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Expression levels of 39 Cyp mRNAs in the mouse brain and neuroblastoma cell lines, C-1300N18 and NB2a – strong expression of Cyp1b1

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ABSTRACT — An analysis of mRNA levels of 39 Cyp enzymes in the mouse brain and neuroblastoma cell lines, C-1300N18 and NB2a was performed using a real-time reverse transcriptase-polymerase chain reaction. Relative expression levels were quantified by normalized β-actin levels, and compared to the mRNA expression level of the cannabinoid receptor (CB1R), which is abundantly expressed in the mouse brain. Mean 2^-ddCts of CB1R in mouse brain, C-1300N18, and NB2a cells were 1.043, 1.003, and 1.005, respectively. Among Cyp mRNAs in the mouse brain, Cyp1b1 mRNA was the most abundantly expressed (2^-ddCt = 0.310), followed by Cyp46a1 mRNA (0.246). The other Cyp mRNAs moderately expressed (0.011 ~ 0.117) were Cyp1a1, 1a2, 2b10, 2c29, 2c50, 2d9, 2d10, 2d12, 2d22, 2d26, 3a11, 3a41, 4f14, 4f15, 4f16, and 4x1. On the other hand, 10 out of 39 Cyp mRNAs (Cyp 2b9, 2b13, 2b19, 2c37, 2c38, 2c55, 3a44, 4a12, 4a14, and 4f18) were not detectable (2^-ddCt < 0.001). In the neuroblastoma cell lines, C-1300N18 and NB2a, Cyp1b1 mRNA was also the most abundant and preferentially expressed, and relative expression levels to CB1R were 4.674 and 5.084, respectively. Thirteen other Cyp mRNAs (Cyp1a1, 1a2, 2a5, 2b10, 2c44, 2c50, 2c55, 2d10, 2d22, 3a11, 4f13, 4f15, and 4f16) were detected in the neuroblastoma cell lines, whereas 17 Cyp mRNAs (Cyp2c29, 2c37, 2c39, 2c40, 2d12, 2d34, 2e1, 3a16, 3a25, 3a41, 3a44, 4a10, 4a12, 4a14, 4f14, 4x1, and 46a1) were not under the current conditions. The pattern of Cyp mRNA expression was similar for both neuroblastoma cell lines. The present results provide fundamental and useful information on the significance of particular Cyp enzymes in the mouse brain and neuroblastoma cell lines, C-1300N18 and NB2a, which may be valuable tools for investigations on the neural expression and function of Cyp1b1.

Key words: C-1300N18 cells, Cyp1b1, Cyp enzymes, Mouse brain, mRNA expression, NB2a cells

INTRODUCTION

Cytochrome P450 (CYP) enzymes are a superfamily of heme-containing enzymes and the most important phase I enzymes involved in the biotransformation by monooxygenation of endogenous and exogenous compounds such as vitamins, hormones, medical drugs, and environmental chemicals. The significance of CYP enzymes has clarified their role as drug-metabolizing enzymes mainly localized in the liver. Total CYP levels in the mammalian brain were previously reported to be extremely low, approximately 0.5-4% those in the liver (Warner *et al.*, 1988; Hedlund *et al.*, 2001). Therefore, CYP enzymes in the brain were originally considered unlikely to contribute significantly to the metabolism of xenobiotics including medical drugs. However, the metabolism of xenobi-

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otics including medical drugs in the brain constitutes a field of recent intensive research in relation to the potential implications of the pharmacological effects of drugs acting on the central nervous system (Ghersi-Egea *et al*., 1994; Miksys and Tyndale, 2002, 2009; Ferguson and Tyndale, 2011). Despite low expression levels, CYP-mediated biotransformation appears to be important for drugs that are transported into the brain and function in the central nervous system. Neural CYP enzymes are considered to play important physiological roles in maintaining neural homeostasis through the biosynthesis and degradation of biologically essential endogenous compounds because previous studies demonstrated that CYP enzyme levels in specific neurons may be as high as or higher than those in hepatocytes and CYP expression is typically distributed to specific populations of neurons (Miksys *et al*., 2000, 2003). The characterization of brain and/or neural CYP enzymes and their localization as well as the identification of endogenous substrates and their metabolic end-products will provide a better understanding of the role of CYP enzymes in brain physiology, development, and diseases (Ghosh *et al*., 2016; Toselli *et al*., 2016). It may also be qualitatively and quantitatively different from that in the liver due to differences in expression or to the presence of unique enzymes in the brain and particular neurons (Stamou *et al*., 2013). Furthermore, useful cell lines for drug metabolism studies in the brain have not yet been established. The present study was undertaken to investigate the mRNA expression of 39 Cyp enzymes in the mouse brain and mouse neuroblastoma cell lines, C-1300N18 and NB2a cells using a real-time reverse transcriptase-polymerase chain reaction.

MATERIALS AND METHODS

Animals

Experiments involving animals were approved by the Institutional Animal Care and Use Committee at the Hokuriku University. Male ddY mice (7 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and acclimated for 1 week. Animals were given water and food *ad libitum*. Animals were euthanized by cervical dislocation, and whole brains were immediately collected on ice, weighed, and then stored at -80ºC before use.

Cell culture

The mouse neuroblastoma cell lines, C-1300N18 and NB2a were provided by the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). C-1300N18 and NB2a cells were maintained in Dulbecco's modified Eagle's medium and RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), respectively, supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) and penicillin/streptomycin (Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37ºC. These cells were seeded on a 6-well plate (20×10^4 cells/well) and cultured for 24 hr.

RNA analysis

Total RNA was extracted from neuroblastoma cells and mouse whole brains using TRIzol reagent (Invitrogen, Grand Island, NY, USA). Reverse transcriptase reactions were performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). A quantitative real-time polymerase chain reaction (qPCR) was performed using an ABI 7500 real-time PCR system (Applied Biosystems) with the KAPA SYBR® Fast qPCR Kit (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions. Primers used for mRNA measurements of Cyp1a1, Cyp1a2, Cyp1b1 (Uno *et al*., 2008), Cyp2a5 (Arpiainen *et al*., 2008), Cyp2b10 (Koike *et al*., 2007), Cyp3a11 (van den Bosch *et al*., 2007), CB1R, CB2R, and β-actin (Yamaori *et al*., 2013) were described previously. The other primers (Cyp2b9, Cyp2b13, Cyp2b19, Cyp2c29, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c44, Cyp2c50, Cyp2c55, Cyp2d9, Cyp2d10, Cuyp2d12, Cyp2d22, Cyp2d26, Cyp2d34, Cyp2e1, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41, Cyp3a44, Cyp4a10, Cyp4a12, Cyp4a14, Cyp4f13, Cyp4f14, Cyp4f15, Cyp4f16, Cyp4f18, Cyp4x1, and Cyp46a1) were designed using qPrimerDepot (http://mouseprimerdepot.nci.nih.gov). Relative expression levels were quantified by the comparative Ct method ($2^{-\Delta\Delta Ct}$), in which the ΔΔCt value of the sample was obtained by subtracting individual values of ($Ct_{P450} - Ct_{\beta\text{-actin}}$) from the mean value of ($Ct_{CB1R} - Ct_{\beta\text{-actin}}$). CB1R, the cannabinoid CB_1 receptor, is abundantly expressed in the brain (Howlett *et al*., 2002) and its mean Ct values for C-1300N18 cells (25.362), NB2a cells (25.139), and mouse brain cells (25.543) were approximately equal. Thus, CB1R was used as the baseline gene.

RESULTS AND DISCUSSION

The relative expression levels of 39 Cyp mRNAs were quantified by normalized β-actin levels, and compared to that of CB1R, which is more abundantly expressed than CB2R in the mouse brain and neuroblastoma cell lines (Table 1). The results obtained clearly demonstrated that

Table 1. Relative expression of Cyp mRNAs in the mouse brain and C-1300N18 and NB2a cell lines.

mRNA	Mouse brain		C-1300N18		NB2a	
	2^-ddCt	Relative Expression	2^-ddCt	Relative Expression	2^-ddCt	Relative Expression
CB1R	1.043	1	1.003	1	1.005	1
CB2R	0.049	0.047	0.183	0.182	0.166	0.165
Cyp1a1	0.087	0.084	0.256	0.255	0.183	0.182
Cyp1a2	0.043	0.041	0.090	0.090	0.099	0.098
Cyp1b1	0.310	0.298	4.689	4.674	5.108	5.084
Cyp2a5	0.002	0.002	0.003	0.003	0.002	0.002
Cyp2b9	BLQ	BLQ	ND	ND	ND	ND
Cyp2b10	0.023	0.022	0.080	0.079	0.065	0.065
Cyp2b13	BLQ	BLQ	ND	ND	ND	ND
Cyp2b19	BLQ	BLQ	ND	ND	ND	ND
Cyp2c29	0.071	0.068	BLQ	BLQ	BLQ	BLQ
Cyp2c37	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Cyp2c38	BLQ	BLQ	BLQ	BLQ	0.001	0.001
Cyp2c39	0.001	0.001	BLQ	BLQ	BLQ	BLQ
Cyp2c40	0.005	0.005	BLQ	BLQ	BLQ	BLQ
Cyp2c44	0.002	0.002	0.052	0.052	0.023	0.023
Cyp2c50	0.014	0.013	0.193	0.193	0.146	0.145
Cyp2c55	BLQ	BLQ	0.091	0.091	0.231	0.229
Cyp2d9	0.065	0.062	0.026	0.026	0.019	0.019
Cyp2d10	0.018	0.018	0.011	0.011	0.013	0.013
Cyp2d12	0.047	0.045	BLQ	BLQ	BLQ	BLQ
Cyp2d22	0.117	0.112	0.002	0.002	0.001	0.001
Cyp2d26	0.045	0.043	0.006	0.006	BLQ	BLQ
Cyp2d34	0.001	0.001	BLQ	BLQ	BLQ	BLQ
Cyp2e1	0.010	0.009	BLQ	BLQ	BLQ	BLQ
Cyp3a11	0.020	0.020	0.009	0.009	0.010	0.010
Cyp3a13	0.002	0.002	ND	ND	ND	ND
Cyp3a16	0.002	0.002	BLQ	BLQ	BLQ	BLQ
Cyp3a25	0.005	0.005	BLQ	BLQ	BLQ	BLQ
Cyp3a41	0.013	0.013	BLQ	BLQ	BLQ	BLQ
Cyp3a44	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Cyp4a10	0.001	0.001	BLQ	BLQ	BLQ	BLQ
Cyp4a12	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Cyp4a14	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Cyp4f13	0.009	0.008	0.020	0.020	0.013	0.012
Cyp4f14	0.011	0.011	BLQ	BLQ	BLQ	BLQ
Cyp4f15	0.018	0.017	0.019	0.018	0.008	0.008
Cyp4f16	0.011	0.011	0.053	0.053	0.049	0.048
Cyp4f18	BLQ	BLQ	0.001	0.001	BLQ	BLQ
Cyp4x1	0.036	0.034	BLQ	BLQ	BLQ	BLQ
Cyp46a1	0.246	0.236	BLQ	BLQ	BLQ	BLQ

Measurements were performed in duplicate (C-1300N18) or triplicate (mouse brain and NB2a)
BLQ, below the limit of quantification (< 0.001) ND, not determined

Cyp1b1 mRNA was the most abundantly expressed in the mouse brain. The relative expression level of Cyp1b1 to CB1R, which is mainly expressed in the brain (Pertwee, 1997; Howlett *et al*., 2002), was approximately one third that of CB1R. CYP1B1 was suggested to be involved in angiotensin-induced hypertension and the resulting renal failure (Jennings *et al*., 2012) as well as in the pathogenesis of glaucoma and other anterior segment dysgene-

sis disorders (Vasiliou and Gonzalez, 2007). CYP1B1 is responsible for the metabolic activation of polycyclic aromatic hydrocarbons, heterocyclic amines, and estradiol to carcinogenic intermediates (Shimada and Fujii-Kuriyama, 2004; Sissung *et al*., 2006). CYP1B1 also plays an important role in the metabolism of estradiol to 4-hydroxyestradiol, which is a reactive metabolite that plays a critical role in estrogen-mediated carcinogenesis (Liehr and Ricci, 1996; Guengerich *et al*., 2003). The enzyme is found at a high frequency in a wide range of human cancer cells of different histogenetic origins (Murray *et al*., 1997), and is known to be involved in the regulation of tumor development (Androutsopoulos *et al*., 2013). In addition, CYP1B1 has been reported to promote cell proliferation and metastasis through the induction of a transcription factor involved in cell growth (Kwon *et al*., 2016). In humans, CYP1B1 is mainly expressed in extrahepatic tissues including the lung, heart, prostate, and brain (Choudhary *et al*., 2005).

In the neuroblastoma cell lines, C-1300N18 and NB2a, Cyp1b1 was primarily and selectively expressed over other Cyp enzymes (Table 1). The relative expression levels of Cyp1b1 were 18.3 and 22.1-fold higher than those of the second dominant Cyps in C-1300N18 (Cyp1a1) and NB2a (Cyp2c55), respectively. In neural tissues, Cyp1b1 expression is conserved in the early embryo during the development of the neural crest and eyes in mice (Tang *et al*., 2009), and microvessels in the human blood-brain barrier (Dauchy *et al*., 2008). In the human neuroblastoma cell lines, UKF-NB-3 and UKF-NB-4, CYP1B1 was detected together with CYP1A1 and 3A4 (Stiborova *et al*., 2014). The preferential expression of Cyp1b1 in C-1300N18 and NB2a cells suggests that both cell lines have potential in investigations on the neural expression and function of Cyp1b1 in mice.

Cyp46a1 mRNA (2^-ddCt = 0.246) was the second most abundant Cyp mRNA in the mouse brain, but was not detectable in C-1300N18 or NB2a cells (Table 1). Cyp46a1, which encodes cholesterol 24-hydroxylase, is known to be primarily expressed in the central nervous system, and is of importance for cerebral cholesterol regulation (Ohyama *et al*., 2006). The rate of cholesterol biosynthesis in the brain is very low, and the half-life of brain cholesterol is markedly longer than that of cholesterol in most of the other organs. Therefore, it is crucial for the disproportionally high level of cholesterol in the mammalian brain to be strictly controlled by the enzyme activity of CYP46A1. A previous study on siRNA suggested that CYP46A1 plays an important role in the induction and/or aggravation of Alzheimer's disease through the degeneration of neural cholesterol homeostasis (Djelti *et al*., 2015).

Five out of 6 Cyp2d mRNAs (Cyp2d9, 2d10, 2d12, 2d22, and 2d26) were primarily detected in the mouse brain. Among them, Cyp2d22 (2^-ddCt = 0.117), an ortholog form of human CYP2D6, showed the highest expression levels and was the third most abundant in 39 Cyp mRNAs (Table 1). Cyp2d9, 2d10, and 2d22 were also detectable in the mouse neuroblastoma cells. CYP2D6 is involved in the metabolism of psychoactive drugs (Toselli *et al*., 2016) and the biosynthesis of the important neurotransmitters, dopamine and 5-hydroxytryptamine (Bromek *et al*., 2011; Idle *et al*., 2003). SH-SY5Y human neuroblastoma cells express CYP2D6, which may play a role in neuroprotection from Parkinson's disease, which generates neurotoxins in the human brain (Mann and Tyndale, 2010). Cyp2d22 is suggested to participate in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease phenotype and nicotine-mediated neuroprotection (Singh *et al*., 2009). Thus, Cyp2d enzymes may also play significant roles in the regulation of neurotransmitters in the mouse brain.

Among 6 out of the 9 Cyp4 family members (Cyp4a10, 4f13, 4f14, 4f15, 4f16, and 4x1) detected in the mouse brain, Cyp4x1 showed the highest expression levels in the mouse brain, but was not detectable in the neuroblastoma cell lines, C-1300N18 and NB2a. A northern blot analysis demonstrated that CYP4X1 mRNA is strongly and specifically expressed in the rat brain stem, hippocampus, and vascular endothelial cells (Bylund *et al*., 2002). Stamou *et al*. (2013) previously reported that CYP4X1 mRNA is equally detectable in the rat liver and brain. Al-Anizy *et al.* (2006) showed the selective expression of Cyp4x1 in the mouse brain, while CYP4X1 mRNA was ubiquitously expressed in adult human tissues (Choudhary *et al*., 2005). It is of interest that CYP4X1 catalyzes the epoxidation of anandamide, an endogenous cannabinoid, but not arachidonic acid (Stark *et al*., 2008). These findings suggest that Cyp4x1 plays a particular role in the mouse brain; however, its physiological role and specific substrates need to be clarified.

In the Cyp2b, 2c, and 3a subfamilies, Cyp2b10, 2c44, 2c50, and 3a11 were detected in the mouse brain and C-1300N18 and NB2a cells, while Cyp2c29, 2c39, 2c40, 3a16, 3a25, and 3a41 were only detectable in the mouse brain. A previous study on the mouse tissue distribution of Cyp mRNA demonstrated that the distribution of Cyp2b10 and 3a11 was the greatest in the lung and liver, respectively, with the second most abundant distribution being observed in the duodenum (Renaud *et al*., 2011). Cyp2c50 has been cloned from the mouse heart and shown to be active in the metabolism of unsaturated fatty

acids including arachidonic acid (Wang *et al*., 2004).

In conclusion, by assessing the expression of Cyp mRNAs, the present study has provided useful and fundamental information on the significance of Cyp enzymes in the mouse brain and neuroblastoma cell lines. In addition, the mouse neuroblastoma cell lines examined have potential as valuable tools for investigations on the expression and function of Cyp1b1 in mouse neural and/or tumor cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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