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Organic Anion Transporter 3 Mediates the Efflux Transport of an Amphipathic Organic Anion, Dehydroepiandrosterone Sulfate, across the Blood-Brain Barrier in Mice

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ABSTRACT:

The present study investigated the efflux transport systems of organic anions across the blood-brain barrier (BBB) using dehydroepiandrosterone sulfate (DHEAS) as a probe. The elimination of DHEAS from the brain after microinjection into the cerebral cortex was characterized in wild-type mice and mice with deficiency of well characterized organic anion transporters, organic anion-transporting polypeptide 1a4 (Oatp1a4)/*Slco1a4* and organic anion transporter 3 (Oat3)/*Slc22a8*, at the BBB. The saturable efflux of DHEAS from the brain was completely inhibited by probenecid, benzylpenicillin, and estrone-3-sulfate and moderately inhibited by taurocholate and *p*-aminohippurate (50–57%). Uptake of DHEAS and estrone-3-sulfate was greater in murine Oat3 cRNA-injected oocytes than that in water-injected oocytes. Efflux of these compounds from the brain was significantly delayed in *Oat3*(−/−) mice compared with that in wild-type mice, indicating that indeed Oat3 is functionally important in vivo. Furthermore, probenecid and taurocholate inhibited DHEAS efflux completely in *Oat3*(−/−) mice. Contrary to the past report in rats that suggested involvement of Oatp1a4, specific uptake of DHEAS and estrone-3-sulfate by murine Oatp1a4 was not detected in vitro, and efflux of both compounds from the brain was not altered in *Oatp1a4*(−/−) mice. There was no significant difference in the uptake of DHEAS by brain slices prepared from wild-type, *Oatp1a4*(−/−), and *Oat3*(−/−) mice. Taken together, these results suggest that Oat3 plays a significant role in the efflux of steroid conjugates across the BBB in mice and that the BBB also expresses other unknown organic anion transporters for the efflux of DHEAS. Transport mechanisms of organic anions at the BBB are far more diverse than they were assumed to be.

Introduction

The blood-brain barrier (BBB), formed by a tight monolayer of endothelial cells, is an interface for the exchange of compounds and gas between the brain interstitial space and blood. Because of highly developed tight junctions between adjacent cells and a paucity of fenestrae from the diffusion barrier, various transport systems play pivotal roles in both the uptake of water-soluble nutrients and peptides from the circulating blood and the efflux of xenobiotic and endogenous compounds from the brain interstitial space (Kusuhara and Sugiyama, 2005). The active efflux system in the BBB keeps the unbound concentrations of exogenous compounds in the brain interstitial space lower than those in the blood to attenuate their effect in the central nervous system and also inactivates neuroactive compounds produced in the brain by transferring them to the blood.

Kinetic studies in rats using microinjection into the cerebral cortex have indicated the importance of transporters in the efflux of various organic anions from the brain across the BBB. Characterization of this process using various organic anions as inhibitors has suggested that the efflux of amphipathic and hydrophilic organic anions is mediated by distinct transporters (Kusuhara and Sugiyama, 2005). Furthermore, a mutual inhibition study suggested that the efflux of amphipathic organic anions, such as bile acids and cyclo-D-Trp-D-Asp-Pro-D-Val-Leu (BQ-123), is mediated by multiple transporters (Kitazawa et al., 1998). On the basis of the substrate specificities and membrane localization, organic anion-transporting polypeptide 1a4 (Oatp1a4/*Slco1a4*) and organic anion transporter 3 (Oat3/*Slc22a8*) have been considered to be responsible for the efflux of amphipathic and hydrophilic organic anions from the brain, respectively, at the BBB (Kusuhara and Sugiyama, 2005). Oatp1a4 is expressed in both luminal and abluminal membranes of brain capillary endothelial cells (Gao et al., 1999; Ose et al., 2010), whereas predominant abluminal expression of Oatp1a4 was also reported in rat BBB (Roberts et al., 2008). Oat3 is localized in the abluminal membrane of mouse BBB (Ohtsuki

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ABBREVIATIONS: BBB, blood-brain barrier; BQ-123, cyclo-D-Trp-D-Asp-Pro-D-Val-Leu; Oatp/OATP, organic anion transporting polypeptide; Oat/OAT, organic anion transporter; Ro 64-0902, [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate; DHEAS, dehydroepiandrosterone sulfate; PAH, *p*-aminohippuric acid; HEK, human embryonic kidney; BEI, brain efflux index; Gadph, glyceraldehyde 3-phosphate dehydrogenase; RT, real time; PCR, polymerase chain reaction; m, mouse; r, rat.

et al., 2004), but we suggested that Oat3 is expressed on both luminal and abluminal membranes of rat BBB (Kikuchi et al., 2003). Both transporters show broad substrate specificities. Of note, Oatp1a4 preferably mediates the uptake of amphipathic organic anions and cardiac glycosides, whereas Oat3 mediates that of both hydrophilic and amphipathic ones (Sweet et al., 2002; van Montfoort et al., 2002; Kobayashi et al., 2004; Ohtsuki et al., 2004; Ose et al., 2010).

In recent studies, *Oatp1a4*(−/−) and *Oat3*(−/−) mice were produced to investigate their role in drug disposition. Disruption of Oatp1a4 and Oat3 genes did not affect the integrity of the BBB or cause any adaptive regulation of the known xenobiotic transporters in the brain, at least at the mRNA level (Ose et al., 2009, 2010). However, the efflux of taurocholate, rosuvastatin, pitavastatin, and Ro 64-0802 from the brain after microinjection was significantly delayed in *Oatp1a4*(−/−) and *Oat3*(−/−) mice, respectively, compared with that in the corresponding wild-type mice (Ose et al., 2009, 2010). This result is consistent with the proposed roles of Oatp1a4 and Oat3 at the BBB.

In this study, we investigated the transporters involved in the efflux transport of organic anions using dehydroepiandrosterone sulfate (DHEAS). DHEAS is the major sulfate conjugate of steroids in the plasma, and it is also one of the *neurosteroids* synthesized de novo in the brain with physiological effects on the central nervous system (Dubrovsky, 2005; Steffensen et al., 2006). In a previous report, Asaba et al. (2000) elucidated the fact that the efflux of DHEAS across the BBB from the brain in rats, the major inactivation pathway of DHEAS in the brain, was saturable and inhibited by amphipathic organic anions, such as taurocholate, but not by hydrophilic ones, such as *p*-aminohippurate (PAH). They suggested that Oatp1a4 was responsible for this process. In this study, we report that Oat3 and unknown organic anion transporters account for the efflux of DHEAS across the BBB, whereas Oatp1a4 unexpectedly makes only a negligible contribution.

Materials and Methods

Chemicals. [^{3}H]DHEAS, [^{3}H]estrone-3-sulfate, and [^{3}H]taurocholate were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{14}C]Carboxyl-inulin (2.5 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled DHEAS, estrone-3-sulfate, probenecid, PAH, benzylpenicillin, and taurocholate were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were commercially available, of reagent grade, and used without further purification.

Animals. *Oatp1a4*(−/−) mice and *Oat3*(−/−) mice were obtained from Deltagen (San Carlos, CA) and maintained by Charles River Laboratories Japan (Yokohama, Japan) and Oriental Yeast (Tokyo, Japan), respectively. *Oatp1a4*(−/−) mice and *Oat3*(−/−) mice were fertile and exhibited no obvious abnormalities. Wild-type (C57BL/6J) mice were supplied by Oriental Yeast. Male mice were used except for the investigation of the efflux of estrone-3-sulfate in wild-type and *Oat3*(−/−) mice. All mice were maintained under standard conditions with a reverse dark-light cycle. Food and water were available ad libitum. All experiments using animals in this study were performed according to the guidelines provided by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan).

In Vitro Transport Study Using Mock- and Oatp1a4-Expressing HEK293 Cells. mOatp1a4-expressing HEK293 cells were constructed as reported previously (Ose et al., 2010). Uptake was initiated by addition of the radiolabeled ligands to the medium in the presence of unlabeled taurocholate (1 μM), DHEAS (0.5 μM), or estrone-3-sulfate (0.5 μM) after cells had been washed three times and preincubated with Krebs-Henseleit buffer (142 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4) at 37°C for 15 to 30 min. The uptake was terminated at designated times by addition of ice-cold Krebs-Henseleit buffer, and cells were washed three times. Cells were then dissolved in 500 μl of 0.2 N NaOH, and the radioactivity associated with the cells and medium was determined in a liquid scintillation counter. Aliquots of cell lysate were used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Ligand uptake is given by the amount of ligand associated with the cell specimens divided by the medium concentration.

In Vitro Transport Study Using mOat3 cRNA-Injected *Xenopus laevis* Oocytes. Water- and mOat3 cRNA-injected *X. laevis* oocytes were kindly provided by Sekisui Medical (Tokyo, Japan). Three days after injection, oocytes were randomly divided into experimental groups (n = 8–9) and preincubated for 30 min at room temperature in Na^+ buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, and 250 mM Tris, pH 7.4). Then oocytes were incubated for 1 h at room temperature in Na^+ buffer containing [^{3}H]DHEAS (0.5 μCi/ml, 5 nM) and unlabeled DHEAS (0.1 μM) or [^{3}H]estrone-3-sulfate (0.5 μCi/ml, 10 nM) and unlabeled estrone-3-sulfate (0.1 μM) in the absence or presence of probenecid (1 mM). Oocyte radioactivity was determined in a liquid scintillation counter. Ligand uptake is given by the amount of ligand associated with the cell specimens divided by the medium concentration.

Microinjection to the Cerebral Cortex of Mouse Brain. The efflux of test compounds from the brain after microinjection into the cerebral cortex was investigated using the BEI method as described previously (Ose et al., 2010). [^{3}H]DHEAS (70 nCi/mouse) with a nonpermeable reference compound, [^{14}C]carboxyl-inulin (2 nCi/mouse), in 0.5 μl of ECF buffer (122 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, pH 7.4) in the presence or the absence of different concentrations of various inhibitors was injected into the Par2 region (3.5 mm lateral to the bregma and 2.5 mm depth). After the microinjection, mice were decapitated, and the radioactivity remaining in the cerebrum was determined. The 100 − BEI (%), which represents the remaining percentage of the test compounds in the cerebrum, was corrected by the recovery of [^{14}C]carboxyl-inulin. The elimination rate constant of the compounds from the brain (k_{el}) was obtained by fitting the 100 − BEI (%) versus time data. A nonlinear least-squares regression program (GraphPad Prism; GraphPad Software Inc., San Diego, CA) was used for the calculation.

In Vitro Uptake Studies Using Brain Slices. The distribution volume of DHEAS in the brain ($V_{d\ brain}$) was determined by the in vitro brain slice uptake technique. Brain slices were prepared as reported previously with a minor modification (Kakee et al., 1997). In brief, a 300-μm-thick slice was cut using a microslicer (DTK-2000; Dosaka EM Co., Ltd., Kyoto, Japan) and kept in oxygenated ECF buffer equilibrated with 95% O_2-5% CO_2. After preincubation for 5 min at 37°C, the brain slice was transferred to 1 ml of oxygenated incubation medium containing unlabeled DHEAS (20 nM), [^{3}H]DHEAS (0.05 μCi/ml, 75 nM), and [^{14}C]carboxyl-inulin (0.01 μCi/ml) at 37°C. Brain slices were collected at designated times, and the radioactivity was determined in a liquid scintillation counter. Ligand uptake was given as the amount of ligand associated with the slice divided by the medium concentration.

Calculation of the Apparent Efflux Clearance ($CL_{app,\ efflux}$). $CL_{app,\ efflux}$ was calculated by multiplying the apparent elimination rate constant (k_{el}) by the distribution volume ($V_{d\ brain}$), as shown in the following equation:

$$CL_{app,\ efflux} = k_{el} \cdot V_{d\ brain}$$

k_{el} was determined by the BEI method, and $V_{d\ brain}$ was obtained from the brain slice uptake studies.

mRNA Expression of Oatp1a4 and Oat3 in Mouse Brain Regions. The mRNA levels of Oatp1a4, Oat3, and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were quantified using the real-time (RT) polymerase chain reaction (PCR) method. Mouse brain regional cDNA was purchased from Genostaff (Tokyo, Japan). These cDNAs were prepared from total tissue, not from brain capillary endothelial cells. RT PCR was performed using SYBR Premix Ex Taq (Takara, Ohtsu, Japan) and a LightCycler system (Roche Diagnostics, Mannheim, Germany). For RT PCR, the following primers were used: mOatp1a4, forward 5′-ATA GCT TCA GGC GCA TTT AC-3′ and reverse 5′-TTC TCC ATC ATT CTG CAT CG-3′; mOat3, forward 5′-CGC CAA GTT CAT CAC AAT C-3′ and reverse 5′-CAT CCC TTC CCA AAT ACA GC-3′; and mGapdh, forward 5′-AAC GAC CCC TTC ATT GAC-3′

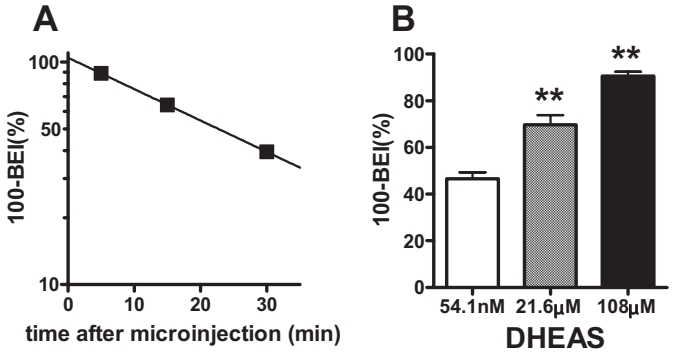

FIG. 1. Time profile of the 100 − BEI (%) of [^{3}H]DHEAS in the cerebrum after intracerebral microinjection and its concentration dependence. A, a solution of [^{3}H]DHEAS (70 nCi/mouse, 1.5 μM) and [^{14}C]carboxyl-inulin (2 nCi/mouse) in 0.5 μl of ECF buffer was injected into the Par2 region of the mouse cerebrum. At designated times, animals were decapitated. The solid line represents the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. ($n = 3$). S.E.s are included within the size of the symbol used to designate the mean. B, a solution of [^{3}H]DHEAS (70 nCi/mouse, 1.5 μM) and [^{14}C]carboxyl-inulin (2 nCi/mouse) in 0.5 μl of ECF buffer with unlabeled DHEAS (1 μM, 500 μM, and 5 mM) was injected into the Par2 region of the mouse cerebrum. Thirty minutes after microinjection, animals were decapitated. The concentrations of total DHEAS are shown as the effective concentration in the cerebrum; each value was calculated by dividing the concentration in the injectate by the dilution factor of 46.2 (Asaba et al., 2000). Each column represents the mean ± S.E. ($n = 4$). **, significantly different from mice injected with 54.1 nM DHEAS as the effective concentration as examined by one-way analysis of variance followed by Dunnett's multiple comparison test ($p < 0.01$).

and reverse 5′-TCC ACG ACA TAC TCA GCA C-3′. For quantification, an external standard curve for each target gene was generated by using serial diluted solutions of its conventional PCR product as template for quantitative RT PCR. This conventional PCR product was purified by agarose electrophoresis, and its DNA content was measured using PicoGreen dsDNA Quantification Reagent (Invitrogen, Carlsbad, CA). Using Light Cycler software, mRNA expression levels for each gene were determined, and mOatp1a4 and mOat3 mRNA expression values were normalized for Gapdh.

Results

Time Profile and Concentration Dependence of DHEAS Efflux after Intracerebral Microinjection. The time profile of the 100 − BEI (%) of DHEAS after intracerebral microinjection is shown in Fig. 1. DHEAS was eliminated from the brain and the apparent elimination rate constant obtained was 0.0325 ± 0.0028 min^{-1}. The concentration dependence of the efflux of DHEAS was examined, and 100 − BEI (%) values determined 30 min after microinjection increased along with the unlabeled DHEAS concentration, showing saturation.

Effect of Various Organic Anions on the Efflux of DHEAS across the BBB. Various unlabeled organic anions were simultaneously injected to characterize the efflux of DHEAS from the brain in mice (Fig. 2). The concentrations of inhibitors were reported to saturate their own efflux from the brain in rats (Kitazawa et al., 1998; Sugiyama et al., 2001; Kikuchi et al., 2003). Taurocholate and PAH moderately inhibited DHEAS efflux from the brain (Fig. 2, A and B), whereas probenecid, estrone-3-sulfate, and benzylpenicillin almost totally inhibited the efflux (Fig. 2, C–E).

Uptake of [^{3}H]DHEAS and [^{3}H]Estrone-3-sulfate by mOat3-Expressing *X. laevis* Oocytes. Because we have not been able to construct mOat3-expressing HEK293 cells for some unknown reason, *X. laevis* oocytes were used as host cells to express mOat3. The uptake of both compounds was significantly greater in mOat3-cRNA-injected oocytes compared with that in water-injected oocytes (Fig. 3). This uptake was inhibited by a potent Oat3 inhibitor, probenecid. DHEAS uptake by mOat3 cRNA-injected oocytes in the presence of probenecid was below the control value, presumably because of an endogenous uptake transporter in oocytes. In addition, for estrone-3-sulfate, probenecid significantly increased its uptake in water-injected oocytes for an unknown reason.

Efflux of DHEAS across the BBB in Wild-Type and *Oat3*(−/−) Mice. The 100 − BEI (%) values were determined 30 min after microinjection in wild-type and *Oat3*(−/−) mice. The efflux of DHEAS was significantly delayed in *Oat3*(−/−) mice, but DHEAS efflux was still observed (Fig. 4A). The k_{el} was 0.0292 ± 0.0028 and 0.0118 ± 0.0028 min^{-1} in wild-type mice and *Oat3*(−/−) mice, respectively (mean ± S.E., $n = 4$). Probenecid and taurocholate almost completely inhibited the efflux of DHEAS from the brains of *Oat3*(−/−) mice, whereas PAH had no effect (Fig. 4A).

To calculate the apparent efflux clearance ($CL_{app, efflux}$), the distribution volume was determined in vitro using brain slices (Fig. 4B). The slice/medium ratio determined after a 60-min incubation was 7.10 ± 1.25 and 7.10 ± 1.49 ml/g brain in wild-type and *Oat3*(−/−) mice, respectively (mean ± S.E., $n = 3$) (Fig. 4B). For inulin, the impermeable brain vascular marker, the distribution volume was

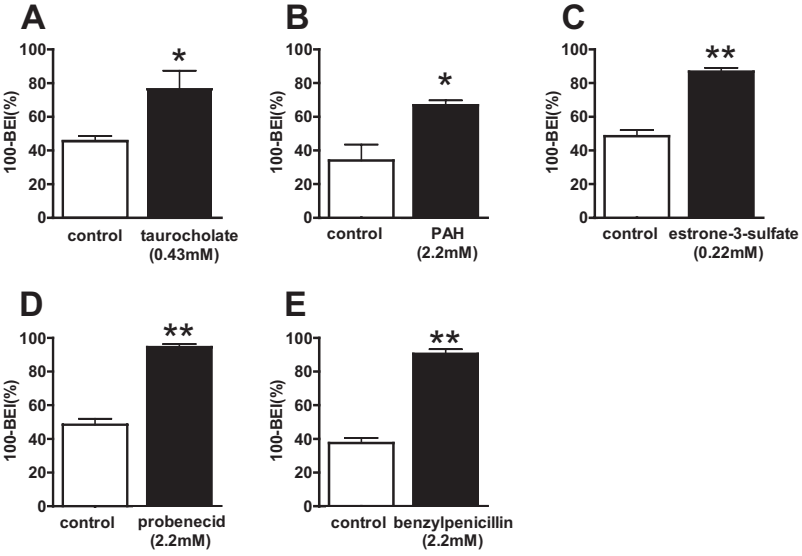

FIG. 2. Inhibition of the efflux of [^{3}H]DHEAS from the brain by various organic anions. ECF buffer containing [^{3}H]DHEAS (140 μCi/ml, 1.5 μM) and [^{14}C]carboxyl-inulin (4 μCi/ml), with or without unlabeled inhibitors (A, taurocholate; B, *p*-aminohippurate; C, estrone-3-sulfate; D, probenecid; E, benzylpenicillin) was microinjected into the Par2 region of mouse cerebrum. The concentrations of inhibitors are shown as the effective concentration in the cerebrum; each value was estimated by the concentration in the injectate divided by the dilution factor of 46.2. Each column represents the mean ± S.E. ($n = 3$–4). *, significantly different from the control as examined by Student's *t* test ($p < 0.05$); **, significantly different from the control as examined by Student's *t* test ($p < 0.01$).

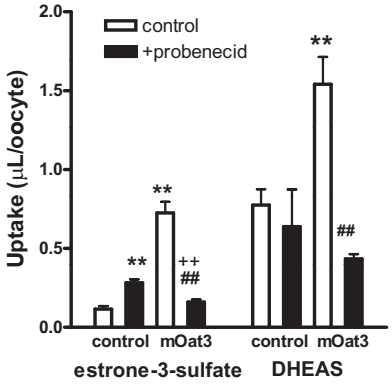

FIG. 3. Uptake of estrone-3-sulfate and DHEAS by water- and mOat3 cRNA-injected oocytes. mOat3 cRNA-injected oocytes were incubated with [^{3}H]estrone-3-sulfate (0.5 μCi/ml, 10 nM) or [^{3}H]DHEAS (0.5 μCi/ml, 5 nM) with 0.1 μM unlabeled compounds at room temperature. After a 1-h incubation, oocytes were washed with ice-cold buffer three times and then dissolved for quantification of the radioactivity in the lysate specimens. Ligand uptake is given by the amount of ligand associated with the cell specimens divided by the medium concentration. Each column represents the mean ± S.E. (n = 7–9). **, significantly different from uptake by water-injected oocytes without probenecid as examined by Student's t test ($p < 0.01$); ##, significantly different from uptake by water-injected oocytes with probenecid as examined by Student's t test ($p < 0.01$). ++, significantly different from uptake by mOat3 cRNA-injected oocytes without probenecid as examined by Student's t test ($p < 0.01$).

1.04 ± 0.15 and 1.16 ± 0.22 ml/g brain in wild-type and *Oat3*(−/−) mice, respectively (mean ± S.E., n = 3). $CL_{app,\ efflux}$ was calculated by multiplying the apparent elimination rate constant (k_{el}) by the distribution volume ($V_{d\ brain}$) to give 207 ± 42 $\mu l \cdot min^{-1} \cdot g^{-1}$ brain for wild-type mice and 83.9 ± 26.4 $\mu l \cdot min^{-1} \cdot g^{-1}$ brain for knockout mice (mean ± S.E., n = 3).

Uptake of [^{3}H]DHEAS and [^{3}H]Estrone-3-sulfate by mOatp1a4-Expressed HEK293 Cells. The uptake of [^{3}H]taurocholate, a typical substrate of Oatp1a4, by mOatp1a4-HEK293 cells was significantly greater than that by vector-transfected HEK293 cells (Fig. 5A). However, for [^{3}H]DHEAS and [^{3}H]estrone-3-sulfate, there was no statistical difference in the uptake between vector-transfected HEK293 cells and mOatp1a4 stable transfectants (Fig. 5, B and C).

Efflux of DHEAS across the BBB in Wild-Type and *Oatp1a4*(−/−) Mice. There was no significant difference in either k_{el} or distribution volume in the brain slices of DHEAS between wild-type and *Oatp1a4*(−/−) mice. The k_{el} was 0.0296 ± 0.0032 and 0.0244 ± 0.0013 min^{-1} in wild-type and *Oatp1a4*(−/−) mice, respectively. Furthermore, impairment of Oatp1a4 did not affect the magnitude of the inhibition by taurocholate and probenecid in the DHEAS efflux compared with that in wild-type mice (Fig. 6A).

The slice/medium ratio determined after a 60-min incubation was 7.41 ± 0.75 and 7.85 ± 0.87 ml/g brain in wild-type and *Oatp1a4*(−/−) mice, respectively (mean ± S.E., n = 3). The distribution volume of inulin was 0.71 ± 0.11 ml/g brain in wild-type mice and 0.82 ± 0.05 ml/g brain in *Oatp1a4*(−/−) mice (mean ± S.E., n = 3). The $CL_{app,\ efflux}$ was calculated as 233 ± 36 and 181 ± 21 $\mu l \cdot min^{-1} \cdot g^{-1}$ brain in wild-type and *Oatp1a4*(−/−) mice, respectively (mean ± S.E., n = 3).

Efflux of Estrone-3-sulfate across the BBB in Wild-Type, *Oatp1a4*(−/−), and *Oat3*(−/−) Mice. The 100 − BEI (%) values of estrone-3-sulfate were determined 30 min after microinjection in wild-type, *Oatp1a4*(−/−), and *Oat3*(−/−) mice. The 100 − BEI (%) values were similar in wild-type and *Oatp1a4*(−/−) mice, 17.9 ± 1.1 versus 15.1 ± 2.3% (mean ± S.E., n = 4). For Oat3, in female mice, efflux of estrone-3-sulfate was significantly delayed in *Oat3*(−/−) mice compared with that in wild-type mice [100 − BEI (%), 40.7 ± 0.9 versus 65.1 ± 2.2% (mean ± S.E., n = 3), $p < 0.05$].

Regional mRNA Expression of Oatp1a4 and Oat3 in Mouse Brain. The mRNA distribution of Oatp1a4 and Oat3 in total tissue of major brain regions was examined by RT PCR. Oatp1a4 and Oat3 were shown to be widely distributed in mouse brain with no large regional differences (Fig. 7, A and B). For Oat3, there was no statistical difference between cerebral cortex and other brain regions.

Discussion

This study investigated the transporters responsible for the brain-to-blood transport of organic anions across the BBB using DHEAS as a probe and the mice with deficiency of well characterized organic anion transporters at the BBB, *Oat3*(−/−) and *Oatp1a4*(−/−) mice, to provide molecular insight into the efflux transport systems of organic anions at the BBB.

After microinjection, the amount of DHEAS remaining in the brain showed a time-dependent decrease. The efflux rate constant of DHEAS in mice was similar to that in rats (0.0268 min^{-1}) (Asaba et al., 2000). Because of the difference in the distribution volume (7.10–7.41 ml/g brain in mice; 4.67 ml/g brain in rats), the apparent efflux clearance, representing the intrinsic efflux activity across the BBB, of DHEAS was greater in mice (207–233 $\mu l \cdot min^{-1} \cdot g^{-1}$ brain) than in rats (118 $\mu l \cdot min^{-1} \cdot g^{-1}$ brain) (Asaba et al., 2000). Saturable mechanisms account for the efflux of DHEAS across the BBB from the brain (Fig. 1 B). Because the 100 − BEI (%) value was near 100% at the concentration of 108 μM as the cerebral concentration in mice, efflux of DHEAS was almost totally saturated at this concentration (Fig. 1B). This result is similar in rats, in which the efflux transport of DHEAS was almost saturated at 100 μM (Asaba et al., 2000). It is notable that the inhibitory effect of PAH showed a clear species difference. PAH significantly delayed DHEAS efflux from the mouse brain (Fig. 2), whereas in rats it showed only a negligible effect (Asaba et al., 2000).

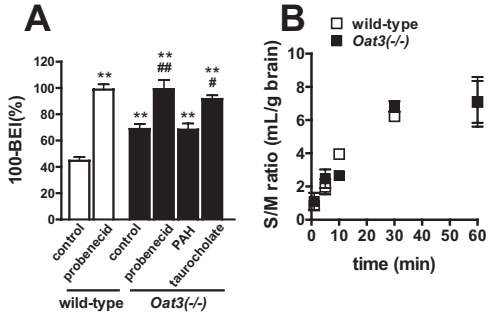

FIG. 4. Comparison of the 100 − BEI (%) of DHEAS in the cerebral cortex and distribution volume of DHEAS in the brain between wild-type and *Oat3*(−/−) mice. A, ECF buffer containing 1 μM unlabeled DHEAS, [^{3}H]DHEAS (140 μCi/ml, 1.5 μM), and [^{14}C]carboxyl-inulin (4 μCi/ml), with or without probenecid (100 mM), PAH (100 mM), and taurocholate (20 mM) was microinjected into the Par2 region of cerebrum of wild-type and *Oat3*(−/−) mice. Mice were decapitated 30 min after microinjection. Each column represents the mean ± S.E. (n = 3–6). **, significantly different from wild-type mice injected without probenecid as examined by one-way analysis of variance followed by Dunnett's multiple comparison test ($p < 0.01$); #, $p < 0.05$; ##, $p < 0.01$, significantly different from *Oat3*(−/−) mice injected without probenecid as examined by one-way analysis of variance followed by Dunnett's multiple comparison test. B, brain slices were incubated with 20 nM unlabeled DHEAS, [^{3}H]DHEAS (0.05 μCi/ml, 75 nM), and [^{14}C]carboxyl-inulin (0.01 μCi/ml) at 37°C. At designated times, the radioactivity in the brain slices and incubation medium was measured. Ligand uptake is given by the amount of ligand associated with the slice specimens divided by the medium concentration. Each point represents the mean ± S.E. (n = 3). Slice/medium (S/M) ratios for inulin were 1.04 ± 0.15 and 1.16 ± 0.22 ml/g brain in wild-type and *Oat3*(−/−) mice, respectively.

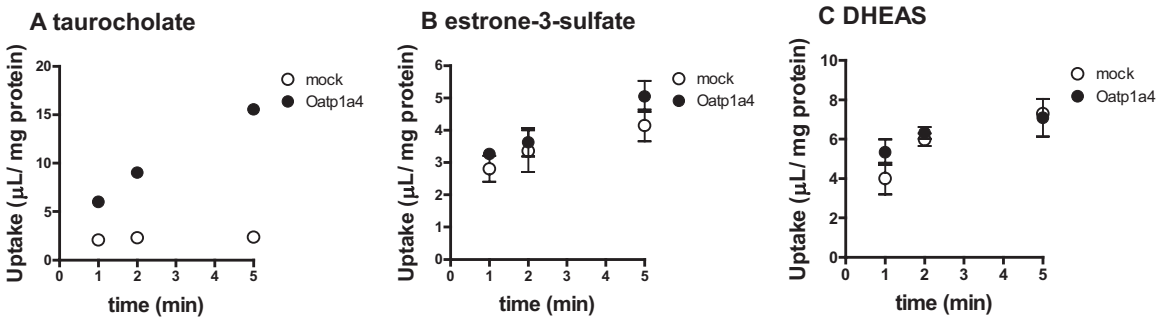

FIG. 5. Time profiles of the uptake of [^{3}H]taurocholate (A), [^{3}H]estrone-3-sulfate (B), and [^{3}H]DHEAS (C) by mock and mOatp1a4-expressed HEK293 cells. Cellular uptake by mock and mOatp1a4-expressed HEK293 cells was determined for [^{3}H]taurocholate (0.1 μCi/ml, 50 nM), [^{3}H]estrone-3-sulfate (0.1 μCi/ml, 2 nM), and [^{3}H]DHEAS (0.1 μCi/ml, 1 nM) at 37°C. Unlabeled taurocholate, estrone-3-sulfate, and DHEAS were added at the concentrations of 1, 0.5, and 0.5 μM, respectively. The uptake was terminated at the designated time by addition of ice-cold buffer. ○, vector-transfected HEK293 cells; ●, mOatp1a4-expressed HEK293 cells. Ligand uptake is given by the amount of ligand associated with the cell specimens divided by the medium concentration. Each point indicates the mean ± S.E. (n = 3).

Using mice with deficiency of Oat3 and Oatp1a4, we directly evaluated the contribution of these transporters in DHEAS efflux. Uptake studies using mouse Oat3-expressing oocytes confirmed that DHEAS is a mOat3 substrate (Fig. 3). DHEAS efflux from the brain was significantly delayed in *Oat3*(−/−) mice (Fig. 4A) with $CL_{app,efflux}$ of DHEAS being 40% of that in wild-type mice, indicating that Oat3 is functionally important in vivo. The degree of reduction in 100 − BEI (%) value of DHEAS by PAH was similar to that in *Oat3*(−/−) mice, suggesting that the effect of PAH is due to inhibition of Oat3. Indeed, the inhibitory effect of PAH was not observed in *Oat3*(−/−) mice (Fig. 4A).

Taurocholate significantly inhibited the efflux of DHEAS both in wild-type and *Oat3* (−/−) mice, and its effect on BEI (%) of DHEAS was similar in both strains. Namely, taurocholate did not inhibit Oat3, but rather another organic anion transporter, which was kept intact in *Oat3* (−/−) mice (Figs. 2A and 4A). Absence of the effect of taurocholate on Oat3 is reasonably explained because its K_m value for rOat3 (790 μM) (Sugiyama et al., 2001) is greater than the effective concentration in the brain after microinjection (430 μM), resulting in insufficient inhibition of Oat3. Furthermore, complete inhibition of DHEAS efflux by probenecid, estrone-3-sulfate, and benzylpenicillin in wild-type mice (Fig. 2, C–E) suggests that the remaining transporter is also sensitive to probenecid, estrone-3-sulfate, and benzylpenicillin.

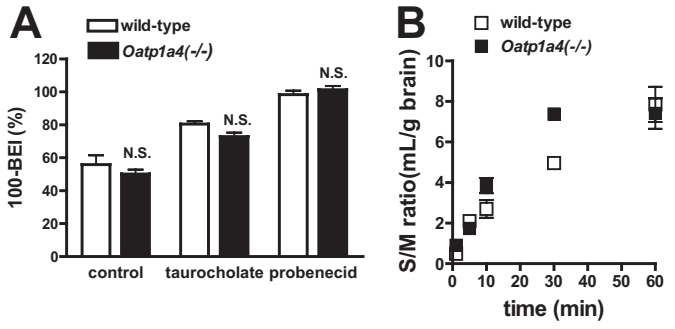

FIG. 6. Comparison of the 100 − BEI (%) of DHEAS in the cerebral cortex and distribution volume of DHEAS in the brain between wild-type and *Oatp1a4*(−/−) mice. A, ECF buffer containing 1 μM unlabeled DHEAS, [^{3}H]DHEAS (140 μCi/ml, 1.5 μM), and [^{14}C]carboxyl-inulin (4 μCi/ml) was microinjected into the Par2 region of the cerebrum of wild-type and *Oatp1a4*(−/−) mice. Mice were decapitated 30 min after microinjection. Each column represents the mean ± S.E. (n = 6–7). B, brain slices were incubated with 20 nM unlabeled DHEAS, [^{3}H]DHEAS (0.05 μCi/ml, 75 nM), and [^{14}C]carboxyl-inulin (0.01 μCi/ml) at 37°C. At designated times, the radioactivity in the brain slices and incubation medium was measured. Ligand uptake is given by the amount of ligand associated with the slice specimens divided by the medium concentration. Each point represents the mean ± S.E. (n = 3). Slice/medium (S/M) ratios for inulin were 0.71 ± 0.11 ml/g brain in wild-type mice and 0.82 ± 0.05 ml/g brain in *Oatp1a4*(−/−) mice. N.S., not significant.

The present study could not detect specific uptake of DHEAS by mOatp1a4 (Fig. 5), although van Montfoort et al. (2002) detected such uptake by mOatp1a4 using mOatp1a4-expressing oocytes. Thus, DHEAS may be a poor substrate of mOatp1a4. Consistent with the in vitro result, there was no significant difference in the efflux clearance of DHEAS between wild-type and *Oatp1a4*(−/−) mice. No compensation by other well defined transporters, such as Oat3, Oatp1c1, P-glycoprotein, and breast cancer resistance protein, was observed in the cerebral cortex of *Oatp1a4*(−/−) mice at the mRNA level (Ose et al., 2010). Consequently, it can be concluded that Oatp1a4 makes a negligible contribution to the efflux of DHEAS from the brain in mice, which is contrary to reports in rats. In addition to DHEAS, on the basis of the discrepancy observed between the in vivo and in vitro K_m values of estrone-3-sulfate, we speculated that the efflux of estrone-3-sulfate is mediated by another transporter distinct from Oatp1a4 (Kusuhara and Sugiyama, 2005). In fact, efflux of estrone-3-sulfate did not show any delay in *Oatp1a4*(−/−) mice, but it was greatly altered in *Oat3*(−/−) mice. This finding is consistent with the in vitro uptake studies, indicating that estrone-3-sulfate is a poor substrate of mOatp1a4. These results suggest that in mice Oat3 plays a major role in the efflux of steroid conjugates from the brain.

The species difference observed in this study can be attributable to the marked difference in transport activity of Oatp1a4. According to previous reports using mOatp1a4- or rOatp1a4-expressing oocytes, transport activity of rat Oatp1a4 for DHEAS and estrone-3-sulfate is much higher than that of mouse Oatp1a4 (Reichel et al., 1999; van Montfoort et al., 2002). Localization of Oatp1a4 at the blood-brain

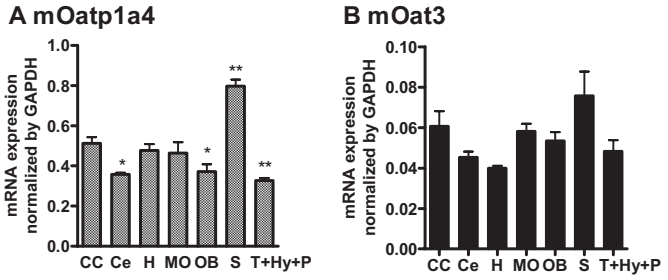

FIG. 7. Regional distribution of Oatp1a4 (A) and Oat3 (B) mRNA in mouse brain. cDNA was prepared by reverse transcription using total RNA of microdissected brain regions. mRNA levels of Oatp1a4 and Oat3 were determined by real-time PCR and normalized by the value of Gapdh (three determinations using one batch of cDNA, mean ± S.E.). CC, cerebral cortex; Ce, cerebellum; H, hippocampus; MO, medulla oblongata; OB, olfactory bulb; S, striatum; T, thalamus; Hy, hypothalamus; P, pons. *, $p < 0.05$; **, $p < 0.01$; significantly different from cerebral cortex as examined by one-way analysis of variance followed by Dunnett's multiple comparison test.

barrier may also differ between mice and rats. In rats, Oatp1a4 was reported to be predominantly expressed at the abluminal membrane of brain capillary endothelial cells(Roberts et al., 2008). In mice, so far there have been no decisive results concerning the comparison of expression levels between the luminal and abluminal membranes. However, our immunohistochemical studies indicated the possibility that Oatp1a4 shows higher expression at the luminal membrane (Ose et al., 2010). Although a difference in expression levels of Oatp1a4 and Oat3 at the blood-brain barrier still needs to be considered, it seems that low transport activity of mOatp1a4 to steroid conjugates results in the emerging role of Oat3.

Involvement of multiple transporters in the brain-to-blood efflux of DHEAS, a neurosteroid, makes it possible to carefully regulate the brain DHEAS concentration. DHEAS modulates synaptic transmission and so has a variety of effects in the central nervous system (Pérez-Neri et al., 2008). Up to now, the physiological effect of DHEAS, notably concerning memory and learning ability, has mainly been investigated in the hippocampus (Dubrovsky, 2005; Steffensen et al., 2006). Because there is no regional difference in mRNA expression of Oat3 (Fig. 7B), we speculate that Oat3 also plays a significant role in DHEAS efflux in the hippocampus as well as cerebral cortex. We examined the expression of transporters in each brain region by using total RNA prepared from dissected brain regions but not from purified brain capillaries. One concern is that our data do not show the exact expression of Oatp1a4 and Oat3 in brain capillaries for each brain region. However, immunohistochemical studies in mouse brain indicate that Oatp1a4 and Oat3 are highly and locally expressed in brain capillary endothelium (Ohtsuki et al., 2004; Ose et al., 2010). Therefore, we considered that expression levels of Oatp1a4 and Oat3 in total brain will mainly reflect expression levels in brain capillaries. Furthermore, the distribution volume of DHEAS determined by the brain slice uptake study was significantly higher than that of inulin, suggesting that DHEAS is also taken up into the brain parenchyma, presumably by transporters.

The present study suggests the involvement of another organic anion transporter, distinct from Oatp1a4, in the efflux of DHEAS across the BBB, in addition to Oat3. We speculate that Oatp2b1 is the candidate transporter because Oatp2b1 is localized on the abluminal membrane of brain capillary endothelial cells in rats (Roberts et al., 2008) and DHEAS is a substrate of OATP2B1 with a low affinity (Kullak-Ublick et al., 2001; Nozawa et al., 2004; Ugele et al., 2008). It should be noted that taurocholate, benzylpenicillin, and probenecid were all reported to inhibit uptake of estrone-3-sulfate by OATP2B1, but benzylpenicillin does not seem to be a potent inhibitor compared with taurocholate and probenecid (Sai et al., 2006). This result contradicts our study in which both benzylpenicillin and probenecid totally inhibited brain efflux of DHEAS. Further studies are necessary to elucidate the unknown transporter mediating the efflux of DHEAS from the BBB.

In conclusion, the brain-to-blood transport of DHEAS involves multiple transporters at the BBB. In addition to expression of well characterized transporters, Oatp1a4 and Oat3, expression of an unknown transporter that mediates the efflux transport of DHEAS across the BBB together with Oat3, is suggested. This study indicates that transport mechanisms of organic anions at the BBB are far more diverse than they were assumed to be.

Authorship Contributions

Participated in research design: Miyajima, Kusuhara, and Sugiyama.

Conducted experiments: Miyajima.

Contributed new reagents or analytic tools: Fujishima and Adachi.

Performed data analysis: Miyajima.

Wrote or contributed to the writing of the manuscript: Miyajima, Kusuhara, and Sugiyama.

Other: Kusuhara and Sugiyama acquired funding for the research.

References

Asaba H, Hosoya K, Takanaga H, Ohtsuki S, Tamura E, Takizawa T, and Terasaki T (2000) Blood-brain barrier is involved in the efflux transport of a neuroactive steroid, dehydroepiandrosterone sulfate, via organic anion transporting polypeptide 2. *J Neurochem* **75:**1907–1916.

Dubrovsky BO (2005) Steroids, neuroactive steroids and neurosteroids in psychopathology. *Prog Neuropsychopharmacol Biol Psychiatry* **29:**169–192.

Gao B, Stieger B, Noé B, Fritschy JM, and Meier PJ (1999) Localization of the organic anion transporting polypeptide 2 (Oatp2) in capillary endothelium and choroid plexus epithelium of rat brain. *J Histochem Cytochem* **47:**1255–1264.

Kakee A, Terasaki T, and Sugiyama Y (1997) Selective brain to blood efflux transport of para-aminohippuric acid across the blood-brain barrier: in vivo evidence by use of the brain efflux index method. *J Pharmacol Exp Ther* **283:**1018–1025.

Kikuchi R, Kusuhara H, Sugiyama D, and Sugiyama Y (2003) Contribution of organic anion transporter 3 (Slc22a8) to the elimination of p-aminohippuric acid and benzylpenicillin across the blood-brain barrier. *J Pharmacol Exp Ther* **306:**51–58.

Kitazawa T, Terasaki T, Suzuki H, Kakee A, and Sugiyama Y (1998) Efflux of taurocholic acid across the blood-brain barrier: interaction with cyclic peptides. *J Pharmacol Exp Ther* **286:**890–895.

Kobayashi Y, Ohshiro N, Tsuchiya A, Kohyama N, Ohbayashi M, and Yamamoto T (2004) Renal transport of organic compounds mediated by mouse organic anion transporter 3 (mOat3): further substrate specificity of mOat3. *Drug Metab Dispos* **32:**479–483.

Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120:**525–533.

Kusuhara H and Sugiyama Y (2005) Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* **2:**73–85.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* **308:**438–445.

Ohtsuki S, Kikkawa T, Mori S, Hori S, Takanaga H, Otagiri M, and Terasaki T (2004) Mouse reduced in osteosclerosis transporter functions as an organic anion transporter 3 and is localized at abluminal membrane of blood-brain barrier. *J Pharmacol Exp Ther* **309:**1273–1281.

Ose A, Ito M, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Hosokawa M, Schuetz JD, and Sugiyama Y (2009) Limited brain distribution of [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802), a pharmacologically active form of oseltamivir, by active efflux across the blood-brain barrier mediated by organic anion transporter 3 (Oat3/Slc22a8) and multidrug resistance-associated protein 4 (Mrp4/Abcc4). *Drug Metab Dispos* **37:**315–321.

Ose A, Kusuhara H, Endo C, Tohyama K, Miyajima M, Kitamura S, and Sugiyama Y (2010) Functional characterization of mouse organic anion transporting peptide 1a4 in the uptake and efflux of drugs across the blood-brain barrier. *Drug Metab Dispos* **38:**168–176.

Pérez-Neri I, Montes S, Ojeda-López C, Ramírez-Bermúdez J, and Ríos C (2008) Modulation of neurotransmitter systems by dehydroepiandrosterone and dehydroepiandrosterone sulfate: mechanism of action and relevance to psychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry* **32:**1118–1130.

Reichel C, Gao B, Van Montfoort J, Cattori V, Rahner C, Hagenbuch B, Stieger B, Kamisako T, and Meier PJ (1999) Localization and function of the organic anion-transporting polypeptide Oatp2 in rat liver. *Gastroenterology* **117:**688–695.

Roberts LM, Black DS, Raman C, Woodford K, Zhou M, Haggerty JE, Yan AT, Cwirla SE, and Grindstaff KK (2008) Subcellular localization of transporters along the rat blood-brain barrier and blood-cerebral-spinal fluid barrier by in vivo biotinylation. *Neuroscience* **155:**423–438.

Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, Artursson P, and Tsuji A (2006) Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab Dispos* **34:**1423–1431.

Steffensen SC, Jones MD, Hales K, and Allison DW (2006) Dehydroepiandrosterone sulfate and estrone sulfate reduce GABA-recurrent inhibition in the hippocampus via muscarinic acetylcholine receptors. *Hippocampus* **16:**1080–1090.

Sugiyama D, Kusuhara H, Shitara Y, Abe T, Meier PJ, Sekine T, Endou H, Suzuki H, and Sugiyama Y (2001) Characterization of the efflux transport of 17β-estradiol-D-17β-glucuronide from the brain across the blood-brain barrier. *J Pharmacol Exp Ther* **298:**316–322.

Sweet DH, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, and Nigam SK (2002) Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice. *J Biol Chem* **277:**26934–26943.

Ugele B, Bahn A, and Rex-Haffner M (2008) Functional differences in steroid sulfate uptake of organic anion transporter 4 (OAT4) and organic anion transporting polypeptide 2B1 (OATP2B1) in human placenta. *J Steroid Biochem Mol Biol* **111:**1–6.

van Montfoort JE, Schmid TE, Adler ID, Meier PJ, and Hagenbuch B (2002) Functional characterization of the mouse organic-anion-transporting polypeptide 2. *Biochim Biophys Acta* **1564:**183–188.

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