuronidations, as UGTs are mainly expressed in the liver (Court et al., 2012). However, several UGT isoforms are also expressed in extrahepatic organs such as the intestine, kidney, lung, and skin. Therefore, extrahepatic tissues can also play an

important role in glucuronidation of exogenous and endogenous substances. For example, kidney could substantially contribute to the elimination of propofol, as glucuronidation clearance of propofol in kidney is relatively comparable to that in liver (Al-Jahdari et al., 2008). Furthermore, it was recently reported that UGT1A1 expressed in the skin could be involved in glucuronidation of bilirubin and play a protective role against neonatal hyperbilirubinemia (Sumida et al., 2013). It was also recently reported that gastrointestinal UGT1A1 played a significant role in bilirubin metabolism (Aoshima et al., 2014).

In the brain, many endogenous compounds play important roles in regulation of physiological function and are implicated to be a key factor of brain disorders. Some of these endogenous compounds have been identified as substrates for UGTs. For example, serotonin, which is a specific substrate for UGT1A6 (Krishnaswamy et al., 2003), is considered to be involved in the synchronization of locomotion in cerebellum (Trouillas, 1993), memory information processing in hippocampus (Sarkisyan and Hedlund 2009), and mood regulation in cerebral cortex and hippocampus (Gross et al., 2002). UGTs have been found in the brain. In rat cerebellum and cerebral cortex, mRNA of Ugt1a1, 1a3, 1a5, 1a6, 1a8, 2a1, 2b1, 2b2, 2b3, 2b6, 2b8, and 2b12, were detected (Shelby et al., 2003). Ugt1a1 was more highly expressed in rat

cerebellum than cerebral cortex. King *et al.* demonstrated mRNA expressions of UGT1A6 and UGT2B7 in human brain by using reverse transcription polymerase chain reaction (RT-PCR) techniques. The glucuronidation activities of human brain microsomes toward morphine and naloxone, the probe substrates for UGT2B7, and 1-naphtol, which is glucuronidated by multiple UGT isoforms (Uchaipichat *et al.*, 2004), were reported (Wahlström *et al.*, 1988; Viani *et al.*, 1990). This suggests that functional UGTs were expressed in human brain. However, a complete expression pattern of human UGTs in total brain and in brain regions has not been clarified. In addition, as the number of the studies on glucuronidation activity of UGTs in the brain was limited, the expression of functional UGTs in human brain remains to be investigated.

Mice and rats are often used for studies on UGTs in the brain because of a difficulty in obtaining human brains. However, it should be noted that there are species differences in UGT expressions between rodents and humans (Shelby *et al.*, 2003; Buckley and Klaassen, 2007, Nakamura *et al.*, 2008). Recently, humanized *UGT1* mice (*hUGT1* mice) in which the original *Ugt1* locus was disrupted and replaced with the human *UGT1* locus have been developed (Fujiwara *et al.*, 2010; Fujiwara *et al.*, 2012). As UGT1A expression patterns in the liver and small intestine of *hUGT1* mice were similar to those of human (Fujiwara *et al.*, 2010; Nakamura

et al., 2008), *hUGT1* mice have been used for offering the knowledge to evaluate pharmacological and toxicological species differences in UGTs (Kutsuno et al., 2013 and 2014). In this study, therefore, the expression pattern of UGT1As in brains from human and *hUGT1* mice were examined. The expression of UGT1A1 and UGT1A6 in brain regions, such as cerebellum, olfactory bulbs, midbrain, hippocampus, and cerebral cortex, of *hUGT1* mice were further quantitatively examined. Additionally, we investigated glucuronidation activity toward estradiol and serotonin in brain microsomes of *hUGT1* mice to examine whether UGTs in brain were functional. The inducibility of UGT mRNAs and glucuronidation activities toward estradiol and serotonin in *hUGT1* mice brain was also examined.

## **Materials and Methods**

### **Chemicals and Reagents**

The 36-51 years female human total brain (containing meningioma) RNA was purchased from OriGene (Rockville, MD). UDPGA, alamethicin, estradiol, and estradiol 3-*O*-glucuronide were purchased from Sigma–Aldrich (St Louis, MO). Serotonin  $\beta$ -D-glucuronide was purchased



from Toronto Research Chemicals (Toronto, ON, Canada). Primers were commercially synthesized at Life Technologies (Carlsbad, CA). KOD-Plus-Neo was purchased from Toyobo Co., Ltd. (Osaka, Japan). 5-Hydroxytryptamine hydrochloride (serotonin) was purchased from Wako Pure Chemical (Osaka, Japan). Human liver microsomes were obtained from BD Gentest (Woburn, MA). Protease inhibitor cocktails were from Roche Diagnostics (Mannheim, Germany). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

### **Animals and tissue collection**

*Tg (UGT1<sup>A1\*28</sup>) Ugt1<sup>-/-</sup> (hUGT1)* mice were developed previously in a C57BL/6 background (Fujiwara et al., 2010). 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. Adult male and female mice were anesthetized by diethyl ether inhalation, and sacrificed by decapitation. Brains were removed and rinsed in cold phosphate-buffered saline (PBS), and were stored at -80°C. Carbamazepine (CBZ) suspended in 5% DMSO and pregnenolone-16alpha-carbonitrile (PCN) suspended in canola oil were

administrated to male *hUGT1* mice (16 weeks) for 7 consecutive days (100 mg/kg, *p.o.* for CBZ and 100mg/kg, *i.p.* for PCN). Brains and livers perfused with ice-cold 1.15% KCl were collected 24 h after the last administration and were used for the RNA extraction and preparation of microsomes. For RNA extraction of brain regions, young male mice (2 weeks) and adult male mice (6 months) were anesthetized by diethyl ether inhalation and sacrificed by decapitation. After brains were quickly removed and rinsed in PBS, brain regions (cerebellum, cerebral cortex, midbrain, hippocampus, and olfactory bulb) were dissected on ice immediately and stored at -80°C.

#### **Total RNA and Reverse Transcription (RT)**

Total RNA of *hUGT1* mouse whole brain and brain regions was extracted with TRIzol reagent (Life Technologies). The cDNA was synthesized from 1 µg total RNA of human and *hUGT1* mouse brain 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 for deactivation of the reverse transcriptase. RT mixture of human brain total RNA was diluted 10-fold in Tris-EDTA (TE) buffer.

#### **RT-PCR**

A 0.5- $\mu$ l portion of RT mixture of *hUGT1* mouse brain or the diluted cDNA of human brain was added to PCR mixtures (25  $\mu$ l). After an initial denaturation at 95°C for 2 min, the amplification was performed by denaturation at 98°C for 10 seconds, annealing at appropriate temperature for 30 seconds, and extension at 72°C for 10 seconds for 45 cycles. The sequences and annealing temperature of primers were shown in Table 1. The PCR products (20  $\mu$ l) were analyzed by electrophoresis with 2% agarose gel and visualized by ethidium bromide staining. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or cyclophilin (CPH) mRNA was used as an internal control for the cDNA quantity and quality.

### **Quantitative RT-PCR**

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 were UGT1A1-S and UGT1A-AS, UGT1A6-S and UGT1A6-AS, cytochrome P450 (Cyp) 2b10-S and Cyp2b10-AS, Cyp3a11-S and Cyp3a11-AS, and CPH-S and CPH-AS (Table 1). After an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 95°C for 5 s, annealing at appropriate temperature for 30 seconds, and extension at 72°C for

30 seconds for 45 cycles. UGT mRNA expression levels were normalized with mouse CYP1B1 mRNA level.

### **Preparation of brain microsomes**

Brain was homogenized in three volumes of homogenization buffer (1.15% KCl /100 mM potassium phosphate buffer, pH 7.4 containing protease inhibitor cocktails). The homogenate was centrifuged at  $9,000 \times g$  for 15 min at 4°C, and the supernatant was collected. The supernatant was centrifuged at  $105,000 \times g$  for 60 min at 4°C, and the pellet was suspended in a stock buffer (20% glycerol / 1.15% KCl /100 mM potassium phosphate buffer, pH 7.4) and was used as the microsomal fraction. All these procedure was performed as quickly as possible.

Protein concentrations of microsomal fractions were measured by the Bradford method using BSA as a standard (Bradford, 1976).

### **Enzyme Assays**

Estradiol and serotonin glucuronide formation was determined according to the reported method with slight modifications (Shibuya et al., 2013; Krishnaswamy et al., 2003). Briefly, a typical incubation mixture (200  $\mu$ l of total volume) contained 100 mM phosphate buffer (pH 7.4), 4 mM MgCl<sub>2</sub>, 5 mM UDPGA, 50  $\mu$ g/ml alamethicin, 1.0 mg/mL brain microsomes and

100  $\mu$ M estradiol or 10 mM serotonin. The reaction was initiated by the addition of UDPGA after a 3-min preincubation at 37°C. Incubation was performed for 12 h for estradiol 3-*O*-glucuronidation in brain microsomes, 60 min for serotonin glucuronidation, and 30 min for estradiol 3-*O*-glucuronidation in liver microsomes. The reaction was terminated by addition of 200  $\mu$ l of cold methanol for estradiol 3-*O*-glucuronidations or acetonitrile for serotonin glucuronidation. After removal of the protein by centrifugation at 12,000 g for 5 min, a 50- $\mu$ l portion of the sample was subjected to HPLC.

#### **HPLC conditions**

Glucuronides were determined by the HPLC system with a LC-10AD pump (Shimadzu, Kyoto, Japan), a FP-2020 fluorescence detector (JASCO, Tokyo Japan), a SIL-10A autosampler (Shimadzu), a SLC-10A system controller (Shimadzu) and a Mightysil RP-18 GP column (4.6  $\times$  150 mm, 5  $\mu$ m; Kanto Chemical, Tokyo, Japan). The mobile phases were 3% acetonitrile containing 20 mM phosphate potassium buffer (pH 7.4) for the serotonin glucuronide and 10 mM H<sub>3</sub>PO<sub>4</sub>-methanol (45:55, v/v) for estradiol 3-*O*-glucuronide. The flow rate was 1.0 ml/min. Glucuronides were detected with a fluorescence detector at 280 nm excitation and 310 nm emission for the estradiol 3-*O*-glucuronide and at 225 nm excitation and 330 nm emission for

the serotonin glucuronide. Quantifications of estradiol 3-*O*-glucuronide and serotonin glucuronide were carried out by comparing the HPLC peak area to those of the authentic standards. The retention times of estradiol 3-*O*-glucuronide and serotonin glucuronide were 6.4 and 3.7 min, respectively.

### **Statistical analysis**

Statistical differences of UGT1A1 and 1A6 expression levels between corresponding regions of 2 week- and 6 month-old *hUGT1* mouse brains were analyzed by a two-way analysis of variance (ANOVA) with post hoc *t*-test comparisons. Statistical differences of UGT1A1 and UGT1A6 expression levels between brain regions in 2 week- and 6 month-old *hUGT1* mice were analyzed by a two-way ANOVA with post hoc Tukey test.  $P < 0.05$  was considered significant.

## **Results**

### **mRNA expression of UGT1As in human brain and *hUGT1* mouse brain**

Although it was reported that mRNA of UGT1A6 and UGT2B7 was expressed in human brain (King et al., 1999), expressions of the other UGTs in human brain have not been clarified yet. In this study, expressions of UGT1A in human and *hUGT1* mouse brain were determined. In

human brain, UGT1A1, UGT1A3, UGT1A6, and UGT1A10 were expressed (Fig. 1A). As UGTs in brains from three different human donors were expressed similarly, large interindividual differences in expression pattern of UGT1As were not observed. In the *hUGT1* mice, UGT1A1, 1A3, 1A6, and 1A10 were expressed in the brain (Fig. 1B), which was in agreement with our previous study (Sakamoto et al., 2015). The expression pattern of UGT1As in the brain was very similar between human and *hUGT1* mice (Fig. 1A and B), indicating that *hUGT1* mice might be useful to understand the expression and function of human UGT1As in brain. As the expression patterns of UGT1As in female and male *hUGT1* mice brain were similar to each other (Fig. 1B), sex differences in expression pattern of UGT1As were not observed.

#### **Expression level of UGT1A1 and UGT1A6 in brain regions of *hUGT1* mouse**

The region- and age-dependent differences in the expression of CYP2D6 involved in the metabolism of endogenous compounds were reported (Miksys et al., 2000). However, the expression of UGTs in human brain regions has not been examined. As UGT1A1, 1A3, 1A6, and 1A10 were expressed in *hUGT1* mouse brain (Fig. 1B), we investigated the expression levels of UGT1A1, 1A3, 1A6, and 1A10 in brain regions of young (2 weeks) and adult (6 months) *hUGT1* mice. Since threshold cycle (Ct) values of UGT1A3 and UGT1A10 were high (Ct > 35), we were

not able to quantitatively analyze the expression of UGT1A3 and UGT1A10. ERV<sub>mch8</sub> (murine leukemia virus type-endogenous retrovirus mapped on chromosome 8), GATA binding protein 3 (Gata3), and serine peptidase inhibitor Kazal type 8 (Spink8) have been known to be highly expressed in cerebellum, midbrain, and hippocampus, respectively (Lee et al., 2011; Nozawa et al., 2009). It was confirmed that those marker genes were specifically expressed in the respective brain regions in our study (Supplemental Figure 1).

In this study, relative expression levels were expressed as the ratio relative to the lowest expression sample in 2 week-old and 6 month-old *hUGT1* mice (White columns in Fig. 2 and 3). The relative expression of UGT1A1 in 2 week-old *hUGT1* mice was between 1.8-6.0 in cerebellum, 1.4-15.0 in midbrain, 1.0-5.9 in olfactory bulb, 2.4-8.1 in hippocampus, and 1.3-5.7 in cerebral cortex, showing a 3.2- to 11.2-fold variability in each region. The expression level of UGT1A1 in midbrain of 2 week-old *hUGT1* mice was statistically higher than that in cerebellum, olfactory bulb, and cerebral cortex of 2 week-old *hUGT1* mice ( $P < 0.05$ , as marked by #). The relative expression of UGT1A1 in 6 month-old *hUGT1* mice was between 21.0-30.6 in cerebellum, 13.3-23.6 in midbrain, 11.1-28.9 in olfactory bulb, 12.6-24.0 in hippocampus, and 15.2-20.1 in cerebral cortex, showing a 1.3- to 2.6-fold variability in each region. UGT1A1 in the



cerebellum, midbrain, olfactory bulb, hippocampus, and cerebral cortex of 6 month-old *hUGT1* mice was expressed significantly higher than that of 2 week-old *hUGT1* mice ( $P < 0.05$ , as marked by \*).

The relative expression of UGT1A6 in 2 week-old *hUGT1* mice was between 1.2-3.6 in cerebellum, 1.0-4.0 in midbrain, and 1.3-2.3 in cerebral cortex, showing small variability in each region. The relative expression of UGT1A6 in 2 week-old *hUGT1* mice was between 1.1-6.9 in olfactory bulb and 1.0-19.2 in hippocampus, showing greater variability in each region. The relative expression of UGT1A6 in 6 month-old *hUGT1* mice was between 2.4-4.0 in hippocampus and 3.0-5.2 in cerebral cortex, showing small variability in each region. The relative expression of UGT1A6 in 6 month-old *hUGT1* mice was between 4.3-19.3 in cerebellum, 1.3-5.5 in midbrain, and 2.5-10.4 in olfactory bulb, showing 4-fold variability in each region. The expression level of UGT1A6 in cerebellum of 6 month-old *hUGT1* mice was statistically higher than that in midbrain, hippocampus, and cerebral cortex of 6 month-old *hUGT1* mice ( $P < 0.05$ , as marked by #). Although the expression levels of UGT1A6 in the midbrain, olfactory bulb, and hippocampus of 6 month-old *hUGT1* mice were similar to that of 2 week-old *hUGT1* mice, the expression levels of UGT1A6 in the cerebellum and cerebral cortex

of 6 month-old *hUGT1* mice were statistically higher than that of 2 week-old *hUGT1* mice ( $P < 0.05$ , as marked by \*).

**Effect of CBZ and PCN on mRNA expression levels of UGT1A1 and UGT1A6 in the brain and liver of *hUGT1* mice**

While CBZ can induce UGTs, CYP2B6, and CYP3A4 in the human liver through constitutive androstane receptor (CAR) (Hewitt et al., 2007), PCN can induce hepatic Ugt and Cyp3a11 through pregnane X receptor (PXR) (Buckley and Klaassen, 2007). Since it has not been reported that whether UGTs could be induced in brains, we investigated the effect of CBZ and PCN on expression levels of UGT1A1 and UGT1A6 in the brain and liver of *hUGT1* mice. CBZ and PCN significantly induced UGT1A1 in the *hUGT1* mouse liver (Fig. 4A). In the brain of *hUGT1* mice, UGT1A1 was slightly induced by CBZ, but not by PCN (Fig. 4B). UGT1A6 was not significantly induced by CBZ in the liver, while it was induced by PCN (Fig. 4C) While there was the considerable interindividual difference in expression levels of UGT1A6 in brains of *hUGT1* mice treated with CBZ, one of the CBZ-treated mice had significantly higher expression of UGT1A6 in the brain than non-treated *hUGT1* mice, although UGT1A6 was not induced in all of PCN-treated mouse brains (Fig. 4D).

The relative levels of UGT1A1 and UGT1A6 mRNA expressions in brain regions, total brain, and liver were compared (Supplemental Figure 2). We found that the expression levels of UGT1A1 and UGT1A6 in the brain were much lower than those in the liver.

CBZ also increased the expressions of Cyp2b10 and 3a11, orthologues of human CYP2B6 and 3A4, in the brain of *hUGT1* mouse (Fig.5A and 5B). While PCN induced the expression of Cyp3a11 in the liver, it did not induce the gene in the brain (Fig. 5B).

#### **Glucuronidation of estradiol and serotonin in *hUGT1* mouse brain microsomes and human liver microsomes**

To examine the expression of functional UGTs in *hUGT1* mouse brains, glucuronidation activities toward estradiol, which is a probe substrate for UGT1A1, and serotonin, which is a probe substrate for UGT1A6 (Court, 2005), were examined using brain microsomes prepared from *hUGT1* mice. Furthermore, the effect of CBZ and PCN on glucuronidation activity toward estradiol and serotonin in *hUGT1* mouse brain microsomes was also investigated in this study. Although the activity toward estradiol 3-*O*-glucuronidation was not detected in more than the half of non-treated *hUGT1* mouse brain microsomes, a few samples of brain microsomes exhibited the activity toward estradiol 3-*O*-glucuronidation (Fig. 6 left). In contrast, all of the

CBZ treated *hUGT1* mice exhibited the estradiol 3-*O*-glucuronidation activity (Fig. 6). The estradiol 3-*O*-glucuronidation activity in brain microsomes of CBZ treated *hUGT1* mice was higher than that of non-treated *hUGT1* mice with the mean values of 0.13 pmol/min/mg for brain microsomes of non-treated *hUGT1* mice and 1.52 pmol/min/mg for brain microsomes of CBZ-treated *hUGT1* mice (Fig.6). PCN treated *hUGT1* mice exhibited no estradiol 3-*O*-glucuronidation activity (Fig. 6).

Serotonin glucuronidation was detected in all but one of the non-treated *hUGT1* mouse brain microsomes (Fig. 7 left). All of the CBZ and PCN treated *hUGT1* mice exhibited the serotonin glucuronidation activity (Fig. 7). The serotonin glucuronidation activities in brain microsomes of *hUGT1* mice treated with CBZ and PCN were higher than that of non-treated *hUGT1* mice with 5.17 pmol/min/mg in non-treated *hUGT1* mouse brain microsomes, 12.7 pmol/min/mg in brain microsomes of *hUGT1* mice treated with PCN, and 31.7 pmol/min/mg in brain microsomes of *hUGT1* mice treated with CBZ.

## Discussion

CYP2D6, which is expressed in human brain, can catalyze *O*-demethylation of

5-methoxytryptamine to generate serotonin (Yu et al., 2003). CYP2D6 has many polymorphisms associated with personality traits, and neurological or psychiatric disorders. A previous study showed that female CYP2D6 ultra-rapid metabolizers were more frequent among patients with eating disorders due to the alteration of brain serotonin concentration (Peñas-Lledó et al., 2010). This study indicated that enzymes capable of catalyzing the generation and elimination of endogenous compounds possibly regulate the local concentration and function of the endogenous compounds in the brain. In the present study, UGT1A1, 1A3, 1A6, and 1A10 were detected in human brain (Fig. 1). The brain microsomes of *hUGT1* mice exhibited glucuronidation activities (Fig. 6 and 7), suggesting that the endogenous compounds that are substrate of these UGTs may be glucuronidated in human brain. Dopamine, which is highly glucuronidated by UGT1A10 (Itäaho et al., 2009), and thyroxine, which is highly glucuronidated by UGT1A1, 1A3, 1A8, and 1A10 (Tong et al., 2007), might be glucuronidated by brain UGTs. Moreover, as the region- and age- dependent differences in expression levels of brain UGT1A1 and 1A6 were observed in *hUGT1* mice (Fig. 2 and 3), UGTs in the brain may be involved in the regulation of the concentration and functions of endogenous compounds in a region- and age-dependent manner.

PCN significantly induced UGT1A1 and UGT1A6 mRNA in the liver (Fig. 4A and C). In

the brain, however, such induction was not observed (Fig. 4B and D). The glucuronidation activities of UGT1A1 and UGT1A6 were not induced by the PCN treatment (Fig. 6 and 7), which was in agreement with our quantitative RT-PCR data (Fig. 4). While PCN induced the expression of Cyp3a11 in the liver, it did not induce the gene in the brain (Fig. 5B). Previously, Lamba *et al.* reported that PXR was not expressed in the human brain regions such as cerebral cortex and cerebellum except for thalamus. These data indicate that PXR might not be functional in the brain. In contrast, CBZ-treated mice showed slightly higher UGT1A1 and UGT1A6 activities in the brain microsomes compared to the control *hUGT1* mice (Fig. 6 and Fig.7), although it was not statistically significant. This finding indicates that CAR activators such as CBZ might increase UGT expressions and activities to affect the function of endogenous compounds in brains. Developmental disorders have been reported in infants who were born from CBZ-administered mothers (Ohnoy and Cohen, 1996). Thyroxine is very important in the development of brain (Dussault and Ruel, 1987). Administered CBZ could be distributed to the infant's brain to induce brain UGTs. Therefore, the reported developmental disorders might have been attributed to a reduced brain thyroxine level, which could be caused by induced UGT functions.

A significant interindividual variability was observed in the responsiveness to a CYP3A

inducer (Fig. 5B). Previously, liver biopsies were collected fourteen patients before and after rifampicin treatment (600 mg/day for 4 days) (Ged et al., 1989). After the rifampicin treatment, there was an 18-fold difference in the induction of CYP3A4 protein. The extent of increase ranged from 160% to 2900% among these patients, indicating that there could be a significant variability in the responsiveness to CYP3A inducers.

Additional discussion can be found online (Supplemental Figure 3, Supplemental Table 1, and Supplemental Discussion).

In the present study, we demonstrated that UGT1A1, 1A3, 1A6, and 1A10 were expressed in human brains. The expression pattern of UGT1As in human brains was similar to that in *hUGT1* mouse brain, indicating that *hUGT1* mice might be useful to study the importance of brain UGTs *in vivo*. The expression levels of UGT1A1 and 1A6 in *hUGT1* mouse brains were different in region- and age-dependent manner. Therefore, UGTs might play important role in function regulation of endogenous compounds in a region- and age-dependent manner.

#### **Authorship contribution**

*Participation in research design:* Kutsuno, Itoh, Tukey, and Fujiwara

*Conducted experiments:* Kutsuno, Hirashima, Sakamoto, Ushikubo, and Fujiwara

*Performed data analysis:* Kutsuno, Hirashima, Michimae, and Fujiwara

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*Wrote or contributed to the writing the manuscript:* Kutsuno, Itoh, Tukey, and Fujiwara



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**Footnotes**

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## Figure Legend

### Figure 1. RT-PCR analysis of UGT mRNA in brains of human and *hUGT1* mouse.

Total RNA samples from three human brains containing meningioma (A) or male and female *hUGT1* mouse brains (B) were analyzed by RT-PCR using prime pairs specific for each UGT1A isoform. M, 100 bp DNA ladder marker.

### Figure 2. The expression level of UGT1A1 in *hUGT1* mouse brain regions.

Total RNA from brains of 2 week-old mice (n=5) and 6 month-old mice (n=5) were analyzed by quantitative RT-PCR. Each column is the mean  $\pm$  S.D. of three independent determinations.

Two-way ANOVA with post hoc *t* test and Tukey test was used to determine statistical significance. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared with the expression in each region of 2 week-old mice. #  $P < 0.05$ , ##  $P < 0.01$  compared between brain regions in 2 week- and 6 month-old *hUGT1* mice (two-way ANOVA with post hoc Tukey test). CB, cerebellum; MB, midbrain; OB, olfactory bulbs; HC, hippocampus; CX, cerebral cortex.

### Figure 3. The expression level of UGT1A1 in *hUGT1* mouse brain regions.

Total RNA from brains of 2 week-old *hUGT1* mice (n=5) and 6 month-old *hUGT1* mice (n=5) were analyzed by quantitative RT-PCR. Two-way ANOVA with post hoc *t* test and Tukey test was used to determine statistical significance. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with the expression in each region of 2 week-old mice (two-way ANOVA with post hoc *t* test). #  $P < 0.05$ , ##  $P < 0.01$  compared between brain regions in 2 week- and 6 month-old *hUGT1* mice (two-way ANOVA with post hoc Tukey test). CB, cerebellum; MB, midbrain; OB, olfactory bulbs; HC, hippocampus; CX, cerebral cortex.

**Figure 4. The effect of CBZ treatment on UGT1A1 expression in brains and livers in *hUGT1* mice.**

Total RNA was isolated from non treated-*hUGT1* mice (n=3) and CBZ-treated *hUGT1* mice (n=3). Complementary DNA was synthesized, and then quantitative RT-PCR was carried out for human UGT1A1 in the *hUGT1* mouse liver (A) and brain (B) and UGT1A6 in the *hUGT1* mouse liver (C) and brain (D). Each column is the mean  $\pm$  S.D. of three independent determinations.

**Figure 5. The effect of CBZ treatment on Cyp3a11 expression in brains in *hUGT1* mice.**

Total RNA was isolated from brains of non treated-*hUGT1* mice (n=3) and CBZ-treated *hUGT1* mice (n=3). Complementary DNA was synthesized, and then quantitative RT-PCR was carried out for mouse Cyp 3a11 in the *hUGT1* mouse brain. Each column is the mean  $\pm$  S.D. of three independent determinations.

**Figure 6. Effects of CBZ treatments on the UGT1A1 activity in the brain microsomes**

**of *hUGT1* mice.** Microsomes from the non-treated *hUGT1* mice (n=12) and CBZ-treated *hUGT1* mice (n=5) were prepared, and estradiol 3-*O*-glucuronide formation was measured. Open circles indicate the microsomes samples that did not exhibit glucuronidation activity toward estradiol. Filled circles indicate the microsomes samples that exhibited glucuronidation activity toward estradiol.

**Figure 7. Effects of CBZ treatments on the UGT1A6 activity in the brain microsomes**

**of *hUGT1* mice.** Microsomes from the non-treated *hUGT1* mice (n=12) and CBZ-treated *hUGT1* mice (n=5) were prepared, and serotonin glucuronide formation was measured. Open circle

indicates the microsomes samples that did not exhibit glucuronidation activity toward serotonin.

Filled circles indicate the microsomes samples that exhibited glucuronidation activity toward serotonin.

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

| Isoforms | Primers    | sequence                              | position    | Annealing temperature (C) | references             |
|----------|------------|---------------------------------------|-------------|---------------------------|------------------------|
| UGT1A1   | UGT1A1-S   | 5'-CCT TGC CTC AGA ATT CCT TC-3'      | 696 - 715   | 62                        | Nakamura et al. (2008) |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| UGT1A3   | UGT1A3-S   | 5'-TGT TGA ACA ATA TGT CTT TGG TCT-3' | 347 - 370   | 62                        | Nakamura et al. (2008) |
|          | UGT1A3-AS  | 5'-CAC AGG ACT GTC TGA GGG ATT TT-3'  | 985 - 1007  |                           | -                      |
| UGT1A4   | UGT1A4-S   | 5'-CCT GCT GTG TTT TTT TGG AGG T-3'   | 502-523     | 54                        | Izukawa et al. (2009)  |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| UGT1A5   | UGT1A5-S   | 5'-ACA ATA TGT CTT TGA TCA TA-3'      | 353 - 372   | 54                        | Nakamura et al. (2008) |
|          | UGT1A5-AS  | 5'-AGA AAC AGC ATG GCA AAG-3'         | 667 - 684   |                           | Nakamura et al. (2008) |
| UGT1A6   | UGT1A6-S   | 5'-AGA GAA TTT CTG CAG GGG TTT T-3'   | 26 - 47     | 54                        | Nakamura et al. (2008) |
|          | UGT1A6-AS  | 5'-TTG GAT TCT TTC AAA AGC-3'         | 195 - 212   |                           | Nakamura et al. (2008) |
| UGT1A7   | UGT1A7-S   | 5'-CCC CTA TTT TTT CAA AAA TGT CTT-3' | 660-683     | 54                        | Izukawa et al. (2009)  |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| UGT1A8   | UGT1A8-S   | 5'-GGT CTT CGC CAG GGG AAT AG-3'      | 498 - 517   | 62                        | Nakamura et al. (2008) |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| UGT1A9   | UGT1A9-S   | 5'-GAA CAT TTA TTA TGC CAC CG-3'      | 646 - 665   | 62                        | Nakamura et al. (2008) |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| UGT1A10  | UGT1A10-S  | 5'-CTC TTT CCT ATG TCC CCA ATG A-3'   | 557 - 578   | 62                        | Nakamura et al. (2008) |
|          | UGT1A-AS   | 5'-ATT GAT CCC AAA GAG AAA ACC AC-3'  | 907 - 929   |                           | Nakamura et al. (2008) |
| Cyp2b10  | Cyp2b10-S  | 5'-GCA AGC CAT GTT GCT CCT AA-3'      | 1405 - 1424 | 60                        | -                      |
|          | Cyp2b10-AS | 5'-CTT GGA GCC CTG GAG ATT TGG-3'     | 1604 - 1624 |                           | -                      |
| Cyp3a11  | Cyp3a11-S  | 5'-CTC AAT GGT GTG TAT ATC CCC-3'     | 1228 - 1248 | 60                        | Fujiwara et al. (2012) |
|          | Cyp3a11-AS | 5'-CCG ATG TTC TTA GAC ACT GCC-3'     | 1630 - 1650 |                           | Fujiwara et al. (2012) |
| GAPDH    | GAPDH-S    | 5'-CCA GGG CTG CTT TTA ACT C-3'       | 56 - 74     | 62                        | Nakamura et al. (2008) |
|          | GAPDH-AS   | 5'-GCT CCC CCC TGC AAA TGA-3'         | 330 - 347   |                           | Nakamura et al. (2008) |
| CPH      | CPH-S      | 5'-CAG ACG CCA CTG TCG CTT T-3'       | 5 - 23      | 62                        | Fujiwara et al. (2012) |
|          | CPH-AS     | 5'-TGT CTT TGG AAC TTT GTC TGC AA-3'  | 115 - 137   |                           | Fujiwara et al. (2012) |

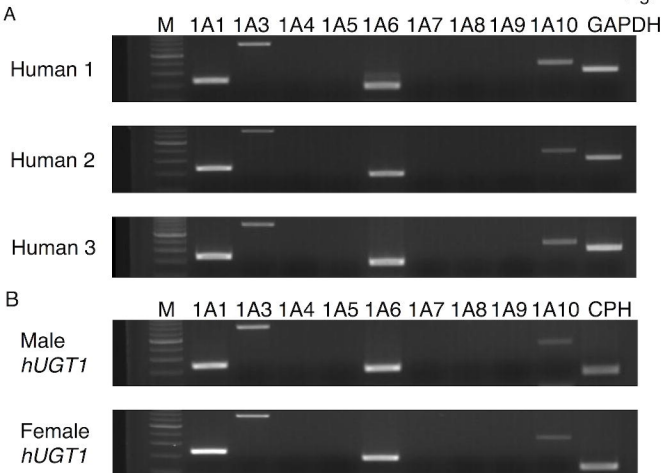


Fig. 2

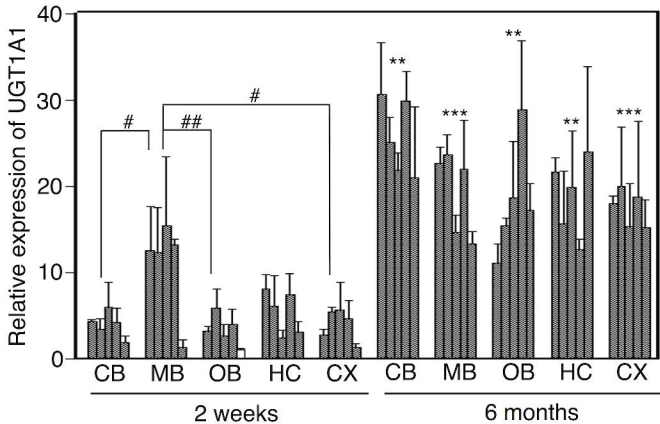
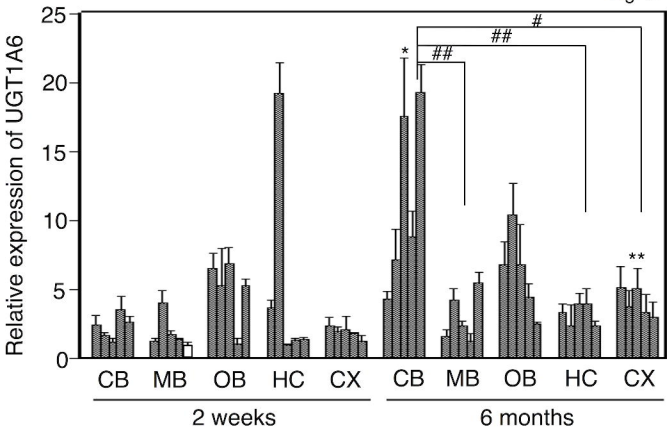




Fig. 3



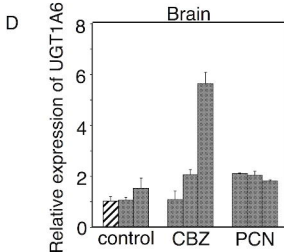
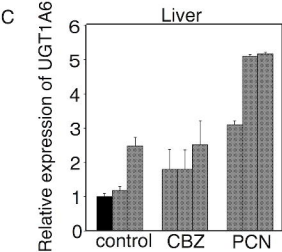
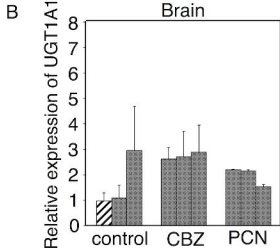
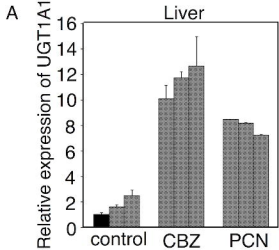
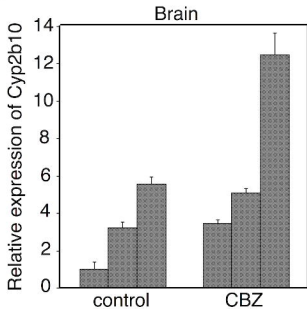


Fig. 5

A



B

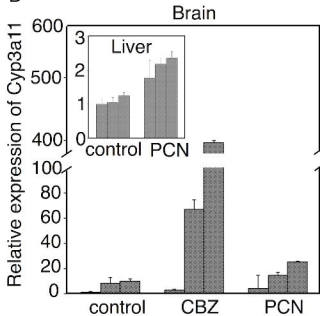




Fig. 7

