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In situ hybridization study of *CYP2D* mRNA in the common marmoset brain

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Abstract: The common marmoset is a non-human primate that has increasingly employed in the biomedical research including the fields of neuroscience and behavioral studies. Cytochrome P450 (CYP) 2D has been speculated to be involved in psycho-neurologic actions in the human brain. In the present study, to clarify the role of CYP2D in the marmoset brain, we investigated the expression patterns of *CYP2D* mRNA in the brain using *in situ* hybridization (ISH). In addition, to identify the gene location of *CYP2D19*, a well-studied CYP2D isoform in the common marmoset, a fluorescence *in situ* hybridization (FISH) study was performed. Consistent with findings for the human brain, *CYP2D* mRNA was localized in the neuronal cells of different brain regions; e.g., the cerebral cortex, hippocampus, substantia nigra, and cerebellum. FISH analysis showed that the *CYP2D19* gene was located on chromosome 1q, which is homologous to human chromosome 22 on which the *CYP2D6* gene exists. These results suggest that CYP2D in the marmoset brain may play the same role as human CYP2D6 in terms of brain actions, and that the *CYP2D19* gene is conserved in a syntenic manner. Taken together, these findings suggest that the common marmoset is a useful model for studying psychiatric disorders related to CYP2D dysfunction in the brain.
Key words: brain, chromosome, common marmoset, *CYP2D*, *in situ* hybridization

Introduction

The search for the mechanisms of and treatments for psycho-neurologic disorders has relied heavily on the development and use of animal models of the respective disorder. The common marmoset (*Callithrix jacchus*), a small-bodied New World primate, has been attracting much attention in the biomedical research field because of its size, comparative ease in handling, high reproductive efficiency, and behavioral similarity to humans [1, 20]. In particular, common marmosets have been used for neuroscientific and behavioral studies including pathological studies of Alzheimer's disease [2, 19], Parkinson's disease [12, 26], Huntington's disease [15], Multiple sclerosis [31] and studies of anxiety and stress [3]. As monkeys are more similar to humans than are rodents, research using monkey models plays an important role in the pre-clinical development of treatments and is useful for psychological analysis of more complex behaviors.

Cytochrome P450 (CYP) is a hemo-protein enzyme

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that mediates the biotransformation of a wide variety of chemical compounds including drugs, toxins and carcinogens. Several isoforms of CYP are well-known to be expressed in the liver, the metabolism of which is mainly mediated by CYP [25]. Recently, it has been reported that CYP2D is constitutively expressed in neuronal cells of the cerebral cortex, cerebellum and hippocampus in the human brain [7], and contributes to the metabolism of psychoactive drugs in the central nervous system of rodents and humans [21, 30]. CYP2D is also involved in the production of neuroactive amines (e.g. serotonin and dopamine) and neurosteroids (e.g. 11-deoxycorticosterone and 16α-, 16β-, 17β-, 2β-, and 6β-hydroxyprogesterone) in the brain [5, 13, 16]. These findings suggest the possible involvement of CYP2D in psycho-neurologic actions through the metabolism of centrally acting substances in the brain.

CYP2D6, a member of the CYP2D subfamily in humans, mediates the metabolism of clinically used medications such as psychoactive drugs (e.g., amphetamines, antidepressants, analgesics and antipsychotics) [8, 34] and neurotoxins [e.g., 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)] [11]. In addition, the association of *CYP2D6* allelic variant with psychiatric phenotypes has been studied [24]. CYP2D6 has been received a good deal of attention owing to the presence of genetic polymorphism that may have a significant impact on the fate of therapeutic drugs [34].

In the common marmoset, eight types of *CYP2D* (*CYP2D19*: NM_001204438.1, *CYP2D19* splicing variant X1: XM_008990847.1, X2: XM_008990848.1 and X3: XM_008990849.1, *CYP2D30*: AY082602.1, *CYP2D6*-like: XM_002763891.3, *CYP2D6*: KJ922563.1 and *CYP2D8*: KJ922562.1) have been registered in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). It has been reported that the mRNAs of *CYP2D6* and *CYP2D8* show the closest relationship with those of *CYP2D19* and *CYP2D6*-like, respectively, and that they are expressed in the marmoset brain [32]. Although functional analyses of CYP2D19 and CYP2D30 have been conducted in the marmoset liver [22, 29], the role of CYP2D in the marmoset brain has not yet been fully clarified.

In the present study, as a first step, we investigated the expression patterns of *CYP2D* mRNA in the marmoset brain and the gene location of *CYP2D19*, which is a well-studied isoform in the common marmoset, using *in situ* hybridization (ISH) and fluorescence *in situ* hybridization (FISH), respectively.

Materials and Methods

Animals

Common marmosets were reared at the RIKEN Brain Science Institute (Saitama, Japan), and kept at 27°C and 50% humidity under a 12h:12h light-dark cycle (light from 8:00 am to 8:00 pm). All marmosets chosen for this study were four males between 1.5 and 3 years old. Marmosets were allowed *ad libitum* access to water and food pellets (CMS-1M, CLEA Japan Inc., Tokyo, Japan) with added vitamin C, D, calcium and acidophilus. Hot water and comb honey were also added to soften the pellets and to improve the animals' preference for the food. Animals were given a piece of Calorie Mate (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) or castella (Castella, Yamazaki Baking Co., Ltd., Tokyo, Japan) as a treat. The research was conducted in accordance with the Declaration of the Helsinki and was approved by the Animal Experiments Committee of RIKEN (Saitama, Japan). All animals were cared for and treated humanely in accordance with the Institutional Guideline for Experiments using Animals (Approval ID: No. H25-2-212 and H25-2-219).

Fluorescence in situ hybridization

The probe for a 3856 bp fragment for the fluorescence *in situ* hybridization (FISH) analysis was designed from positions 201242 to 205097 of the genomic *CYP2D19* DNA (GenBank accession number: NW_003187933.1). The probe was labeled with Cy3-dUTP (Thermo Fisher Scientific, Waltham, MA, USA) using nick translation according to the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN, USA). Chromosome preparations made from marmoset fibroblasts taken for FISH analysis were obtained from Chromosome Science Lab. Ltd. (Hokkaido, Japan). The chromosome slides were exposed to ultraviolet (UV) light after staining with Hoechst 33258 (Tocris Bioscience, Ellisville, MO, USA) to visualize chromosome banding patterns. Hybridization was carried out at 37°C overnight, and hybridization signals of the Cy3-labeled cDNA fragments were captured using Leica CW4000 FISH (Cambridge, UK) imaging software mounted on a Leica DMRA2 microscope and were analyzed with the CW4000 application program produced by Leica Microsystems Imaging Solutions Ltd. (Cambridge, UK).

In situ hybridization

Paraffin-embedded blocks and sections of brain and liver tissues from marmosets for *in situ* hybridization (ISH) were obtained from Genostaff (Tokyo, Japan). The brain and liver were dissected after perfusion, fixed with tissue fixative (Genostaff), then embedded in paraffin and sectioned at 6 μm. The hybridization protocol was conducted as previously reported [27]. The probe for a 1,273 bp fragment for the ISH analysis was designed from positions 202550 to 203822 of the *CYP2D19* genomic DNA (GenBank accession number: NW_003187933.1). The probes were labeled with a digoxygenin RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) overnight and the sections were then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Mutoh Pure Chemicals, Tokyo, Japan) and mounted with CC/Mount (Diagnostic Biosystems Inc., Pleasanton, CA, USA).

Results

Mapping of the CYP2D19 gene

Marmoset metaphase spreads were consistently seen to contain 22 pairs of autosomes and two sex chromosomes (Fig. 1A). FISH experiments showed that the marmoset *CYP2D19* gene is located on chromosome 1q (Figs. 1A and 1B).

Expression patterns of CYP2D mRNA in the brain and liver

We assessed the expression pattern of *CYP2D* mRNA in the marmoset brain and liver using ISH. The expression patterns of *CYP2D* mRNA in the brain and liver are shown in Figs. 2A and 2B, respectively. Among the brain regions, signals were detected in neurons of the hippocampus (Figs. 3A–3D), cortex (Figs. 3E and 3F), substantia nigra (Figs. 3G and 3H), and cerebellar Purkinje cells (Figs. 3I and 3J). The signal of *CYP2D* mRNA was moderate in the cortex and relatively high in the hippocampus and substantia nigra. No signals were detected in any regions of the brain or liver using the sense probe (data not shown).

Specificity of CYP2D19 probe

BLAST search showed that the *CYP2D19* probe used in this ISH study had 99, 100, 93, 94% sequence similarity to the mRNA nucleotide sequences of *CYP2D19* (*CYP2D6*), *CYP2D19* (*CYP2D6*) splicing variants (X1, X2 and X3), *CYP2D30* and *CYP2D8*, respectively.

Discussion

In the present study, to study whether the common marmoset could be a useful animal model for the analysis of CYP2D function in the brain or CYP2D-dependent emotional disease model, we examined the chromosomal location of the *CYP2D19* gene and localization of the *CYP2D* transcript in the brain.

The location of *CYP2D19* gene was verified by FISH analysis. Marmoset chromosome 1 shares similarity with three segments of the human DNA; partial chromosome 13, 9, and 22 [28]. The *CYP2D19* gene was located in a region of chromosome 1q that is homologous to human chromosome 22, which contains the human *CYP2D6* gene [14]. These results suggest that *CYP2D19* gene is conserved in a syntenic manner on the marmoset chromosome.

Next, we clarified the localization of *CYP2D* mRNA in the marmoset brain. *CYP2D* mRNA was localized in the neuronal cells from different regions of the marmoset brain, i.e., the cerebral cortex, hippocampus, substantia nigra and cerebellum (Fig. 3). As the results of BLAST showed that the probe used in this ISH study had at least 93% sequence similarity to the mRNA nucleotide sequences of *CYP2D* isoforms of the marmoset, the ISH probe was able to detect the mRNAs of the *CYP2D* isoforms expected to be expressed in the marmoset brain and liver. In our supplementary experiment, we confirmed that *CYP2D30* mRNA was not detected by RT-PCR in the brain and liver of the marmosets examined (data not shown). In addition, it has been reported that *CYP2D8* (*CYP2D6*-like) is more abundantly expressed than *CYP2D6* (*CYP2D19*) in the marmoset brain [32]. Therefore, the signal of *CYP2D* mRNA detected by the *CYP2D19* probe used in this study may reflect the expression of both *CYP2D19* and *CYP2D8*, mainly *CYP2D8*, in the marmoset brain.

The results of our ISH study were consistent with the localization of *CYP2D* mRNA in the human brain [7, 11]. The distribution of *CYP2D* mRNA was heterogenous between the marmoset brain areas. The signal of *CYP2D* mRNA was moderate in the cortex, and relatively high in the hippocampus and substantia nigra (Fig. 3). These results were similar to the distribution of *CYP2D1*

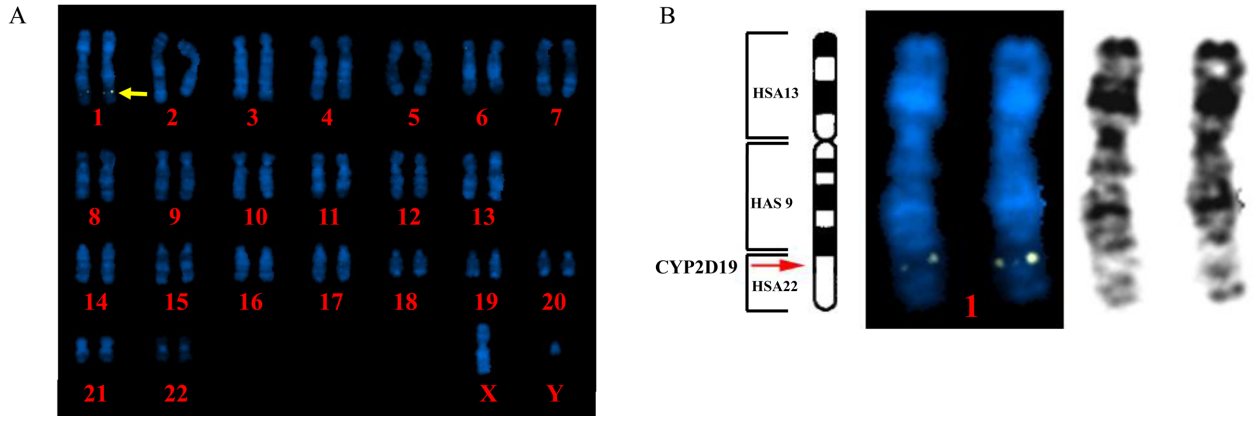

Fig. 1. Fluorescence *in situ* hybridization analysis of metaphase chromosomes of the common marmoset. Metaphase spreads were hybridized with probes detecting the *CYP2D19* genomic fragments. Loci encoding *CYP2D19* are indicated by the yellow arrow (A) and by red arrow (B), showing the regions of homology with the human (HSA) chromosome.

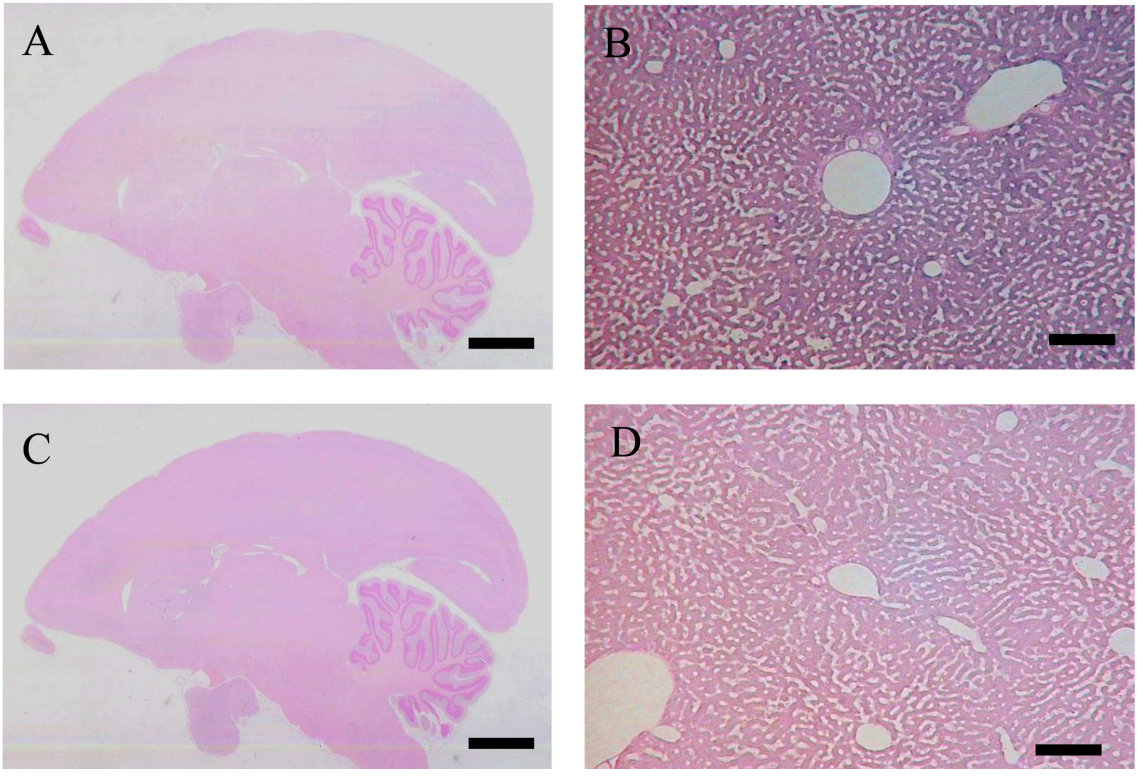

Fig. 2. Expression patterns of *CYP2D* mRNA in the marmoset brain and liver obtained using *in situ* hybridization (ISH) analysis. Representative sections of the marmoset brain (A and C) and liver (B and D) are shown. A and B: the sections hybridized with the antisense probe, C and D: with the sense probe. Scale bar in A and C: 2.5 mm. Scale bar in B and D: 200 μm.

mRNA in the rat brain [23]. CYP2D isoforms in the brain of human and rat are involved in the metabolism of centrally acting drugs [21] and endogenous neuroactive compounds such as monoamine neurotransmitters (e.g. dopamine and serotonin) and neurosteroids (e.g. allopregnanolone, a γ-amino butylic acid A receptor modulator) [4, 6, 10]. On the other hand, Uehara *et al.* has reported the N-demethylase activity of the MPTP neurotoxin in brain microsomes of the common marmoset, the activity of which is inhibited by quinidine, a spe-

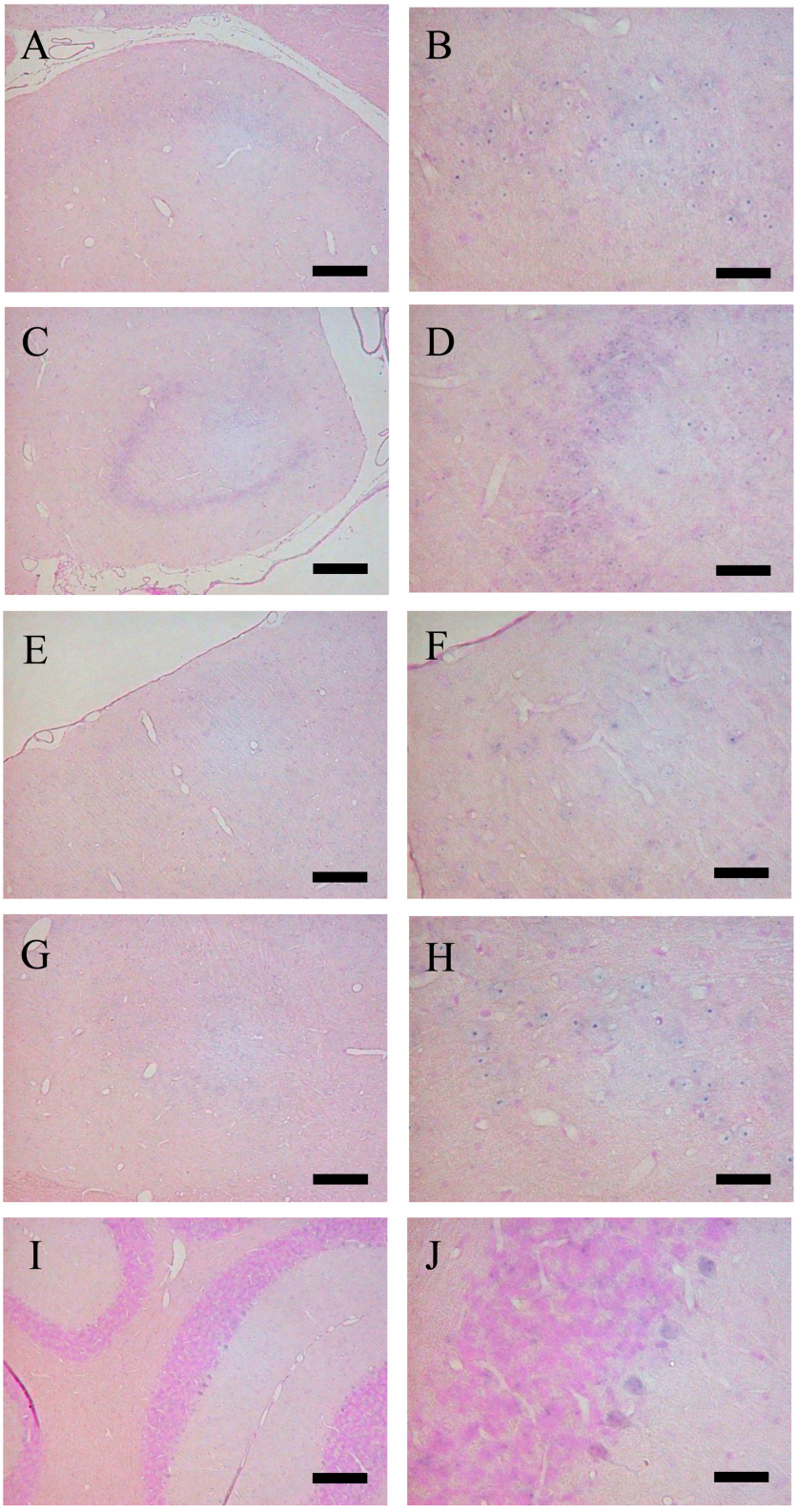

Fig. 3. Expression patterns of *CYP2D* mRNA in the marmoset brain regions obtained using *in situ* hybridization analysis. Representative sections of hippocampus (A, B, C, D), cortex (E, F), substantia nigra (G, H), and cerebellum (I, J) are shown. Scale bar in A, C, E, G, and I: 200 μm. Scale bar in B, D, F, H, and J: 50 μm.

cific inhibitor of CYP2D [33]. Therefore, it is suggested that functional CYP2D proteins may heterogeneously exist in the marmoset brain where by participate in the local metabolism of the exogenous and endogenous compounds at their action site.

In addition to the association of *CYP2D6* genetic polymorphism with psychopathology in human [24], it has been reported that early life stress in the marmoset monkey produces long-term changes in the hippocampal expression of genes involved in synaptic plasticity and implicated in mood disorders [17, 18]. Unraveling the role of CYP2D in the early stress model marmoset brain might lead to a better understanding of the molecular mechanisms underlying psychiatric disorders in the primates.

Unfortunately, we could not examine the level and distribution of each mRNA of *CYP2D8* and *CYP2D19* between the marmoset brain regions in the present study. NADPH-cytochrome P450 reductase is also well-known to assist the catalytic reaction of CYP. This enzyme in cooperation with CYP plays important roles in fear conditioning and memory [9]. Regional quantitative real time PCR using specific primers and the ISH study with specific probes to these *CYP2D* isoforms and to NADPH-cytochrome P450 reductase would offer substantial aid in clarifying the function of CYP2D in the marmoset brain. Further study is needed to clarify the involvement of CYP2D in the psycho-neurologic actions of the marmoset brain.

In this study, we showed the expression patterns of *CYP2D* in the brain of the common marmoset. Our results indicate that the common marmoset can be a useful model for the analysis of the role of CYP2D in the brain, for the study of mechanisms underlying psychiatric disorders related to CYP2D dysfunction, and for the development of new drugs based on novel approaches.

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