Title: 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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Running title: Oat3-mediated efflux of DHEAS across the BBB

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The number of pages: 37
The number of tables: 0
The number of figures: 7
The number of references: 26
The number of words in Abstract: 248
The number of words in Introduction: 638
The number of words in Discussion: 1284

Abbreviations: BBB: blood-brain barrier, DHEAS: dehydroepiandrosterone sulfate, K_m: Michaelis-Menten constant, Oat: Organic anion transporter, Oatp: Organic anion transporting polypeptide, PAH: p-amino hippuric acid
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 following microinjection into the cerebral cortex was characterized in wild-type mice and knockout mice of well-characterized organic anion transporters at the BBB, Oatp1a4/Slc10a4 and Oat3/Slc22a8. The saturable efflux of DHEAS from the brain was completely inhibited by probenecid, benzylpenicillin, and estrone-3-sulfate, and moderately by taurocholate and p-aminohippurate (50~57%). Uptake of DHEAS and estrone-3-sulfate was greater in murine Oat3 cRNA-injected oocytes than that by water-injected oocytes. Efflux of these compounds from the brain was significantly delayed in Oat3(-/-) mice compared with 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 suggests 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 it was 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 form 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 that in the blood to attenuate their effect in the central nervous system (CNS), 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 importance of the transporters in the efflux of various organic anions from the brain across the BBB. Characterization of this process using various organic anions as inhibitor have 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 BQ-123, is mediated by multiple transporters (Kitazawa et al., 1998).

Based on the substrate specificities and membrane localization, it has been considered that organic anion transporting polypeptide 1a4 (Oatp1a4/Slco1a4) and organic anion transporter 3 (Oat3/Slc22a8) is 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., 2009b), 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 et al., 2004), but in rats, 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. Notably, 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., 2009b).

Recently, 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, and cause any adaptive regulation of the known xenobiotic transporters in the
brain, at least at the mRNA level (Ose et al., 2009a; Ose et al., 2009b). However, the efflux of taurocholate, rosuvastatin, pitavastatin, and Ro64-0802, from the brain following microinjection was significantly delayed in Oatp1a4(-/-) and Oat3(-/-) mice, respectively, compared with the corresponding wild-type mice (Ose et al., 2009a; Ose et al., 2009b). This is consistent with the proposed roles of Oatp1a4 and Oat3 at the BBB.

This study 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). Previously, Asaba et al. (2000) elucidated 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 Oatp1a4 to be responsible for this process. In this study, we reported that Oat3 and unknown organic anion transporters account for the efflux of DHEAS across the BBB, whereas Oatp1a4 unexpectedly makes only a negligible contribution.
Methods

Chemicals

[^3]H]DHEAS,[^3]H]estrone-3-sulfate,[^3]H]taurocholate was purchased from PerkinElmer Life Science Products (Boston, 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 (Tokyo, Japan). 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 carried out according to the guidelines provided by the Institutional Animal Care
Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo).

**In vitro transport study using mock- and Oatp1a4-expressing HEK293 cells**

mOatp1a4-expressing HEK293 cells were constructed, as previously reported (Ose et al., 2009b). Uptake was initiated by adding 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 NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl₂, pH 7.4) at 37 ºC for 15-30 min. The uptake was terminated at designated times by adding ice-cold Krebs-Henseleit buffer, and cells were washed 3 times. Cells were then dissolved in 500 μL 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, with bovine serum albumin as a standard (Lowry et al., 1951). 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 *Xenopus 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, 2mM KCl, 1mM MgCl\(_2\), 10mM HEPES, 1mM CaCl\(_2\), 250mM Tris, pH 7.4). Then, oocytes were incubated for 1 hour at room temperature in Na\(^+\) buffer containing \[^{3}\text{H}]\)DHEAS (0.5\(\mu\text{Ci/mL}, 5\text{nM}) and unlabeled DHEAS (0.1 \(\mu\text{M})\), or \[^{3}\text{H}\)estrone-3-sulfate (0.5\(\mu\text{Ci/mL}, 10\text{nM}) and unlabeled estrone-3-sulfate (0.1 \(\mu\text{M})\), in the absence or presence of probenecid (1mM). 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. 2009b). \[^{3}\text{H}]\)DHEAS (70nCi/mouse) with a non-permeable reference compound \[^{14}\text{C}]\)carboxyl-inulin (2nCi/mouse), in 0.5\(\mu\text{L} \)ECF buffer (122 mM NaCl, 25 mM NaHCO\(_3\), 10 mM D-glucose, 3mM KCl, 1.4 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 0.4 mM K\(_2\)HPO\(_4\), 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 \(^{14}\text{C}\)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 Prizm) 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). Briefly, a slice, 300μm thick, 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 1mL oxygenated incubation medium containing unlabeled DHEAS (20nM), \(^{3}\text{H}\)DHEAS (0.05μCi/mL, 75 nM) and \(^{14}\text{C}\)carboxyl-inulin (0.01μCi/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 ($k_e$) by the distribution volume ($V_{d,brain}$), as shown below:

$$CL_{app,efflux} = k_e \times V_{d,brain}$$

$k_e$ 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 polymerase chain reaction (RT-PCR) method. Mouse brain regional cDNA was purchased from Genostaff (Tokyo, Japan). These cDNA 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’; mGapdh, forward 5’- AAC GAC CCC TTC ATT GAC -3’ 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 (Molecular Probes, Eugene, OR).

Using Light Cycler software, mRNA expression levels for each gene was 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 Figure 1. DHEAS was eliminated from the brain and the apparent elimination rate constant obtained was 0.0325±0.0028min⁻¹. The concentration-dependence of the efflux of DHEAS was examined. 100-BEI (%) values determined 30 minutes 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 (Figure 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 (Figure 2A and 2B), whereas probenecid, estrone-3-sulfate and benzylpenicillin almost totally inhibited the efflux (Figures 2C, 2D and 2E).
Uptake of [$^3$H]DHEAS and [$^3$H]estrone-3-sulfate by mOat3-expressing Xenopus laevis oocytes

Because we have not been able to construct mOat3 expressing HEK293 cells for some unknown reason, Xenopus laevis oocytes were used as host cells to express mOat3. The uptake of both compounds was significantly greater in mOat3-cRNA oocytes compared with water-injected oocytes. 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

100-BEI(%) values were determined 30 minutes after microinjection in wild-type and Oat3(-/-) mice. The efflux of DHEAS was significantly delayed in Oat3(-/-) mice, but DHEAS efflux was still observed (Figure 4A). The $k_{el}$ was $0.0292\pm0.0028$ and $0.0118\pm0.0028\ min^{-1}$ in wild-type mice and Oat3(-/-) mice, respectively (mean$\pm$S.E., n=4). Probenecid and taurocholate almost completely inhibited the efflux of DHEAS from the brain of Oat3(-/-) mice, whereas PAH had not effect (Figure 4A).
To calculate the apparent efflux clearance \( (CL_{app,efflux}) \), the distribution volume was determined \textit{in vitro} using brain slices (Figure 4B). The slice-to-medium ratio determined following a 60-minute incubation was 7.10±1.25 and 7.10±1.49 mL/g brain in wild-type and \textit{Oat3(-/-)} mice, respectively (mean±S.E., n=3) (Figure 4 B). For inulin, the impermeable brain vascular marker, the distribution volume was 1.04±0.15 and 1.16±0.22 mL/g brain in wild-type and \textit{Oat3(-/-)} mice, respectively (mean±S.E., n=3).

\( CL_{app,efflux} \) was calculated by multiplying the apparent elimination rate constant \( (k_e) \) by the distribution volume \( (V_{d,brain}) \) to give 207±42 μL/min/g brain for wild-type and 83.9±26.4 μL/min/g brain for knockout mice (mean±S.E., n=3).

**Uptake of \[^3H\]DHEAS and \[^3H\]estrone-3-sulfate by mOatp1a4-expressed HEK293 cells**

The uptake of \[^3H\]taurocholate, a typical substrate of Oatp1a4, by mOatp1a4-HEK293 cells was significantly greater than that by vector-transfected HEK 293 cells (Figure 5 A). However, for \[^3H\]DHEAS and \[^3H\]estrone-3-sulfate, there was no statistical difference in the uptake between vector-transfected HEK293 cells and mOatp1a4 stable transfectants (Figures 5B and 5C).

**Efflux of DHEAS across the BBB in wild-type and \textit{Oatp1a4(-/-)} mice**
There was no significant difference both in \( k_{el} \) and 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 wild-type mice (Figure 6A).

The slice-to-medium ratio determined following a 60-minute 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/min/g 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 minutes 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 to 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 (Figures 7A and 7B). 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 knockout mice of well-characterized organic anion transporters at the BBB, Oat3(-/-) and Oatp1a4(-/-) mice, to provide a molecular insight into the efflux transport systems of organic anions at the BBB.

Following 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 μL/min/g brain) than in rats (118 μL/min/g brain) (Asaba et al. 2000). Saturable mechanisms account for the efflux of DHEAS across the BBB from the brain (Figure 1 B). Since 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 (Figure 1B). This is similar in rats, in which the efflux transport of DHEAS was almost saturated at 100μM (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 (Figure 2), whereas, in rats, it showed only a negligible effect (Asaba et al., 2000).

Using knockout mice 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 (Figure 3). DHEAS efflux from the brain was significantly delayed in Oat3(-/-) mice (Figure 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 would be due to inhibition of Oat3. Indeed, inhibitory effect of PAH was not observed in Oat3(-/-) mice (Figure 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 another organic anion transporter, which was kept intact in Oat3 (-/-) mice (Figure 2A and 4A). Absence of the effect of taurocholate on Oat3 is reasonably explained because its Km value for rOat3 (790µM, Sugiyama et al., 2001) is greater than the effective concentration in the brain after microinjection (430µM), resulting in an insufficient inhibition of Oat3.
Furthermore, complete inhibition of DHEAS efflux by probenecid, estrone-3-sulfate and benzylpenicillin in wild-type mice (Figure 2 C–E) suggests that the remaining transporter is also sensitive to probenecid, estrone-3-sulfate and benzylpenicillin.

The present study could not detect a specific uptake of DHEAS by mOatp1a4 (Figure 5), although van Montfoort detected such uptake by mOatp1a4 using mOatp1a4-expressing oocytes (van Montfoort et al., 2002). 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 (P-gp) and breast cancer resistance protein (Bcrp), was observed in the cerebral cortex of Oatp1a4(-/-) mice at the mRNA level (Ose et al., 2009b). 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, based on 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). Actually, efflux of estrone-3-sulfate did not show any delay in Oatp1a4(-/-) mice, but it
was greatly altered in Oat3(-/-) mice. This 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.

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 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., 2009b). Although 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 brain DHEAS concentration. DHEAS modulates synaptic transmission and so has a variety of effects in the central nervous system (Perez-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). Since there is no regional difference in mRNA expression of Oat3 (Figure 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 does 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., 2009b). Therefore, we considered that expression levels of Oatp1a4 and Oat3 in total brain will mainly reflect expression levels in brain capillaries. Furthermore, 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 Oatp2b1 to be the candidate transporter. This is 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 are all reported to inhibit uptake of estrone-3-sulfate by OATP2B1, but benzylpenicillin does not seem to be a potent inhibitor compared to taurocholate and probenecid (Sai et al., 2006). This contradicts with our study where 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 well characterized transporters, Oatp1a4 and Oat3, the expression of an unknown transporter is suggested which mediates the efflux transport of DHEAS across the BBB together with Oat3. This study indicates that transport mechanisms of organic anions at the BBB are far more diverse than it was 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.
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Sugiyama D, Kusuhara H, Shitara Y, Abe T, Meier PJ, Sekine T, Endou H, Suzuki H and


Footnotes

This study was supported partly by a Grant-in-Aid for Scientific Research (A) [20249008] to Y. S., and partly by a Grant-in-Aid for Scientific Research (B) [20390046] to H. Ku. from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
Titles and legends to figures

Figure 1. Time-profile of the 100-BEI(%) of $[^{3}\text{H}]$DHEAS in the cerebrum after intracerebral microinjection and its concentration-dependence.

A. A solution of $[^{3}\text{H}]$DHEAS (70 nCi/mouse, 1.5 µM) and $[^{14}\text{C}]$carboxyl-inulin (2 nCi/mouse) in 0.5µL 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 $[^{3}\text{H}]$DHEAS (70nCi/mouse, 1.5 µM) and $[^{14}\text{C}]$carboxyl-inulin (2nCi/mouse) in 0.5µL ECF buffer with unlabeled DHEAS (1µM, 500µM, 5mM) 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).

Figure 2. Inhibition of the efflux of $[^{3}\text{H}]$DHEAS from the brain by various organic anions. ECF buffer containing $[^{3}\text{H}]$DHEAS (140 µCi/ml, 1.5 µM) and
[\textsuperscript{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).

Figure 3. Uptake of estrone-3-sulfate and DHEAS by water- and mOat3-cRNA-injected oocytes.

mOat3 cRNA-injected oocytes were incubated with [\textsuperscript{3}H]estrone-3-sulfate (0.5 µCi/ml, 10 nM) or [\textsuperscript{3}H]DHEAS (0.5 µCi/ml, 5 nM) with 0.1 µM unlabeled compounds at room temperature. After an 1-hour 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 ($p<0.01$, respectively).

##, significantly different from uptake by water-injected oocytes with probenecid as examined by Student’s t test ($p<0.01$, respectively).

++, significantly different from uptake by mOat3 cRNA-injected oocytes without probenecid as examined by Student’s t test ($p<0.01$, respectively).

Figure 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 1µM unlabeled DHEAS, [3H]DHEAS (140 µCi/ml, 1.5 µM) and [14C]carboxyl-inulin (4 µCi/ml), with or without probenecid (100 mM), PAH (100 mM) and taurocholate (20mM) was microinjected into the Par2 region of cerebrum of wild-type and Oat3(-/-) mice. Mice were decapitated 30 minutes 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 ANOVA followed by Dunnett’s multiple comparison test ($p<0.01$).

#,##, significantly different from Oat3(-/-) mice injected without probenecid as examined by one-way ANOVA followed by Dunnett’s multiple comparison test ($p<0.05$, $p<0.01$, respectively).
B. Brain slices were incubated with unlabeled DHEAS 20nM, [3H]DHEAS (0.05 µCi/ml, 75 nM) and [14C]carboxyl-inulin (0.01 µCi/ml) at 37 ºC. 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). 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.

Figure 5. Time-profiles of the uptake of [3H]taurocholate, [3H]estrone-3-sulfate and [3H]DHEAS by mock- and mOatp1a4-expressed HEK293 cells.

Cellular uptake by mock- and mOatp1a4-expressed HEK293 cells was determined for [3H]taurocholate (0.1 µCi/mL, 50nM), [3H]estrone-3-sulfate (0.1 µCi/mL, 2 nM) and [3H]DHEAS (0.1 µCi/mL, 1 nM) at 37 ºC. Unlabeled taurocholate, estrone-3-sulfate, and DHEAS were added at the concentration of 1 µM, 0.5 µM and 0.5 µM, respectively. The uptake was terminated at the designated time by addition of ice-cold buffer. Open and closed circles represent vector transfected HEK293 cells and mOatp1a4-expressed HEK 293 cells, respectively. Ligand uptake is given by the amount of ligand associated
with the cell specimens divided by the medium concentration. Each point indicates mean ± S.E. (n=3).

Figure 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 1µM unlabeled DHEAS, [^3H]DHEAS (140 µCi/ml, 1.5 µM) and [^14C]carboxyl-inulin (4 µCi/ml) was microinjected into the Par2 region of the cerebrum of wild-type and *Oatp1a4* (-/-) mice. Mice were decapitated 30 minutes after microinjection. Each column represents the mean ± S.E. (n=6-7).

B. Brain slices were incubated with 20nM unlabeled DHEAS, [^3H]DHEAS (0.05 µCi/ml, 75 nM) and [^14C]carboxyl-inulin (0.01 µCi/ml) at 37 ºC. 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). 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.
Figure 7. Regional distribution of Oatp1a4 and Oat3 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 ± SE).


*,**, significantly different from cerebral cortex as examined by one-way ANOVA followed by Dunnett’s multiple comparison test (p<0.05, p<0.01).
Figure 1

A

100-BEI(%) vs. time after microinjection (min)

B

100-BEI(%) vs. DHEAS concentrations

- 54.1 nM
- 21.6 μM
- 108 μM

** indicates statistical significance.
Figure 2

A. Control vs. taurocholate (0.43mM)

B. Control vs. PAH (2.2mM)

C. Control vs. estrone-3-sulfate (0.22mM)

D. Control vs. probenecid (2.2mM)

E. Control vs. benzylpenicillin (2.2mM)
Figure 3

Uptake (μL/oocyte)

- control
- +probenecid

estrone-3-sulfate
DHEAS

control mOat3
control mOat3

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Figure 4

A

![Bar chart showing 100-BEI(%) for different conditions: control, probenecid, PAH, taurocholate for wild-type and Oat3(-/-) mice.](chart_a.png)

B

![Graph showing S/M ratio (mL/g brain) vs time (min) for wild-type and Oat3(-/-) mice.](chart_b.png)
Figure 5

A taurocholate

B estrone-3-sulfate

C DHEAS

Uptake (μL/mg protein) vs. time (min) for mock and Oatp1a4 conditions.
Figure 6

A

- **wild-type**
- **Oatp1a4(-/-)**

100-BEI (%)

- control
- taurocholate
- probenecid

N.S.

B

- **wild-type**
- **Oatp1a4(-/-)**

S/M ratio (mL/g brain)

time (min)
Figure 7

A mOatp1a4

B mOat3

mRNA expression normalized by GAPDH

CC Ce H MO OB S T+Hy+P

mRNA expression normalized by GAPDH

CC Ce H MO OB S T+Hy+P