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Brain-to-blood efflux transport of estrone-3-sulfate at the blood-brain barrier in rats

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Abstract

Efflux transport of estrogens such as estrone-3-sulfate (E_1S), and estrone (E_1) across the blood-brain barrier (BBB) was evaluated using the Brain Efflux Index (BEI) method. The apparent BBB efflux rate constant (K_{eff}) of [^{3}H]E_1S, and [^{3}H]E_1 was $6.63 \times 10^{-2} \pm 0.77 \times 10^{-2}$ min^{-1}, and $6.91 \times 10^{-2} \pm 1.23 \times 10^{-2}$ min^{-1}, respectively. The efflux transport of [^{3}H]E_1S from brain across the BBB was a saturable process with Michaelis constant (K_m) of 96.0 ± 34.4 μM and 93.4 ± 22.0 μM estimated by two different methods. By determining [^{3}H]E_1S metabolites using high performance liquid chromatography (HPLC) after intracerebral injection, significant amounts of [^{3}H]E_1S were found in the jugular venous plasma, providing direct evidence that most of [^{3}H]E_1S is transported from brain across the BBB in intact form. To compare the apparent efflux clearance across the BBB of E_1S with that of E_1, the brain distribution volume of E_1S and E_1 was estimated using the brain slice uptake method. The apparent efflux clearance of [^{3}H]E_1S was determined to be 74.9 ± 3.8 μl/(min·g brain) due to the distribution volume of 1.13 ± 0.06 ml/g brain. By contrast, the apparent efflux clearance of E_1 was more than 227 ± 3 μl/(min·g brain), since the distribution volume of [^{3}H]E_1 at 60 min was 3.28 ± 0.13 ml/g. The E_1S efflux transport process was inhibited by more than 40% by coadministration of bile acids (taurocholate, and cholate), and organic anions (sulfobromophthalein, and probenecid), whereas other organic anions did not affect the E_1S efflux transport. The [^{3}H]E_1S efflux was significantly reduced by 48.6% after preadministration of 5 mM dehydroepiandrosterone sulfate. These results suggest that E_1S is transported from brain to the circulating blood across the BBB *via* a carrier-mediated efflux transport system.

Keywords: Efflux transport; Estrone-3-sulfate; Organic anion transporting polypeptide; Blood-brain barrier

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Introduction

Estrogens, the female hormones, play an important role in the expression of genes to maintain sex differences and homeostasis in the brain [1,2]. Very recently, Shimada et al. [3] were able to show that estrone (E_1) is present in male and female rat brain using gas chromatography-tandem mass spectrometry (GC-MS-MS). Moreover, neurons and astrocytes isolated from rat cerebral cortex produce neurosteroids including E_1 [4]. Sulfation is a major pathway in the metabolism of steroid hormones [5] and estrogen sulfotransferase, which converts E_1 to estrone-3-sulfate (E_1S), is found to be present in the male rat brain, but not in the female rat brain by Western blot [6]. Therefore, E_1S seems to be present in the male rat brain as a metabolite of E_1. Although no physiological role for E_1S has been established yet, E_1S has been used to treat senile dementia of Alzheimer type (SDAT) [7]. It is given as an estrogen replacement therapy and has an ameliorative effect [7]. This therapy has improved the memory, orientation, and calculating ability of SDAT patients, whereas SDAT patients not receiving such therapy did not show any improvement [7]. Therefore, it will be important for successful estrogen replacement therapy that E_1S is efficiently transported from the circulating blood to brain. To maintain therapeutic drug concentrations in the brain, it is necessary not only to increase the influx rate but also to reduce the efflux rate across the blood-brain barrier (BBB). E_1S can be expected to penetrate the BBB due to its relatively high lipophilicity, with an octanol-water partition coefficient of 230 [8]. Nevertheless, the distribution of E_1S in the brain is very limited compared with E_1 [8]. In fact, the percentage extracted in rat brain using the brain uptake index method was 100% for E_1 but only 6.5% for E_1S [8].

BBB plays a key role for the influx of nutrients [9] and hormones [10] as well as the efflux transport of peptide hormones, and drugs such as corticotropin-releasing hormone [11], vinblastine [12], and vincristine [13]. Moreover, the development of the Brain Efflux Index (BEI) method [14] allows us to characterize the specific efflux transport systems at the BBB for compounds such as *p*-aminohippuric acid [15], 3′-azido-3′-deoxythymidine, and 2′, 3′-dideoxyinosine [16], and cerebral neurotransmitters such as L-glutamic acid, and L-aspartic acid [17]. Previous studies have focused on E_1S transport from the circulating blood to brain [8,18], but our knowledge of the efflux transport of E_1S from brain to the circulating blood is still incomplete. It is, therefore, important to investigate the E_1S efflux transport system across the BBB because it will not only help us to understand the disposition of E_1S in the brain but also give us a better overall understanding of BBB function.

The purpose of the present study is to characterize the efflux transport process of E_1S, and E_1 at the BBB using the BEI method. We focused on E_1S because it is quantitatively the most abundant estrogen in human plasma [19] and seems to be the major metabolite of E_1 in the brain.

Materials and methods

Animals

Male Wistar rats weighing 210–260 g were purchased from Charles River (Yokohama, Japan). This study was approved by the Animal Care Committee Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

[6,7-^{3}H(N)]Estrone-3-sulfate ammonium salt ([^{3}H]E_1S, 53.0 Ci/mmol), [2,4,6,7-^{3}H(N)]estrone ([^{3}H]E_1, 100 Ci/mmol), and [carboxyl-^{14}C]inulin ([^{14}C]inulin, 1.92 mCi/g) were purchased from DuPont NEN (Boston, MA); sodium taurocholate (TCA), sodium cholate (CA), *p*-aminohippuric acid (PAH), γ-aminobutylic acid (GABA), 5-hydroxyindoleacetic acid (5-HIAA), *p*-nitrophenyl sulfate (PNP-S), and probenecid were obtained from Wako Pure Chemical (Osaka, Japan); 4-hydroxy-3-methoxy-phenylglycol-4-sulfate (MHPGS) was purchased from Research Biochemicals International (Natick, MA); sulfobromophthalein sodium hydrate (BSP), and homovanilic acid (HVA) were obtained from Nacalai Tesque (Kyoto, Japan); phenolsulfonphthalein (PSP) was from Tokyo Kasei (Tokyo, Japan); E_1S, dehydroepiandrosterone sulfate (DHEAS), and xylazine hydrochloride were from Sigma Chemical Co. (St. Louis, MO); Ketaral 50 (ketamine hydrochloride) was obtained from Sankyo Co. (Tokyo, Japan) and used as an anesthetic. All other chemicals were of reagent grade available commercially and used without further purification.

Brain efflux index (BEI) study

The in vivo brain efflux experiments were performed by the intracerebral microinjection technique reported previously [14,15]. Rats were anesthetized with an intramuscular injection of ketamine (235 mg/kg), and xylazine (2.3 mg/kg) and their heads were fixed in a stereotaxic frame (SR-6, Narishige, Tokyo, Japan). This frame was used to determine the coordinates of the rat brain coinciding with Parietal Cortex, Area 2 (Par2) region. Par2 region was selected to administer a test substrate because only less than 3% of inulin as the reference compound was found in the contralateral cerebrum, cerebellum, and cerebrospinal fluid (CSF) [14]. Therefore, the apparent elimination of test substrate reflects the efflux process across the BBB [14]. After exposing the skull, a 1.0 mm hole was made in the skull, 0.20 mm anterior and 5.5 mm lateral to the bregma using a dental drill (Osada Electric, Tokyo, Japan). A 5.0 μl-microsyringe (Hamilton, Reno, NE) fitted with a needle (100 μm i.d., 350 μm o.d.; Seiseido Medical Industry, Tokyo, Japan) was inserted into the Par2 region through a hole to a depth of 4.5 mm. [^{3}H]E_1S (80 nCi) or [^{3}H]E_1 (80 nCi) and [^{14}C]inulin (4 nCi) dissolved in 0.50 μl of the 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, 10 mM HEPES, pH 7.4, 290 mOsml/kg) was administered to rat brain over 1 min. At appropriate time, CSF was collected from the cisterna magna, as reported previously [14], and then the brain was removed. The ipsilateral (left) cerebrum, contralateral (right) cerebrum, and cerebellum were weighed and dissolved in 2.5 ml of 2 N NaOH at 50°C for 3 h. Then, samples were mixed with 14 ml liquid scintillation cocktail (Hionic-fluor, Packard, Meriden, CT). The associated radioactivity was determined in a liquid scintillation counter (LSC 5000, Aloka, Tokyo, Japan) with the automatic external standard for quenching correction. To examine the inhibitory effect on [^{3}H]E_1S efflux transport, inhibitor was co-administrated with [^{3}H]E_1S and [^{14}C]inulin. Moreover, in the case of DHEAS, which has a very limited solubility, 50 μl of the inhibitor (0.1 or 5 mM DHEAS) was preadministered into the Par2 region over 1 min just prior to microinjection of [^{3}H]E_1S solution. This is because this kind of preadministration study minimizes the effect of dilution of the inhibitor on the BEI method [14].

Determination of BEI from the brain

The BEI was defined by equation (1) [14] and the percentage of substrate remaining in the ipsilateral cerebrum was determined using equation (2).

$$\text{BEI (\%)} = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$\text{100-BEI (\%)} = \frac{\begin{array}{c}\text{(amount of test substrate in the brain/}\\ \text{amount of reference in the brain)}\end{array}}{\begin{array}{c}\text{(concentration of test substrate injected/}\\ \text{concentration of reference injected)}\end{array}} \times 100 \quad (2)$$

As the percentage of E_1S remaining in the brain is given by (100-BEI), K_{eff}, the apparent BBB efflux rate constant, was estimated by fitting the semilogarithmic plot of (100-BEI) *versus* time data to the nonlinear least-squares regression analysis program, MULTI [20]. Moreover, the apparent efflux clearance across the BBB, $CL_{BBB,eff}$, was obtained from equation (3).

$$CL_{BBB,eff} = K_{eff} \times V_{brain} \quad (3)$$

where V_{brain} represents the distribution volume of substrate in the brain, determined by the in vitro brain slice uptake study as described below.

Estimation of kinetic parameters

Efflux transport rate at the BBB ($R_{BBB,eff}$) was determined by two different methods. In the first method, the equation (4) was used,

$$R_{BBB,eff} = CL_{BBB,eff} \times C = [V_{max} \times C/(K_m + C)] + P_{non} \times C \quad (4)$$

where V_{max}, K_m, and P_{non} represent the maximum efflux transport rate at the BBB, the Michaelis constant, and the non-saturable efflux clearance, respectively. C is the concentration of E_1S in the brain. As the injectate was diluted 30.3-fold by diffusion into brain following 0.50 μl of an intracerebral microinjection solution [14], C was calculated from the E_1S concentration in the injectate divided by this dilution factor.

In the second method, $R_{BBB,eff}$ expressed in nmol/(min·g brain) was determined at each concentration from the equation (5) according to Banks et al. [21,22];

$$R_{BBB,eff} = (A\text{-}M)S/itW \quad (5)$$

where M is the dpm/brain in an individual rat 20 min after injection, S is the amount of E_1S injected, i is the amount of $[^3H]E_1S$ injected, t is time from injection to decapitation (in minutes) and W is the brain weight (in grams).The amount of $[^3H]E_1S$ for transport (A) was taken to be equal to the counts remaining in the brain of animals sacrificed with an overdose of anesthetic before receiving their intracerebral injections. $R_{BBB,eff}$ was plotted against C.

$$R_{BBB,eff} = \frac{V_{max} \times C^n}{K_m + C^n} \quad (6)$$

where n is Hill's coefficient.

Analytical method

The amount of [^{3}H]E_1S metabolism in the brain and plasma after intracerebral microinjection was determined by high performance liquid chromatography (HPLC). At 5 min after intracerebral microinjection with 2 μCi of [^{3}H]E_1S, blood was collected through the ipsilateral jugular vein, and the rat was immediately decapitated. The ipsilateral cerebrum was homogenized with 2.5 ml tetrahydrofuran. After centrifugation of blood, and homogenate at 310 × g for 10∼15 min, 400 μl of plasma was mixed well with 1.0 ml tetrahydrofuran. The tetrahydrofuran phase was dried under nitrogen gas, and the residue was reconstituted with 300 μl of mobile phase. An aliquot (200 μl) of each sample (plasma, and brain) was subjected to HPLC using an analytical column (Capcell Pak C18 UG120, 250 × 4.6 mm, Shiseido, Tokyo, Japan). A guard column (4.0 × 10 mm, Shiseido, Tokyo, Japan) was placed between the injector and the analytical column. The mobile phase consisted of MeOH-0.1% trifluoroacetic acid (55:45, vol/vol) at a flow rate of 1.0 ml/min. The absorbance of the eluate was monitored at 267 nm in collected fractions, each of 0.5 ml. Then, samples were mixed with liquid scintillation cocktail (Hionic-fluor, Packard) and the radioactivity was determined in a liquid scintillation counter (LSC 5000, Aloka).

Brain slice uptake study

The distribution volume of [^{3}H]E_1S in the brain was determined the in vitro brain slice uptake study as reported previously [14]. It is difficult to determine the distribution volume of drug in the brain in vivo since BBB restricts to distribute the drugs to the brain which is different from other organs. Therefore, brain slice uptake study was chosen for a better method. Moreover, the distribution volume determined in vitro brain slice uptake was closed to that estimated in vivo brain microdialysis using new quinolone antibacterial agents [23]. After preincubation of brain slices (300 μm) for 5 min at 37°C, the uptake was initiated by transferring to 50 ml of oxygenated ECF buffer containing 0.20 μCi/ml of [^{3}H]E_1S and 0.04 μCi/ml [^{14}C]inulin at 37°C. At appropriate time, brain slices and an aliquot (500 μl) of incubation medium were collected. The associated radioactivity was measured by liquid scintillation counting. The volume of adhering water was determined as the zero-time intercept of the [^{14}C]inulin uptake profile, *i.e.*, 0.132 ml/g brain (n=4). The metabolized and unmetabolized forms of [^{3}H]E_1S in the uptake experiments were determined by HPLC as described above.

Statistical analysis

Unless otherwise indicated, all data represent the mean ± S.E.M. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by modified Fisher's least squares difference method.

Results

Efflux transport of [^{3}H]E_1S and [^{3}H]E_1 from the brain

Fig. 1 shows the time course of the percentage of [^{3}H]E_1S, and [^{3}H]E_1 remaining in rat brain. A total of 75.2% of [^{3}H]E_1S was eliminated from the ipsilateral cerebrum within 20

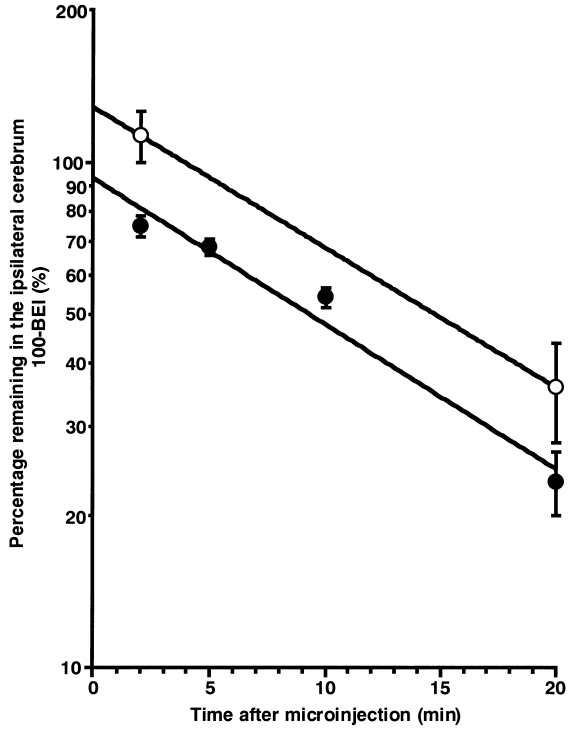

Fig. 1. Time-courses of [^{3}H]E_1S, and [^{3}H]E_1 in the ipsilateral cerebrum following intra-cerebral microinjection in the presence of [^{14}C]inulin as an internal reference. A mixture of [^{3}H]E_1S (●), or [^{3}H]E_1 (○) (80 nCi) and [14C]inulin (4 nCi) dissolved in 0.50 μl ECF buffer was injected into Par2 region of rat brain. The solid line was obtained using the nonlinear least-squares regression analysis program. Each point represents the mean ± SEM (n = 3).

min. The K_{eff} value of [^{3}H]E_1S was $6.63 \times 10^{-2} \pm 0.77 \times 10^{-2}$ min^{-1} (mean ± S.D.). The efflux of [^{3}H]E_1 was similar to the [^{3}H]E_1S and the K_{eff} was $6.91 \times 10^{-2} \pm 1.23 \times 10^{-2}$ min^{-1} (mean ± S.D.). During the course of the efflux study, less than 0.5% of both [^{3}H]E_1S and [^{3}H]E_1, relative to the administered dose, was found in the contralateral cerebrum, cerebellum, and CSF, indicating that diffusion into the rest of central nervous system from the injection site was very limited. Fig. 2 illustrates the HPLC chromatograms of [^{3}H]E_1S present in the ipsilateral cerebrum and ipsilateral jugular venous plasma 5 min after intracerebral microinjection of 2 μCi of [^{3}H]E_1S. [^{3}H]E_1S was detected in the jugular venous plasma (81.6%) and the ipsilateral cerebrum (86.1%), indicating that most of the E_1S was transported in its intact form across the BBB.

Uptake of [^{3}H]E_1S by brain slices

The distribution volume of E_1S in the brain was determined by in vitro brain slice uptake study (Table 1). There was not significant difference in the slice-to-medium concentration (S/M) ratio for intact [^{3}H]E_1S between incubation periods of 30 and 60 min, giving a steady-state S/M ratio of 1.13 ± 0.06 ml/g brain as the distribution volume of the brain. Incorporating the

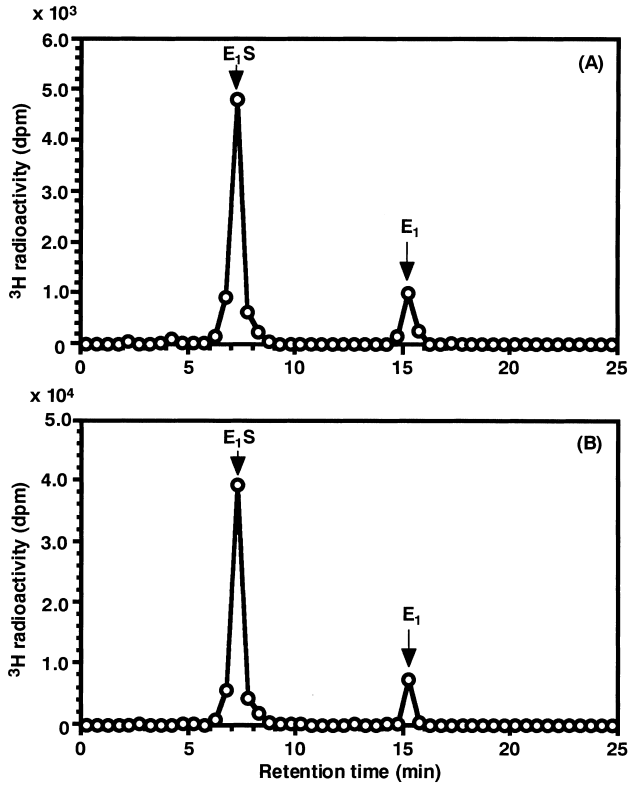

Fig. 2. Identification of [^{3}H]E_1S in (A) jugular venous plasma, and (B) ipsilateral cerebrum by using HPLC. An aliquot of [^{3}H]E_1S (2 μCi) was injected into Par2 of rat brain. Venous blood was collected from the ipsilateral jugular vein at 5 min. Ipsilateral cerebrum was removed at 10 min. HPLC analysis was performed at a flow rate of 1.0 ml/min. Each point represents radioactivity in the respective fraction. The arrows show the expected elution times of E_1S, and E_1.

K_{eff} ($6.63 \times 10^{-2} \pm 0.77 \times 10^{-2}$ min^{-1}, Fig. 1) and the distribution volume in the brain (1.13 ± 0.06 ml/g brain) into equation (3), the apparent BBB efflux clearance of E_1S was 74.9 ± 3.8 μl/(min·g brain). The distribution volume of E_1 was estimated by using the value of the S/M ratio of [^{3}H]E_1 which was hydrolyzed from [^{3}H]E_1S. The S/M ratio for [^{3}H]E_1 at

Table 1
Uptake of [^{3}H]E_1S by brain slices

Incubation time (min)	Slice-to-medium concentration ratio (ml/g brain)	
	E_1S	E_1
30	1.01 ± 0.06	2.17 ± 0.28
60	1.24 ± 0.04	3.28 ± 0.13*

Rat brain slices were incubated with ECF buffer including 0.05 μCi/ml of [^{3}H]E_1S, and 0.01 μCi/ml [^{14}C]inulin at 37°C. The radioactivity of [^{3}H]E_1S, and [^{3}H]E_1 in the brain slices and incubation medium was determined by HPLC at 30, and 60 min. Each value represents the mean ± S.E.M. (n = 4).

* $p < 0.01$, significantly different from the value of E_1 at 30 min.

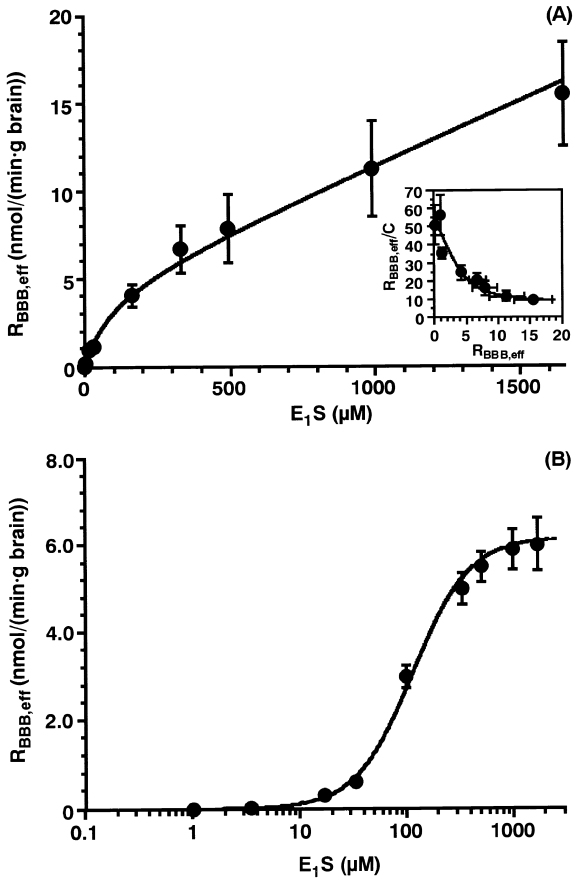

Fig. 3. Concentration-dependent efflux transport of E_1S from brain across the BBB using method 1 (A) and method 2 (B). Two different methods (A: *method 1*:, B: *method 2*) are compared. The solid line was estimated from equation (4) or (6) using the nonlinear least-squares regression analysis program. The cerebral concentration was estimated from the injectate concentration divided by the dilution factor, *i.e.*, 30.3, which was reported previously [15]. Inset: Eadie-Scatchard plot for [^{3}H]E_1S efflux at the BBB. Each point represents the mean ± SEM (n = 3–7). The K_m values were 96.0 ± 34.4 μM (*method 1*) and 93.4 ± 22.0 μM (*method 2*) as a cerebral concentration, the V_{max} were 4.66 ± 1.77 nmol/(min·g brain) (*method 1*) and 6.31 ± 1.44 nmol/(min·g brain) (*method 2*), and a P_{non} was 7.18 ± 1.72 μl/(min·g brain) (*method 1*).

60 min was 3.28 ± 0.13 ml/g brain (Table 1). As the steady state S/M ratio of E_1 might be greater than the S/M ratio of E_1 at 60 min, the apparent BBB efflux clearance of E_1 could be estimated to be at least 227 ± 3 μl/(min·g brain).

The concentration-dependent efflux transport of E_1S

The efflux transport rate of E_1S as a function of the E_1S concentration is shown in Figs. 3A and B. The [^{3}H]E_1S efflux rate was saturable on increasing the unlabeled E_1S concentration in the injectate, indicating a concentration-dependence. The Eadie-Scatchard plot exhibited

Table 2
Coadministration effect of various organic anions on [^{3}H]E_1S efflux from rat brain

Inhibitors	Injectate concentration (mM)	Concentration in the brain[a] (mM)	No. studied	BEI value[b] (%)
Control	3.0×10^{-3}	1.0×10^{-4}	9	76.0 ± 2.9 (100)
p-Aminohippuric acid	100	3.3	5	68.2 ± 1.9 (89.7)
γ-Aminobutylic acid	100	3.3	4	71.1 ± 2.1 (93.6)
5-Hydroxyindoleacetic acid	100	3.3	4	66.8 ± 3.2 (87.9)
Homovanilic acid	100	3.3	4	65.3 ± 0.5 (85.8)
4-Hydroxy-3-methoxy-phenylglycol-4-sulfate	100	3.3	4	69.2 ± 3.5 (91.1)
p-Nitrophenyl sulfate	100	3.3	5	61.9 ± 6.0 (81.3)
Phenolsulfonphthalein	20	0.66	6	69.4 ± 4.5 (91.3)
Sulfobromophthalein	20	0.66	4	45.4 ± 1.5 (59.8)*
Taurocholate	20	0.66	5	35.1 ± 2.9 (46.2)**
Cholate	20	0.66	5	36.6 ± 2.9 (48.1)**
Probenecid	100	3.3	4	29.4 ± 1.7 (38.6)**

[a] The brain concentration was estimated from the injectate concentration divided by the dilution factor, *i.e.*, 30.3, which was reported previously [14].

[b] Data, determined 20 min after intracerebral microinjection, are mean ± S.E.M. values (percent of control).

* $p < 0.05$, * $p < 0.01$, significantly different from control.

both saturable and non-saturable [^{3}H]E_1S efflux transport processes (Fig. 3A inset). Nonlinear least-squares regression analysis provided a K_m of 96.0 ± 34.4 μM (*method 1*) and 93.4 ± 22.0 μM (*method 2*) as a cerebral concentration, a V_{max} of 4.66 ± 1.77 nmol/(min·g brain) (*method 1*) and 6.31 ± 1.44 nmol/(min·g brain) (*method 2*), and a P_{non} of 7.18 ± 1.72 μl/(min·g brain) (*method 1*). These values represent the mean ± S.D.

Coadministration effect of various compounds on [^{3}H]E_1S efflux from rat brain

[^{3}H]E_1S efflux transport was significantly inhibited by coadministration of 20 mM bile acids (0.66 mM in the brain) such as TCA (by 53.8 ± 3.8%) and CA (by 51.9 ± 3.8%), and by organic anions such as 20 mM BSP (by 40.3 ± 2.0%) and 100 mM probenecid (3.3 mM in the brain, by 61.4 ± 2.3%) (Table 2). By contrast, other organic anions such as 100 mM PAH, 100 mM GABA, 100 mM 5-HIAA, 100 mM HVA, 100 mM MHPGS, 100 mM PNP-S, and 20 mM PSP did not affect the [^{3}H]E_1S efflux transport (Table 2).

Preadministration effect of DHEAS on [^{3}H]E_1S efflux from rat brain

Since the solubility of DHEAS is limited, a drug solution (0.50 μl) containing [^{3}H]E_1S was given by microinjection into the Par2 region following preadministration of injectate (50 μl) containing 0.1 mM or 5 mM DHEAS. Although [^{3}H]E_1S efflux transport was not inhibited by 0.1 mM DHEAS, 5 mM DHEAS significantly reduced [^{3}H]E_1S elimination from brain by 48.6 ± 7.8% (Table 3). The BEI value at 0.1 mM DHEAS was identical to the control even when 50 μl was injected (Table 3), supporting that preadministration does not affect the transport functions at the BBB as was reported previously [17].

Table 3
Preadministration effect of DHEAS on [^{3}H]E_1S efflux from rat brain

Inhibitor	Injectate concentration (mM)	No. studied	BEI value[a] (%)
Control		8	61.6 ± 3.4 (100)
DHEAS	0.1	4	61.2 ± 2.4 (99.4)
	5	4	31.7 ± 4.8 (51.5)*

Inhibitor solution (50 μl) was administered to the Par2 region of rat brain, just before injection of [^{3}H]E_1S (6.0 μM).
[a] Data, determined 20 min after intracerebral microinjection, are mean ± S.E.M. values (percent of the control).
* $p < 0.05$, significantly different from control.

Discussion

In the present study, in vivo evidence has been provided to show that E_1S is transported *via* a carrier-mediated efflux transport process from brain to the circulating blood across the BBB. Rapid elimination of E_1S, with a $t_{1/2}$ of 10.5 min, from rat brain after intracerebral injection suggests the presence of a specific transport system(s) being responsible for the transcellular transport across the BBB (Fig. 1). Although estrone sulfatase, which converts E_1S to E_1, is present in rat brain [24], significant amounts of E_1S in intact form were detected in the jugular venous plasma (Fig. 2). The other peak in the plasma was due to E_1, which is a desulfated form of E_1S. This detection of E_1 may be due to a number of possibilities. One is that E_1 itself is eliminated from the brain since the apparent efflux clearance of E_1 from brain across the BBB (*i.e.*, 227 ± 3 μl/(min·g brain)) was at least 3-fold greater than that of E_1S (*i.e.*, 74.9 ± 3.8 μl/(min·g brain)). Another possibility is that E_1S is metabolized primarily in the liver to estrogen [25], and E_1 is released into the circulating blood.

The efflux transport process of E_1S from brain to the circulating blood across the BBB was saturable and concentration-dependent, with a K_m value of 96.0 μM (*method 1*) and 93.4 μM (*method 2*) as a cerebral concentration (Figs. 3A and B). This result supports the hypothesis that E_1S is transported *via* a carrier-mediated efflux transport process across the BBB. The K_m values determined using two different methods were not statistically different (Figs. 3A and B). The K_m value for E_1S efflux transport process was relatively close to that for E_1S uptake by isolated rat hepatocytes (16 μM) [26]. However, it was higher than that (0.8 μM) reported by Schwenk et al. [25]. Both oatp1 and oatp2, which transport E_1S, are well known to play important roles in the detoxification of drugs in the liver and are present in the brain [27,28]. The oatp1- and oatp2-cRNA injected *Xenopus laevis* oocytes mediated E_1S uptake with a K_m of 4.5 μM and 11 ± 3 μM, respectively [28,29]. These values are relatively similar to that at the BBB.

The [^{3}H]E_1S efflux from brain was significantly inhibited by TCA, CA, BSP and probenecid, whereas PSP did not affect [^{3}H]E_1S efflux (Table 2). This pattern of inhibition was in good agreement with that of oatp1- and oatp2- mediated steroid hormone transport in the liver and kidney [29–31]. Moreover, preadministration of 5 mM DHEAS significantly reduced [^{3}H]E_1S elimination by 48.6% (Table 3). DHEAS is called "neurosteroid" and acts as a functional antagonist at $GABA_A$ receptor [32]. The oatp1 and oatp2 have been identified as

a carrier protein for DHEAS in rat liver [31,33,34]. Recently, Gao et al. [35] reported that the oatp2 is identified by *in situ* hybridization histochemistry, and by immunofluorescence microscopy to exist at the rat BBB, but not oatp1. Positive signal *in situ* hybridization were scattered diffusely over all brain region including cerebral cortex. However, it is not yet known whether oatp2 localizes in the luminal or abluminal side of the BBB and acts as the influx transporter from the circulating blood to brain or efflux one from brain to the circulating blood. Taken together, our findings suggest that oatp2 is involved in an efflux transport process for E_1S from brain to the circulating blood across the BBB.

P-Glycoprotein (P-gp), which functions as an ATP-dependent pump, undergoes efflux transport of several drugs such as vincristine and vinblastine and is expressed on the luminal (blood) side of brain capillary endothelial cells [12,13]. Moreover, Kusuhara et al. [36] reported that multidrug resistance-associated protein (MRP) is expressed at the BBB by Northern and Western blot analysis. These ATP-binding cassette (ABC) transporters are responsible for the asymmetrical transport of some compounds at the BBB. Therefore, the ABC transporters such as P-gp and MRP is also possible to be involved in the efflux transport of E_1S from the barin across the BBB.

Kakee et al. [15] reported the efflux transport of PAH, which is well known to be actively excreted at the kidney *via* organic anion transporter, across the BBB using the BEI method. However, the efflux transport system for E_1S at the BBB differs from that for PAH since the efflux transport of E_1S across the BBB was not inhibited by PAH. Although 5-HIAA, and HVA are endogenous cerebral organic anions [37], these compounds did not affect the E_1S efflux transport. Recently, a PNP-S transport system was found in rat hepatocytes [38], but this transporter may not be responsible for [^{3}H]E_1S efflux transport at the BBB since PNP-S, and MHPGS failed to inhibit [^{3}H]E_1S efflux (Table 2).

In the present study, we chose male rats as experimental animal due to the presence of estrogen sulfotransferase in male rat brain but not in female rat brain [6]. However, the efflux kinetics for E_1S might be different between male and female rats since E_1S is one of the female hormones. At present, it is not known whether the efflux kinetics are the same or different. Further studies including similar work with female rats are required to address these issues.

In conclusion, this is the first in vivo demonstration of a significant E_1S efflux transport process from the brain to the circulating blood at the BBB. It can be postulated that E_1S is transported by oatp2 at the BBB. This finding is very important for increasing the net influx of E_1S across the BBB since drug diffusion through the brain parenchyma is known to be significantly limited [39,40]. It is also important in helping us better understand how the BBB functions with regard to steroid hormone.

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