In Vitro Study of the Functional Expression of Organic Anion Transporting Polypeptide 3 at Rat Choroid Plexus Epithelial Cells and Its Involvement in the Cerebrospinal Fluid-to-Blood Transport of Estrone-3-Sulfate

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ABSTRACT

The cerebrospinal fluid-to-blood efflux transport of estrone-3-sulfate (E1S) via the blood-cerebrospinal fluid barrier (BCSFB) may play an important role in regulating E1S levels in the brain. Here, we investigated the efflux transport of E1S at the BCSFB using conditionally immortalized rat choroid plexus epithelial cells (TR-CSFB) and identified the responsible transporter. The \[^{3}H\]E1S uptake by TR-CSFB cells was composed of saturable and nonsaturable components, and the \(K_{m}\) and \(V_{max}\) values of the saturable component were determined to be 16.8 ± 5.1 \(\mu\)M and 12.3 ± 2.3 pmol/min/mg of protein, respectively. \[^{3}H\]E1S uptake was inhibited by probenecid, cholate, taurocholate, sulfobromophthalein, dehydroepiandrosterone sulfate, triiodothyronine, thyroxin, and digoxin but not by \(p\)-aminohippuric acid, \(\gamma\)-aminobutyric acid, or methotrexate, suggesting the involvement of organic anion transporting polypeptide (oatp) in the uptake. Reverse transcription-polymerase chain reaction analysis revealed that oatp3 was expressed in TR-CSFB cells and isolated rat choroid plexus, although oatp1 was not detected in either. Xenopus laevis oocytes expressing oatp3 exhibited \[^{3}H\]E1S uptake activity with a \(K_{m}\) of 8.09 ± 2.83 \(\mu\)M and \(V_{max}\) of 8.02 ± 0.87 pmol/h/oocyte. Moreover, oatp3 is localized at the brush-border membrane of choroid plexus epithelial cells. These results suggest that oatp3 is involved in the E1S efflux transport at the BCSFB.

Estrone-3-sulfate (E1S) is produced as a metabolite of estrone by sulfation in the brain (Platia et al., 1984; Connolly and Resko, 1989). E1S has also been used to treat senile dementia of the Alzheimer’s type as a form of estrogen replacement therapy (Honjo et al., 1989). Our recent report shows that E1S is excreted from cerebrospinal fluid (CSF) via the blood-cerebrospinal fluid barrier (BCSFB) (Kitazawa et al., 2000). Thus, the elimination of E1S from the CSF via the BCSFB could play an important role in regulating estrone levels in the central nervous system (CNS). Furthermore, E1S undergoes very limited distribution to the brain compared with estrone (Steingold et al., 1986). The limited distribution of E1S may result from the operation of an efflux system transporting E1S to the circulating blood. However, the responsible molecule for E1S efflux transport at the BCSFB has not been identified yet.

The BCSFB consists of a tight monolayer of choroid plexus epithelial cells. An organic anion transport system at the BCSFB plays a key role in the clearance of endogenous and exogenous anions from the CSF to blood (Forn, 1972; Wong et al., 1993; Suzuki et al., 1997; Gao and Meier, 2001). Two organic anion transporter families have been identified. One is the organic anion transporting polypeptide (oatp) family, and the other is the organic anion transporter (OAT) family. At the BCSFB, histochemical studies have shown that oatp1 and OAT3 are localized at the brush-border membrane of choroid plexus epithelial cells (Angeletti et al., 1997; Nagata

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ABBREVIATIONS: E1S, estrone-3-sulfate; BCSFB, blood-cerebrospinal fluid barrier; TR-CSFB, conditionally immortalized rat choroid plexus epithelial cells; RT-PCR, reverse transcription-polymerase chain reaction; oatp, organic anion transporting polypeptide; CSF, cerebrospinal fluid; BSP, sulfobromophthalein; CNS, central nervous system; OAT, organic anion transporter; \[^{3}H\]E1S, \[^{3}H\]estrone-3-sulfate ammonium salt; \[^{14}C\]inulin, [carboxyl-\(^{14}\)C]inulin; DHEAS, dehydroepiandrosterone sulfate; \(T_{3}\), triiodothyronine; \(T_{4}\), thyroxin; PAH, \(p\)-aminobhippuric acid; ECF, extracellular fluid; MRP, multidrug resistance-associated protein; PBS, phosphate-buffered saline; PFA, paraformaldehyde.
et al., 2002), and oatp2 is localized at the basolateral membrane (Gao and Meier, 2001). These organic anion transporters transport $E_S$, and therefore it is necessary to investigate the contribution of each transporter to the $E_S$ efflux transport at the BCSFB to clarify the efflux mechanism.

Furthermore, another oatp subtype, oatp3, has been identified (Abe et al., 1998) and is expressed abundantly in the brain and at a lower level in the liver, unlike oatp1 and -2 (Abe et al., 1998; Walters et al., 2000). A human ortholog of rat oatp3, OATP-A, is also expressed in the brain and at a lower level in the liver (Walters et al., 2000). These reports suggest that this transporter may play a role in CNS function in both rats and humans, although its physiological function in the brain remains unclear. Oatp3 also transports $E_S$, so that it is of great interest to determine whether oatp3 is expressed at the BCSFB and is involved in the $E_S$ efflux transport.

In the present study, the $E_S$ transport function at the BCSFB was characterized using conditionally immortalized rat choroid plexus epithelial cells (TR-CSFB). The expression of oatp subtypes at the BCSFB was evaluated in TR-CSFB cells and isolated rat choroid plexus by means of reverse transcription-polymerase chain reaction (RT-PCR). $E_S$ transport by oatp3 was analyzed using the Xenopus laevis oocyte expression system. In addition, the localization of oatp3 at the BCSFB was determined by immunohistochemical studies.

### Materials and Methods

**Animals.** Adult male Wistar rats, weighing 230 to 280 g, were purchased from Charles River Laboratories (Yokohama, Japan). Mature female $X. laevis$ were purchased from Hamamatsu (Hamamatsu, Japan) and maintained in a controlled environment as described by Goldin (1992). All experiments were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

**Reagents.** [6,7-3H](N$\varepsilon$)estrone-3-sulfate ammonium salt ([3H]$E_S$, 43.1 Ci/mmol) and [carboxy-14C]cimetidine ([14C]cimetidine, 1.92 mCi/g) were purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled $E_S$, dehydroepiandrosterone sulfate (DHEAS) and digoxin were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium taurocholate, sodium cholate, l-thyroxin sodium salt ($T_4$), 3,5,3'-triiodo-L-thyronine sodium salt ($T_3$), $\gamma$-aminobutyric acid (GABA), $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and probenecid were purchased from Wako Pure Chemicals (Osaka, Japan). Sodium cholate, L-thyroxin sodium salt (T4), 3,5,3'-triiodo-L-thyronine ($T_3$), GABA, and AMPA were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium taurocholate, sodium cholate, l-thyroxin sodium salt ($T_4$) and sodium chloride were used as control cells.

**E$_S$ Uptake Study with TR-CSFB Cells.** TR-CSFB cells established from transgenic rats harboring the temperature-sensitive SV40 large T-antigen gene were cultured at $33^\circ$C in an atmosphere of 5% CO$_2$ in air in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 4.5 g/l glucose, 100 U/ml penicillin, 100 g/ml streptomycin, and 10% fetal bovine serum (Moregate, Bulimba, Australia) (Kitazawa et al., 2001). For the transport study, TR-CSFB cells were seeded on 24-well plates (BD Biosciences, Franklin Lakes, NJ) at a density of 1.0 × 10$^5$ cells/well and cultured for 48 h at 33°C. Briefly, TR-CSFB cells were washed three times with extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO$_3$, 3 mM KCl, 1.4 mM CaCl$_2$, 1.2 mM MgCl$_2$, 0.4 mM KH$_2$PO$_4$, 10 mM d-glucose, and 10 mM HEPES, pH 7.4, at 37°C. Na$^+$-free ECF buffer was prepared by equimolar replacement of NaCl and NaHCO$_3$ with choline chloride and choline bicarbonate. Uptake was initiated by applying ECF buffer containing 1.25 $\mu$Ci of [3H]$E_S$, 0.25 $\mu$Ci of [14C]cimetidine, and the indicated concentration of unlabeled $E_S$ or inhibitor. [14C]cimetidine was used to correct for water adhesion. To terminate the transport reactions, cells were rinsed three times with ice-cold ECF buffer. The cells were then solubilized with 1% Triton X-100 solution, and the radioactivity was measured using a liquid scintillation counter equipped with an appropriate channel crossover correction for [3H] and [14C] (LS-6500; Beckman Coulter, Inc., Fullerton, CA). Cell-associated protein was determined with use of a DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

**Reverse Transcription-Polymerase Chain Reaction Analysis.** Total RNA was isolated from the indicated tissues of 6-week-old male Wistar rats using TRIzol Reagent (Invitrogen, Carlsbad, CA) as indicated by the manufacturer. The RNA was reverse-transcribed using oligo(dT) primer and ReverTra Ace (Toyobo, Osaka, Japan). For the amplification of oatp gene products in TR-CSFB cells, oligonucleotide primers were selected from conserved regions of oatp1 (GenBank accession no. L19031), oatp2 (GenBank accession no. U88036), and oatp3 (GenBank accession no. AF041105): 5'-TGG-GATCCATGGGTGGAGCAATTG-3' (oatp3 1463–1488) and 5'-TTTCCAGCTCCCATAGTAAC-3' (oatp3 1861–1883). PCR was performed using Ex-Taq DNA polymerase (Takara, Kyoto, Japan) with use of the following thermal cycle program: 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final elongation of 72°C for 10 min. For the further determination of oatp1 and oatp3 mRNA expression in the rat brain, liver, isolated choroid plexus, and TR-CSFB cells, oatp1, oatp3, and multidrug resistance-associated protein 1 (MRP1)–specific primers were designed as follows: oatp1, 5'-TGGAGGGTTGTGCTGCACTTATTT-3' (oatp1 688–714) and 5'-GGTGTGTTAATGGACCACTGC-3' (oatp1 1346–1363); oatp3, 5'-CGAGGAGGTCTGGTCTGCCAATAG-3' (oatp3 602–625) and 5'-AGTTATATACACACTTATAGAAGGACCC-3' (oatp3 1087–1062); and MRP1, 5'-GGCTGTTGTTGTGAACTGATG-3' and 5'-AGCTCGTCTCCATATG-3' (Decleves et al., 2000). PCR was performed using Ex-Taq DNA polymerase with use of the following thermal cycle program: 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and a final elongation of 72°C for 10 min. PCR for MRP1 was performed with use of the following thermal cycle program: 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final elongation of 72°C for 7 min. PCR-amplified products were subcloned into pBluescript SKII+ (Stratagene, La Jolla, CA) or pGEM-T Easy vector (Promega, Madison, WI) and sequenced using a DNA Sequencer (model 4200; LI-COR, Lincoln, NE). Sequences were compared with the GENETYXWIN software package version 4.0 (Soft-ware Development, Tokyo, Japan).

$\text{[3H]}E_S$ Uptake by oatp3 cRNA-Injected $X. laevis$ Oocytes. pGEM-HE N vector containing oatp3 was linearized by NotI digestion, and capped cRNA was synthesized in vitro with T7 RNA polymerase (Stratagene). Transcribed cRNA (25 ng) was injected into defolliculated $X. laevis$ oocytes. Injected oocytes were cultured for 3 days in Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO$_3$)$_2$, 0.4 mM CaCl$_2$, 0.8 mM MgSO$_4$, 2.4 mM NaHCO$_3$, and 10 mM HEPES, pH 7.4) supplemented with 100 g/ml streptomycin and 100 U/ml benzylpenicillin. The uptake (60 min) of [3H]$E_S$ was assayed at 20°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, and 5 mM HEPES, pH 7.4). Oocytes injected with water were used as control cells.

**Immunohistochemical Analysis.** Anti-oatp3 antibody was raised against a 9 amino acid peptide of oatp3 (KITVKKSEC, position 643–651) as described previously (Ito et al., 2002). A 6-week-old male Wistar rat was perfused with ice-cold PBS for 5 min, followed by 4% paraformaldehyde (PFA) for 10 min via the left ventricle. After the perfusion, the brain was removed and stored overnight in 4% PFA at 4°C. Before sectioning, the brain was infused with 0.5 M sucrose. Then, cryostat sections (15 $\mu$m in thickness; CM1900; Leica, Wetlza, Germany) were fixed with 2% PFA/0.1% Triton X-100 for 30 min on ice. After incubation in Histofine (Nichirei Co., Tokyo, Japan) for 1 h at room temperature the sections were reacted with 5 $\mu$g/ml
anti-oatp3 antibody (Ito et al., 2002), 5 µg/ml normal rabbit IgG, and 1:20 of MRPr1 (Kamiya Biomedical, Thousand Oaks, CA) in 0.1% bovine serum albumin/PBS for 1 h at 4°C and then with fluorescein- or rhodamine-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark) at a 1:100 dilution in 0.1% bovine serum albumin/PBS for 1 h at room temperature. Nuclei were stained with 6.6 µM propidium iodide, and sections were viewed by confocal laser microscopy (TCS SP, Leica).

Data Analysis. For kinetic studies, the $K_m$ and the $V_{max}$ of $[^{3}H]$E1S uptake by TR-CSFB cells or oatp3-expressing oocytes were estimated from the following equations using the nonlinear least-squares regression analysis program MULTI (Yamaoka et al., 1981):

$$v = \frac{V_{max} [S]}{K_m + [S]} + \frac{K_d}{[S]}$$

where $v$, $[S]$, and $K_m$ are the uptake rate, the concentration of E1S, and the saturation constant, respectively.

Unless otherwise indicated, all data represent the mean ± S.E.M. values. The statistical significance of differences among means of more than two groups was determined by one-way analysis of variance followed by the modified Fisher’s least-squares difference method.

**Results**

$[^{3}H]$E1S Uptake by TR-CSFB Cells. The time course of $[^{3}H]$E1S uptake by TR-CSFB cells, an in vitro model of the BCSFB, is shown in Fig. 1. The $[^{3}H]$E1S uptake increased linearly up to 5 min and reached a steady state over 20 min. The substitution of Na+ by choline had no significant effect on the $[^{3}H]$E1S uptake at 5 min (Fig. 1, inset). As shown in Fig. 2, the $[^{3}H]$E1S uptake by TR-CSFB cells was concentration-dependent and composed of saturable and nonsaturable components (Fig. 2, inset). The apparent $K_m$ and $V_{max}$ values of the saturable component were found to be $16.8 ± 5.1 \mu M$ and $12.3 ± 2.3 pmol/min/mg$ of protein, respectively (mean ± S.D.). The uptake clearance ($K_v$) of the nonsaturable component is $0.56 ± 0.01 \mu l/(min \cdot mg \text{ protein})$ (mean ± S.D.).

Fig. 2. Concentration-dependence of E1S uptake by TR-CSFB cells. $[^{3}H]$E1S uptake (115 nM) in the presence of unlabeled E1S was measured at 37°C for 5 min. Inset, Eadie-Scatchard plot of the same data. Each point represents the mean ± S.E.M. (n = 4). The $K_m$ and $V_{max}$ values of the saturable component are $16.8 ± 5.1 \mu M$ and $12.3 ± 2.3 pmol/min/mg$ of protein, respectively (mean ± S.D.). The uptake clearance ($K_v$) of the nonsaturable component is $0.56 ± 0.01 \mu l/(min \cdot mg \text{ protein})$. These results indicate that Na+ -independent carrier-mediated transport is involved in $[^{3}H]$E1S uptake by TR-CSFB cells.

As shown in Table 1, the $[^{3}H]$E1S uptake was 88.1% self-inhibited by E1S and was also significantly inhibited by up to 84.7% by substrates of oatps, such as probenecid, cholate, taurocholate, BSP, DHEAS, and T3. T4 and digoxin inhibited the uptake moderately (up to 31.5%). In contrast, PAH, methotrexate, and γ-aminobutyric acid did not affect the uptake, suggesting that oatps contributes to $[^{3}H]$E1S uptake by TR-CSFB cells.

**TABLE 1**

Effect of various compounds on uptake of $[^{3}H]$E1S by TR-CSFB cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>C/M Ratio</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.8 ± 0.55</td>
<td>100 ± 5.04</td>
<td></td>
</tr>
<tr>
<td>E1S</td>
<td>1.29 ± 0.07</td>
<td>11.9 ± 0.60**</td>
<td></td>
</tr>
<tr>
<td>Probenecid</td>
<td>4.14 ± 0.34</td>
<td>38.3 ± 3.17**</td>
<td></td>
</tr>
<tr>
<td>Cholate</td>
<td>6.44 ± 0.89</td>
<td>59.5 ± 8.23**</td>
<td></td>
</tr>
<tr>
<td>Taurocholate</td>
<td>4.56 ± 0.22</td>
<td>42.1 ± 2.60**</td>
<td></td>
</tr>
<tr>
<td>BSP</td>
<td>3.51 ± 0.57</td>
<td>32.5 ± 5.25**</td>
<td></td>
</tr>
<tr>
<td>DHEAS</td>
<td>1.65 ± 0.24</td>
<td>15.3 ± 2.25**</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>0.53 ± 0.41</td>
<td>59.1 ± 4.58**</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>6.76 ± 0.18</td>
<td>75.0 ± 2.00**</td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.025</td>
<td>68.5 ± 1.55**</td>
<td></td>
</tr>
<tr>
<td>PAH</td>
<td>9.16 ± 0.32</td>
<td>84.6 ± 2.93</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>1.06 ± 1.48</td>
<td>98.7 ± 13.7</td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td>1.06 ± 1.08</td>
<td>98.3 ± 9.97</td>
<td></td>
</tr>
</tbody>
</table>

*, P < 0.05; **, P < 0.01, significantly different from the control uptake.
RT-PCR Analysis of oatp3 and oatp1 in TR-CSFB Cells and Isolated Rat Choroid Plexus. RT-PCR using the common primer set for oatp1, -2, and -3 gave one amplified product from TR-CSFB cells, and the nucleotide sequence of the product was identical with that of oatp3 (GenBank accession no. AF041105; data not shown), suggesting that oatp3 is expressed at the BCSFB. As shown in Fig. 3A, the expected 488-base pair fragments were detected in rat brain, isolated choroid plexus, and TR-CSFB cells by RT-PCR with the specific primer set for oatp3. The nucleotide sequence of the amplified product in the isolated rat choroid plexus was identical with that of oatp3, except for one nucleotide displacement. On the other hand, in the case of oatp1 (Fig. 3B), no product was observed in rat brain, isolated choroid plexus, or TR-CSFB cells, whereas a 661-base pair product was amplified in rat liver. The nucleotide sequence of the product was identical with that of oatp1 except for two nucleotide displacements. Moreover, TR-CSFB cells expressed MRP1, which is reported to be expressed in choroid plexus epithelial cells (Nishino et al., 1999; Rao et al., 1999) (Fig. 3C). These results suggest the predominant expression of oatp3 at the BCSFB, compared with oatp1.

Oatp3-Mediated Uptake of E1S in the X. laevis Oocyte Expression System. E1S transport by oatp3 was examined by using the X. laevis oocyte expression system. The oatp3-expressing oocytes exhibited 12-fold greater [3H]E1S uptake than that of water-injected oocytes: 1.67 ± 0.61 versus 0.14 ± 0.02 µ/l/(h/oocyte). As shown in Fig. 4, the [3H]E1S uptake by the oatp3-expressing oocytes was saturable, with a \( K_m \) of 8.09 ± 2.83 µM and a \( V_{max} \) of 8.02 ± 0.87 pmol/h/oocyte, indicating that oatp3 mediates E1S transport at the BCSFB.

Immunohistochemical Study of oatp3 in Rat Choroid Plexus. The localization of oatp3 in rat choroid plexus was evaluated by an immunohistochemical study using anti-oatp3 antibody (Ito et al., 2002). As shown in Fig. 5A, oatp3 immunoreactivity (green) was detected in rat choroid plexus, and this immunostaining was observed along the surface of choroid plexus epithelial cells. Such characteristic immunostaining did not appear when normal rabbit IgG was used as a negative control (Fig. 5B). For further determination of oatp3 localization in the epithelial cells, double immunostaining was conducted with MRP1 antibody, which reacts with MRP (Fig. 5, C–E). Immunostaining by MRP1 antibody (Fig. 5, D and E; green) was observed at the basolateral membrane (Rao et al., 1999), and the oatp3 immunoreactivity (Fig. 5, C and E; red) did not overlap with MRP immunoreactivity (Fig. 5E), suggesting that oatp3 is localized on the brush-border membrane of choroid plexus epithelial cells.

Discussion

In this study, we demonstrated the involvement of oatp3 in E1S transport and its expression and localization at the BCSFB. To evaluate in detail the properties of E1S transport at the BCSFB, we used TR-CSFB cells. TR-CSFB cells have been found to possess the characteristic properties of choroid plexus epithelial cells, such as transthyretin expression and apical localization of Na+, K+-ATPase (Kitazawa et al., 2001), and they express MRP1 mRNA (Fig. 3C). The isolated
rat choroid plexus possesses [3H]E1S uptake activity with a $K_m$ of 18.1 μM, and this uptake was Na⁺-independent and significantly inhibited by cholate, taurocholate, BSP, and DHEAS, but not by PAH (Kitazawa et al., 2000). In the present study, TR-CSFB cells exhibited Na⁺-independent [3H]E1S uptake, and the $K_m$ value was found to be 16.8 μM (Figs. 1 and 2). The uptake in TR-CSFB cells was inhibited by various inhibitors in the same manner as that in isolated choroid plexus and was unaffected by PAH (Table 1). These results suggest that a similar organic anion transport system for E1S is expressed in both TR-CSFB cells and in the isolated rat choroid plexus.

The organic anion transporters reported to be expressed in rat brain are oatp1, oatp2, oatp3, OAT1, and OAT3 (Jacqueemin et al., 1994; Noe et al., 1997; Sekine et al., 1997; Abe et al., 1998; Kusuhara et al., 1999). The Na⁺-independent and the inhibitory effects on [3H]E1S transport by TR-CSFB cells shown in Fig. 1 and Table 1 are consistent with oatp-mediated transport, because oatp is an Na⁺- and PAH-insensitive anion transporter (Kanai et al., 1996; Sugiyama et al., 2001). OAT3 also mediates E1S transport and has been reported to be involved in benzylpenicillin transport at the BCSFB (Kusuhara et al., 1999; Nagata et al., 2002). Nevertheless, the transport by OAT1 and OAT3 is inhibited by PAH and methotrexate, with the $K_m$ values of OAT1 and OAT3 for PAH being 14.3 and 64.7 μM, respectively (Sekine et al., 1997; Kusuhara et al., 1999). In addition, the transport activity of OAT1 is reduced under Na⁺-free conditions (Sekine et al., 1997). E1S transport by TR-CSFB cells is Na⁺-independent and was not significantly inhibited by PAH or methotrexate, suggesting that oatp is predominantly involved in E1S transport by TR-CSFB cells.

Substrate specificities among oatp1, -2, and -3 are similar, except for digoxin, which is a selective substrate of oatp2 ($K_m = 0.24$ μM) (Noe et al., 1997; Kakyo et al., 1999; Cattori et al., 2001). Digoxin inhibits [3H]E1S uptake by 31.5% (Table 1), suggesting the participation of oatp2 in its uptake. This observation is consistent with previous immunohistochemical results showing that oatp3 is expressed at the BCSFB (Gao et al., 1999). However, the inhibitory effects shown in Table 1 also suggest a contribution by other oatp subtypes, such as oatp1 and -3, because the [3H]E1S uptake is inhibited by up to 84.7% by substrates for oatps. RT-PCR analysis (Fig. 3) revealed oatp3 expression in TR-CSFB cells and isolated choroid plexus, whereas no oatp1 was detected, suggesting that oatp3, rather than oatp1, is involved in [3H]E1S transport in TR-CSFB cells and the BCSFB. Immunohistochemical studies by Angeletti et al. (1997) and Gao et al. (1999) have shown that oatp1 is localized at the BCSFB. One possible explanation for this discrepancy is cross-reactivity of the antibody between oatp1 and -3, because the antibody was raised against the C terminus of oatp1, which is conserved in oatp3.

In a previous transport study using the oocyte expression system, the $K_m$ value for E1S transport by oatp3 from rat intestine was reported to be 268 μM (Cattori et al., 2001), which is 15- and 16-fold lower than those of isolated rat choroid plexus and TR-CSFB cells, respectively. We evaluated the [3H]E1S uptake mediated by oatp3 from rat choroid plexus in an oocyte expression system, and the $K_m$ value for [3H]E1S transport by oatp3 was found to be 8.09 μM (Fig. 4), supporting the hypothesis that oatp3 mediates [3H]E1S uptake by isolated rat choroid plexus and TR-CSFB cells. The difference in the $K_m$ is presumably caused by the difference in the nucleotide sequences, because oatp3 from rat intestine (GenBank accession no. AF083469) contains 5 nucleotide changes that result in a difference of four amino acids from oatp3 isolated from rat retina and choroid plexus (retinal and choroid plexus clone residue listed first: Q33K, I55T, F70L, and K543E) (Cattori et al., 2001). Polarized localization of transporters controls vectorial transport via the BCSFB (Gao and Meier, 2001). oatp3 is localized at the surface of rat choroid plexus epithelial cells (Fig. 5A), and its localization is complementary to immunostaining by MRPr1 (Fig. 5E). Rao et al. (1999) have reported that MRPr1 reacts with MRP localized at the basolateral blood side) membrane of rat choroid plexus epithelial cells, suggesting that oatp3 is localized at the brush-border membrane (CSF side) of these cells. As far as E1S transport from the CSF via the BCSFB is concerned, oatp3 could be involved in the first E1S uptake step into epithelial cells at the brush-border membrane. Then, on the opposite basolateral side, MRP1 and/or oatp2 seem to be involved in E1S excretion from epithelial cells, because MRP1 and oatp2 are localized at the basolateral membrane (Gao et al., 1999; Rao et al., 1999), and MRP1 mediates E1S efflux transport while oatp2 bi-directionally transports organic anions (Li et al., 2000; Qian et al., 2001).

Recently, OAT3 was also reported to be localized at the brush-border membrane of rat choroid plexus epithelial cells (Nagata et al., 2002). Furthermore, the involvement of OAT3 in organic anion transport at the choroid plexus was shown using OAT3 knockout mice (Sweet et al., 2002). However, the relative contributions by oatp3 and OAT3 to organic anion efflux transport at the BCSFB are still unknown. As far as E1S efflux transport is concerned, oatp3 is a major efflux transporter because E1S is a substrate of both oatp3 and OAT3, and PAH (1 mM), which inhibits OAT3 but not oatp3, inhibited E1S uptake by TR-CSFB cells and isolated rat
chordoid plexus only by 15.4% and 1.8%, respectively (Table 1) (Kitazawa et al., 2001).

$^{3}[^{3}H]E_{1}$S uptake by TR-CSFB cells is inhibited by DHEAS, $T_{3}$, and $T_{4}$, suggesting that the transport process mediated by oatp3 also operates for DHEAS, $T_{3}$, and $T_{4}$. DHEAS is a steroid hormone sulfate that exhibits a variety of effects in the central nervous system, including neuromodulation and neuroprotection (Zwain and Yen, 1999). Thyroid hormones also exhibit neuroprotective effects and are essential for normal brain function, particularly during development (Rami and Kriegstein, 1992; Porterfield and Hendrich, 1993; Tremont and Stern, 1997). These observations support the hypothesis that transport via oatp3 at the BCSFB plays an important role in maintaining CNS function.

In conclusion, the present study shows that oatp3 is expressed at the brush-border membrane of rat choroid plexus epithelial cells and is involved in $E_{1}$S efflux transport in TR-CSFB cells as an in vitro BCSFB model. This is the first in vitro evidence to demonstrate the functional expression and possible physiological role of oatp3 in the brain. Oatp3 may function as part of the CSF-to-blood transport system for organic anions via the BCSFB to maintain homeostasis of the CSF.

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References

Abbreviations

$T_{3}$, $T_{4}$, and DHEAS

$T_{3}$, $T_{4}$, and DHEAS

$T_{3}$, $T_{4}$, and DHEAS

Biosynthesis and metabolism of dehydroepiandrosterone: From placenta to brain.

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