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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;

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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

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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

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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

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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

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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 β-D-glucuronide was purchased

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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^{A1*28}) Ugt1$^{-/-}$ (*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

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A 0.5-μl portion of RT mixture of *hUGT1* mouse brain or the diluted cDNA of human brain was added to PCR mixtures (25 μl). 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 µl) 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 CPH 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 × g for 15 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 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 μl of total volume) contained 100 mM phosphate buffer (pH 7.4), 4 mM $MgCl_2$, 5 mM UDPGA, 50 μg/ml alamethicin, 1.0 mg/mL brain microsomes and

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100 μM 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 μl 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-μl 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 × 150 mm, 5 μm; 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_3PO_4-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

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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

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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_{mch8} (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

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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

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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 Ugts 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).

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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

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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

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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

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 ± 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.

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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 ± S.D. of three independent determinations.

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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 ± 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.

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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. 1

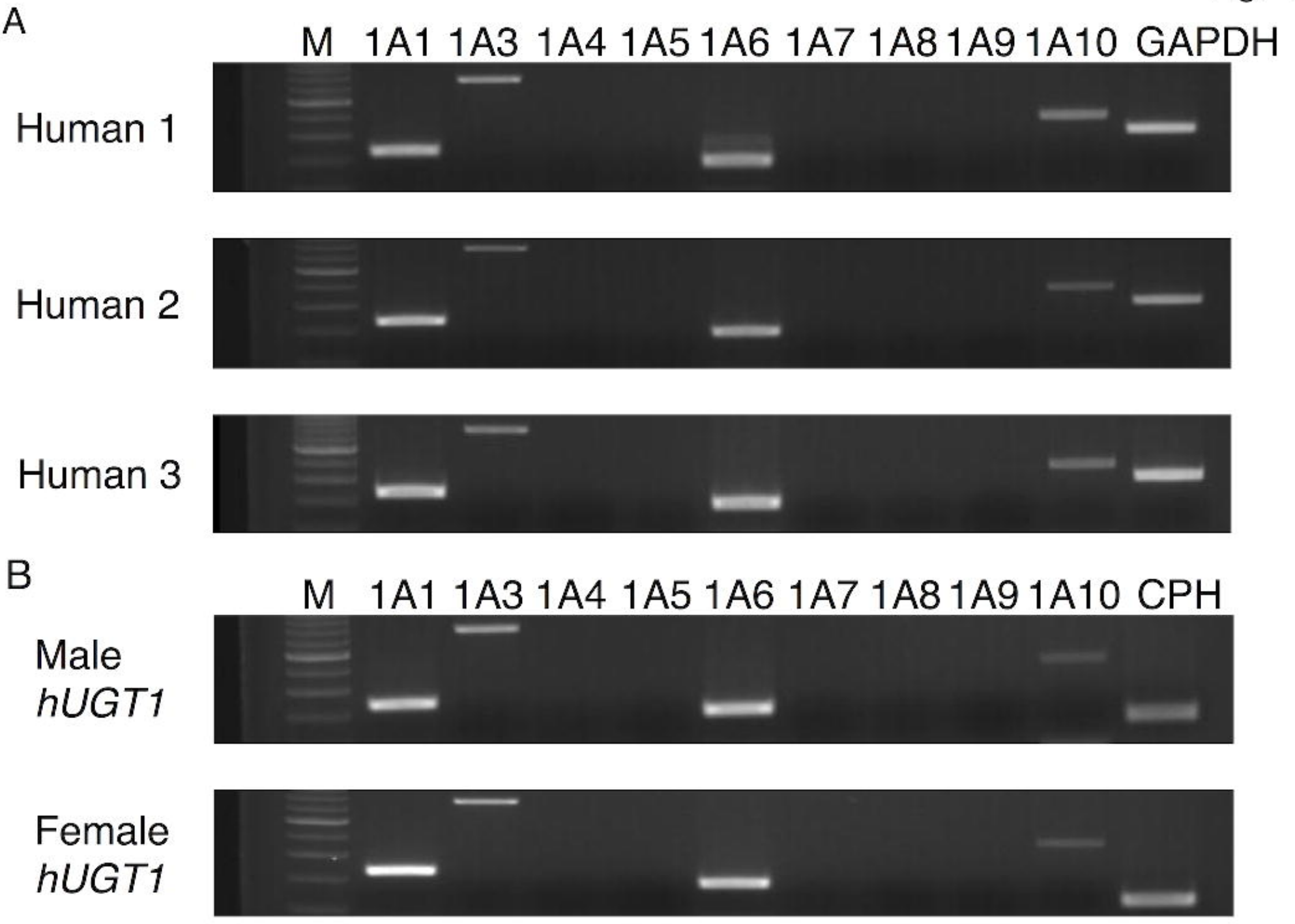

A
M 1A1 1A3 1A4 1A5 1A6 1A7 1A8 1A9 1A10 GAPDH
Human 1
Human 2
Human 3
B
M 1A1 1A3 1A4 1A5 1A6 1A7 1A8 1A9 1A10 CPH
Male
hUGT1
Female
hUGT1

Fig. 2

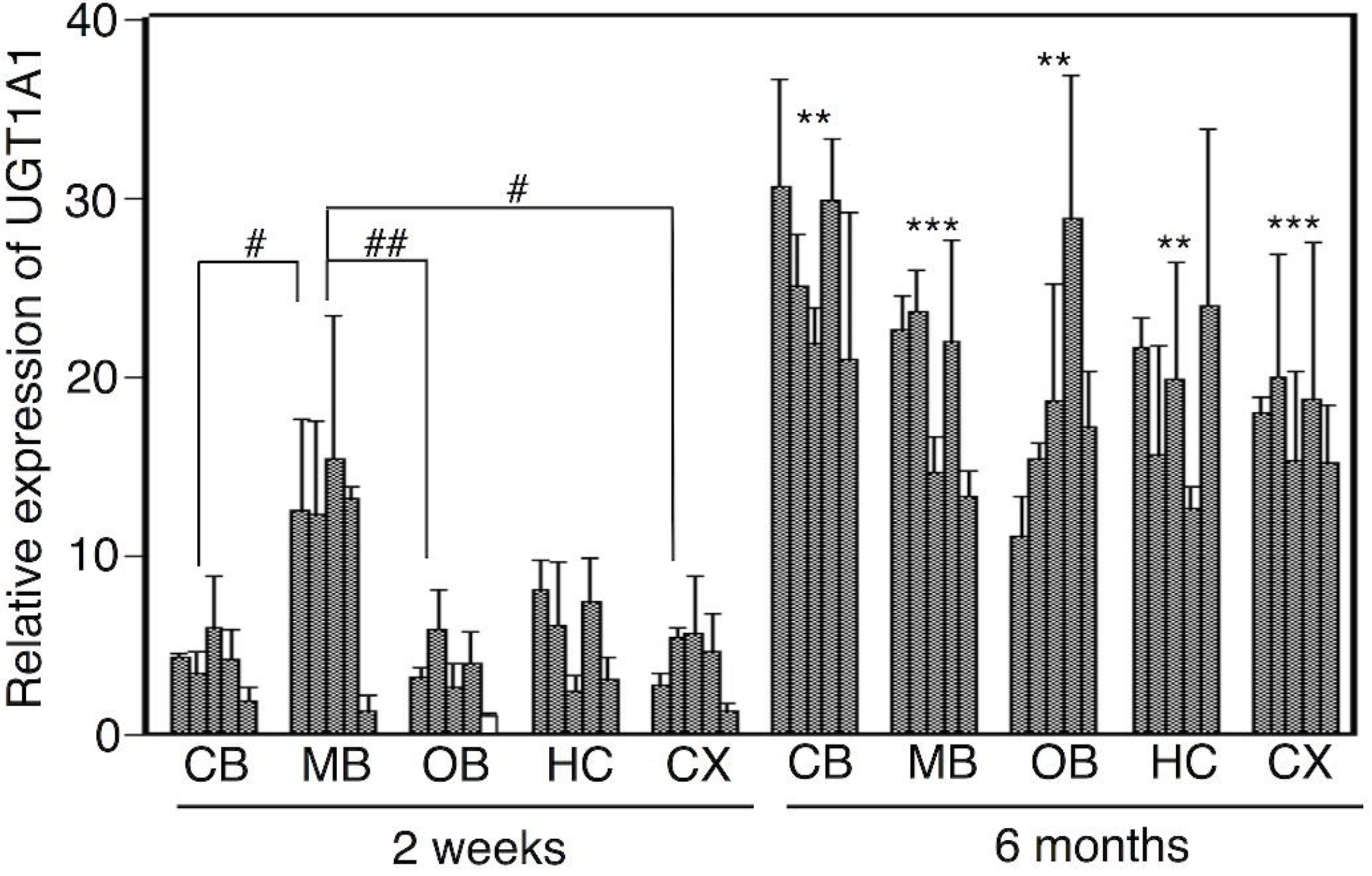
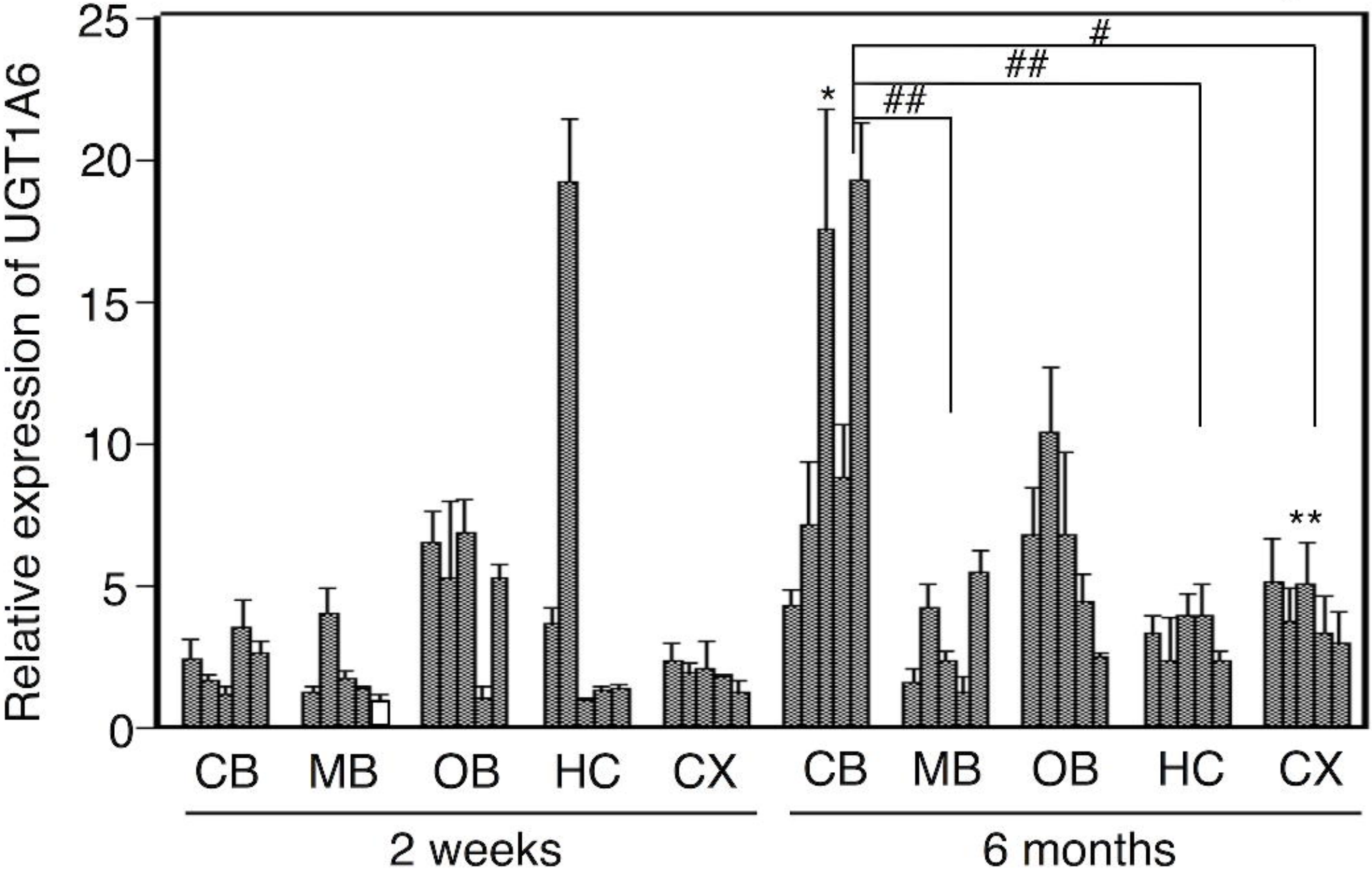

Relative expression of UGT1A1
40
30
20
10
0
#
##
#
**
***
**
**
***
CB
MB
OB
HC
CX
CB
MB
OB
HC
CX
2 weeks
6 months

Fig. 3


Relative expression of UGT1A6
25
20
15
10
5
0
*
**
#
##
##
CB
MB
OB
HC
CX
CB
MB
OB
HC
CX
2 weeks
6 months

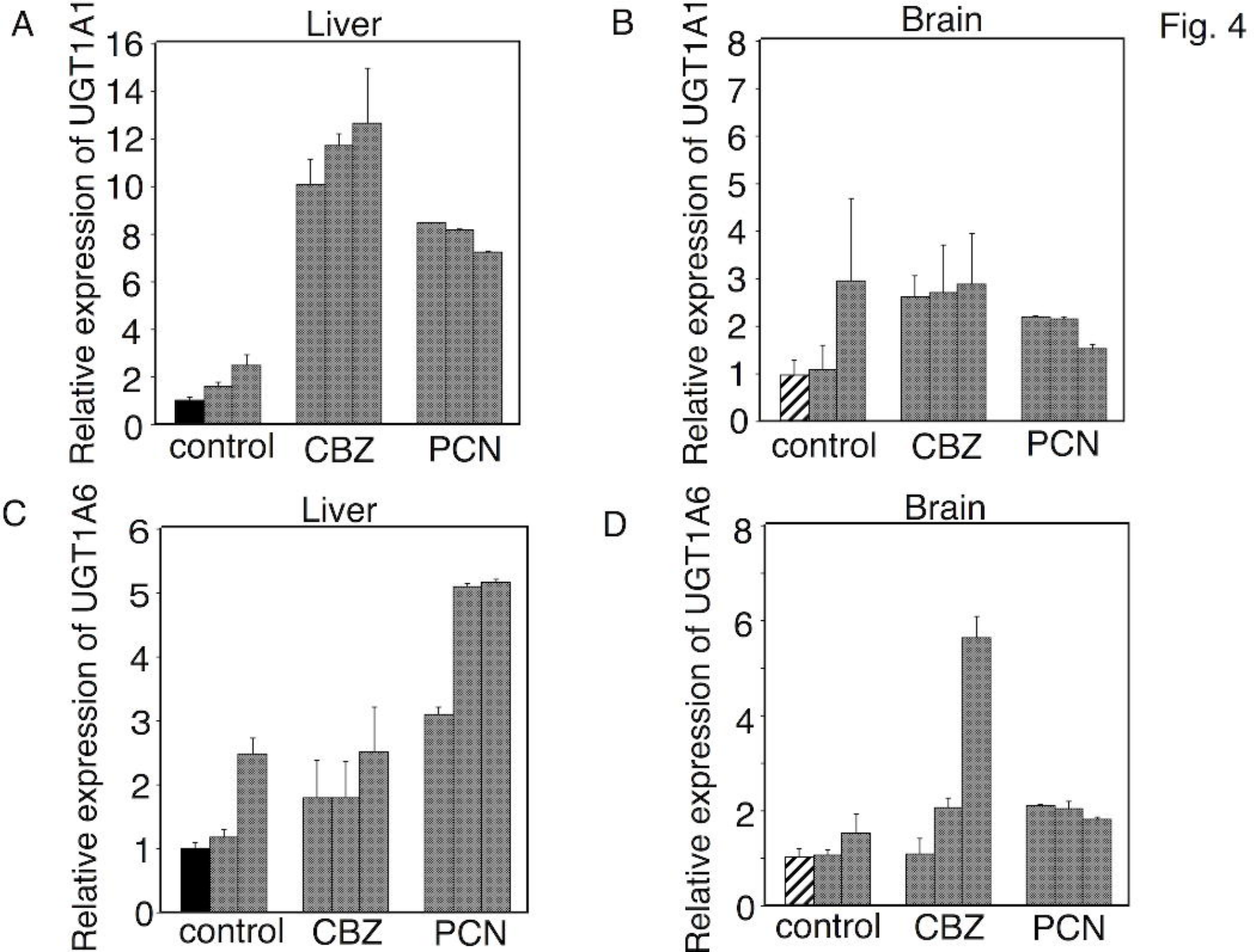

A
Liver
Relative expression of UGT1A1
16
14
12
10
8
6
4
2
0
control
CBZ
PCN
B
Brain
Relative expression of UGT1A1
8
7
6
5
4
3
2
1
0
control
CBZ
PCN
C
Liver
Relative expression of UGT1A6
6
5
4
3
2
1
0
control
CBZ
PCN
D
Brain
Relative expression of UGT1A6
8
6
4
2
0
control
CBZ
PCN

Fig. 4

Fig. 5

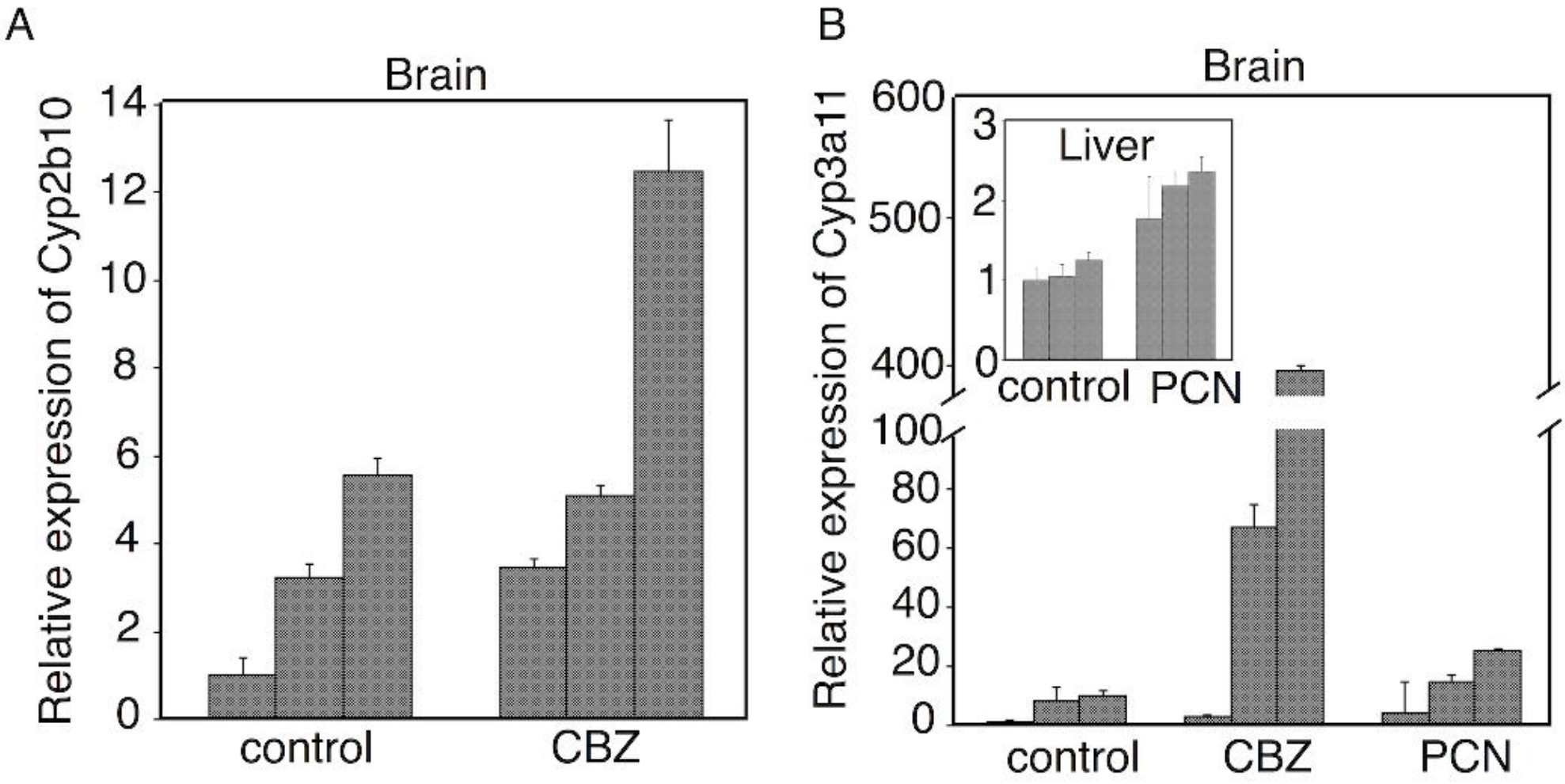

A
Brain
Relative expression of Cyp2b10
0
2
4
6
8
10
12
14
control
CBZ
B
Brain
Relative expression of Cyp3a11
400
500
600
100
80
60
40
20
0
Liver
0
1
2
3
control
PCN
control
CBZ
PCN

Fig. 6

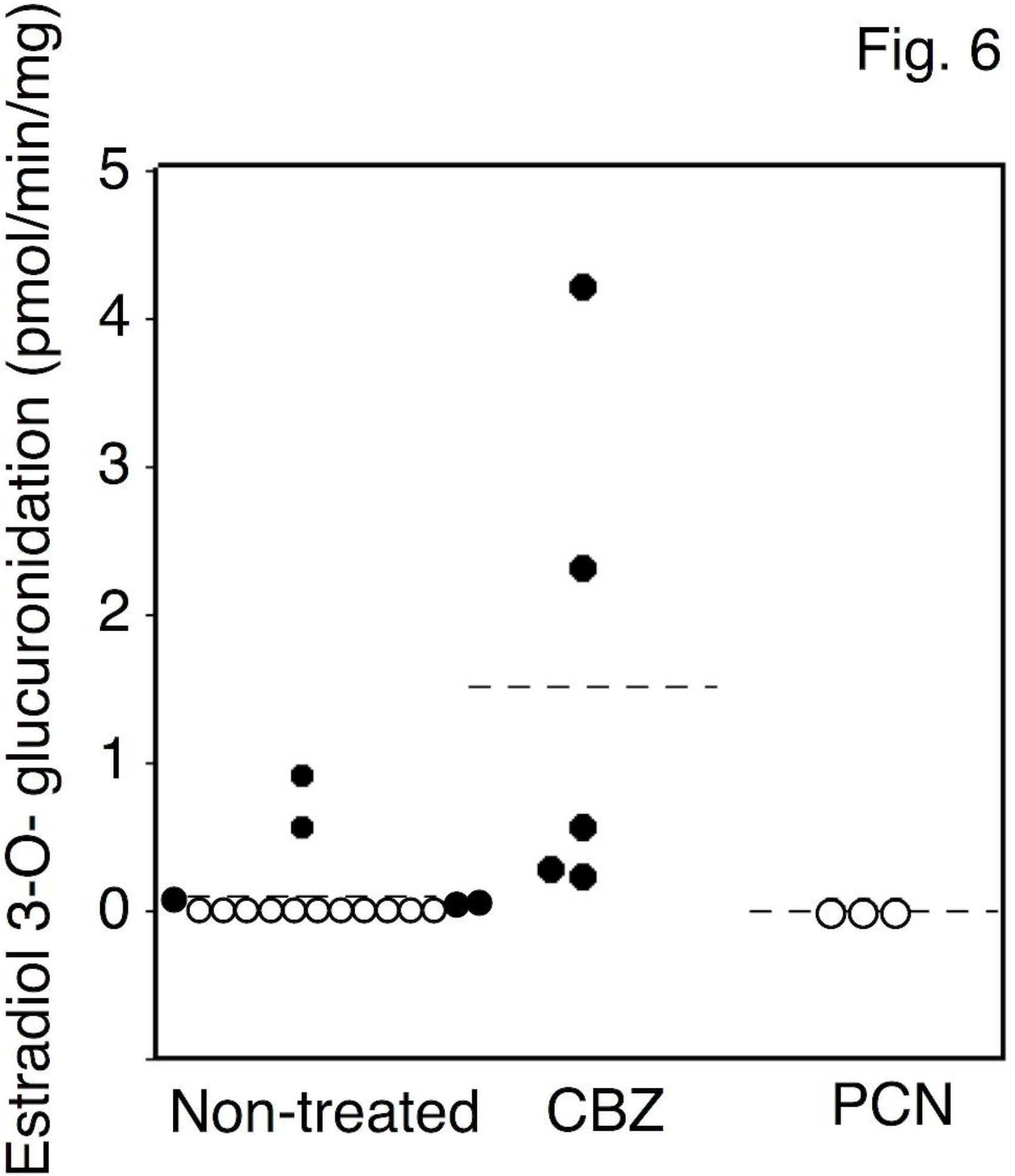
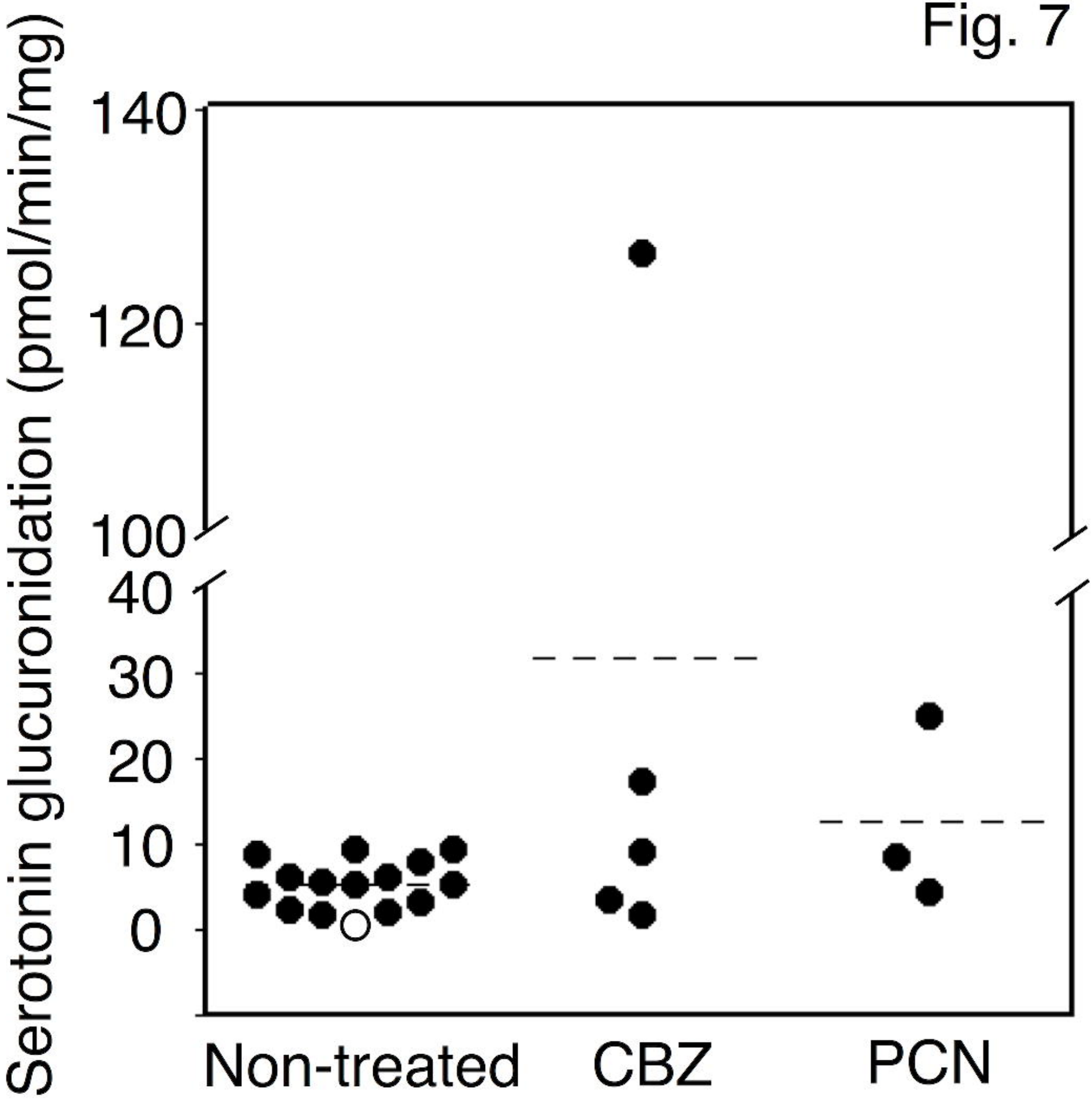

Estradiol 3-O- glucuronidation (pmol/min/mg)
5
4
3
2
1
0
Non-treated
CBZ
PCN

Fig. 7

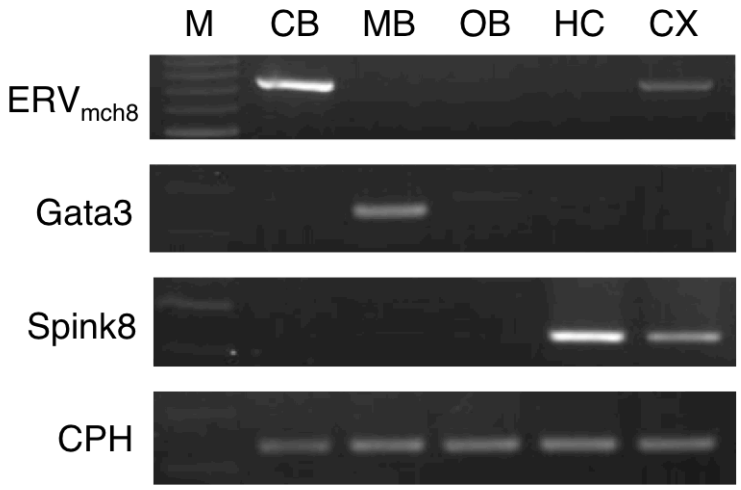

Serotonin glucuronidation (pmol/min/mg)
140
120
100
40
30
20
10
0
Non-treated
CBZ
PCN

Journal: Drug Metabolism and Disposition

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

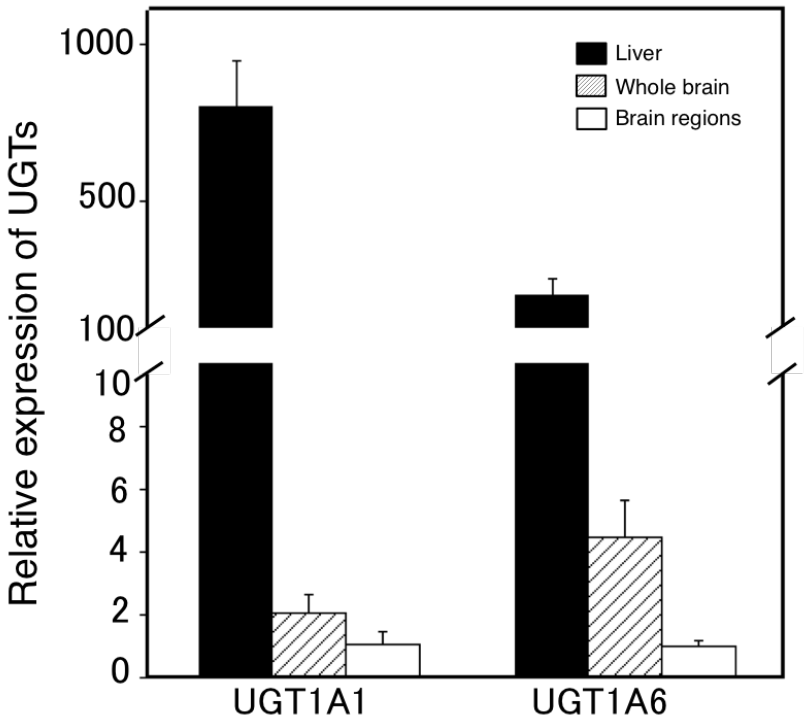
Yuki Kutsuno, Rika Hirashima, Masaya Sakamoto, Hiroko Ushikubo, Hirofumi Michimae, Tomoo Itoh, Robert H. Tukey, and Ryoichi Fujiwara

Supplemental Fig. 1



Supplemental Figure 1. Expression of brain region-specific marker genes in the brain.
Total RNA was prepared from brain regions and was used for the RT-PCR using primer pairs specific for brain region-specific markers, ERV_{mch8} (murine leukemia virus type-endogenous retrovirus mapped on chromosome 8), GATA binding protein 3 (Gata3), and serine peptidase inhibitor Kazal type 8 (Spink8). Forward and reverse primers used were: ERV_{mch8}-S, 5'-GAA GTT GAA AAG TCC ATC ACT AA -3' and ERVmch8-AS, 5'-TCT GGG TCT CTT GAA ACT GT-3'; Gata3-S, 5'-TCT CAC TCT CGA GGC AGC ATG T -3' and Gata3-AS, 5'- GTA CCA TCT CGC CGC CAC AG -3'; Spink8-S, 5'-CTG CTG TCC TTC TGA CGT CTT -3' and Spink8-AS, 5'-GCT CCC CGG TCA TGT GAA A-3'. M, 100 bp DNA ladder marker; CB, cerebellum; MB, midbrain; OB, olfactory bulbs; HC, hippocampus; CX, cerebral cortex.

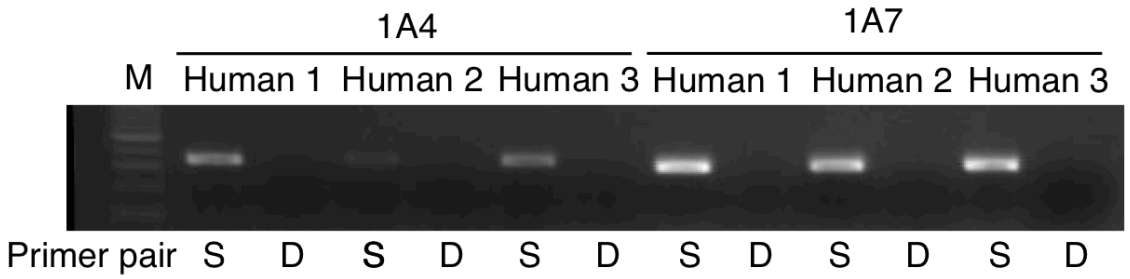
Supplemental Fig.2



Supplemental Figure 2. Comparison of mRNA expression levels of UGT1A1 and 1A6 between liver and brain in *hUGT1* mice.

Relative expression levels of UGT1A1 and UGT1A6 in the liver (black columns in Fig. 4), the total brain (shaded columns in Fig. 4), and the brain regions (white columns in Fig. 2 and 3) were shown.

Supplemental Fig. 3



Supplemental Figure 3. Location of primer pairs and detection of UGT1A4 and UGT1A7 by RT-PCR

Total RNA samples from three human brains containing meningioma were analyzed by RT-PCR using primer pairs specific to UGT1A4 and UGT1A7. Forward and reverse primers used were: UGT1A4 ex1-S, 5'-ACG CTG GGC TAC ACT CAA GG-3' and UGT1A4 ex1-AS, 5'-TCT GAA TTG GTC GTT AGT AAC T-3'; UGT1A4-S and UGT1A-AS (Table 1); UGT1A7 ex1-S, 5'-TGG CTC GTG CAG GGT GGA CTG-3' and UGT1A7 ex1-AS, 5'-TTC GCA ATG GTG CCG TCC AGC-3'; UGT1A7-S and UGT1A-AS (Table 1). The location of primers is indicated at the bottom of each gel. S and D indicate that the used primer pairs were located in the same exon and different exons, respectively. M, 100bp DNA ladder marker.

Supplemental discussion

A previous study reported that RT-PCR using the sense and antisense primers located in the same exon could overestimate the mRNA expression due to amplification of genomic DNA and pre-mRNA (Izukawa et al., 2009). Thus, RT-PCR using the primer pair located in the different exons provides more reliable data than that using the primer pair located in the same exon. A limited number of studies on UGT expression in human brain have been carried out by RT-PCR (Supplemental Table 1). King et al. demonstrated that UGT1A6 and UGT2B7 were detected in human brain by using RT-PCR techniques with the sense and antisense primers located in the different exons. In a following study, it was reported by using quantitative RT-PCR techniques with primer pairs located in the same exon that mRNAs of UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A10, 2A2, 2A3, 2B7, and 2B11 were expressed in human brain (Court et al., 2012). The latter study was partly not consistent with the present study. As the interindividual difference in the expression pattern of UGT1As in human was not observed (Fig. 1A), the discrepancy of these results might be due to the difference of experimental condition such as primers. Actually, UGT1A4 and UGT1A7 mRNA were detected by using primer pairs located in the same exon in the present study (Supplemental Figure 3), while they were not detected by

using primer pairs located in different exons (Fig. 1A). Because we examined the expression of UGT isoforms in human brain by using the primer pairs located in different exons, our data could be more reliable than past data. Thus, we demonstrated that UGT1A1, 1A3, 1A6, and 1A10 were expressed in human brain in the present study.

It was reported that CYP activity was lost during the preparation of intestinal microsomes due to degradation by proteases (Kaminsky and Fasco, 1992). In another study, Abe et al. demonstrated that UGT proteins in UGT-expressing cells treated with protein kinase C inhibitors were degraded during the preparation of cell homogenates in the absence of a detergent. In the present study, several brain microsomes of *hUGT1* mouse brains did not exhibit glucuronidation activities toward estradiol and serotonin (Fig. 6 and 7). Therefore, the difference of the glucuronidation activity toward estradiol and serotonin in each microsome might have been partially caused by degradation during microsomes preparation. The preparation methods of brain microsomes should be improved to assess the glucuronidation activity of brain UGTs.

Supplemental Table 1. UGT1A mRNA expression in human brain determined by RT-PCR and location of primer pairs

UGT1A Isoforms	Location of primer pairs		References
	Forward	Reverse	
UGT1A1, 1A3, 1A4, 1A6, 1A7, and 1A10	Exon 1	Exon 1	Court et al. (2012)
UGT1A6	Exon 1	Exon 2	King et al. (1999)
UGT1A1, 1A3, 1A6, and 1A10	Exon 1	Exon 2	This study