Brain Cyclosporine A Levels Are Determined By Ontogenic Regulation Of *Mdr1a* Expression

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Running Title**:** Ontogeny of p-glycoprotein function

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Abstract

Cyclosporine A (CyA) toxicity is a common occurrence in pediatric organ transplant patients. We hypothesized that reduced *mdr1a* expression in newborn and developing mice would affect CyA accumulation within organs and/or toxicity. For functional studies, CyA was administered (5 mg kg^{-1} i.p.) to 1, 12, 19 day and adult male and female *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice. Peak blood CyA was lower in 1, 12 and 19-day-old (1000 ng ml^{-1}) versus adult (1500 ng ml^{-1}) mice but was similar in *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice. Kidney *mdr1a* expression (measured by quantitative PCR) increased 2.5-fold in 19-day-old male and female mice and increased a further 4-fold in adult females compared to adult males. Liver *mdr1a* expression increased 6-fold by day 12 compared to neonatal mice. Thereafter, maintenance of hepatic *mdr1a* expression in females and a reduction to neonatal levels in males was observed. Kidney/blood (8-9 fold) and liver/blood (12-15 fold) CyA were highest on day 12 and 19 and not dependent on maturational changes in *mdr1a* mRNA levels. Adults had higher brain expression of *mdr1a* mRNA (3-fold), a corresponding 5-fold increase in immunodetectable P-glycoprotein and 80% lower brain accumulation of CyA compared to 1-day-old mice. Conversely, in *mdr1a*-null mice, brain/blood CyA was similar in newborn and adults. A similar pattern was observed for the brain accumulation of the mdr1a substrate ^{3}H-digoxin. We conclude that the risk for CNS drug toxicity could be higher in neonates or young children as a consequence of under-developed P-glycoprotein.

The genes *ABCB1* (*MDR1*) in humans and *abcb1a* (*mdr1a*) and *abcb1b* (*mdr1b*) in mouse encode the drug efflux transporter protein P-glycoprotein (P-gp). P-gp is normally expressed in endothelial or epithelial cells of several organs including the brain, liver, intestine and kidney (Gottesman and Pastan, 1993). Evidence supports a role for the P-gp transporter in the disposition or elimination of endogenous substances including corticosteroids, estrogens and bilirubin (Karssen et al., 2001; Watchko et al., 2001; Kim and Benet, 2004). This transporter also plays a key role in the disposition and excretion of a wide array of clinically used drugs and provides protection against the accumulation of toxic xenobiotics within tissues (Schinkel et al., 1994; Mayer et al., 1996; Johnson et al., 2001). P-gp is a major component of the blood-brain drug permeability barrier, which prevents the accumulation, of drugs (e.g. cyclosporine A (CyA), digoxin, dexamethasone and antineoplastics) in the central nervous system (Cordon-Cardo et al., 1989; Schinkel et al., 1994; Schinkel et al., 1995; Beaulieu et al., 1997). One of the most striking examples of this barrier function is the 20-50-fold increase in CyA and digoxin accumulation that is observed in brains of adult mice completely deficient in the *mdr1a* gene compared to their wild-type littermates (Schinkel et al., 1995).

It is well known that maturational changes contribute to altered drug pharmacokinetics in pediatric versus adult populations (Strolin Benedetti and Baltes, 2003). In earlier studies, developmental changes in MDR1/mdr1 protein levels have been examined in mice, rats or humans using the monoclonal antibody C219. The findings of reduced C219 immunoreactivity from those studies have suggested decreased P-gp in the brain, liver and kidney in foetal or immature animals compared to adults (Schuetz et al., 1995; Matsuoka et

al., 1999; Mahmood et al., 2001; Watchko et al., 2001). In support of the protein data, two recent studies have identified by semi-quantitative PCR that *mdr1a* expression in the mouse brain and *mdr1a* and *mdr1b* expression in the rat brain, liver and kidney increased with maturation (Tsai et al., 2002; Rosati et al., 2003). These findings suggested that under-developed MDR1 might reduce drug elimination or increase drug entry into the brain of neonates and young children compared to adults. However, the earlier investigations did not determine if reduced P-gp in neonates had functional consequences for the tissue disposition or elimination of P-gp substrates.

The major goal of this study was to perform *in vivo* functional studies in 1-day-old to adult *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice to determine if newborn and young mice had a greater propensity than adults to accumulate mdr1a substrates within blood/plasma and tissues. CyA, is used extensively as an immunosuppressant agent in pediatric solid organ transplant recipients (Hoyer and Vester, 2004; Pape et al., 2004) and can produce serious toxic side effects including nephrotoxicity and neurotoxicity, which can hinder successful immunotherapy and adversely affect pediatric patient health (Jeruss et al., 1998; Pape et al., 2004; Taque et al., 2004). Based on this clinical/toxicological relevance in pediatric drug therapies, CyA was chosen as our primary substrate for the mdr1a functional studies. Digoxin is a well-characterized substrate for mdr1a (Schinkel et al., 1995; Mayer et al., 1996; Schinkel et al., 1997; Goralski et al., 2003) and was used as additional substrate to confirm maturational changes in function of this transporter. Also, we have used quantitative PCR (Q-PCR) to accurately determine the importance of gender as a variable that affects the developmental expression patterns of the *mdr1a* and *mdr1b* genes in mouse brain, liver and kidney. Our

major findings have revealed that maturational expression of the *mdr1a* gene was gender and organ dependent. Most striking was our novel demonstration that a reduction in *mdr1a* mRNA expression was associated with decreased P-gp in the brain of neonates and allowed for greater accumulation of CyA and digoxin within that organ. If P-gp follows a similar maturational expression profile in the human brain there could be an increased risk for CNS toxicity in neonates exposed to drugs known to be P-gp substrates.

Methods

The Dalhousie University Committee on Laboratory Animals approved all experimental procedures involving mice according to the guidelines of the Canadian Council on Animal Care. FVB wild-type and *mdr1a*$^{-/-}$ mice were kept on a 12-hour day/night cycle, were housed in cages lined with pine bedding and had free access to water and Purina mouse chow. The 1-day-old pups were kept with the mother during the experimental procedures.

DNA isolation for gender determination of 1-day-old pups and mdr1a genotyping. Tail clips were digested with lysis buffer that contained 0.1 M Tris (pH 8.0), 5 mM EDTA, 0.2% SDS, 0.2 M NaCl and 100 μg/ml proteinase K for 30 min at 50°C. DNA was precipitated with isopropanol, washed with 70% ethanol and then resuspended in 50-100 μl of sterile distilled water. Male gender of 1-day-old pups was determined by amplification of a 185 base pair fragment of the Y-chromosome specific zinc finger protein (zfy). The gender of adult control mice was independently verified by comparison of anogenital distance or the presence of mammary glands. The PCR reaction mix (25 μl) contained 4 μl of resuspended tail DNA, $(NH_4)_2SO_4$ buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1μM *zfy* primers (**Table I**) and 1.25 units of Taq polymerase. The amplification protocol consisted of an initial denaturation at 95 °C for 30 seconds followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 1 min. PCR amplification of tail DNA with wild-type and mutant specific primers (**Table I**) were used to confirm the *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ genotypes according to previously published methodology (Johnson et al., 2001). The primers for mitochondrial expoxide hydrolase (*mEH*) amplify a 367 bp region from mouse genomic DNA and were included in all PCR reactions as a gender-independent positive control. Separation

of PCR products on a 2.5% 0.5x TAE agarose gel was used to ensure the formation of a single product at the correct size.

RNA isolation and Quantitative-PCR (Q-PCR) analyses. Total RNA was isolated from the brain, liver and kidneys of 1, 12 and 19-day-old and 6-week-old adult male and female FVB wild-type mice using TriZol^{TM} reagent (Invitrogen. Carlsbad, CA) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed using Stratascript^{TM} Reverse Transcriptase (Stratagene, Cedar Creek, TX) with 12.5 ng/ul random hexamers pd$(N)_6$ and 1.0 mM dNTPs according to the supplier's instructions. One μl of the cDNA product was amplified by Q-PCR using 125 nM of gene specific primers (**Table I**) in a total volume of 20 μl with Brillant SYBR Green QPCR Master Mix (Stratagene) using the MX3000p thermocycler (Stratagene). The amplification protocol consisted of a 10 min hot start at 94°C, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 18 s, and elongation at 72°C for 30 s. Melting curves followed by separation of PCR products on a 2.5% 0.5x TAE agarose gel were used to ensure the formation of a single product at the appropriate size. The threshold cycle (C_T) values were obtained with a threshold of 3 standard deviations above background. Relative gene expression normalized to ribosome polymerase II (*rpII*) expression was calculated using the $-2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene expression in 1-day-old male mice was assigned an arbitrary value of 1 and was used as the reference to which all other groups were compared.

Western blots. Isolation of crude membrane fractions from brain, kidney and liver and separation of proteins by SDS-polyacrylamide gel electrophoresis was performed according to previously published methods (Jette et al., 1996). The Lowry assay (Lowry et al., 1951)

was used to determine total protein content and the membrane fractions were stored at -80ºC. Crude membranes were diluted in 2x laemmli buffer and heated to 37ºC for 5 min. Ninety μg (brain and kidney) or 30 μg (liver) of total protein was separated by electrophoresis on a 7.5% polyacrylamide gel and then transferred overnight to nitrocellulose membrane. The blots were blocked in 3% skim milk-tris buffered saline with tween-20 (TBS-T, 0.1%) for 1 hr at room temperature, then incubated with the monoclonal antibody C219 (Idlabs, London, Ontario) (1:125) in 3% skim milk-TBS-T (0.1%) for 2 hr at room temperature and then anti-mouse horseradish peroxidase-conjugated secondary antibody (1:25000) in 3% skim milk-TBS-T (0.1%) for 1 hr at room temperature. Immunoreactivity was detected by incubation with Fluorescent ECL-plus™ reagent (Amersham Biosciences) and visualized directly with a Storm 840 phospor imager (Amersham Biosciences).

CyA and ^{3}H-Digoxin tissue distribution. Sandimune® i.v. (50 mg/ml) was diluted 1:100 in a sterile 5% glucose solution. ^{3}H-digoxin (1 μg ml^{-1}) was made up in 5% sterile glucose solution and contained 1.75% ethanol (v/v) and 3.25% cremoforEl (v/v). Mice were injected i.p. with 5 mg kg^{-1} of the diluted CyA solution or 10 μg kg^{-1} of the ^{3}H-digoxin solution. The total volume of injection was proportional to weight (10 μl/g body weight). After 2 hr, mice were anaesthetized with enflurane and were killed by decapitation. Trunk blood, brain, liver and kidney were collected for CyA and digoxin determination. Tissues were weighed, rinsed and homogenized in phosphate-buffered saline (pH 7.4) with a Polytron homogenizer.

CyA determination. The protocol for CyA determination was based on previously established methodology (Volosov et al., 2001). Blood samples (1 ml) were collected into microfuge tubes containing 3.2 mg of EDTA and were stored at –20°C until preparation for

LC/MS. CyA was extracted from 100 µl of whole blood or 100 µl of brain, liver and kidney homogenate. Due to the small blood volume obtained from 1-day-old mice, the extractions were performed from 10 µl of whole blood diluted to 100 µl in PBS. One hundred µl of zinc sulfate (0.1 M) was added to each sample and vortexed for 5 sec. Three hundred µl of acetonitile containing 30 ng/ml of the internal standard ascomycin was added to precipitate blood and tissue protein. Samples were vortexed for a further 10 s, centrifuged for 10 min in a microcentrifuge (10 000x g) and the supernatants were transferred to a clean vial. Thirty five µl of each sample was then separated on a Zorbax SB-C_{18} analytical column (4.6 x 75mm), 3.5-micron particle size at 70°C isocratically at a flow rate of 1.6 ml/minute. The mobile phase for separations contained 65% methanol and 35% ammonium acetate for 4 min. The ammonium adducts were then eluted into the tandem mass spectrometer with 100% methanol for 1 min 50 s (1.4 ml min) and quantified utilizing the heated nebulizer (APC1) source and the multiple reaction monitoring (MRM) scan mode of the MS/MS (AP1 3000). The ammonium adducts, induced by ammonium acetate, of CyA and internal standard ascomycin collected by the first quadrupole (Q1) and their characteristic products ions are collected by the third quadrupole (Q3). The peak height of the ion transitions was used to quantify the CyA levels by comparing to a calibration curve. Data analysis was performed by Analyst software version 1.3.1. (Applied Biosystems MDS SCIEX 2003).

^{3}H-digoxin determination. For ^{3}H-digoxin measurement 50 µl of plasma, 100 µl of liver and kidney homogenate and 200 µl of brain homogenate (in duplicate) were added to 4 ml of Beckman ready-safe scintillation cocktail and counted for total disintegrations per minute (DPM) using a Beckman LS5000TA scintillation counter (Beckman instruments, Fullerton,

CA, U.S.A.). *Data analysis*. All groups represent the mean ± s.e.m. of at least 3-5 mice. A two-way analysis of variance (ANOVA) was used to measure the effect of the main variables (age and gender) and the interaction of those variables on the expression of *mdr1a* and *mdr1b* in the brain, liver and kidney. A three-way ANOVA was used to measure the effect of age, gender and *mdr1a* genotype and the interaction of those variables on the blood and tissue distribution of CyA and digoxin. An ANOVA with a p value ≤ 0.05 was considered significant. A Tukey's multiple comparison test was used for *post-hoc* analysis of the significant ANOVA.

Drugs and Chemicals. Sandimune® i.v. (Novartis) was obtained from the IWK Health Centre Pharmacy. ^{3}H-digoxin (250 μCi) was obtained from Perkin Elmer (Boston, MA). Oligonucleotide primers were synthesized by SigmaGenosys Canada (Missisauga,ON). All other reagents were of the finest grade available and were purchased from commercial suppliers.

Results

We have examined the effect of gender and maturation and the interaction of those variables on the levels of *mdr1a* and *mdr1b* genes in the mouse brain, liver and kidney. Due to the inherent difficulties of gender determination by morphological examination of newborn pups, we have developed a PCR-based assay for unequivocal sex determination. This assay is based upon amplification of a 185 base pair PCR fragment from the Y-chromosome specific gene (zinc finger protein, *zfy*). As shown in the representative gender-typing assay (**fig 1)** the *zfy* PCR product was detectable in adult male but not female mice. For the 1-day-old pups, the presence of the PCR product indicated male gender, whereas the absence of the PCR product indicated female gender. As a gender independent control, a fragment of *mEH* DNA was amplified from male and female mice in a separate PCR reaction.

The expression of *mdr1a* mRNA normalized to the control gene *ribosomal polymeraseII* (*rpII*) in the brain was dependent on age but not on gender (**fig. 2A**). *Mdr1a* mRNA in the brain was at its lowest level in 1-day-old mice, but increased with maturation to a level 2.5-3 fold higher in 19-day-old and 6-wk-old mice. Conversely, brain *mdr1b* mRNA levels did not increase significantly with age and were also not affected by gender (**fig. 2B**). The ratio of *mdr1a/mdr1b* expression increased from 70 in newborn to 130 in adult mice and indicated that the *mdr1a* gene was the primary expressed P-gp isoform in all age groups (**fig. 2C**). The level of the control gene *rpII* was similar for both genders and in all age groups (**fig. 2D**).

In the kidney, 1-day-old mice had the lowest relative expression of *mdr1a* mRNA (**fig. 3A**). In 19-day-old male and female mice, *mdr1a* mRNA levels increased 2.5 fold compared to the 1-day-old mice. In adult female but not male mice, *mdr1a* mRNA was increased a further 4 -

fold and revealed gender-dependent expression in adult mice. A similar gender differential was observed for *mdr1b* mRNA levels in adult female versus male kidney (**fig. 3B**). The *mdr1a* and *mdr1b* genes were expressed to a similar degree in the kidney (**fig. 3C**). The level of the control gene *rpII* was similar in kidneys from males and females and in all age groups (**fig. 3D**).

Similar to the brain and kidney, 1-day-old male and female mice had the lowest levels of *mdr1a* mRNA in the liver (**fig. 4A**). *Mdr1a* mRNA level then increased 6-8 fold in 12-day-old male and female mice compared to the newborn mice. Thereafter, *mdr1a* mRNA levels declined in male mice and remained elevated in female mice. This resulted in a 5-fold higher expression of *mdr1a* mRNA in the liver of adult female compared to adult male mice. *Mdr1b* appeared to be primarily a neonatal expressed gene in the liver as its expression was highest in 1-day-old mice (**fig. 4B**). The amount of *mdr1b* mRNA declined by 75 % in the liver of 12-day and 19-day-old mice. In adult mice, the levels of hepatic *mdr1b* remained 50 – 60 % lower than observed in newborn mice. Unlike *mdr1a*, the level of *mdr1b* mRNA in the liver was not dependent on gender. In all age groups, liver *mdr1a* levels were substantially higher than *mdr1b* levels (**fig. 4C**). The difference (300-800 fold) was most predominant in the 12-19 day old mice. The level of the control gene *rpII*, was similar in liver from male and female mice and in all age groups (**fig. 4D**).

Western blots on crude membranes obtained from brain, liver and kidney of 1-day-old and adult mice confirmed that maturational and gender changes in *mdr1a* expression correlated with P-gp expression (**Fig 5**). In the brain of 1-day-old mice, P-gp expression was about 20% of the levels observed in adult mice. Similar to *mdr1a* mRNA expression, no gender

difference was observed for P-gp expression in the brain. P-gp was not detectable in the kidney membrane fractions prepared from 1-day-old mice but was detectable in the kidney membrane fractions prepared from adult mice. Consistent with *mdr1a* mRNA expression in that organ, P-gp expression was substantially higher in the adult females compared to males. In crude membrane fractions prepared from the liver, P-gp was detectable in 1 day-old male and female mice. The level of immunodetectable P-gp decreased in adult males and did not increase further in adult females. The gender difference in liver P-gp expression in adult mice was consistent with the gender difference in liver *mdr1a* mRNA expression.

To establish conditions for the functional studies we performed an initial experiment in which adult male wild-type mice were administered a therapeutically relevant dose (5 mg kg^{-1}) of CyA by i.p. injection. Similar to the kinetics of CyA in humans (Jorga et al., 2004), peak drug levels (1000-1200 ng ml^{-1}) in mouse blood were obtained two hours after dosing and 24 hr trough levels were in the range of 100-200 ng ml^{-1} (**fig. 6**). For subsequent experiments, blood and tissue cyclosporine concentrations were measured 2 hr after dosing (C2). We have also validated the CyA analysis for detection of that drug in tissue samples as the assay procedures were initially developed for the detection of CyA in blood (**Table II**). Thus, CyA was added to brain, liver and kidney homogenates to yeild low and high CyA concentration that corresponded to range of expected CyA levels in those tissues. The average measured CyA concentrations in brain, liver and kidney were within 5% of the predicted values. The lower limit of detection was 500 pg (5 ng/ml) CyA per 100 µl of homogenate. Thus, the mouse served as an appropriate model for examining CyA kinetics and the described HPLC-tandem MS assay provides a highly sensitive method for

determining blood and tissue CyA in this species.

In order to confirm *mdr1a*-dependent effects and to reveal *mdr1a*-independent effects, the effects of ontogeny on CyA disposition were examined in *mdr1a*-expressing and *mdr1a*-null mice. Furthermore, within each age group, the *mdr1a*$^{-/-}$ mice served as a reference point for complete loss of mdr1a function. Blood CyA concentrations measured 2 hr after administration (C2) were on average 1/3 lower in 1-, 12- and 19-day-old mice compared to adult mice but were not dependent on gender (**fig. 7A**). C2 blood CyA was similar in *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice at all ages and indicated that peak blood CyA after i.p. dose was not influenced by *mdr1a* mRNA levels at any age. CyA was highly concentrated in liver (8-12 fold) and kidney (4-8 fold) compared to the blood compartment (**fig. 7B and 7C**). The accumulation of CyA in the liver and kidney was elevated in the 12- and 19-day-old mice. However, this effect was not due to maturational changes in *mdr1a* mRNA levels as *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice had similar accumulation of CyA in those organs in all age groups studied. Furthermore, the increased level of *mdr1a* in the liver and kidney of adult female mice was not associated with a decrease in CyA accumulation. In the brain (**fig. 7D**), CyA accumulation was inversely related to age in wild-type mice. The 1-day-old mice had 3-4-fold higher brain/blood concentration ratios than adult wild-type mice, which was consistent with the pattern of *mdr1a* mRNA and P-gp expression during maturation. Conversely, brain/blood CyA was similar in 1-day, 19-day and adult *mdr1a*$^{-/-}$ mice with a marked increase in brain/blood CyA in the 12-day-old *mdr1a*$^{-/-}$ mice.

To provide further support for maturational changes in function of mdr1a, a tissue distribution study was performed under the same conditions with the mdr1a substrate ^{3}H-

digoxin (**Fig. 8**). Similar to CyA, plasma ^{3}H-digoxin DPM measured 2 hr after dosing was comparable in 1-19-day-old male and female *mdr1a*$^{+/+}$ and *mdr1a*$^{-/-}$ mice (**fig 8A**). An effect of the *mdr1a* gene deletion became unmasked in adult mice where the *mdr1a*$^{-/-}$ mice had 2-fold higher plasma digoxin compared to the wild-type male and female controls. The liver/plasma digoxin ratio peaked (6-fold) in 12-day-old mice, whereas the kidney/plasma ratio was higher in 1- and 12-day-old mice and decreased thereafter (**fig. 8B and C**). However, the maturational effect on digoxin accumulation in the liver and kidney in the 1-19 day-old mice was similar in male and female mice of both genotypes indicating that the change was not gender or *mdr1a*-dependent. Although not significant, the liver/plasma and kidney/plasma ^{3}H-digoxin levels tended to be higher in the *mdr1a*$^{-/-}$ mice compared to the *mdr1a*$^{+/+}$ mice. Comparable to the functional studies with CyA, ^{3}H-digoxin accumulation in the brain of *mdr1a*$^{+/+}$ mice was 5 times higher in 1-day-olds compared to adults and was consistent with the maturational increase in *mdr1a* mRNA and P-gp levels in that organ. In the *mdr1a*$^{-/-}$ mice, digoxin accumulation in the brain was similar in 1-day-old compared to adults and peaked in the 12-day-old mice. The fold difference of CyA or ^{3}H-digoxin accumulation in the brain of *mdr1a*$^{-/-}$ mice versus the age-matched *mdr1a*$^{+/+}$- mice was lowest in 1-day-old and increased with maturation; and indication that the mdr1a transporter is functionally active in newborn mice albeit at a reduced level compared to adults (**Table III**).

Discussion

We present a novel characterization of gender and age-dependent regulation of *mdr1a* and *mdr1b* mRNA levels and function of the P-gp transporter in the mouse brain liver and kidney. In mice and humans, P-gp expression is an early marker of blood-brain barrier development (Qin and Sato, 1995; Schumacher and Mollgard, 1997). In a previous report antibody C219 detectable P-gp was 5-fold higher in the brains of adult FVB mice compared newborn mice (Watchko et al., 2001; Tsai et al., 2002). We have confirmed a similar maturational increase in brain P-gp. This increase in brain P-gp was most likely attributable to brain *mdr1a* expression, which also increased post-natally and was 3-fold higher in adult compared to 1-day-old mice. *Mdr1a* is expressed (qualitatively) to a higher degree than *mdr1b* in the adult mouse brain (Croop et al., 1989; Schinkel et al., 1994). Similar to adult mice, our results have established that *mdr1a* is the predominant P-gp isoform expressed in newborn and maturing mice. The higher level of *mdr1a* compared to *mdr1b* mRNA in the adult mouse brain is consistent with functional data, whereby deletion of the *mdr1b* gene did not result in increased brain accumulation of the P-gp substrate digoxin (Schinkel et al., 1997).

The phenotype of increased brain accumulation and/or toxicity of P-gp substrates including CyA, digoxin, dexamethasone, ivermectin, vincristine and vinblastine has been documented in adult *mdr1a*$^{-/-}$ mice (Schinkel et al., 1994; Schinkel et al., 1995; Johnson et al., 2001). However, the level of protection provided by *mdr1a* against drug accumulation in the brains of newborn mice has not been examined. Based on our observations of decreased *mdr1a* mRNA levels and P-gp, newborn mice should be less protected than adults. Consistent with this prediction, newborn wild-type mice achieved levels of CyA and ^{3}H-digoxin accumulation

in the brain that were about 50% of those levels achieved in newborn *mdr1a*$^{-/-}$ mice that lack expression of that transporter. In contrast, the relative accumulation of CyA and ^{3}H-digoxin in the brains of adult *mdr1a*$^{+/+}$ mice decreased to about 10% compared to the adult *mdr1a*$^{-/-}$ mice. These results indicated that level of protection afforded by *mdr1a* against drug accumulation in the brain is lowest in newborn mice and is enhanced as the expression of that gene and its product P-gp increase during maturation. Our novel finding that newborn *mdr1a*$^{+/+}$ but not *mdr1a*$^{-/-}$ mice had increased brain accumulation of CyA (3-fold) and ^{3}H-digoxin (5-fold) compared to the respective adult mice further supports the conclusion that the reduced brain expression of *mdr1a* in the neonate directly contributed to greater accumulation of those mdr1a substrates in that organ. The 12-day-old *mdr1a*$^{-/-}$ mice had a peak in brain CyA and ^{3}H-digoxin levels suggesting a maturational change in the blood-brain barrier permeability to drugs that is unmasked only when *mdr1a* expression is completely absent. This change may reflect maturational expression patterns of other drug transporters (e.g. oatp2, mrp1, mrp2 and bcrp) that are present in the blood-brain barrier (Bauer et al., 2005).

Use of calcineurin inhibitors (CyA and tacrolimus) for suppression of organ rejection in pediatric renal transplant patients can precipitate central nervous system (CNS) toxicity, behavioural disturbances and interruptions in immunosuppressive therapy (Awan et al., 1999; Parvex et al., 2001; Yamauchi et al., 2002; Taque et al., 2004). Approximately 10% -15% of pediatric renal transplant patients will exhibit significant clinical CNS toxicity characterized by post-transplant seizures (Awan et al., 1999). Although the etiology of CNS toxicity is multi-factorial, young age and drug inhibition of P-gp function in the brain has been

implicated (Jeruss et al., 1998; Awan et al., 1999). Our data further suggest that the risk for adverse CNS toxicity may be higher in young patients as a consequence of not yet fully developed P-gp. Consistent with this hypothesis, a G2677T mutation in exon 21 of the human MDR1 transporter has been associated a greater frequency of adverse CNS effects during immunotherapy with tacrolimus (Yamauchi et al., 2002).

Biological differences between males and female are well known to affect drug pharmacokinetics (Schwartz, 2003). We have identified for the first time that the developmental pattern of *mdr1a* mRNA expression in the liver and kidney is highly dependent on gender. In females, expression of the *mdr1a* gene increased in liver (5-fold) and kidney (6-fold) with age and was maintained in adulthood. In males, kidney expression increased 2.5-fold with maturation whereas hepatic *mdr1a* expression was maximal in 12-day-old males and declined thereafter. Overall females maintained a 4-5-fold higher *mdr1a* expression in the kidney and liver as compared to adult males. Similarly, adult females maintained higher levels of P-gp expression in the kidney and liver as compared to males. This pattern of expression is not restricted to the *mdr1a* transporter as similar gender-dependent expression of the oatp2 transporter mRNA or protein occurs in rat or mouse liver during maturation (Guo et al., 2002; Cheng et al., 2005). A previous study reported that *mdr1a* mRNA expression in rat liver and kidney increased minimally (2-3 fold) during maturation (Rosati et al., 2003). In comparison to our results, those findings could reflect a species difference in developmental expression of the *mdr1a* transporter. Furthermore, gender of rats was not identified, or included as a variable in the earlier study by Rosati and Coworkers and could also contribute to the differences in findings. In line with the gender

related expression difference of *mdr1a* mRNA and P-gp in the kidney, adult male mice tended to have higher plasma and kidney levels of ^{3}H-digoxin as compared to the adult wild-type female mice. Consistent with the findings of an earlier study (Schinkel et al., 1994), we also demonstrated a gender-dependent (female > male) expression of the *mdr1b* isoform in the kidney but not the liver. Higher expression levels of liver and kidney drug transporters such as P-gp may be required for disposition and elimination of female sex steroids. This idea is supported by a recent study, which showed that estradiol, estrone, and estriol were transported substrates and inducers of MDR1 expressed in MDCK cells (Kim and Benet, 2004). Although not the focus of this study, female sex hormones could contribute to the increased hepatic and kidney *mdr1a* expression in female mice. Unlike the kidney and liver our findings show that the brain levels of *mdr1a* or *mdr1b* genes and P-gp or accumulation of CyA and digoxin in that organ was not gender-dependent. Those results are supported by earlier findings that showed gender independence of verapamil, morphine and quinidine uptake into the brain of adult mice (Dagenais et al., 2001).

In our functional studies we observed that 12- and 19-day-old mice had higher liver/blood and kidney/blood CyA levels than newborn and adult mice. The increase in organ CyA accumulation could be especially important in the kidney as one of the main side effects of therapy with that drug in children is nephrotoxicity (Pape et al., 2004). A recent study has demonstrated that MDR1 3435TT genotype of the donor kidney was a major risk factor for CyA nephrotoxicity and was not explained by CyA dose or blood levels of the drug (Hauser et al., 2005). These data indicate that increased kidney levels of CyA might be associated with certain MDR1 polymorphisms. In comparison with the toxicological data from that

human study, our functional studies in mice indicated that maturational or gender changes in *mdr1a* were not a limiting factor for determining peak CyA levels in the blood, or accumulation of that drug in the liver and kidney. However, it remains to be determined if a lower level of *mdr1a* expression decreases elimination of CyA from the kidney or liver over time resulting in higher tissue trough levels.

Within each of the younger age groups (1-, 12- or 19-day-old) CyA or ^{3}H-digoxin accumulation in the liver and kidney was similar in wild-type versus *mdr1a*-null mice. This indicates that changes in drug accumulation in those organs of young mice could not be explained by maturational changes in *mdr1a* mRNA levels. However, a phenotype of increased plasma, liver and kidney ^{3}H-digoxin but not CyA began to appear in adult *mdr1a*$^{-/-}$ mice as compared to the wild-type mice. Schinkel and coworkers demonstrated similar results for CyA and digoxin in adult *mdr1a*$^{+/+}$ versus *mdr1a*$^{-/-}$ mice 4 or 8 hr after dosing with those drugs (Schinkel et al., 1995). Thus, the effect of *mdr1a* expression on blood and tissue disposition of P-gp substrates was age-, organ- and drug-dependent.

In summary, we have demonstrated that maturation of *mdr1a* expression in the mouse liver and kidney is dependent on gender. Importantly, our functional studies have demonstrated that CyA and ^{3}H-digoxin accumulation in the brain was highly dependent upon *mdr1a* expression: in contrast, accumulation of these drugs in the liver and kidney was not affected significantly by the reduced *mdr1a* expression levels in these organs of newborn mice. Considering these findings, it will be important to address if the risk for CNS toxicity due to increased brain drug levels is higher in young patients as a consequence of decreased MDR1 expression and function.

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Footnotes

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Figure Legends

Figure 1. Representative figure showing PCR gendertyping of 1-day-old mice. **A)** The detection of the 185 bp PCR product of the Y-chromosome specific gene indicates male (**M**) gender. The absence of the *zfy* product indicates female (F) gender. **B)** As a gender independent control, a fragment of microsomal epoxide hydrolase (*mEH*) DNA was amplified from 1-day-old male and female mice in a separate PCR reaction.

Figure 2. The effect of maturation and gender on brain expression of *mdr1a* and *mdr1b* mRNA. The relative expression of *mdr1a* (**A**) and *mdr1b* (**B**) was determined by QPCR. The ratio of *mdr1a* to *mdr1b* threshold cycle C_t values in the brain is shown in panel **C**. For each group, *mdr1a* and *mdr1b* expression was normalized to the control gene *ribosome polymeraseII* (*rpII*) (**D**). The 1-day-old male mice were chosen as the reference group (expression = 1.0) to which all other groups were compared. Each bar represents the mean ± s.e.m. of 3-4 mice. Data were compared by a two-way ANOVA followed by Tukey's HSD *post-hoc* analysis of the significant ANOVA. ‡ $p < 0.05$, significantly higher gene expression (independent of gender) as compared to the 1-day-old mice.

Figure 3. The effect of maturation and gender on kidney expression of *mdr1a* and *mdr1b* and mRNA. The relative expression of *mdr1a* (**A**) and *mdr1b* (**B**) was determined as described in the legend to figure 2. The ratio of *mdr1a* to *mdr1b* expression in the kidney and the expression of the housekeeping gene *rpII* are shown in panels **C and D** respectively. Each bar represents the mean ± s.e.m. of 3-4 mice. Data were compared by a two-way

ANOVA followed by Tukey's HSD *post-hoc* analysis. ‡ $p < 0.05$, significantly higher gene expression (independent of gender) as compared to the 1-day-old mice. * $p < 0.05$, significantly higher gene expression in adult females compared to all other age groups. † $p <$ 0.05, significantly higher gene expression in adult female compared to adult male mice.

Figure 4. The effect of maturation and gender on liver expression of *mdr1a* and *mdr1b* mRNA. The relative expression of *mdr1a* (**A**) and *mdr1b* (**B**) was determined as described in the legend to figure 2. The ratio of *mdr1a* to *mdr1b* expression in the liver and the expression of the housekeeping gene *rpII* are shown in panels **C and D** respectively. Each bar represents the mean ± s.e.m. of 3-4 mice. Data were compared by a two-way ANOVA followed by Tukey's HSD *post-hoc* analysis. ‡ $p < 0.05$, significantly lower gene expression compared to 1-day-old mice (independent of gender). * $p < 0.05$, significantly higher gene expression as compared to the 1-day-old-male or female mice. † $p < 0.05$, significantly higher gene expression in adult female compared to adult male mice.

Figure 5. Maturation and gender affect P-gp expression. Crude membrane fractions were prepared from brain, liver and kidney of 1-day-old and adult male and female mice. P-gp expression in these tissues was determined by Western blot analysis using the antibody C219.

Figure 6. Blood CyA levels versus time after dosing. 6-week-old male mice received a single dose of Sandimune® (5 mg kg^{-1}) by i.p. injection. 10-15 µl blood samples were taken from the end of the tail of each mouse at 0.5, 1.0, 2,0, 4.0, 8.0 and 24 hr after the CyA dose.

Each symbol represents the mean ± s.e.m. of 3 mice. The standard errors are too small to be observed for the 8 hr and 24 hr time points.

Figure 7. The effect of maturation on the blood and tissue distribution of CyA. 1-, 12-, 19-day and 6-week male and female mdr1a$^{+/+}$ and mdr1a$^{-/-}$ mice were administered 5 mg kg CyA by i.p. injection. Blood (**A**) liver/blood (**B**), kidney/blood (**C**) and brain/blood (**D**) CyA levels were measured 2 hr later. Each bar represents the mean ± s.e.m of 3 to 4 mice. Data were compared by a three-way ANOVA followed by Tukey's HSD *post-hoc* analysis of the significant ANOVA. * $p < 0.05$, significantly different as compared to all other age groups (independent of gender or genotype). † $p < 0.05$, significantly higher in mdr1a$^{-/-}$ mice compared to mdr1a$^{+/+}$ mice of the same age (independent of gender). ‡ $p < 0.05$, significantly different compared to 1-day-old mice of the same genotype (independent of gender). $^{\#}$ $p < 0.05$, significantly different compared to 1-day-old mice (independent of gender or genotype).

Figure 8. The effect of maturation on the blood and tissue distribution of ^{3}H-digoxin. 1, 12, 19 day and 6 wk male and female mdr1a$^{+/+}$ and mdr1a$^{-/-}$ mice were administered 10 μg kg^{-1} of ^{3}H-digoxin by i.p. injection. Plasma (**A**) liver/plasma (**B**), kidney/plasma (**C**) and brain/plasma (**D**) DPM levels were measured 2 hr later. Each bar represents the mean ± s.e.m of 3 to 4 mice. The groups were compared by a three-way ANOVA followed by Tukey's HSD *post-hoc* analysis. * $p < 0.05$, significantly different as compared to all other age groups (independent of gender or genotype). † $p < 0.05$, significantly higher in mdr1a$^{-/-}$ mice compared to mdr1a$^{+/+}$ mice of the same age group (independent of gender). ‡ $p < 0.05$,

significantly different compared to 1-day-old mice of the same genotype (independent of gender). $^{\#}$ $p < 0.05$, significantly different compared to 1-day-old mice (independent of gender or genotype). ** $p < 0.05$ significantly different compared to adult male or female wild-type mice.

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Table I. PCR primers

Gene	Genbank Accession#	PCR primer sequences listed 5′ to 3′	PCR product size
Genotyping and gendertyping primers			
zfy	AC139318.5	Fw cct att gca tgg aca gca gtc tta tg Rv gac tag aca tgt ctt aac atc tgt cc	185 bp
Abcb1a wild-type	NM_011076	Fw cag ctc cat cca aca act tc Rv gac aca ggt act gtc cac ag	411 bp
Abcb1a mutant	NM_011076	Fw atg tcc tgc ggg taa ata gc Rv cgt cag gac att gtt gga gc	481 bp
mEH	AC119911.10	Fw aag tga gtt tgc atg gcg cag c Rv ccc ttt agc ccc ttc cct ctg	367 bp
Quantitative PCR primers			
Abcb1a	NM_011076	Fw gag tga ggc cga taa aag agc cat gtt Rv tca tct gtg agc cgg gtg ttg agc tcc c	248 bp
Abcb1b	NM_011075	Fw gct gtt ggc gta ttt ggg atg ttt cg Rv gat gat cag agt act gtt ggg tcc ac	210 bp
RpII	U37500	Fw ctg gac cta ccg gca tgt tc Rv gtc atc ccg ctc cca aca c	132 bp

Table II. Validation of CyA measurements in tissue homogenates.

Brain		Liver		Kidney	
[CyA] ng/ml added	[CyA] ng/ml detected	[CyA] ng/ml added	[CyA] ng/ml detected	[CyA] ng/ml added	[CyA] ng/ml detected
10	10.4 ± 0.76[a]	100	96 (n = 2)[b]	100	100.2 (n =2)[b]
100	98.5 ± 3.12[a]	1000	994 ± 80[a]	1000	1056 ± 31[a]

CyA was added to tissue homogenates to achieve a final concentration of 10, 100 or 1000 ng/ml. Samples were then extracted and CyA was measured by HPLC-MS/MS to validate measurement of that drug in the tissue homogenates. [a] These values are expressed as the mean (n=3) ± s.e.m. [b] These values represent the mean value of two samples.

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Table III. Fold difference in brain/blood (CyA) and brain/plasma (digoxin) accumulation between *mdr1a*$^{-/-}$ versus *mdr1a*$^{+/+}$ mice of different ages

Substrate	Day 1	Day 12	Day 19	6 week
CyA	2.0	5.5	8.6	8.0
Digoxin	2.4	6.0	4.7	10.6

Fold difference CyA *mdr1a*$^{-/-}$/*mdr1a*$^{+/+}$ = (brain/blood CyA *mdr1a*$^{-/-}$)/(brain/blood CyA *mdr1a*$^{+/+}$). Fold difference digoxin DPM *mdr1a*$^{-/-}$/*mdr1a*$^{+/+}$ = (brain/blood digoxin *mdr1a*$^{-/-}$)/(brain/blood digoxin *mdr1a*$^{+/+}$). As there was no statistical difference between male and female mice, the data from these groups was combined.

Figure 1

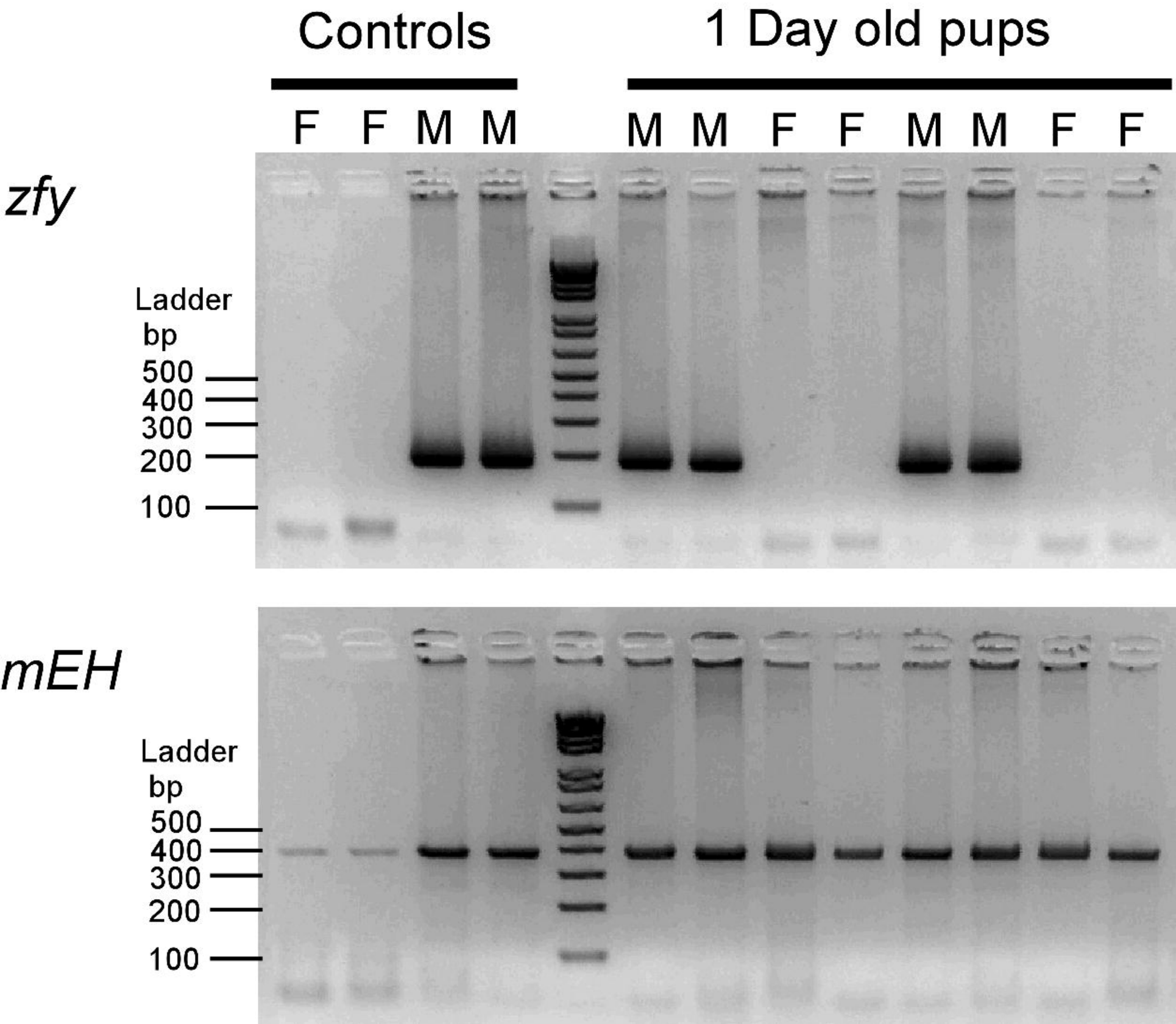

Controls
1 Day old pups
F F M M M M F F M M F F
zfy
Ladder
bp
500
400
300
200
100
mEH
Ladder
bp
500
400
300
200
100

Figure 2

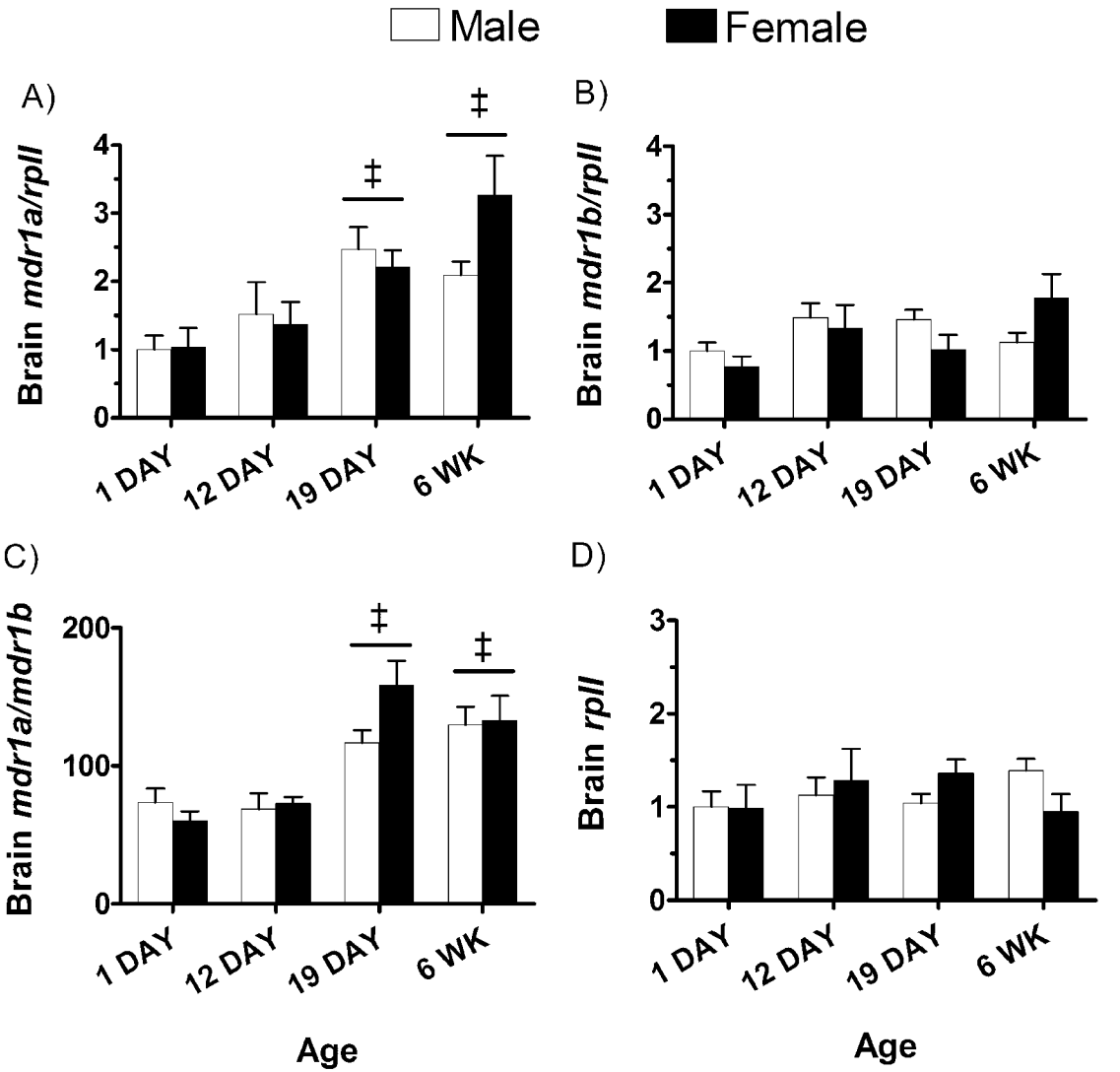

Male
Female
A)
Brain mdr1a/rpII
4
3
2
1
0
‡
‡
1 DAY
12 DAY
19 DAY
6 WK
B)
Brain mdr1b/rpII
4
3
2
1
0
1 DAY
12 DAY
19 DAY
6 WK
C)
Brain mdr1a/mdr1b
200
100
0
‡
‡
1 DAY
12 DAY
19 DAY
6 WK
Age
D)
Brain rpII
3
2
1
0
1 DAY
12 DAY
19 DAY
6 WK
Age

Figure 3

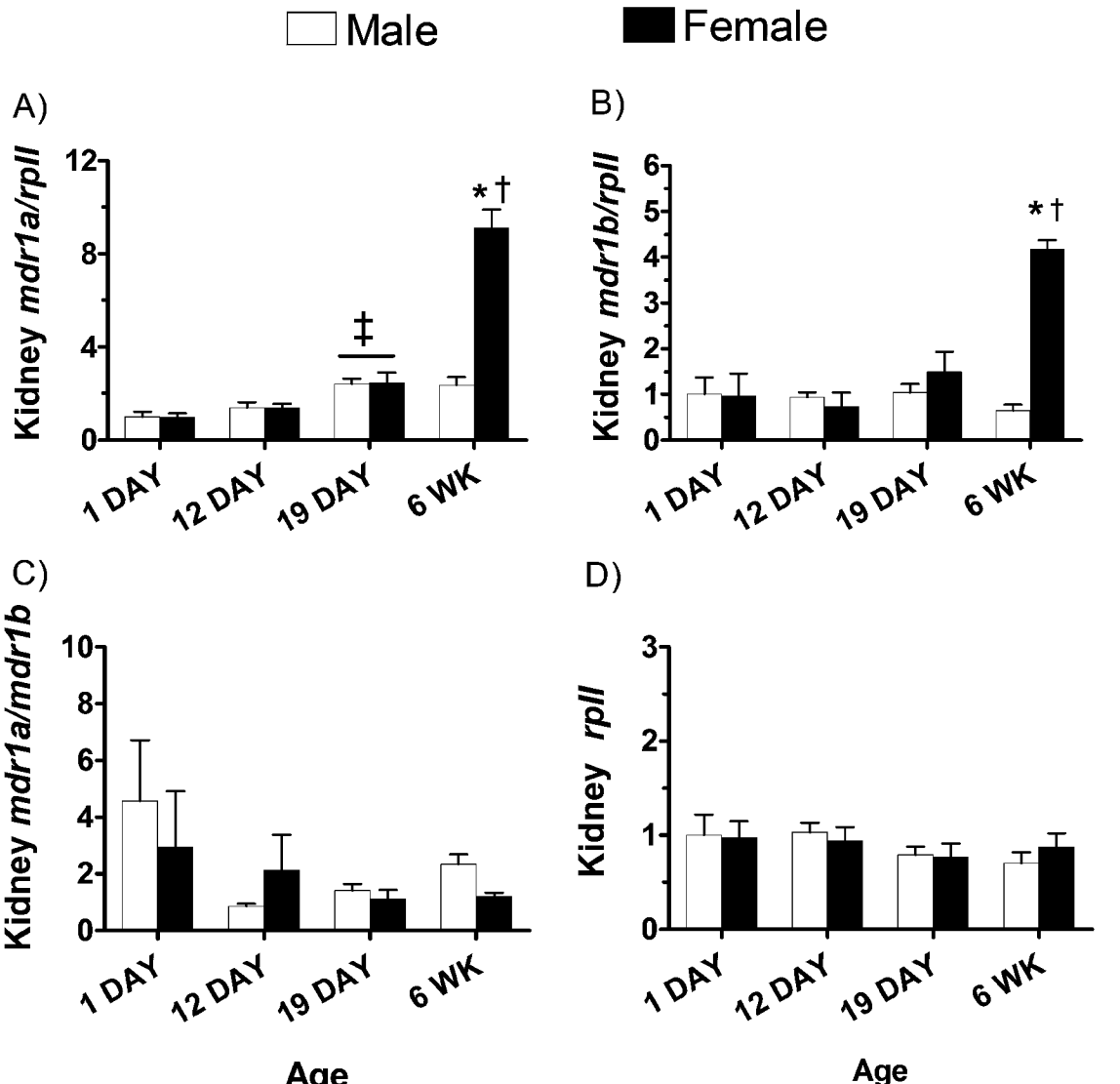

Male
Female
A)
Kidney mdr1a/rpII
12
8
4
0
*†
‡
1 DAY
12 DAY
19 DAY
6 WK
B)
Kidney mdr1b/rpII
6
5
4
3
2
1
0
*†
1 DAY
12 DAY
19 DAY
6 WK
C)
Kidney mdr1a/mdr1b
10
8
6
4
2
0
1 DAY
12 DAY
19 DAY
6 WK
Age
D)
Kidney rpII
3
2
1
0
1 DAY
12 DAY
19 DAY
6 WK
Age

Figure 4

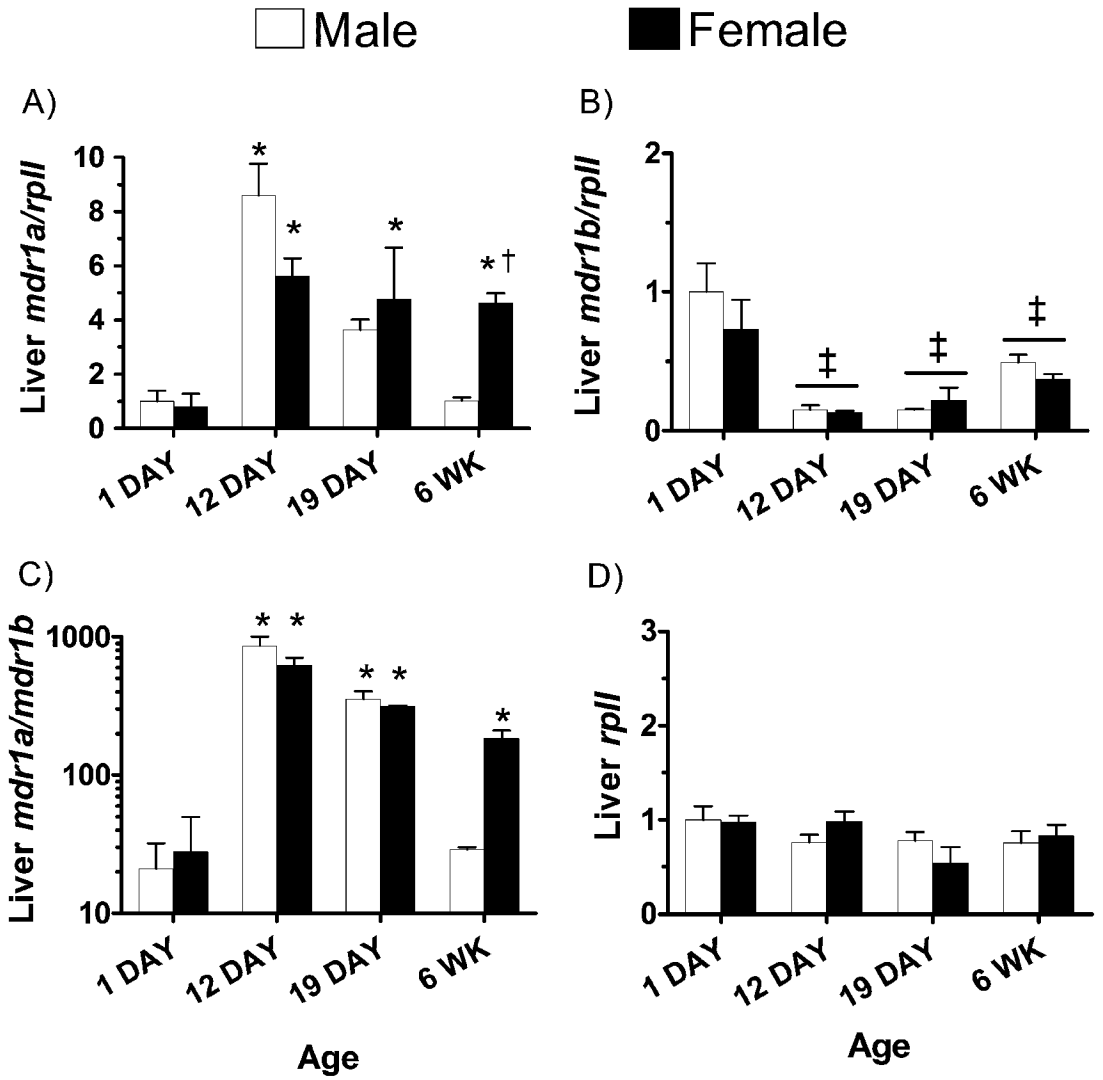

Male
Female
A)
Liver mdr1a/rpII
10
8
6
4
2
0
*
*
*
*†
1 DAY
12 DAY
19 DAY
6 WK
B)
Liver mdr1b/rpII
2
1
0
‡
‡
‡
1 DAY
12 DAY
19 DAY
6 WK
C)
Liver mdr1a/mdr1b
1000
100
10
* *
* *
*
1 DAY
12 DAY
19 DAY
6 WK
Age
D)
Liver rpII
3
2
1
0
1 DAY
12 DAY
19 DAY
6 WK
Age

Figure 5

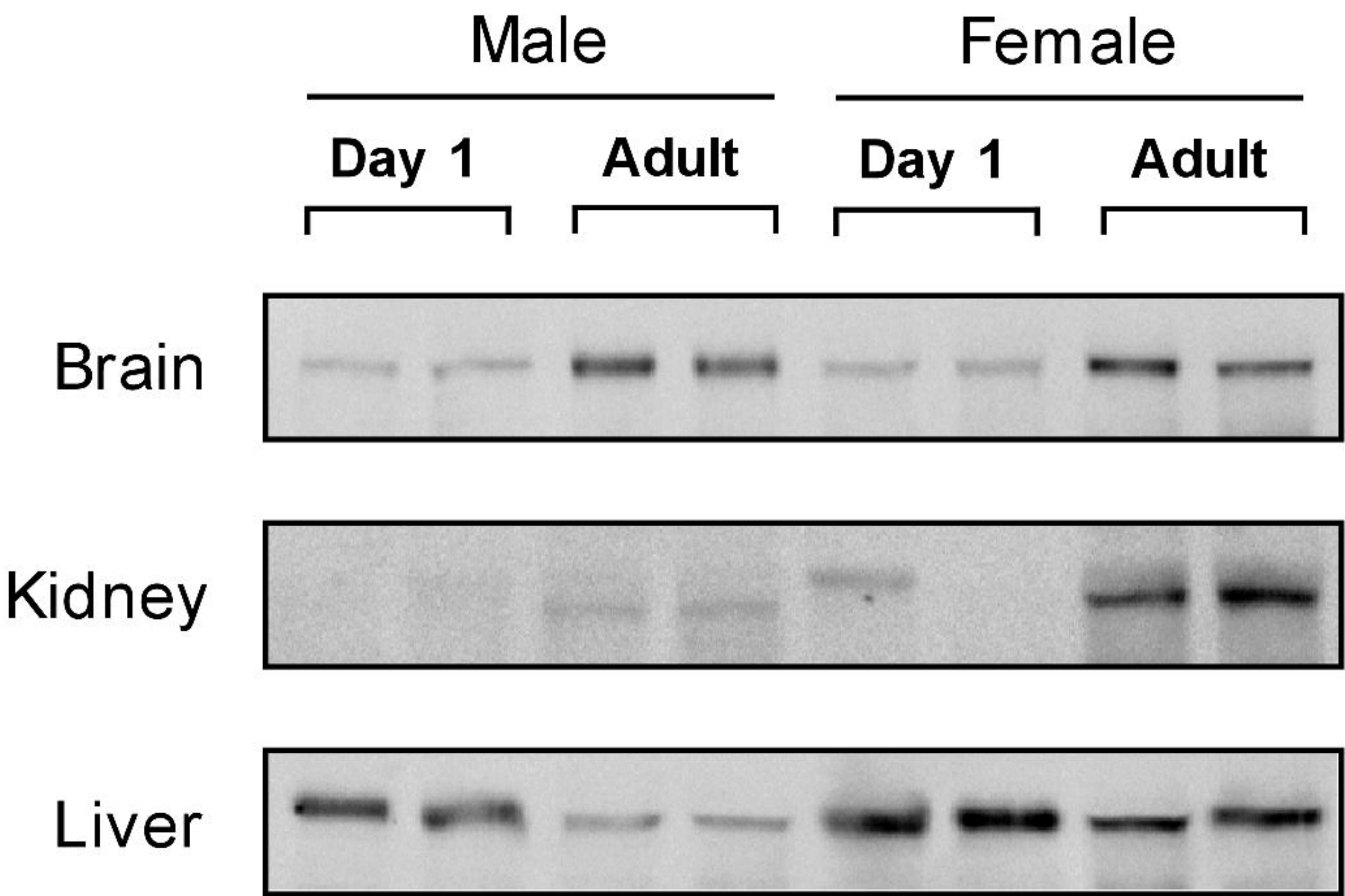


Figure 6

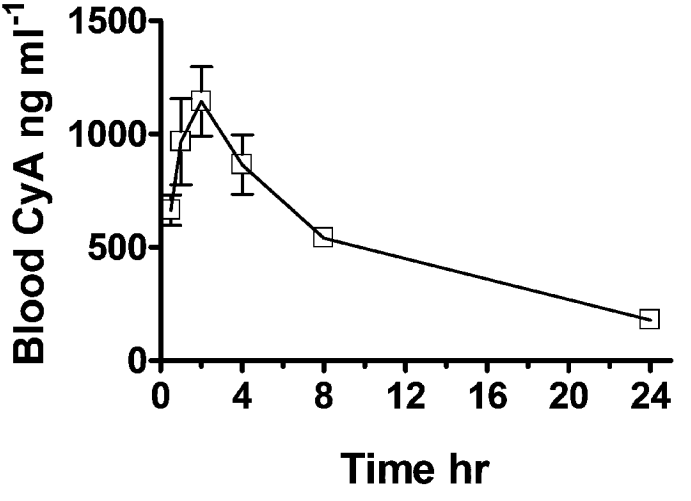


Figure 7

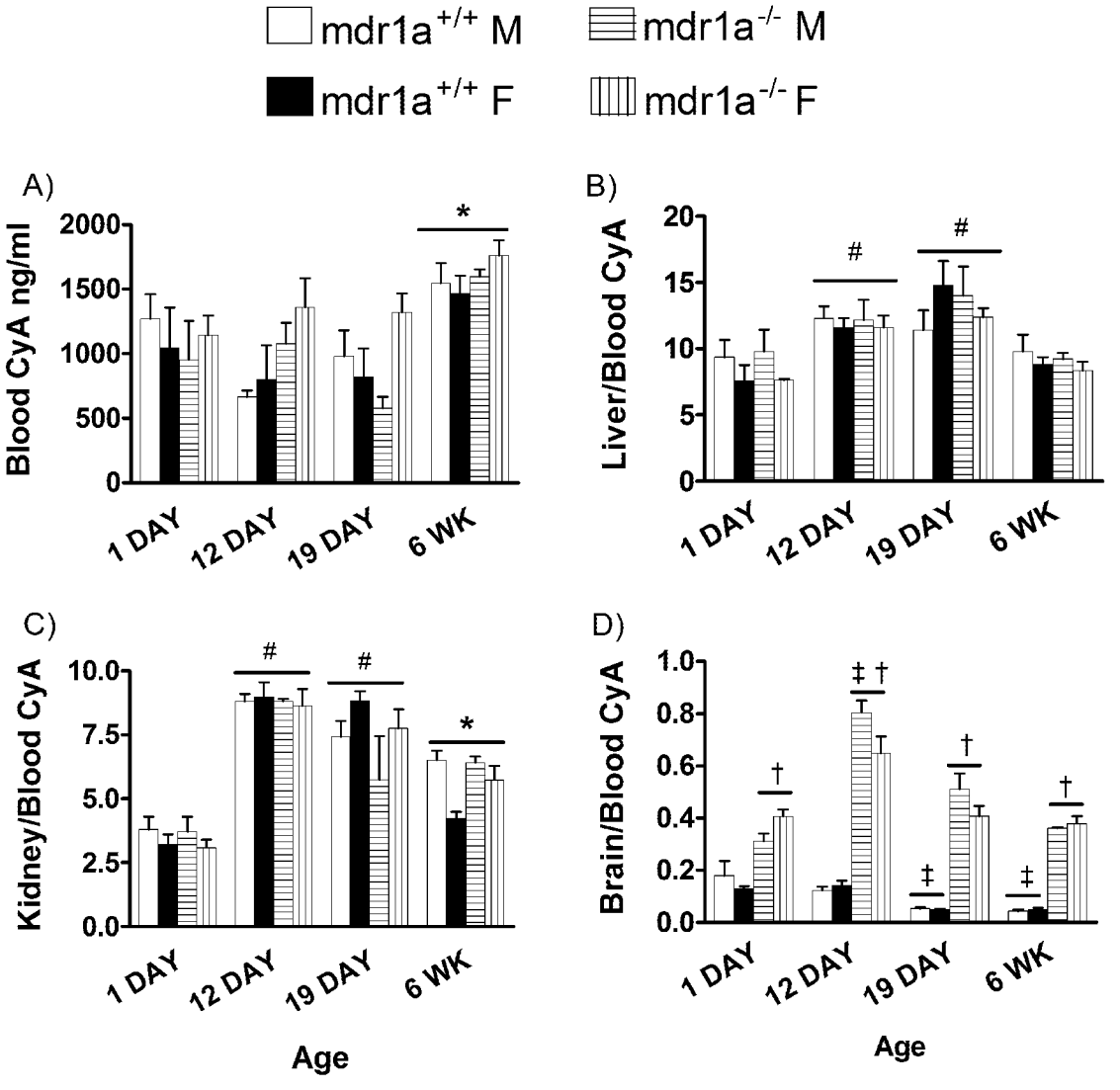
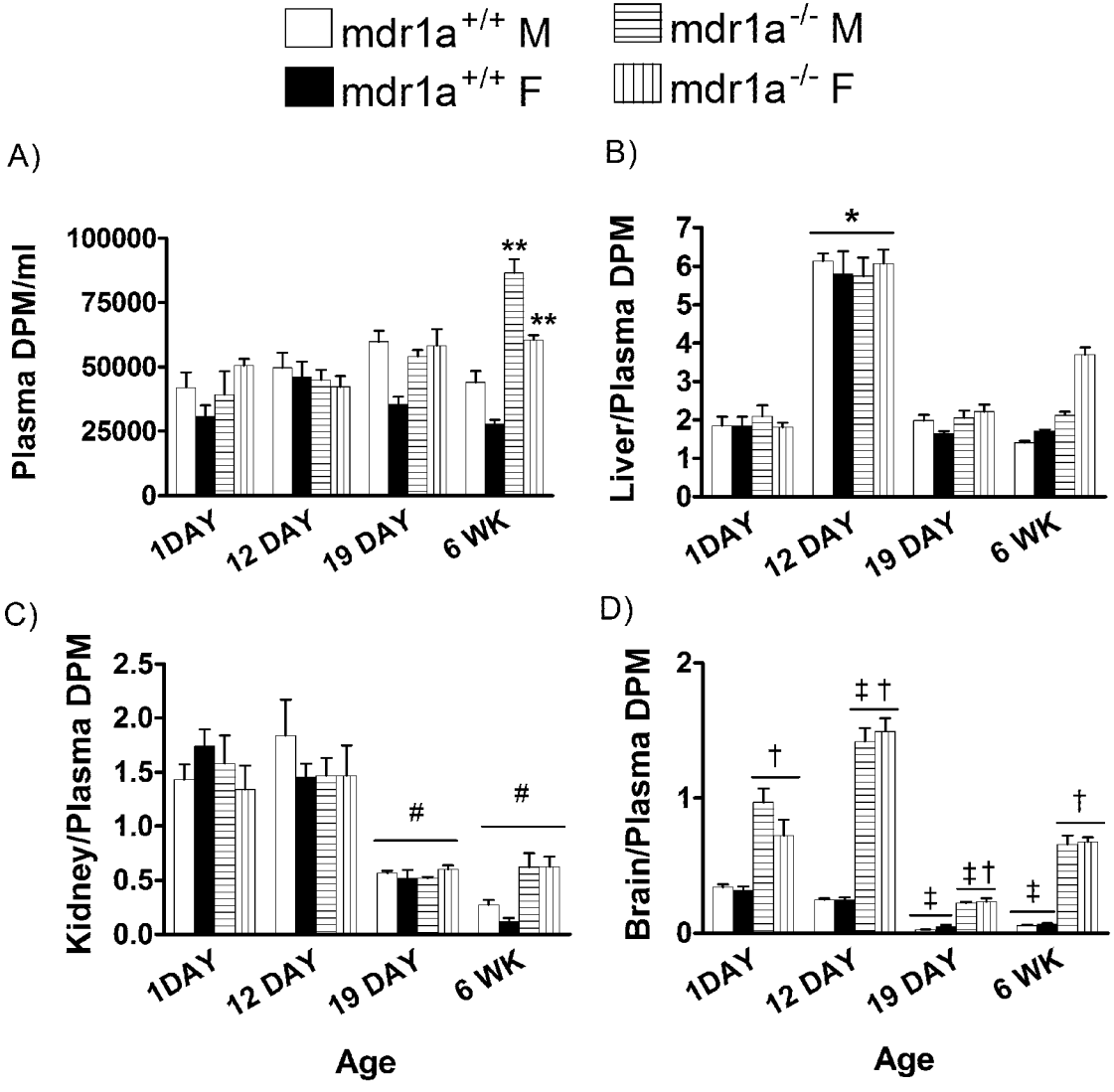

mdr1a+/+ M
mdr1a-/- M
mdr1a+/+ F
mdr1a-/- F
A)
Blood CyA ng/ml
2000
1500
1000
500
0
*
1 DAY
12 DAY
19 DAY
6 WK
B)
Liver/Blood CyA
20
15
10
5
0
#
#
1 DAY
12 DAY
19 DAY
6 WK
C)
Kidney/Blood CyA
10.0
7.5
5.0
2.5
0.0
#
#
*
1 DAY
12 DAY
19 DAY
6 WK
Age
D)
Brain/Blood CyA
1.0
0.8
0.6
0.4
0.2
0.0
†
‡ †
‡
†
‡
†
1 DAY
12 DAY
19 DAY
6 WK
Age

Figure 8


mdr1a+/+ M
mdr1a+/+ F
mdr1a-/- M
mdr1a-/- F
A)
Plasma DPM/ml
100000
75000
50000
25000
0
**
**
1DAY
12 DAY
19 DAY
6 WK
B)
Liver/Plasma DPM
7
6
5
4
3
2
1
0
*
1DAY
12 DAY
19 DAY
6 WK
C)
Kidney/Plasma DPM
2.5
2.0
1.5
1.0
0.5
0.0
#
#
1DAY
12 DAY
19 DAY
6 WK
Age
D)
Brain/Plasma DPM
2
1
0
†
‡ †
‡
‡ †
‡
†
1DAY
12 DAY
19 DAY
6 WK
Age