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# Human CYP2D6 and mouse CYP2Ds: organ distribution in a humanized mouse model.

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Running Title: CYP2D organ expression in humanized mice.

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Non-standard abbreviations: CYP, cytochrome P450; DDT, dithiothreitol; EDTA, ethylenediaminetetra-acetic acid disodium salt; MAB, monoclonal antibody; PAB, polyclonal antibody; TBST, tris buffered saline triton-X.

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Abstract

Polymorphic cytochrome P450 2D6 (CYP2D6) metabolizes several classes of therapeutic drugs, endogenous neurochemicals, and toxins. A CYP2D6-humanized transgenic mouse line was previously developed to model CYP2D6 poor and extensive metabolizer phenotypes. Human CYP2D6 was detected in the liver, kidney and intestine of these animals. In this study we investigated further the cellular expression and relative tissue levels of human CYP2D6 in these transgenic mice in liver, intestine, kidney and brain. In addition, we compared this to the expression of mouse CYP2D enzymes in these organs. In humans, these organs are of interest with respect to CYP-mediated drug metabolism, toxicity and disease. The expression of human CYP2D6 and mouse CYP2D enzymes in humanized and wild type mice was quantified by immunoblotting, and detected at the cellular level by immunocytochemistry. The cell-specific expression of human CYP2D6 in liver, kidney and intestine in humanized mice was similar to that reported in humans. The expression patterns of mouse CYP2D proteins were similar to those in humans in liver and kidney, but substantially different in intestine. Human CYP2D6 was not detected in brain of transgenic mice. Mouse CYP2D proteins were detected in brain, allowing, for the first time, a direct comparison of CYP2D expression among mouse, rat and human brain. This transgenic mouse model is useful for investigating CYP2D6-mediated metabolism in liver, kidney and especially the intestine where expression patterns demonstrated substantial species differences.

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The cytochrome P450 enzymes constitute a superfamily of heme-thiolate enzymes that are responsible for metabolism of many clinically used drugs and other xenobiotic compounds, and a variety of endogenous compounds. Human CYP2D6 metabolizes up to 20% of all clinical drugs. Substrates belong to several therapeutic classes that include centrally acting compounds such as tricyclic antidepressants, SSRIs, beta blockers, monoamine oxidase inhibitors, neuroleptics, opioids, drugs of abuse such as ecstasy (methylenedioxymethamphetamine), neurotoxins and endogenous neurochemicals (Yu et al., 2004; Zanger et al., 2004).

The *CYP2D* gene locus has been mapped to human chromosome 22q13.1 and comprises the functional *CYP2D6* gene and two inactive pseudogenes *CYP2D7p* and *CYP2D8p* (Nelson et al., 2004). The *CYP2D6* gene is polymorphic and there are more than 90 known allelic variants to date (www.imm.ki.se/CYPalleles/cyp2d6.htm). From 5 to 10% of Caucasians are poor metabolizers (Zanger et al., 2004) and this can lead to individual variation in response to many drugs due to reduced or absence of metabolism by CYP2D6. There have been several reports of association of genetic variation in *CYP2D6* with risk for diseases and cancer, for example, Parkinson's Disease (Kurth and Kurth, 1993), lung cancer, liver cancer and melanoma (Agundez, 2004). Interindividual differences in response to drugs metabolized by CYP2D6 may also be influenced modestly by hormonal state, diet (Llerena et al., 1996; Wadelius et al., 1997), and by xenobiotic regulation of expression of the enzyme in liver and extrahepatic organs such as brain, kidney and intestine (Miksys et al., 2002).

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Drug-metabolizing CYPs are expressed at high levels in the liver, and this organ controls the systemic levels of most drugs and xenobiotics and their metabolites. However, CYPs are also expressed in extrahepatic organs, where in situ metabolism may profoundly influence the effect of a drug or endogenous compound, or the chemical toxicity of a reactive intermediate, in the target organ. Some organs of interest are intestine, kidney and brain, in which CYP2D6 is expressed, and has been implicated in organ toxicity and/or disease. The intestine is exposed to orally administered xenobiotics before the liver, and has the capacity to metabolize many of these compounds through pathways mediated by CYPs including CYP2D6 (Prueksaritanont et al., 1995; Madani et al., 1999). This organ may contribute to first pass metabolism of many oral drugs, affecting their bioavailability and activation or deactivation. CYP2D6 has been identified in human kidney (Nishimura et al., 2003), and tissue injury by some xenobiotics is dependent on renal CYP metabolism to toxic metabolites (Liu et al., 2003; Liu and Baliga, 2003). CYP2D6 is expressed constitutively in neurons in human brain (Siegle et al., 2001; Chinta et al., 2002; Miksys et al., 2002), raising the possibility of an endogenous function of CYP2D6 in the metabolism of neurochemicals. While CYP2D6 activity has not been characterized in human brain, the kinetics of CYP2D-mediated dextromethorphan O-demethylase activity have been well described in rat brain tissues (Tyndale et al., 1999).

A CYP2D6 humanized mouse line has been developed where the transgenic and wild type mouse lines act as models of human extensive and poor metabolizers respectively (Corchero et al., 2001; Yu et al., 2004). The transgene includes the complete human

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wild type CYP2D6 gene and its regulatory sequence, and is present in the mice at 5±1 copies per haploid genome (Corchero et al., 2001). The mouse CYP2D genes are also expressed in these animals. There are at least nine different mouse CYP2D genes (Nelson et al., 2004), but some of the isozymes have not been characterized for expression or function. One isozyme, CYP2D22, has been suggested to be the functional orthologue of CYP2D6 (Blume et al., 2000). Debrisoquine hydroxylase activity is enhanced in *CYP2D6* transgenic mice compared with wild type mice (Corchero et al., 2001), indicating that CYP2D6 in humanized mice is functional. The transgenic mouse line has been used, for example, to establish that CYP2D6 is a 5-methoxyindolethylamine O-demethylase (Yu et al., 2003). This model has the potential for investigating human CYP2D6-mediated metabolism of drugs, xenobiotics, and endogenous compounds, extrahepatic first-pass metabolism by CYP2D6 and oral bioavailability of drugs, and also the role of CYP2D6-mediated metabolism in organ-specific toxicity, pathologies and disease. CYP2D6 expression was detected in liver, kidney and intestine of the transgenic mice (Corchero et al., 2001). In this paper we examined in detail the tissue and cellular expression of CYP2D6 protein in liver, intestine, kidney, and brain of humanized mice. In addition, we described the relative expression levels and distribution of mouse CYP2D enzymes in liver, intestine, kidney and brain. We compared the distribution of CYP2D6 in humanized mice to the distribution reported in human tissues, and to the distribution of mouse CYP2D enzymes in wild type mice in order to further validate this mouse model of polymorphic expression of human CYP2D6.

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## Methods

### Animals

Adult male *CYP2D6* transgenic mice and non-transgenic wild type mice (Corchero et al., 2001) (FVB/N) (25-30 g, 2-4 months) were maintained under conditions of controlled temperature (23 ± 1°C) and lighting (lights on 6:00 AM-6:00 PM), with food and water provided ad libitum. Animals were sacrificed by exsanguination under $CO_2$ anesthesia. Handling of animals was in accordance with animal study protocols approved by the National Cancer Institute Animal Care and Use Committee.

### Immunoblotting

Liver, kidney, intestine (separated into duodenum, jejunum, ileum and colon) and brain were dissected then frozen in liquid nitrogen and stored at -80°C. Microsomal membranes were prepared from liver, intestine and kidney of wild-type and transgenic mice (n=3-4/group) by homogenizing tissues in 100 mM Tris (pH 7.4) with 0.1 mM EDTA, 0.1 mM DDT and 0.32 M sucrose, centrifugation at 10,000 g for 30 min, followed by centrifugation of the supernatant at 100,000 g for 90 min. Total membranes were prepared from cerebellum by homogenizing in the same buffer followed by centrifugation at 3,000 g for 5 min, followed by centrifugation of the supernatant at 110,000 g for 90 min (Miksys et al., 2000). Pellets were resuspended in 100 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1 mM DTT, 1.15% w/v KCl and 20% v/v glycerol, aliquoted and stored at -80°C. The protein content of each sample was assayed with a Biorad protein assay kit (Biorad Laboratories Canada Inc., Mississauga, Ontario, Canada) and proteins were separated by SDS polyacrylamide gel electrophoresis in 4.5% stacking

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gel and 10% separating gel (12 x 14cm), transferred to nitrocellulose membranes and probed for human and mouse CYP2D proteins. Membranes were blocked with 1% skim milk in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Triton X-100, 0.1% BSA) then probed with either (1) monoclonal antibody (MAB) (Table 1) MAB-2D6 Cat. No. 458246 (BD Gentest, BD Biosciences, Bedford, MA, USA) diluted 1:2,500 with TBST, followed by peroxidase-conjugated anti-mouse IgG (Pierce Chemical Co., Rockford, IL, USA) diluted 1:10,000 with TBST or (2) polyclonal antibody (PAB) (Table 1) rabbit PAB-2D6 Cat. No. 458210 (BD Gentest) diluted 1:2,000 with TBST, followed by peroxidase-conjugated anti-rabbit IgG (Chemicon, Temecula, CA, USA) diluted 1:2000 with TBST. Bands were visualized by chemiluminescence (Pierce Chemical Co.) and the films (Ultident, St. Laurent, QC, Canada) were digitized and analyzed using MCID Elite image analysis software (Imaging Research Inc. St. Catharines, ON, Canada). The relative optical density of each band was corrected for background and expressed as arbitrary density units. Dilutions of liver, kidney, intestine and brain membranes were blotted to determine the best protein concentration for a distinct, linear signal. Human lymphoblast expressed CYP2D6 (BD Gentest), human liver microsomes (Dr. T. Inaba, University of Toronto) and membrane preparations from human cerebellum (Dr. D. Mash, Director of the University of Miami Brain Endowment Bank) were used as positive controls. Neither antibody showed cross-reactivity on blots loaded with 0.2 pmol expressed human CYP2B6, CYP2E1 or CYP2A6 (BD Gentest).

Immunocytochemistry

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Tissues were fixed in 4% paraformaldehyde in phosphate buffer, and frozen sections were collected. Liver sections were stained with seven different antibodies in order to identify those that could differentiate between human CYP2D6 and mouse CYP2D enzymes. Each antibody was titrated to determine the optimum working conditions. Two polyclonal antibodies (Table 1), M-PAB-2D6, a rabbit anti-CYP2D6 peptide (a gift from A. Cribb and Merck & Co. NJ, USA (Cribb et al., 1995)), and PAB-2D6 (the same PAB used for immunoblotting), a rabbit anti-human CYP2D6 Cat. No. 458210 (BD Gentest,), were used in the range of 1:1000 to 1:5000 diluted in 10 mM phosphate buffered saline (PBS) with 0.1% w/v BSA and 2% v/v normal horse serum (NHS). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase in PBS. Sections were blocked 1h with PBS with 1% w/v skim milk, 0.1% BSA and 10% NHS, incubated 48h with antibody, incubated 1h with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlington, ON, Canada) diluted 1:750 in PBS with 0.1% BSA and 2% NHS, followed by the avidin-biotin complex (ABC) technique (Vector Laboratories) and reaction with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide (Vector Laboratories). Control sections were incubated in PBS with 0.1% BSA and 2% NHS without primary antibody. Five monoclonal antibodies (Table 1), IH-MAB-2D6 Cat. No. 458366 (BD Gentest), MAB-2D6 (the same MAB used for immunoblotting) Cat. No. 458246 (BD Gentest), MAB-2D6 clones 4-74-1, 50-1-3, and 512-1-8 (Gelboin et al., 1997), were used in the range of 1:250 to 1:2000. Immunocytochemical staining was carried out using the M.O.M. (mouse on mouse) system (Vector Laboratories) followed by ABC and reaction with DAB. Frozen sections of kidney and intestine were immunostained with MAB 4-74-1 diluted 1:500. Frozen sections of brain were

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immunostained with MABs 4-74-1, 50-1-3 and 512-1-8 at concentrations of 1:150 to 1:2000, and with M-PAB-2D6 (from A. Cribb) at 1:1000.

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## Results

All mouse CYP2Ds have high amino acid sequence identity (65-75%) with human CYP2D6 (Blume et al., 2000; Nelson et al., 2004), but by screening seven antibodies, we were able to distinguish between mouse and human CYP2D enzymes by both immunoblotting and by immunocytochemistry. A summary of antibodies used for immunoblotting and immunocytochemistry, their cross-reactivity with human CYP2D6 and mouse CYP2Ds, and each organ examined are given in Table 1. In wild type mice, multiple banding patterns were observed on immunoblots, most likely due to expression of several CYP2D isozymes. For both antibodies used for immunoblotting, we found no cross-reactivity with CYPs 2A6, 2B6 or 2E1, and the manufacturer reports no cross-reactivity with CYPs 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2E1, 3A4, 3A5, 3A7 and 4A11. The predominant mouse CYP2D proteins in liver, kidney and intestine had lower mobilities than human CYP2D6, and this reflects their higher molecular weights (Swiss-Prot or EBI data-bases).

### Liver Immunoblotting

Linear conditions were established for the detection of CYP2Ds with MAB-2D6 (Fig 1A) and PAB-2D6, and 0.4 μg of protein were used for all subsequent experiments. With MAB-2D6, human CYP2D6 was detected in livers from transgenic mice, but no signal was detected in wild-type liver (Fig. 1B), indicating that the antibody was selective for human CYP2D6. In liver of transgenic mice, CYP2D6 was expressed at a level similar (75%) to that in human liver (assessed by densitometry of 3 different immunoblots),

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illustrated in Figure 1D, where equal amounts of microsomal protein from transgenic mouse liver and from human liver gave MAB-CYP2D6 signals of similar intensity. With PAB-2D6, mouse CYP2Ds were detected in wild type mice, and both mouse CYP2Ds and human CYP2D6 were detected in transgenic mice (Fig. 1C). Figure 1D shows more clearly that the human CYP2D6 in transgenic mouse liver co-migrated with CYP2D6 from human liver microsomes and CYP2D6 expressed in human lymphocytes. Mouse CYP2Ds were detected with the PAB-2D6 as two distinct bands. The predominant band migrated more slowly than human CYP2D6.

Liver Immunocytochemistry

Of the seven different antibodies tested, MABs 4-74-1 and 50-1-3 were the most selective for human CYP2D6 (Table 1). They produced intense staining in livers from transgenic mice, but in livers from wild type mice, staining levels were the same as seen in control sections that were incubated in the absence of primary antibody (Fig. 2A, B, C). MAB 512-1-8 stained liver from transgenic mice strongly, but there was also some moderate staining of livers from wild type mice, suggesting some cross-reactivity with mouse CYP2Ds. MAB-2D6 (used for immunoblotting) did not stain liver from either transgenic or wild type mice, suggesting that this antibody only recognizes the denatured human CYP2D6 on immunoblots and not the protein in its native form in fixed, frozen sections. The remaining three antibodies (IH-MAB-2D6, PAB-2D6 and M-PAB-2D6) resulted in equally intense staining of livers from wild type and transgenic mice, suggesting that they detected both human CYP2D6 and mouse CYP2D (Fig. 2D, E, F). Both CYP2D6 in transgenic mice and mouse CYP2Ds in wild type mice exhibited

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pericentral expression patterns in liver sections (Fig. 2). CYP2D6-selective MAB 4-74-1 was used for subsequent immunocytochemistry on kidney and intestine.

Brain Immunoblotting

To establish whether human CYP2D6 was expressed in brains of transgenic mice, the same MAB-2D6 that differentiated between human CYP2D6 and mouse CYP2Ds in liver was used (Table 1). Mouse cerebellar membranes were used for immunoblotting as human CYP2D6 RNA and protein (Siegle et al., 2001; Chinta et al., 2002; Miksys et al., 2002) and rat CYP2D RNA and proteins (Miksys et al., 2000) are expressed at moderate to high levels in cerebellum. Linear conditions were established. We were unable to detect human CYP2D6 in transgenic mouse cerebellar membranes (100 μg) with MAB-2D6 (Fig. 3A). With PAB-2D6 (Table 1), mouse CYP2Ds were detectable in cerebellum (80 μg) of both wild type and transgenic mice (Fig. 3B). As CYP2D6 in human cerebellum was detected by both MAB-2D6 and PAB-2D6 (Fig. 3C, D), this suggests that human CYP2D6 was not expressed at detectable levels in brains of transgenic mice. As in liver, several mouse CYP2D bands were detected in both wild type and transgenic mice (Fig. 3D). Unlike in liver, the most intense mouse CYP2D band detected in brain migrated further than expressed human CYP2D6 and the single CYP2D6 band detected in human cerebellum (Fig. 3D).

Brain Immunocytochemistry

To confirm the immunoblotting results, we used immunocytochemistry to investigate the presence of CYP2D6 in brains of transgenic mice with CYP2D6-selective MABs that

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detected CYP2D6 in sections of livers of transgenic mice (Table 1). We were unable to detect any staining of human CYP2D6 in brain with the CYP2D6-selective MABs 4-74-1, 50-1-3 and 512-1-8 even at the highest concentrations of primary antibody, which were more than 10 times higher than concentrations used to detect CYP2D6 in liver (Fig. 4A, B, C, Fig. 5A, B, C). This suggests that human CYP2D6 was either not present, or present at extremely low, undetectable levels in brains of transgenic mice. With M-PAB-2D6, we were able to detect distinct cellular staining in brains from all animals (Table 1), indicating that mouse CYP2Ds were expressed in brain at detectable levels (Fig. 4D, E, F, Fig. 5D, E, F). There was no difference in pattern or intensity of staining between wild type and transgenic mice. This again suggests that human CYP2D6 was not expressed in brains of transgenic mice. Table 2 summarizes the regional and cellular distribution of mouse brain CYP2D. Included in this table for comparison are the immunostaining levels with the same M-PAB-2D6 antibody of brain regions from rat (Miksys et al., 2000) and human (Miksys et al., 2002). Immunostaining of mouse CYP2Ds was particularly evident in neurons of layers II-V of the cerebral cortex (Fig. 4D, E, F), and in the dentate gyrus and pyramidal cells of the CA1-3 regions of the hippocampus (Fig. 5D, E, F).

Kidney Immunoblotting

Linear conditions were established for detection of CYP2Ds with both MAB-2D6 and PAB-2D6 (Table 1), and 20 μg protein was used for subsequent assays. Human CYP2D6 was detected in kidney of transgenic mice with MAB-2D6, but no signal was detected in kidney of wild type mice (Fig. 6A). With PAB-2D6, mouse CYP2Ds were

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detected in kidney of wild type mice, and both mouse CYP2D and human CYP2D6 were detected in kidney of transgenic mice (Fig 6B). As was found in liver, the predominant mouse CYP2D band had a lower mobility than human CYP2D6, and a second less intense mouse CYP2D band had a similar mobility to human CYP2D6 (Fig. 6B). To confirm that the higher mobility band in transgenic kidney was human CYP2D6, expressed CYP2D6 was added to kidney membranes from a wild type mouse. As seen in lane 1 of Figure 6B, the expressed CYP2D6 had a higher mobility than the predominant mouse CYP2D band, and co-migrated with CYP2D6 in kidney of transgenic mice. In kidney, CYP2D6 was expressed at 2.5% of the levels detected in transgenic mouse liver.

Kidney Immunocytochemistry

Human CYP2D6 was detected in kidney of transgenic mice with MAB 47-4-1 (Table 1), and there was no immunostaining of kidney of wild type mice with this antibody (Fig. 7). In the kidney cortex, immunostaining was more intense in the proximal tubules than in the distal tubules, and the glomeruli of the Bowman's capsules were strongly stained (Fig. 7B, D). In the medulla, the loops of Henle were more intensely stained than the adjacent cortical distal convoluted tubules (Fig. 7B, E).

Intestine Immunoblotting

Linear conditions were established for detection of CYP2D with both MAB-2D6 and PAB-2D6 (Fig. 8A), and 15 μg protein were used subsequently for all regions. CYP2D6 was detected with MAB-2D6 in intestine of transgenic mice, and this co-migrated with

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expressed human CYP2D6 and transgenic mouse liver (Fig. 8B). No signal was detected with MAB-2D6 in intestine of wild type mice (Fig. 8B). Expression of CYP2D6 was highest in jejunum followed by ileum and colon and lowest in duodenum (Fig. 8B). With PAB-2D6, mouse CYP2Ds were detected in intestine of wild type mice, and both human CYP2D6 and mouse CYP2Ds were detected in intestine of transgenic mice (Fig. 8C). As was seen in liver and kidney, the predominant mouse CYP2D band had a lower mobility than human CYP2D6, and a second weaker mouse CYP2D band had a similar mobility to human CYP2D6 (Fig. 8C). In wild type mice, CYP2D expression was highest in colon, followed by ileum, jejunum and lowest in duodenum (Fig. 8C). This was a different regional pattern of expression to that observed for human CYP2D6 in transgenic mice (Fig. 8B). In duodenum, jejunum, ileum and colon, CYP2D6 was expressed at <1%, 13%, 6% and 2% respectively of the levels detected in liver of transgenic mice.

Intestine Immunocytochemistry

Human CYP2D6 was detected with MAB 47-4-1 (Table 1) in all intestinal regions of transgenic mice (Fig. 9B, C and F), and no signal was detected in wild type mice (Fig. 9 A, E). As was observed by immunoblotting, the intensity of staining was weak in duodenum, maximal in jejunum and decreased distally from ileum to colon. Staining was confined to the epithelial enterocyte cells at the surface of tips of the colon mucosa (Fig.9 B, C) and villi of jejunum (Fig.9 F). There was no CYP2D6 observed in the lamina propria in the core of the villi, in the crypts of Lieberkuhn, or in goblet cells (Fig.9 B, C, F).

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## Discussion

In this study we showed that human CYP2D6 is expressed in liver, kidney and intestine of CYP2D6-humanized mice, and that the cellular localization for the most part is similar to that observed in human organs. We observed a striking difference in the pattern of expression of human CYP2D6 and mouse CYP2D along the length of the intestine. CYP2D6 protein and enzyme activity have been detected at low levels in human intestine, and are differentially expressed along the length of the gastrointestinal tract (de Waziers et al., 1990; Prueksaritanont et al., 1995; Madani et al., 1999), with the same pattern that we observed for CYP2D6 in transgenic mice. Expression was highest in the jejunum, and decreased distally to the colon. Mouse intestine can metabolize bufuralol (Emoto et al., 2000), but CYP2D protein expression has not been described. We found that mouse intestinal CYP2D followed a distinctly different expression pattern to human CYP2D6; it was highest in the colon, and decreased proximally, with lowest expression in the proximal small intestine, the duodenum. The upper part of the small intestine is thought to be a site of CYP-mediated first-pass metabolism in humans. Several CYPs are expressed in mucosal enterocytes (Zhang et al., 1999; Ding and Kaminsky, 2003), and we found that, like other CYPs (Murray and Burke, 1995), CYP2D6 cellular expression was restricted to enterocytes at the surface of the intestinal mucosa, and concentrated at the tips of the villi. This is an ideal situation for CYPs to act as a barrier to systemic uptake of potentially toxic xenobiotics. These compounds can be inactivated in situ to metabolites that are bound within the enterocytes and lost when these cells are sloughed off. Alternately, CYPs may activate xenobiotics to

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toxicants or carcinogens. This transgenic mouse line should serve as a good model for investigation of the possible role of CYP2D6 in intestinal toxicities, diseases and cancers.

Hepatic CYP2D6 expression and activity has been previously established in this mouse line (Corchero et al., 2001). In addition, we observed that both CYP2D6 in transgenic mice and mouse CYP2Ds in wild type mice exhibited pericentral expression patterns in liver sections (Fig. 2), typical of most human CYPs (Oinonen and Lindros, 1998), and mouse CYP2D (Blume et al., 2000).

The kidney is frequently exposed to xenobiotics and their metabolites, and the cells of the proximal renal tubules are potential targets for metabolic toxicity. Human kidney expresses CYP2D6, and exhibits CYP-mediated drug metabolism (Lohr et al., 1998; Nishimura et al., 2003). In kidney of transgenic mice, we found that CYP2D6 expression was highest in the proximal tubules of the cortex, as was observed in mice (Manns et al., 1989) and rats (Duclos-Vallee et al., 2000). Very little is known about the cellular localization or function of CYP2D6 in human kidney, but other CYPs show a similar high expression in renal proximal tubules (Lohr et al., 1998; Lasker et al., 2000). Nephrotoxicity and tissue injury from some xenobiotics have been shown to be at least partly dependent on renal CYP metabolism (Liu et al., 2003; Liu and Baliga, 2003; Van Vleet and Schnellmann, 2003). This transgenic mouse model may serve to advance our knowledge of the function of renal CYP2D6 in the metabolism of clinical drugs and its potential role in nephrotoxicities.

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We investigated whether CYP2D6 was expressed in the brain of this transgenic mouse line because of the polymorphic expression of CYP2D6 in neurons in human brain (Miksys et al., 2002), the discovery of endogenous neurochemical substrates of CYP2D6 (Yu et al., 2003; Yu et al., 2004), and the implication in some studies of a role for CYP2D6 in neurological diseases (Kurth and Kurth, 1993; Saitoh et al., 1995; Tanaka et al., 1998). We did not detect human CYP2D6 in transgenic mouse brain by either immunoblotting or by immunocytochemistry. This was not due to lack of sensitivity of the assays, as we were able to detect mouse CYP2D by both techniques, and CYP2D6 in human brain by immunoblotting. This is not unprecedented; in a transgenic-*Cyp3A4* mouse model, CYP3A4 protein is expressed in intestine but not in liver (Granvil et al., 2003), a site of high expression levels in humans. The lack of expression of CYP2D6 in brain could be for several reasons for example there could be species differences in tissue-specific transcription factors, where the mouse brain transcription factors are unable to activate human *CYP2D6* gene transcription. The transgene contains the entire CYP2D6 sequence, starting at –2453; this may not include the transcriptional information necessary for brain-specific transcription and expression. In addition, the mode of insertion of the CYP2D6 transgene could be such that it interrupts the three-dimensional structure essential for brain-specific activation of transcription. As our interest is focused on protein expression, we elected not to pursue this further, for example by assaying for CYP2D6 mRNA levels in brain.

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We were able to detect mouse CYP2Ds in brain by both immunoblotting and by immunocytochemistry. While one isozyme, CYP2D22, has been identified in brain (Blume et al., 2000), little has been reported of CYP2D expression and localization throughout mouse brain. One study suggests that mouse brain microsomes can metabolize MPTP, and that this is mediated by CYP2D primarily localized in neuronal cells (Upadhya et al., 2001). We have also found CYP2D enzyme activity in multiple brain regions of rat (Tyndale et al., 1999), where CYP2D is also predominantly neuronal (Miksys et al., 2000). In the current study, we also found that mouse brain CYP2D protein was predominantly expressed in neurons, and that the pattern of expression among brain regions was similar to the pattern we observed in rats (Table 2) (Miksys et al., 2000). In human brain, CYP2D6 is also expressed neuronally, and the pattern of expression is similar to that in rat and mouse in most brain regions (Table 2). For example, in mouse, rat and human brain, there is high expression of CYP2D in pyramidal neurons of frontal cortex layers II – VI, and lower expression in globus pallidus than in the caudate and putamen. In contrast, in cerebellum of mice and rats (Miksys et al., 2000), CYP2D is expressed moderately to highly in Purkinje cells, whereas in cerebellum of humans the Purkinje cells exhibit very low levels of CYP2D6 expression (Miksys et al., 2002).

All of the organs examined displayed multiple mouse CYP2D bands on immunoblots, suggesting the expression of more than one mouse CYP2D isozyme. Genes encoding at least nine different mouse CYP2D isozymes have been identified (Nelson et al., 2004), but not all of them have been characterized for protein expression. The

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migrations of the CYP2D immunoreactive bands relative to CYP2D6 were different among brain and liver and kidney. This suggests that mouse CYP2D isozymes are differentially expressed among organs and that there may be a brain-specific isoform of mouse CYP2D. In rat, there are six isozymes, CYP2D1-5, and 18 (Nelson et al., 1993; Kawashima et al., 1996), and these are differentially expressed among organs with CYP2D4 being the most prevalent in brain (Miksys et al., 2000), and CYP2D18 being expressed in brain but not in liver (Kawashima et al., 1996). More detailed studies are needed to clarify which mouse CYP2D isozymes are expressed in brain, liver, kidney and intestine and which, if any, are functionally homologous to human CYP2D6.

The expression of human CYP2D6 in liver, kidney and intestine of CYP2D6-humanized mice demonstrated overall similar tissue- and cell-specific patterns to those reported in humans. In addition, we described the expression patterns of mouse CYP2Ds in liver, kidney, intestine and brain, and compared these with the expression of CYP2Ds in rat and human tissues. Of note, the pattern of intestinal expression differed substantially between mouse CYP2D and human CYP2D6 indicating that this may be a particularly interesting model for human CYP2D6 intestinal metabolism. Human CYP2D6 was not detected in brain of the humanized mice, but mouse CYP2D protein was readily detected and this is the first description of the brain region- and cell-specific expression of mouse brain CYP2D proteins. This detailed study of CYP2Ds validated the use of these transgenic mice as a model to investigate CYP2D6-mediated metabolism in specific organs, its contribution to organ-specific first-pass metabolism, its role in in situ

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activation and/or inactivation of xenobiotic toxicants, and the potential role of CYP2D6 in certain organ diseases, pathologies and cancers.

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Footnotes

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Figure Legends:

Figure 1. Immunoblotting of human CYP2D6 and mouse CYP2Ds in liver. A. Dilution series (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 μg microsomal protein) of transgenic liver blotted with MAB-2D6; 0.4 μg microsomal protein was used for subsequent liver blots. B. With MAB-2D6, there was no signal for livers from four wild type (Wt) mice, but for livers from four transgenic (Tg) mice a single band was detected that co-migrated with 30 fmol expressed CYP2D6 (2D6). C. With PAB-2D6, mouse CYP2Ds were detected as two bands in wild type livers, and mouse CYP2Ds and CYP2D6 (arrow) were detected in transgenic livers. D. With MAB-2D6, there was no signal from wild type liver, and a single band was detected from transgenic liver that co-migrated with 0.4 μg human liver microsomes (hL) and expressed CYP2D6 (2D6). With PAB-2D6, two bands were detected from wild type and transgenic livers. For transgenic liver, the lower band (arrow) included CYP2D6, and co-migrated with human liver and expressed CYP2D6.

Figure 2. Immunocytochemistry of human CYP2D6 and mouse CYP2Ds in liver. A. With MAB 4-47-1, wild type (Wt) liver did not stain above control levels (C). B. With MAB 4-47-1, transgenic liver (Tg) showed intense staining concentrated in hepatocytes around the central vein (v). C, F. Control (Cont) sections processed without primary antibody. D, E. With M-PAB-2D6, wild type and transgenic livers showed similar intense staining that was strongest in hepatocytes surrounding the central vein (v). Bar: 200 μm.

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Figure 3. Immunoblotting of mouse CYP2Ds in brain. A. With MAB-2D6, there was no signal for cerebellar membranes (100 μg) from either four wild type (Wt) or four transgenic (Tg) mice. B. With PAB-2D6, mouse CYP2Ds were detected in both wild type and transgenic cerebellar membranes (80 μg). C. MAB-2D6 detected CYP2D6 in human cerebellar vermis (CBV) and cerebellar hemisphere (CBH) membranes (80 μg) and these co-migrated with expressed CYP2D6 (30 fmol). D. With PAB-2D6, when the SDS-PAGE gel was electrophoresed for an extended time, several mouse CYP2D protein bands were detected in cerebellar membranes from wild type and transgenic mice. The predominant band had a higher mobility than expressed CYP2D6 and CYP2D6 in human cerebellum (arrow, CBH).

Figure 4. Immunocytochemistry of mouse CYP2Ds in frontal cortex. A, B. With MAB 4-74-1, there was no staining of either wild type (Wt) or transgenic (Tg) frontal cortex above control (no primary MAB 4-74-1 antibody) levels (C). D, E. With M-PAB-2D6, frontal cortex of both wild type and transgenic mice showed immunoreactivity in pyramidal neurons of layers II to V. F. Control, no primary M-PAB-2D6 antibody. Bar: 20 μm (inset: pyramidal cells layer III, bar: 200 μm).

Figure 5. Immunocytochemistry of mouse CYP2Ds in hippocampus. A, B. With MAB 4-74-1, there was no staining of either wild type (Wt) or transgenic (Tg) hippocampus above control (no primary MAB 4-74-1 antibody) levels (C). D, E. With M-PAB-2D6, neurons in the granular layer of the dentate gyrus (black arrows) and neurons in the pyramidal layer of the CA1, 2 and 3 regions (white arrows) were stained very strongly in

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hippocampus of both wild type and transgenic mice. F. Control, no primary M-PAB-2D6 antibody. Bar: 200 μm.

Figure 6. Immunoblotting of human CYP2D6 and mouse CYP2Ds in kidney. A. With MAB-2D6, there was no signal from kidney microsomes (20μg microsomal protein) of four wild type (Wt) mice, but there was a strong band detected in kidney from four transgenic (Tg) mice that co-migrated with 30 fmol expressed CYP2D6. B. With M-PAB-2D6, mouse CYP2Ds were detected in kidneys (20μg microsomal protein) of four wild type mice, and mouse CYP2Ds and CYP2D6 were detected in kidneys of transgenic mice. The predominant mouse CYP2D had a lower mobility than CYP2D6 (arrow). The first lane is microsomes from wild type mouse 1 (lane 2) plus 30 fmol of expressed CYP2D6, confirming that the higher mobility band in transgenic mice (arrow) was CYP2D6.

Figure 7. Immunocytochemistry of human CYP2D6 in kidney with MAB 4-74-1. A. There was no staining of CYP2D in wild type kidney. B. There was strong staining of CYP2D6 in cortex (C) and medulla (M) of transgenic kidney. C. Control, no MAB 4-74-1 primary antibody.

D. In transgenic kidney, staining was stronger in the proximal tubules (PT) than in the distal tubles (DT) of the cortex. Glomeruli (arrows) of the Bowmann's capsules were also stained. E. There was strong staining of the loops of Henley (LH) in the medulla of the transgenic mice, with moderate staining of the distal convoluted tubules (DC). A, B, C, Bar: 1 mm, D, E, Bar: 200 μm.

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Fig. 8. Immunoblotting of human CYP2D6 and mouse CYP2Ds in intestine. A. With MAB-2D6, there was no signal for intestinal regions from wild type mice, but a single band was detected in transgenic intestine that co-migrated with 30 fmol CYP2D6 and 0.4 μg transgenic liver microsomes (Liv). Strongest staining was seen in jejunum followed by ileum, colon and then duodenum. B. With PAB-2D6, mouse CYP2Ds were detected as two bands in intestinal regions of wild type mice, and both CYP2D6 and mouse CYP2Ds were detected in transgenic mice. In wild type mice, staining was strongest in colon followed by ileum, jejunum and then duodenum. As in liver and kidney, the predominant mouse CYP2D band had a lower mobility than CYP2D6. Bar charts depict means + SD (n= 4 mice) of the optical density (arbitrary units) of immunoreactive bands from intestinal regions of wild type and transgenic mice.

Fig. 9. Immunocytochemistry of human CYP2D6 intestine with MAB 4-74-1.
A. There was no staining of colon in wild type mice. B. There was strong staining of CYP2D6 in colon of transgenic mice. C. In transgenic colon, the staining was most intense in the enterocytes at the luminal surface of the intestinal mucosa (arrow). There was no staining of the goblet cells (asterisk). D, G. Control sections of colon (D) and jejunum (G) incubated without MAB 4-74-1 primary antibody. E. There was no staining of jejunum of wild type mice. F. There was strong staining in the jejunum of transgenic mice, confined to the enterocytes at the surface and tips of the villi (arrows). Bar: 200 μm.

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Table 1. Antibodies used for immunoblotting and immunocytochemistry, and their cross-reactivity with human CYP2D6 and mouse CYP2D. + :immunoreactive, – :not immunoreactive, nd: not determined, * :used to generate data.

| | **MAB-2D6*** BD458246 | | **PAB-2D6*** BD458210 | | **MAB* 4-47-1** | ***M-PAB-2D6**[a] | MAB 512-1-8[b] | IH-MAB-2D6 BD458366 | MAB 50-1-3[c] |
|---|---|---|---|---|---|---|---|---|---|
| | IB | ICC | IB | ICC | ICC | ICC | ICC | ICC | ICC |
| Human 2D6 | + | – | + | + | + | + | + | + | + |
| Mouse 2d | – | – | + | + | – | + | +/- | + | – |
| Liver Tg | + | – | + | + | + | + | + | + | + |
| Liver Wt | – | – | + | + | – | + | +/- | + | – |
| Brain Tg | – | nd | + | nd | – | + | – | nd | – |
| Brain Wt | – | nd | + | nd | – | + | – | nd | – |
| Kidney Tg | + | nd | + | nd | + | nd | nd | nd | nd |
| Kidney Wt | – | nd | + | nd | – | nd | nd | nd | nd |
| G.I. Tg | + | nd | + | nd | + | nd | nd | nd | nd |
| G.I. Wt | – | nd | + | nd | – | nd | nd | nd | nd |

[a] Cribb et al., 1995; [b,c] Gelboin et al., 1997

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Table 2. Immunocytochemical staining of mouse, rat and human brain CYP2D with M-PAB-2D6. (++++) very strong, (+++) strong, (++) moderate, (+) weak, (—) no staining

| Brain Region and cell type | Mouse | Rat[a] | Human[b] |
|---|---|---|---|
| Cortex (frontal, temporal, parietal, occipital): | | | |
| layer I neurons | — | — | — |
| layers III and V neurons | ++ | ++ | ++ |
| layers II, IV, VI neurons | + | ++ | ++ |
| Piriform cortex | ++ | +++ | |
| Lateral olfactory tract | — | — | |
| Corpus callosum | — | — | |
| Striatum | | | |
| caudate-putamen neurons of matrix | +++ | ++ | ++/+ |
| globus pallidus neurons | ++ | ++ | + |
| stria terminalis | — | | |
| internal capsule | — | — | — |
| Subgeniculate nucleus neurons | ++ | | |
| Ventral lateral geniculate nucleus neurons | +++ | | |
| neuropil | ++ | | |
| Medial geniculate nucleus | ++ | | |
| Reticular thalamic nucleus | ++ | ++ | |
| Ventral posterior thalamic nucleus | ++ | ++ | |
| Zona incerta | ++ | | |
| Hippocampus | | | |
| dentate gyrus granular cell layer | +++ | — | + |
| polymorphic layer, large neurons | ++ | ++ | — |
| CA1, 2, 3 pyramidal cell layer | +++ | — | + |
| molecular layer | +/++ | ++ | — |
| polymorphic layer | +/++ | ++ | — |
| subiculum, large neurons | +++ | | + |
| amygdalohippocampal area, neurons | ++ | +++ | |
| Substantia nigra compacta, reticulata, neurons | +++ | ++ | ++ |
| Pontine nuclei, neurons | +++ | | |
| Facial nucleus, large neurons | +++ | ++ | |
| Lateral vestibular nucleus, large neurons | ++++ | | |
| Dorsal cochlear nucleus, neurons | +++ | +++ | |
| Interposed cerebellar nucleus | +++ | | |
| Cerebellum | | | |
| molecular layer | ++ | +++ | + |
| small neurons | +++ | +++ | |
| granular cell layer | +++ | + | + |
| Purkinje cells | ++ | +++ | +/— |
| white matter | — | — | — |

[a] Miksys et al., 2000; [b] Miksys et al., 2002

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Figure 1

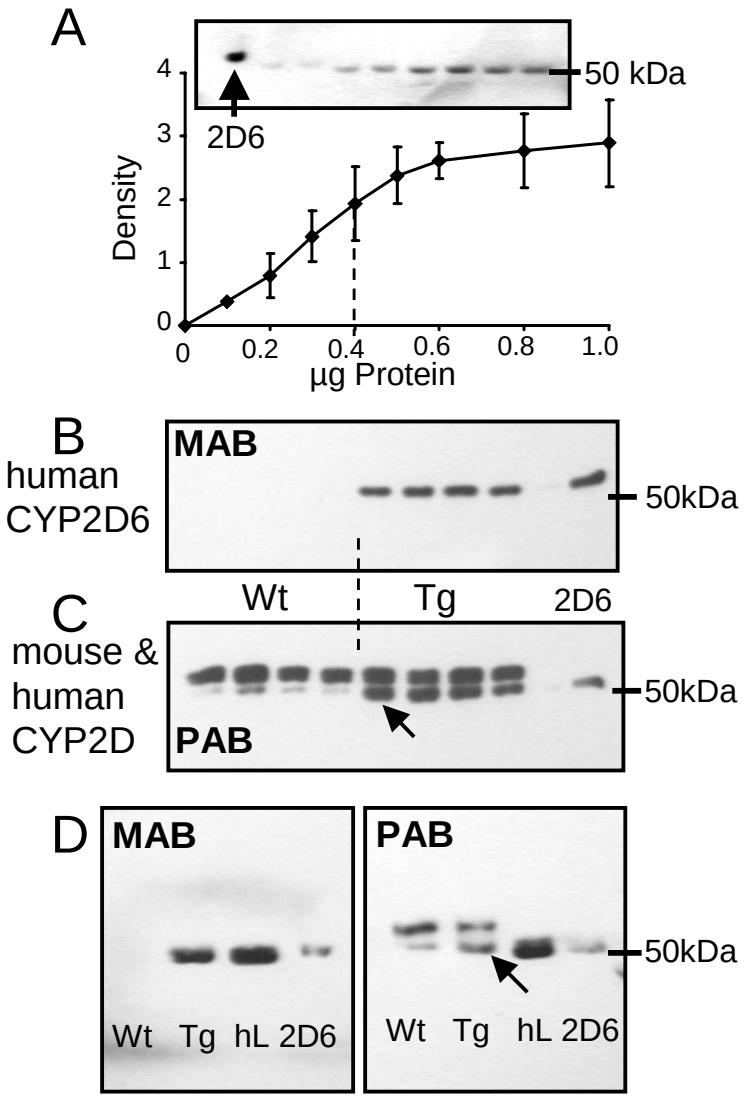


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Figure 2

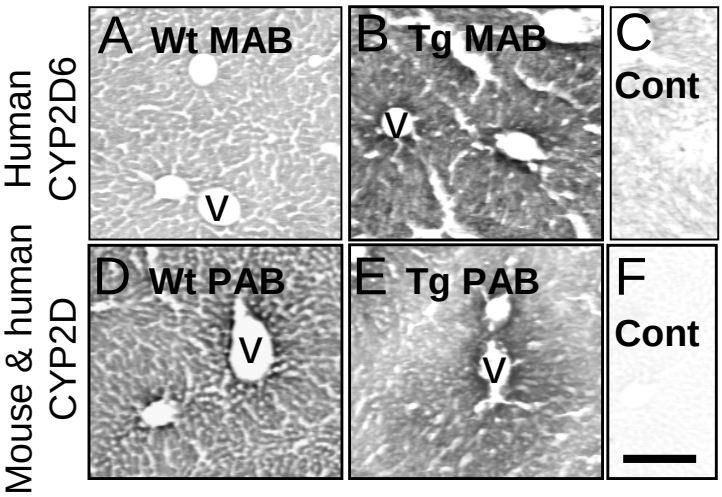


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Figure 3

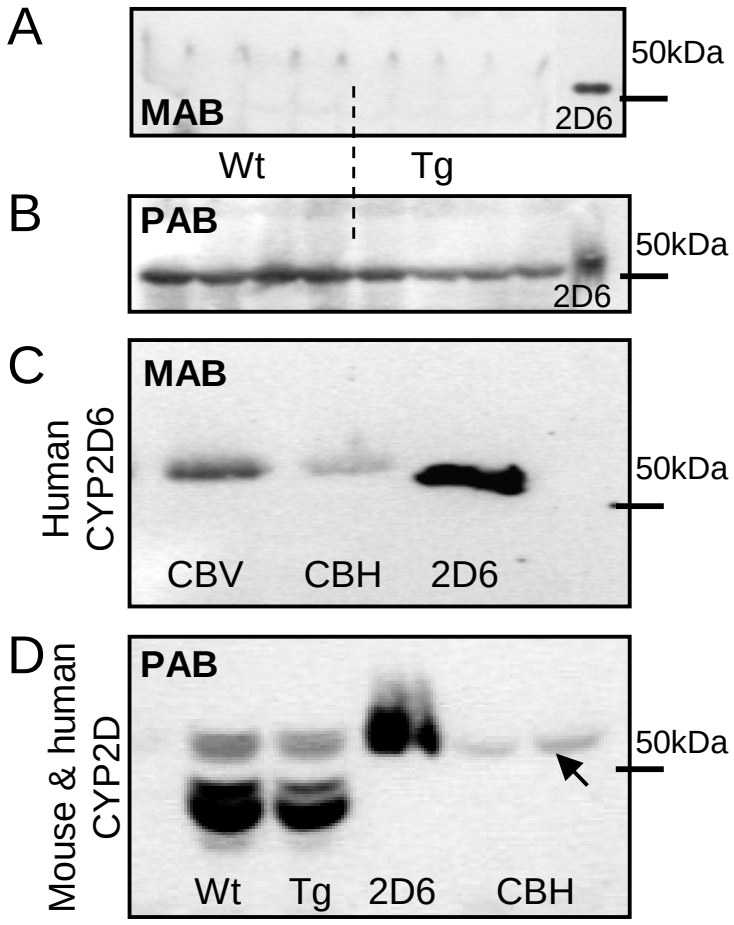


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Figure 4

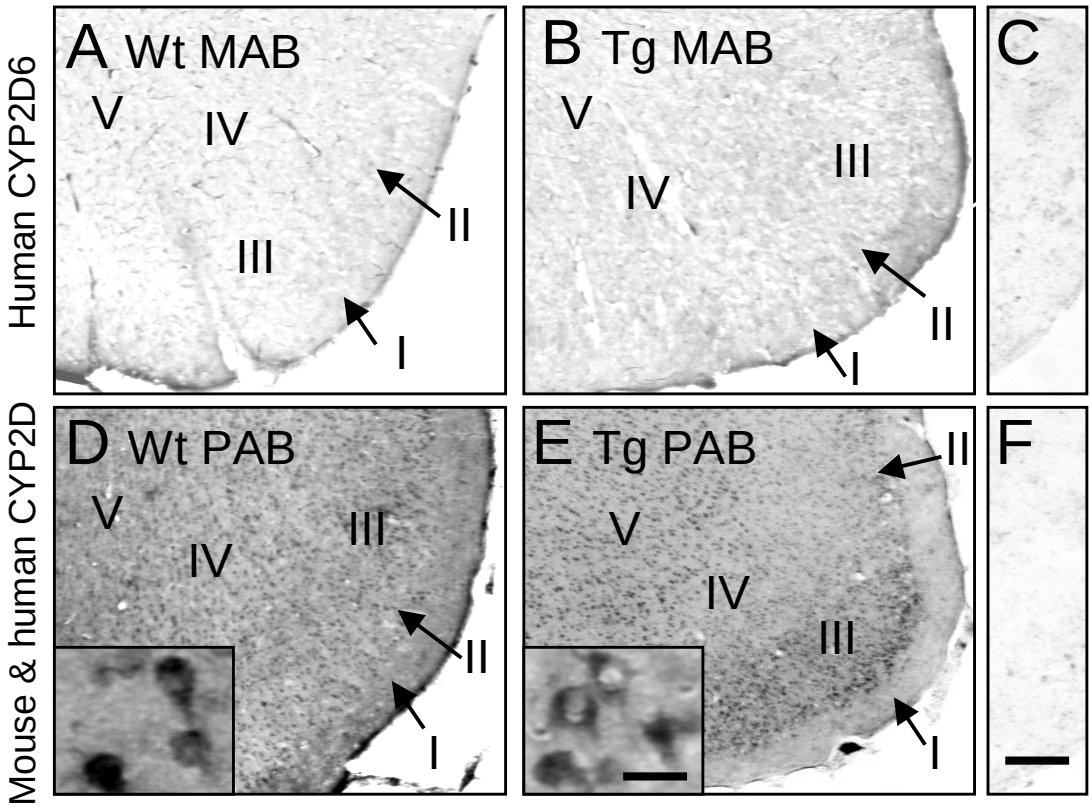


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Figure 5

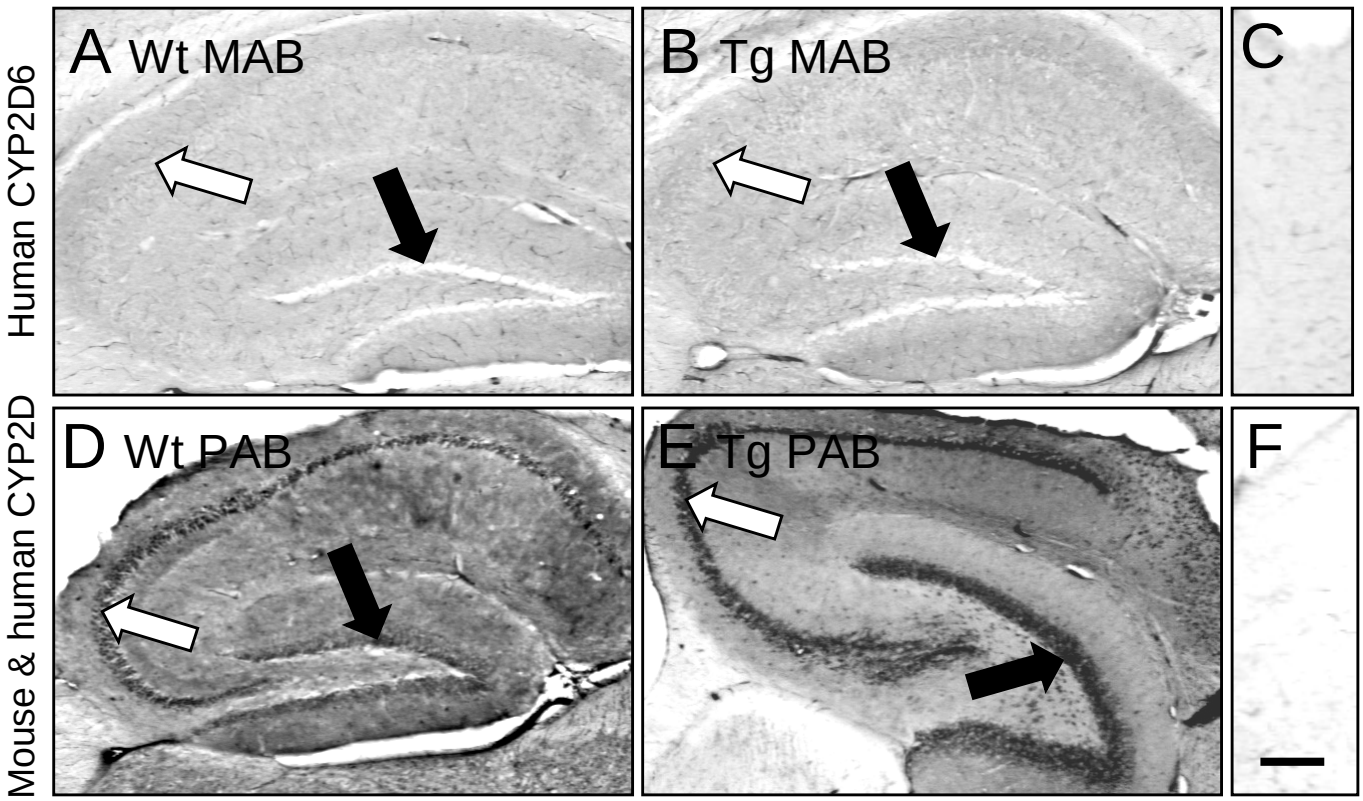


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Figure 6

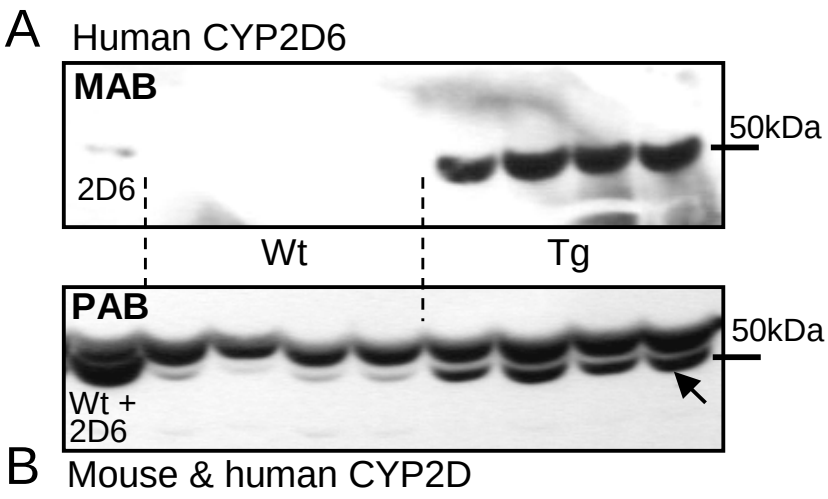


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Figure 7

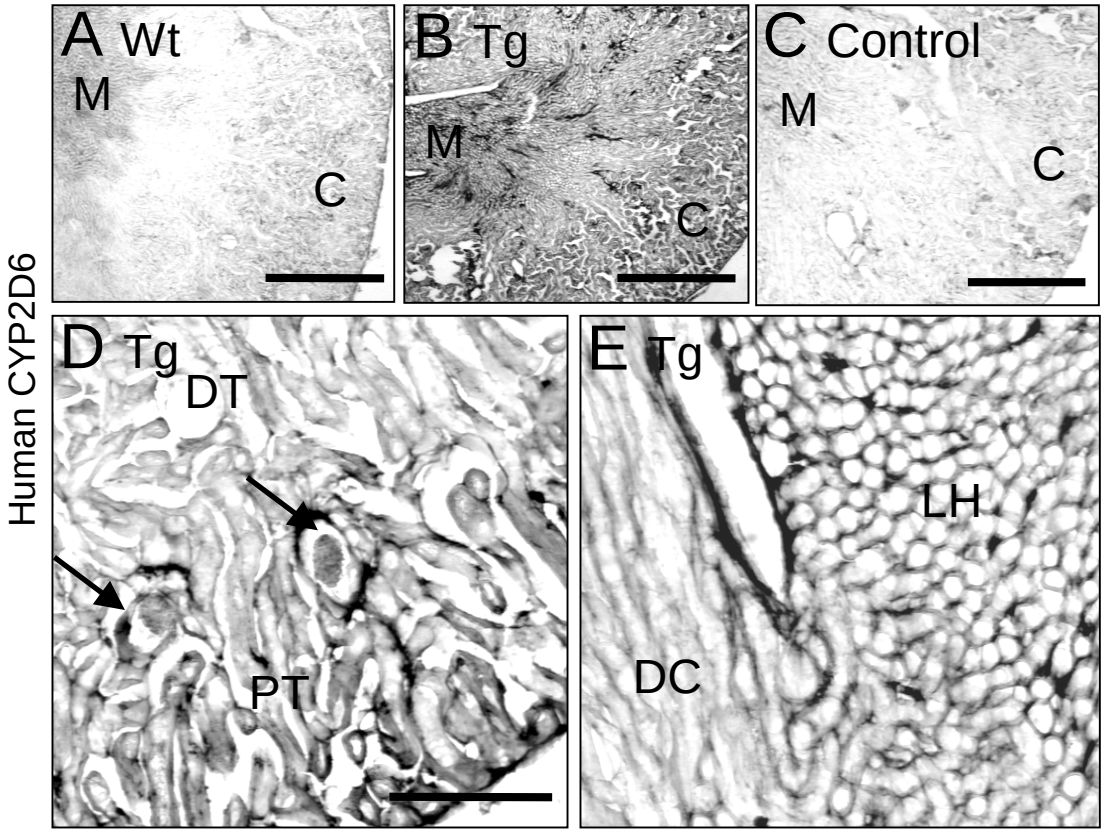


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Figure 8

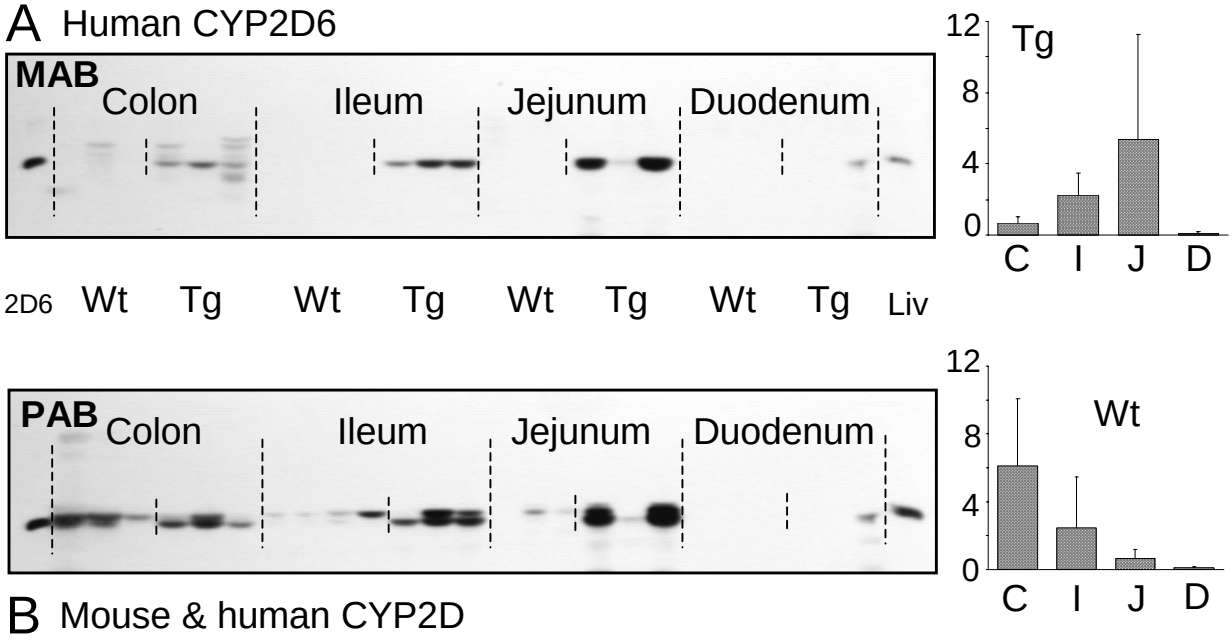


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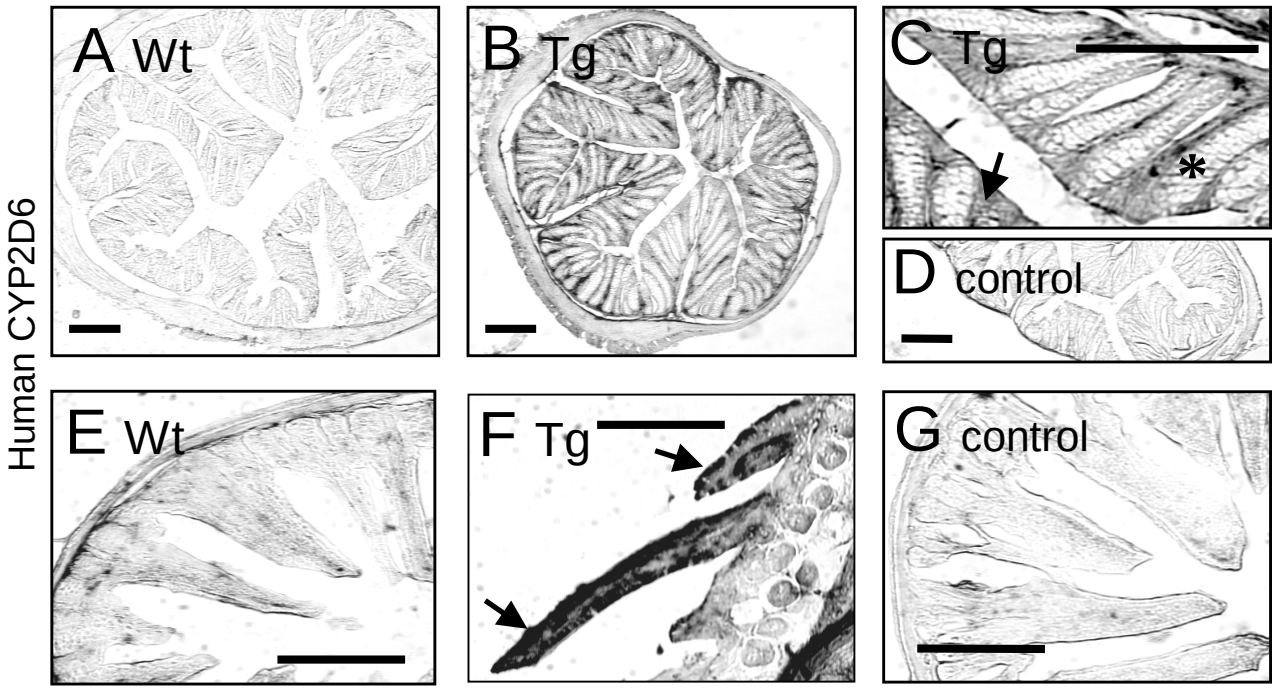
Figure 9

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