Impaired Organic Anion Transport in Kidney and Choroid Plexus of Organic Anion Transporter 3 (Oat3 (Slc22a8)) Knockout Mice*

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Douglas H. Sweet, David S. Miller, John B. Pritchard, Yuko Fujiwara, David R. Beier, and Sanjay K. Nigam

From the Departments of Pediatrics, Medicine (Division of Nephrology/Hypertension), and Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California 92039; the Laboratory of Pharmacology and Chemistry, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709; the Division of Hematology/Oncology, Children’s Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115; and the Genetics Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

To begin to develop in vivo model systems for the assessment of the contributions of specific organic anion transporter (OAT) family members to detoxification, development, and disease, we carried out a targeted disruption of the murine organic anion transporter 3 (Oat3) gene. Surviving Oat3−/− animals appear healthy, are fertile, and do not exhibit any gross morphological tissue abnormalities. No Oat3 mRNA expression was detected in kidney, liver, or choroid plexus (CP) of Oat3−/− mice. A distinct phenotype manifested by a substantial loss of organic anion transport capacity in kidney and CP was identified. Uptake sensitive to inhibition by bromosulfophthalein or probenecid was observed for taurocholate, estrone sulfate, and para-aminohippurate in renal slices from wild-type mice, whereas in Oat3−/− animals transport of these substances was greatly reduced. No discernable differences in uptake were observed between hepatic slices from wild-type and Oat3−/− littermates, suggesting Oat3 does not play a major role in hepatic organic anion uptake. Cellular accumulation of fluorescein was reduced by ~75% in CP from Oat3−/− mice. However, capillary accumulation of fluorescein-methotrexate was unchanged, indicating the effects of Oat3 loss are restricted to the entry step and that Oat3 is localized to the apical membrane of CP. These data indicate a key role for Oat3 in systemic detoxification and in control of the organic anion distribution in cerebrospinal fluid.

Active transport of endogenous metabolites and xenobiotics from blood to urine across the cells of the renal proximal tubule is an important protective mechanism. Accordingly, there are efficient excretory transport systems in the kidney comprising groups of organic anion transporters (OATs) and organic cation transporters (OCTs), which are subfamilies within the amphiphilic solute transporter branch (SLC22A) of the major facilitator superfamily (1–4). In the adult, these transporters are also expressed in other barrier epithelia such as the intestine, placenta, retinal pigment epithelium, and the choroid plexus (CP) (5–12). Their expression in the CP (located in the ventricles of the brain), coupled with evidence that neurotransmitters (e.g. choline) and neurotransmitter metabolites (e.g. 5-hydroxyindoleacetic acid (from serotonin) and homovanillic acid (from dopamine)) are substrates for the OATs and OCTs, suggests that these transporters actively regulate the composition of brain extracellular fluid by controlling the flux of xenobiotics and central nervous system by-products from cerebrospinal fluid (CSF) to blood (7). Moreover, during development the spatiotemporal pattern of renal OAT expression suggests that these genes may be useful in understanding the mechanisms of proximal tubule maturation (13). Transient OAT expression in unexpected sites (e.g. spinal cord, bone, skin) during development may indicate that these transporters play a critical role in the formation or preservation of extrarenal tissues, as well (13). Thus, elucidation of the specific mechanisms regulating OAT expression may provide insight into the processes controlling development, CSF-blood equilibrium, and drug handling capacity in the kidney.

Four members of the organic anion transporter family have been characterized thus far: Oat1, Oat2, Oat3, and Oat4 (5, 14–18). Oat1, originally described as novel kidney transporter, NKT, (14, 19, 21; GenBank™ accession no. MMU52842), has been localized to the basolateral membrane of renal proximal tubules and to the apical membrane of CP through direct observation of an Oat1/green fluorescent protein fusion construct and by immunohistochemistry on adult rat kidney sections (6, 14, 22, 23). Uptake by Oat1 is trans-stimulated by glutarate, demonstrating that it functions as an organic anion/dicarboxylate exchanger, consistent with its localization in the basolateral membrane of proximal tubule cells (16, 23). Initial characterization studies of Oat2 (originally described as novel liver transporter (Ref. 17)), Oat3, and Oat4 indicated that, unlike Oat1, uptake mediated by these transporters is not subject to trans-stimulation (5, 18, 24), possibly indicating that they function as facilitative transporters rather than exchangers. Mechanistically this would suggest that these transporters...
are located in the apical membrane in proximal tubule; however, human OAT3 has recently been localized to the basolateral membrane by immunocytochemistry (25). Thus, more thorough investigations of Oat1- and Oat4-mediated transport are clearly required to resolve this issue.

Oat3 (Scl22a8) was originally identified as a gene of unknown specificity that had sequence homology to the transporter genes Oat1 and Oat2 (26,27). It was subsequently demonstrated that its expression is absent in the juvenile cystic kidney (jck) mouse model and markedly reduced in the kidneys of mice homozygous for the osteosclerosis (oc) mutation (26, 27). It was, therefore, designated as “reduced in osteosclerosis transporter,” or Roct. However, it is now known that Roct shares a 92 and 64% identity at the amino acid level with the human OAT3 and OAT1 genes.

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**EXPERIMENTAL PROCEDURES**

**Oat3 Genomic Clone Isolation and Targeting Vector Construction**—A BAC clone carrying the Oat3 gene was isolated from the 129/Sv-derived CitbCj7 library (Research Genetics, Inc., Huntsville, AL). A targeting construct for Oat3 was generated in the vector pPNT in which an internal fragment of the gene containing exon 3 was deleted and replaced with a neomycin (Neo)-selectable marker (Fig. 1A). This was done by cloning a 6-kb EcoRI fragment containing exons 1 and 2 upstream of the Neo cassette (which is in an antisense orientation with respect to Oat3 transcription) and a 2-kb HindIII-XhoI fragment containing exons 4 and 5 downstream of the cassette. These fragments were inserted into pPNT such that the herpes simplex virus thymidine kinase cassette (used for counter selection) is upstream and in an antisense orientation with respect to the genomic sequences (Fig. 1A).

Exon 3 deletion introduces a subsequent frameshift and premature stop codon such that direct splicing of exons 2 and 4 would result in a truncated peptide (281 versus 537 amino acids) with a scrambled amino acid sequence after residue 111.

**Generation and Identification of Oat3−/− Mice**—The targeting construct was linearized by NotI digestion and electroporated into CJ-7 embryonic stem cells (a gift from Dr. Tom Gridley, Jackson Laboratory, Bar Harbor, ME). Transfectants were selected in G418 (200 mg/ml) and ganciclovir (2 μM) and expanded for Southern blot analysis. Homologous recombinants were identified using the G7 probe, which is distal to the genomic sequences contained in the targeting construct (Fig. 1A). The G7 probe detects a 6-kb XbaI wild-type allele fragment and a 3-kb XbaI recombinant allele fragment. One embryonic stem cell line carrying both a wild-type and a targeted allele was identified in the first 35 clones analyzed; this was injected into blastocysts and a founder line established. Male chimeras were mated to C57BL/6 females, and heterozygous offspring were intercrossed to generate homozygous mutants.

Mice were genotyped by polymerase chain reaction (PCR) analysis of their genomic DNA. Genomic DNA was isolated from tail snips by overnight digestion with 400 μg/ml proteinase K in SNE buffer (20 mM Tris-Cl, pH 8, 5 mM EDTA, pH 8, 400 μg/ml NaCl, and 1% w/v SDS) followed by extraction with phenol-chloroform-isooamyl alcohol and precipitation with isopropanol. Twenty nanograms of genomic DNA was used as template for PCR reactions using three different forward primers, one specific for exon 3 of the Oat3 gene (Oat3for) and two specific for the neomycin cassette present in the exon 3 deletion construct (Neo1for and Neo2for), each paired with a single reverse primer located in the intron region just prior to exon 4 of Oat3 (KO3’). PCR products for the Oat3for/KO3’ (a), Neo1for/KO3’ (b), and Neo2for/KO3’ (c) primer pairs are 200, 200, and 230 bp, respectively. Identification of wild-type (wt), heterozygous Oat3+/− (het), and Oat3−/− knockout (KO) offspring are shown.

Fig. 1. Targeted disruption of Oat3 gene. A, the genomic locus (exons 1–5) and targeting construct for Oat3 are shown. When hybridized with XbaI-digested genomic DNA, the G7 probe detects a 6-kb wild-type fragment and a 3-kb mutant fragment. The positions of the PCR primers used to detect the wild-type allele and targeted allele are shown (arrowheads). R, EcoRI; H, HindIII; X, XbaI; XhoI. B, the Oat3 allelic pattern was analyzed by PCR of genomic DNA. Three different forward primers, one specific for exon 3 of the Oat3 gene (Oat3for) and two specific for the neomycin cassette present in the exon 3 deletion construct (Neo1for and Neo2for), were each paired with a single reverse primer located in the intron region just prior to exon 4 of Oat3 (KO3’). PCR products for the Oat3for/KO3’ (a), Neo1for/KO3’ (b), and Neo2for/KO3’ (c) primer pairs are 200, 200, and 230 bp, respectively. Identification of wild-type (wt), heterozygous Oat3+/− (het), and Oat3−/− knockout (KO) offspring are shown.
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Northern Analysis—Approximately 10 μg of total kidney and liver RNA from wild-type, heterozygous, and Oat3−/− littermates was separated by electrophoresis on a 1% agarose formaldehyde gel in MOPS buffer, capillary transferred overnight to a charged nylon membrane (Osmonics, Westborough, MA) with 20× SSC, and UV-cross-linked at 20,000 J/cm² with a Stratalinker (Stratagene, La Jolla, CA). The blot was cut into identical halves and exposed to probes generated by random prime labeling using the Rediprime II kit (Amersham Biosciences), hybridized overnight to a region of interest at high magnification (×4) from each of the sections. No morphological abnormalities were observed in any of the animals examined.

Fig. 2. Histopathological analysis of wild-type and Oat3−/− mouse tissues. Paraffin sections of formalin-fixed tissues from three wild-type and four Oat3 knockout animals were stained with hematoxylin and eosin and examined by light microscopy. Panels A, C, and E are low magnification (×4) images of kidney, liver, and choroid plexus, respectively, from a representative wild-type animal. Panels B, D, and F are low magnification (×4) images of kidney, liver, and choroid plexus, respectively, from a representative Oat3−/− animal. Insets show a region of interest at high magnification (×40) from each of the sections.

Fig. 3. Northern blot analysis of Oat3 expression in kidney and liver. A, approximately 10 μg of total kidney (K) and liver (L) RNA from wild-type (wt), heterozygous (het), and Oat3−/− (KO) littermates was separated by electrophoresis and transferred to a nylon membrane. The membrane was cut into identical halves and exposed to probes generated using either rat Oat1 or mouse Oat3 cDNA as template. No Oat3 mRNA expression was detected in kidney of Oat3−/− mice, but it was readily detected in wild-type and to a lesser degree in heterozygous littermates. No Oat3 signal was detected in liver. Oat1 gene expression was readily detected in the kidney, but not in the liver, of all three animals. The blots were stripped and reprobed with human β-actin to confirm the integrity of the RNA. The experiment was repeated in two independent sets of wild-type, heterozygous, and Oat3−/− littermates with similar results. B, to examine sexual dimorphism of Oat3 expression in mice, a blot containing total kidney and liver RNA from a male (M) and a female (F) wild-type mouse, a male Oat3−/− mouse, and a male and a female wild-type rat was prepared and screened. Oat3 expression was detected in the kidney of the male and female wild-type mice and rats. Importantly, a faint Oat3 signal was also detected in the male rat liver, but not in the liver of the male mouse. Inclusion of male Oat3 knockout RNA demonstrated specificity of the probes and screening of the blot for β-actin monitored sample integrity.

RT-PCR—RT-PCR analysis of CP was adapted from procedures previously described (7). Total RNA was isolated from several freshly collected lateral CP from adult rat and wild-type and Oat3−/− mice using the Absolutely RNA RT-PCR Miniprep kit (Stratagene) according to the manufacturer’s protocols (including treatment with DNase I). After denaturation for 5 min at 70 °C in the presence of 0.5 μg of oligo(dT) primer (Invitrogen), CP RNA was reverse transcribed for 1 h at 42 °C with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a 25-μl reaction (containing 25 units of RNasin and 0.5 mM amounts of each dNTP). One microliter of the reverse transcription reaction was used as template for subsequent PCR with the following intron-spanning Oat1, Oat2, and Oat3 genespecific primer pairs: Oat1for, 5′-GTGCGTATCCACACCCGTG-3′; Oat1rev, 5′-GCTGGACTGACAGTGATGAC-3′; Oat2for, 5′-GGCAGAACATGGTGGATCAG-3′; Oat2rev, 5′-GGGACCAAGGCTAATAT-3′; Oat3for, 5′-TGCAGACAGGCGACTGAC-3′; Oat3rev, 5′-CTGATGAGCAGACGGTG-3′.

Cycle parameters were: denaturing at 95 °C for 15 min; followed by 35 cycles of 95 °C denaturing for 20 s, 58 °C annealing for 20 s, and 68 °C extension for 20 s. Products were visualized on a 1% agarose gel stained with ethidium bromide.
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Transport Assays—Xenopus oocyte isolation procedures and uptake assay were performed as reported previously (23, 29, 30). Briefly, ovaries were removed from tricaine methanesulfonate anesthetized adult female Xenopus laevis and follicle-free stage V and stage VI oocytes were isolated by treatment with collagenase A. After an overnight recovery period in Barth’s buffer at 18 °C, oocytes were microinjected with 20 ng of capped cRNA synthesized from linearized cDNA (mMessage mMachine in vitro transcription kit, Ambion, Inc., Austin, TX). Three days after injection, oocytes were randomly divided into experimental groups (n = 5) and incubated for 1 h at room temperature in oocyte Ringer 2 (in mM: 82.5 NaCl, 2.5 KCl, 1 NaHCO3, 2.5 CaCl2, 1 MgCl2, 1 pyruvic acid, 5 HEPES, pH 7.6) containing 10 μM [3H]para-aminomhippurate (PAH, 1 μCi/μl), 90 nM [3H]estrone sulfate (ES, 1 μCi/μl), or 500 nM [3H]taurocholate (TC, 1 μCi/ml) in the absence or presence of 1 mM probenecid (Pro). Oocyte radioactivity was measured in disintegrations/min (dpm) in a Packard 1600TR liquid scintillation counter with external quench correction.

Renal and hepatic tissue slice preparation and uptake assays were performed according to standard protocols (31). Four- to 6-month-old mice were euthanized by CO2 inhalation, and the liver and kidneys were immediately placed in ice-cold saline. Tissue slices (500 μm) were cut with a Stadie-Riggs microtome and maintained in ice-cold modified Cross and Taggart saline (in mM: 95 NaCl, 80 mannitol, 5 KCl, 0.74 CaCl2, and 9.5 Na2PO4, pH 7.4). Slices were incubated for 1 h with substrate (1 μM taurocholate or para-aminomhippurate, 100 nM estrone sulfate, 10 μM tetraethylammonium (TEA)) in the presence and absence of inhibitors (1 mM bromo-phenylalanine (BPA)) or probenecid, 200 μM quinine sulfate (Q)). Conditions for the PAH experiments were optimized for Oat1 by the addition of 10 μM glutarate to the uptake buffer. After incubation the slices were removed from the uptake medium, blotted, weighed, dissolved in 1 ml of 1 M NaOH, neutralized with 1 ml of 1 M HCl, and assayed by liquid scintillation spectroscopy. Duplicate medium samples were also assayed as tissue samples, and data are presented as tissue to medium (T/M) ratios (i.e. dpm/mg of tissue divided by dpm/μl of medium).

Choroid plexus isolation procedures were performed as described previously (6). Briefly, adult male and female wild-type and Oat3−/− mice were euthanized with CO2. Lateral CP were dissected immediately and transferred to ice-cold artificial cerebrospinal fluid (aCSF) (in mM): 103 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, 2.5 CaCl2, 10 glucose, and 1 sodium pyruvate, pH 7.4), previously gassed with 95% O2, 5% CO2. A forty-five min accumulation of 1 μM fluorescein (FL) or 2 μM fluorescein-methoxetate (FL-MTX) was measured in CP incubated in 1 ml of aCSF in Teflon incubation chambers maintained in Ziploc plastic bags containing 95% O2, 5% CO2 at room temperature until imaging.

Confocal Fluorescence Microscopy—CP were imaged as described previously (6, 7, 32) using an inverted Zeiss model 510 laser scanning confocal microscope fitted with a 40× water immersion objective (numerical aperture, 1.2). Samples were illuminated with the 488-nm line of an argon laser; a 510-nm dichroic filter was in the light path, and a long pass emission filter (515 nm) was positioned in front of the detector. Single confocal images (512 × 512 × 8 bits; 4 frames line-averaged) were obtained and stored for later analysis. For FL and FL-MTX transport studies, cellular and capillary fluorescence intensities were measured from the stored confocal images as described previously using NIH ImageJ 1.25 (23, 32). Briefly, for each CP, 5–10 adjacent cellular and capillary areas were selected. After background subtraction, the average pixel intensity for each area was calculated and the values reported graphically for each CP are the means ± S.E. for all selected areas (n = 5–10). Values reported in the text are mean ± S.E. of the individual mean values for each CP as determined above (n = 4–6 animals/group).

Statistics—The renal slice data were compared using unpaired Student’s t test. Differences in mean values between the control and inhibited conditions were considered significant when p < 0.05.

Materials—[3H]TA (2 Ci/mmol), [3H]ES (40 Ci/mmol), and [3H]PAH (4 Ci/mmol) were obtained from PerkinElmer Life Sciences. [3H]TEA (55 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Unlabeled TC, ES, PAH, TEA, BSP, Pro, and Q were obtained from Sigma. FL and FL-MTX were purchased from Molecular Probes (Eugene, OR). All other chemicals were of reagent grade.

RESULTS

Oat3 Gene Targeting and Phenotypic Analysis of Oat3−/− Mice—Exon 3 of the murine Oat3 gene, which corresponds to putative transmembrane domain 2 in the Oat3 protein, was replaced by an inverted neomycin cassette via homologous recombination in CJ-7 embryonic stem cells (Fig. 1A). Southern analysis of selected embryonic stem cell clones confirmed specific targeting of the Oat3 allele, and chimeric mice were generated by blastocyst injection. Homozygous Oat3−/− mice crossed with the F2 generation of chimeric Oat3 mice crossed with C57BL/6J animals were subsequently backcrossed 4 generations with the C57BL/6J strain. Offspring from heterozygous
pairings were genotyped by PCR assay (Fig. 1B). Identified Oat3/+/H11002+/H11002 mice appear healthy and normal, do not exhibit shortened life expectancy as compared with wild-type littermates, and are fertile, and an Oat3 knockout colony has been established. Histological study of Oat3/+/H11002+/H11002 mice and wild-type littermates, with an emphasis on kidney, liver, and choroid plexus, did not reveal any gross morphological abnormalities (Fig. 2).

Analysis of Oat3 mRNA Expression—No Oat3 mRNA expression was detected in the kidney of Oat3−/− mice by Northern analysis, but an ~2.2–2.4 kb band corresponding to Oat3 (18, 25) was readily detected in wild-type littermates and to a lesser degree in heterozygous Oat3+/− mice (Fig. 3A). No Oat3 signal was observed in the liver. The blot was stripped and re-exposed to a human β-actin probe to confirm the integrity of RNA transferred to the blot (Fig. 3A). The experiment was repeated in a second set of littermates and yielded similar results (data not shown). Expression of Oat1, a gene known to be expressed exclusively in the kidney and choroid plexus of adult rats, was also examined (6, 16). In both sets of animals, Oat1 gene expression was detected in the kidney, but not in the liver, of wild-type, Oat3+/−, and Oat3−/− littermates (Fig. 3A). Differences in Oat3 expression between male and female wild-type mice and rats were also examined (Fig. 3B). As reported recently (33), a very low level of Oat3 expression was detectable in liver from a male wild-type rat. However, such expression was not observed in male mouse liver (Fig. 3B). Screening of the blot for β-actin confirmed sample integrity.

To determine whether Oat1, Oat2, and/or Oat3 are expressed in CP, total RNA was isolated from plexus tissue and used as template for reverse transcription. Subsequent PCR reactions were performed with Oat1-, Oat2-, and Oat3-specific primers using 1 μl of the CP-RT reactions as template. PCR reaction products were detected for Oat1 (417 bp), Oat2 (325 bp), and Oat3 (338 bp) in CP from wild-type rat and mouse, providing direct evidence that these genes are expressed in CP of both species and may play a role in organic anion clearance.

Renal Slices

Hepatic Slices

Fig. 6. Organic anion uptake in renal and hepatic slices. Tissue slices from wild-type and Oat3−/− littermates were incubated for 1 h with substrate (1 μM [3H]taurocholate or [3H]para-aminobiphenyl, 100 nm [3H]estrone sulfate) in the presence and absence of inhibitors (1 mM bromosulfophthalein or probenecid). Substantial inhibitor-sensitive uptake of taurocholate, estrone sulfate, and PAH was observed in slices from wild-type mouse kidneys. In contrast, renal uptake of each of the substrates was significantly reduced in the knockout animals. Quinine sulfate (Q)-sensitive renal uptake of the organic cation [14C]TEA was unaffected by Oat3 loss, demonstrating the proper functioning of this related transport system in knockout animals. No significant differences in uptake were measured between hepatic slices from wild-type and Oat3−/− littermates. Experiments were repeated in 3–4 wild-type and knockout littermate pairs, and representative results are shown. Data were calculated as tissue to medium T/M ratios (i.e. dpm/mg tissue divided by dpm/μl medium) and are presented as mean values ± S.E. (3 slices/treatment). Statistical comparisons (unpaired t test): *, significantly lower than corresponding (wild-type or knockout) control, p < 0.05; **, significantly lower than corresponding control, p < 0.01.
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Fig. 7. Confocal images showing FL accumulation in isolated wild-type and Oat3−/− choroid plexus tissue. The CP is composed of capillary projections surrounded by a single layer of cells that protrude into the cerebrospinal fluid-filled ventricles of the brain. The orientation is such that the CSF bathes the apical membrane of the cell and the basal membrane is toward the underlying fenestrated capillary. A and C, transmitted light images of wild-type and Oat3−/− CP, respectively, showing the tissue structure. B and D, corresponding fluorescence micrographs of the CP shown in A and C. Confocal images were acquired 45 min after exposure to 1 µM FL in the aCSF medium. Panel B, in wild-type CP, note the intracellular concentration of FL above the medium concentration and the fluorescence intensity of the capillaries higher than the cells. Panel C, FL accumulation is markedly lower in the cells and capillaries of Oat3−/− CP. The positions of representative cells and capillaries (cap) are indicated by arrows. A 20-µm bar is shown.

from CSF (Fig. 4). No PCR product was detected for Oat3 in CP from Oat3 knockout mice (Fig. 4). Control reactions with non-RT RNA and RT cDNA without primers were all negative (data not shown).

Organic Anion Transport in Xenopus Oocytes—Although the specificity of organic anion transport mediated by Oat1 and Oat3 cloned from rat and human has been well characterized using several expression systems (16, 18, 25, 28, 29, 34), little data are available for the mouse orthologs. Therefore, we measured the uptake of PAH, TC, and ES in oocytes expressing murine Oat1 and Oat3 (Fig. 5). Oocytes expressing Oat1 (141-fold increase) and Oat3 (152-fold increase) exhibited substantial PAH uptake over that measured in water-injected control oocytes (Fig. 5). This PAH accumulation was completely blocked by the classical organic anion transport inhibitor probenecid and demonstrated that functional transporters were expressed in each group of injected oocytes. However, despite functional transporter expression, TC and ES uptake were both negligible in murine Oat1-expressing oocytes, demonstrating that TC and ES are not readily transported by Oat1 (Fig. 5). In marked contrast, murine Oat3 supported significant uptake of TC (38-fold increase) and ES (726-fold increase) that was probenecid-sensitive (Fig. 5). Additionally, probenecid-sensitive FL uptake was observed in oocytes expressing Oat1 and Oat3, confirming FL is a substrate for these transporters (data not shown). These data indicate that for the mouse, rat, and human forms of Oat1 and Oat3 transport characteristics differ little from species to species.

Transport Function in Oat3−/− Mice—Transport of [3H]TC, [3H]ES, and [3H]PAH was investigated in renal and hepatic slices of wild-type and Oat3−/− littermates (Fig. 6). Substantial uptake of taurocholate was observed in slices from wild-type kidneys that was significantly inhibited in the presence of 1 mM either BSP or Pro (54 and 52% reduced, respectively). In marked contrast, TC uptake in knockout animals was essentially reduced to the inhibited levels seen in wild-type littermates, with no further significant reduction in uptake observed in the presence of the inhibitors (Fig. 6). When directly compared, this loss in transport was found to be significant with the renal taurocholate T/M values being 6.52 ± 1.06 for wild-type versus 3.47 ± 0.35 for Oat3−/− (n = 4, p < 0.05). Similarly, estrone sulfate accumulation was significantly reduced in Oat3−/− kidney slices as compared with wild-type (Fig. 6), with T/M values of 5.52 ± 0.79 for wild-type versus 2.76 ± 0.20 for knockout animals (n = 3, p < 0.05). Addition of BSP or Pro significantly reduced uptake of ES in the wild-type slices (63 and 56% inhibited) and in the knockout slices (33 and 37% inhibited, as compared with uninhibited Oat3−/− control). PAH uptake was observed in both wild-type and Oat3−/− renal slices; however, uptake in the knockout animals was again significantly reduced (T/M wild-type of 5.22 ± 0.31 versus only T/M Oat3−/− of 1.94 ± 0.34, n = 4 and p < 0.001). The addition of 1 mM BSP inhibited uptake by ~85 and ~66% in wild-type and Oat3−/− slices, respectively, and addition of 1 mM probenecid also reduced uptake by ~85 and ~72%, respectively (Fig. 6). Proper functioning of the renal organic cation transport system in Oat3−/− animals was confirmed by demonstrating that inhibitable uptake of the classical organic cation [14C]TEA was unchanged by Oat3 loss. No significant differences in uptake were observed between hepatic slices from wild-type and Oat3−/− littermates for any of the compounds examined (Fig. 6). [3H]TC uptake was inhibited by BSP (~60–70% reduction) and Pro (~10–30% reduction). There was marked uptake of [3H]ES that was also significantly inhibited by BSP (~45–54%...
FIG. 8. Quantitation of FL and FL-MTX uptake in intact CP. Fluorescence levels in cells and vessels of CP from 4 wild-type and 4 Oat3−/− mice were measured (n = 5–10 adjacent cellular and capillary areas/CP) as described under "Experimental Procedures." Cellular and capillary FL levels were significantly reduced in CP from Oat3−/− mice as compared with wild-type. No difference in capillary accumulation of FL-MTX was observed between wild-type and Oat3−/− CP. Data are given as mean ± S.E. for each animal.

Breen et al. (32) recently demonstrated that FL transport across intact rat CP could be followed using confocal microscopy. They found evidence for a two-step mechanism, involving mediated uphill transport at both the apical and basolateral membranes. Such transport was sensitive to inhibition by a number of organic anions, including PAH and probenecid. The same pattern of FL distribution was seen in CP from wild-type mice as in rats, i.e. substantial accumulation of FL was observed in the cells and underlying capillaries with fluorescence intensity in capillaries > cells > medium (Fig. 7B). Uptake of 1 μM FL was nearly completely inhibited by 200 μM probenecid (data not shown). In marked contrast, FL uptake appeared to be substantially reduced in the cells and capillaries of CP from Oat3−/− mice (Fig. 7D). Measurement of cellular and capillary fluorescence intensities (n = 5–10 adjacent cellular and capillary areas/CP) in CP from 4 wild-type and 4 Oat3−/− littersmates showed a 75% reduction in cellular fluorescence and a 60% reduction in capillary fluorescence (Fig. 8; wild-type CP averaged 79 ± 2 and 205 ± 7 units for cells and capillaries, respectively; corresponding values for Oat3−/− littersmates were 24 ± 2 and 79 ± 24, both significantly lower than corresponding wild-type values, p < 0.01).

The large organic anion, FL-MTX, is also actively transported from bath to capillaries by rat CP, but this substrate does not share any steps with FL. Indeed, uptake into the cells appears to be by simple diffusion, but efflux at the basolateral membrane was uphill and carrier-mediated. A similar pattern of FL-MTX distribution was also seen in CP from mouse (Fig. 9). However, no differences in the transport of FL-MTX were found between wild-type and Oat3−/− littersmates (Fig. 9). Measurement of luminal fluorescence intensities (n = 5–10 measurements/CP) in CP from 4 wild-type and 4 Oat3−/− littersmates showed no differences (Fig. 8; mean fluorescence intensity in CP from wild-type mice was 208 ± 6, that in Oat3−/− littersmates was 210 ± 4).

DISCUSSION

One hallmark of the OAT family members is their ability to transport a wide variety of organic compounds, requiring only a hydrophobic backbone and negative charge. This property results in distinct, yet greatly overlapping substrate "specificities" for Oat1–4 and, thus, makes identification of transporter-specific substrates and inhibitors difficult. This in turn prevents a clear assessment of the individual contribution of each transporter to tissue transport capacity as a whole. However, as investigations continue some diagnostic compounds are being identified (Table I). For example, PAH is highly transported by both Oat1 (Km = 14–70 μM; Refs. 15 and 16) and Oat3 (Km = 65–87 μM; Refs. 18 and 25), but transport by; or inhibition of; either Oat2 or Oat4 is negligible (5, 24). Oat3 (Km = 3 μM) and Oat4 (Km = 1 μM) each transport estrone sulfate (5, 18), whereas Oat1 and Oat2 do not (this work and Ref. 24). Importantly, within the OAT family (Oat1–4), taurocholate is apparently an Oat3-specific substrate with no transport of, or inhibition of transport by, taurocholate observed for Oat1, Oat2, or Oat4 (5, 24, 25). Thus, the continuing discovery of diagnostic substrates, the increasing knowledge of OAT tissue expression patterns, and the development of OAT knockout mouse lines, together, are providing tools for establishing a greater under-

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2 D. S. Miller, unpublished observations.
standing of how each of these transporters contribute functionally to the homeostasis of protected body compartments, detoxification after xenobiotic exposure, and drug-drug interaction in the clinical setting.

When interpreting the present data in terms of the contributions of individual transporters to the measured tissue uptake, two important points must be considered. First, in the renal and hepatic tissue slice in vitro transport model, only the basolateral membrane is accessible for study. Second, although not strongly homologous to OATs, another branch of the amphiphilic solute carrier family, the organic anion transporting polypeptides (SLC21A; Oatp1, Oatp2, Oatp3, and Oatp4) are also involved in the disposition of organic anions within the body. Some Oatps are found in kidney, liver, and brain, and they are known to transport TC, ES, and BSP, but not PAH (Table I). Thus, only those transporters expressed in the appropriate tissue and localized to the experimentally accessible membrane would be expected to contribute to the measured uptake.

In the kidney, Oat1–4 are all expressed (5, 16–18), with Oat1 and OAT3 having been localized to the basolateral membrane of proximal tubule cells (23, 25, 35). For the Oatp family, only Oatp1 expression has been conclusively demonstrated in the kidney (36–39). However, Oatp1 has been localized to the apical membrane of the proximal tubule and therefore would not contribute to the basolateral uptake of organic anions (40). Although there is some controversy as to whether Oatp3 is expressed in kidney, and renal Oatp4 expression has not been examined, the absence of any additional inhibition of taurocholate uptake by BSP in the Oat3−/− mice supports the notion that kidney-expressed Oatps participate minimally, if at all, in basolateral OA uptake (Fig. 6). Thus, it appears that the movement of TC, ES, and PAH across the basolateral membrane of proximal tubule cells measured in these experiments is mediated by the OAT family members 1, 3, and 4 (Oat2 does not transport these compounds; Table I). The significant (p < 0.05) drop in TC uptake combined with the lack of any inhibitory effect of BSP and probenecid in the Oat3−/− renal slices indi-
icates that renal taurocholate uptake is largely mediated by Oat3 and that Oat3\(^{-/}\) mice have a demonstrable OA-deficient transport phenotype (Fig. 6). The significant \((p < 0.05)\) reduction in estrone sulfate transport in Oat3\(^{-/}\) mice also supports this interpretation, with the additional drop in ES transport in the presence of BSP and Pro potentially because of Oat4 expression in the basolateral membrane of proximal tubule cells (Fig. 6 and Table I). Although there is a significant \((p < 0.001)\) decrease in PAH uptake associated with Oat3 loss, there is nonetheless a large inhibitor-sensitive transport component left in renal slices from knockout animals, presumably representative of intact Oat1 transport function. The residual Oat4-mediated ES uptake and Oat1-mediated PAH uptake, along with unaltered organic cation (TEA) transport, in Oat3\(^{-/}\)-renal slices confirms that the observed OA transport-deficient phenotype in these animals is the result of specific Oat3 loss, as opposed to a generalized, non-descriptive disruption of transport function.

Recently it was reported that Oat3 is also expressed in the liver of male, but not female, rats (33); however, in our studies no Oat3 expression was detected in mouse liver RNA from wild-type or heterozygous Oat3\(^{-/}\) male mice (Fig. 3, A and B). Regardless, to avoid the possibility that using female wild-type mice as control animals in hepatic transport studies would mask any actual change in OA transport as a result of Oat3 loss, only data using male wild-type littersmates are presented in Fig. 6. Therefore, basolaterally expressed Oat2 would be the only OAT present in liver (Table I) and none of the compounds used in this study are known substrates for Oat2 (17, 24). Thus, all of the uptake measured in hepatic slices should be attributable to non-OAT transporters. As such, Oatp1-4 have all been detected in liver and Oatp1, Oatp2, and Oatp4 have been localized to the basolateral membrane by immunocytochemistry (36, 37, 39–43). This interpretation is further supported by the lack of any significant difference in uptake between wild-type and Oat3\(^{-/}\) mice (Fig. 6) for taurocholate and estrone sulfate and the complete lack of hepatic PAH uptake (PAH is not a substrate for Oat2 or Oatp1-4; see Table I).

As indicated in Fig. 4, Oat1, Oat2, and Oat3 expression has been detected in rat and murine CP. In rat CP, apical uptake of the organic anions PAH, 2,4-dichlorophenoxyacetic acid, and FL has been demonstrated to occur at least in part via the indirect sodium-coupled exchange mechanism utilized by Oat1 (6, 32). Furthermore, Oat1 and Na\(^{+}\)-K\(^{-}\)-ATPase have been demonstrated to be targeted to the apical membrane in rat CP (6, 44). Thus, the CP is unique in that, to accomplish the extraction of OAs from CSF to blood, the tissue exhibits a reversal of functional polarity as compared with other excr-

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Impaired Organic Anion Transport in Kidney and Choroid Plexus of Organic Anion Transporter 3 (Oat3 (Slc22a8)) Knockout Mice
Douglas H. Sweet, David S. Miller, John B. Pritchard, Yuko Fujiwara, David R. Beier and Sanjay K. Nigam

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