Active Efflux across the Blood-Brain Barrier: Role of the Solute Carrier Family

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Summary: The brain uptake of xenobiotics is restricted by the blood-brain brain barrier formed by brain capillary endothelial cells. Active efflux transport systems in the blood-brain barrier work as a detoxification system in the brain by facilitating removal of xenobiotic compounds from the brain. Drugs, acting in the brain, have to overcome such efflux mechanisms to achieve clinically significant concentration in the brain. Multiple transporters are involved in this efflux transport in the brain capillaries. In the past few years, considerable progress has been made in the cloning of these transporters and their functional characterization after heterologous expression. Members of the solute carrier family (SLC) play an important role in the efflux transport, especially for organic anions, which include organic anion transporting polypeptides (OATP/*SLCO*) and organic anion transporters (OAT/*SLC22A*). It is believed that coordination of the members of SLC family, and ABC transporters, such as P-glycoprotein, multidrug resistance protein, and breast cancer-resistant protein (BCRP/*ABCG2*), allows an efficient vectorial transport across the endothelial cells to remove xenobiotics from the brain. In this review, we shall summarize our current knowledge about their localization, molecular and functional characteristics, and substrate and inhibitor specificity. **Key Words:** Efflux, blood-brain barrier, organic anion transporter, OAT, OATP, ABC transporter.

INTRODUCTION

Brain capillary endothelial cells (BCEC) are characterized by the paucity of fenestra and pinocytotic vesicles and the highly developed tight junction between adjacent endothelial cells formed by a complex of integral membrane proteins (claudin, occuldin, and junctional adhesion molecules), and peripheral membrane proteins such as zonula occludens proteins.[1–3] These anatomical features restrict the nonspecific permeation of xenobiotics via the paracellular leakage and allow the BCEC to act as a static wall between the brain and circulating blood.[4] Due to this barrier function, the brain capillaries are referred to as the blood-brain barrier (BBB). In addition to the BBB, there is another barrier between the CSF and the blood in the ventricles called the blood-CSF barrier (BCSFB).[5] The capillaries in the choroid plexus, a tiny organ located in the ventricles that produces CSF, are leaky, and compounds in the blood circulation have free access to the basolateral surface of the choroid plexus epithelial cells. However, choroid plexus epithelial cells form a tight monolayer and act as a static wall behind the capillaries of the choroid plexus. Because the transcellular route across the BCEC or choroid plexus epithelial cells is the major pathway for exchanging compounds between the brain and CSF, and blood, compounds with a low intrinsic permeability across the lipid bilayer because of their large molecular weight or high hydrophilicity exhibit poor brain penetration without specific transport systems.

In addition, it is well accepted that active efflux mechanisms at the BBB restrict the brain penetration of xenobiotics.[6–9] P-glycoprotein (P-gp) is a well-known transporter acting as a gate keeper protein for xenobiotics at the luminal membrane of the BCEC.[10,11] P-gp is a primary active transporter, so-called ABC transporter, located on the luminal membrane of the BCEC and extrude hydrophobic neutral and cationic compounds, and certain kinds of organic anions to the blood side. Knockout of the P-gp gene causes a significant increase in the brain concentration of P-gp substrates.[11–14]

Apart from lipophilic compounds, transporters are involved both in uptake and efflux in the transcellular transport from the brain side to the blood-side. A number of transporter genes have been identified during the last decade, and almost 300 genes have been classified into

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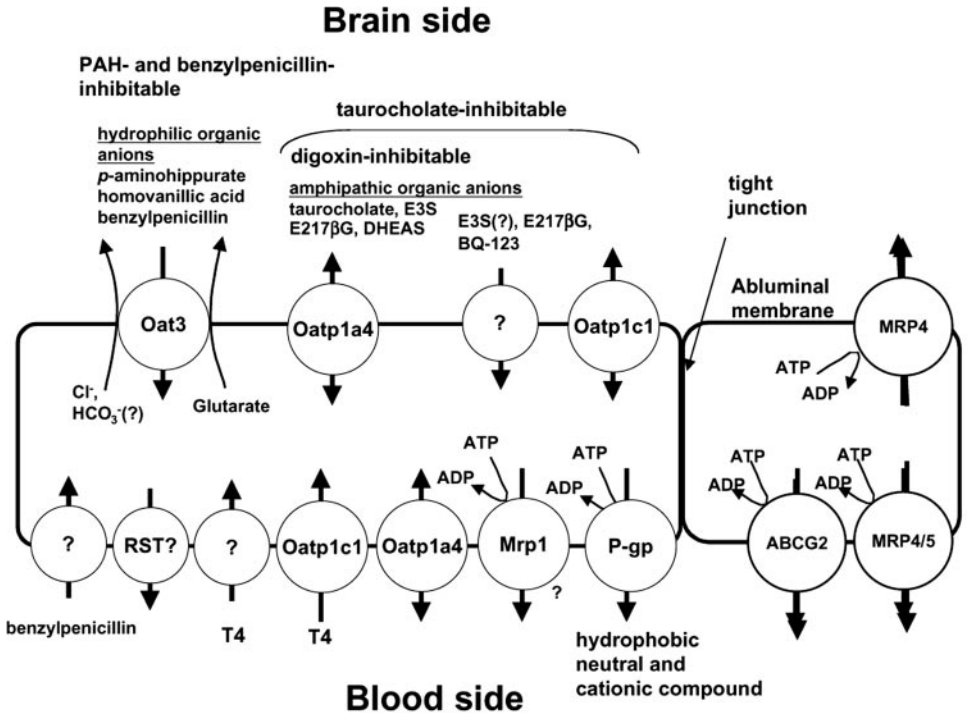

FIG. 1. Schematic diagram of the efflux transport mechanisms at the blood-brain barrier. *In vivo* experiments employing BEI method has suggested that the efflux mechanism for hydrophilic organic anions is accounted for by Oat3, and that for amphipathic organic anions is accounted for partly by Oatp1a4 (taurocholate- and digoxin-inhibitable pathway) and partly by unknown transporter. The unknown transporter is involved in the efflux of E3S, E217βG, and BQ-123. On the luminal membrane, Oatp1a4 and Oatp1c1 are involved in the uptake of amphipathic organic anions and peptide, and thyroxine (T4). E3S = estrone-3-sulfate; E217βG = estradiol 17β glucuronide.

43 families of the solute carrier (SLC) family (the list is available from the web site of BIOPARADIGMS URL: http://www.gene.ucl.ac.uk/nomenclature/). The details of the SLC members are available in the special issue of the *European Journal of Physiology* (volume 447, no. 5, 2004). The members of the SLCO/SLC21 and SLC22A family are characterized by their multispecificity and have been identified at the BBB. Cumulative studies have demonstrated that they play important roles in the efflux transport of organic compounds, especially organic anions, across the BBB. Fig 1 illustrates the efflux transport mechanisms at the BBB. The present manuscript focuses on the role of members of the SLCO/SLC21 and SLC22 in efflux transport at the BBB, which are summarized in Table 1.

SLCO/SLC21

Currently, the SLCO/SLC21 family includes fourteen members in rodents and human. Review articles of the Oatp/OATP family are available.[15,16] Of the fourteen members, Oatp1a4, Oatp1a5, and Oapt1c1, have been shown to be expressed in the brain capillaries (Table 1).

The molecular characteristics of Oatp1a4 and Oatp1a5

Oatp1a4 was originally isolated from brain and retina cDNAs using homology cloning.[17,18] The cDNA consists of 661 amino acids with an apparent molecular mass of 92 kDa, and hydropathy plot analysis predicts 12 putative transmembrane domains. Northern blot analysis revealed abundant expression in the brain where it is widely expressed.[17,18] Gao et al.[19] used an immunofluorescence method to show that its membrane localization was on both the luminal and abluminal membrane of the brain capillaries, and on the basolateral membrane of the choroid plexus epithelial cells.

Functional expression in *Xenopus laevis* oocytes and mammalian cells has revealed its broad substrate specificity including amphipathic organic anions such as bile acids and steroid conjugates, cardiac glycosides such as digoxin and ouabain, and bulky organic cations, such as N-(4,4-azo-n-pentyl)-21-deoxyajmalinium, N-methyl-quinidine, N-methyl-quinine and rocuronium, as well as anionic peptides, such as BQ-123, [D-Pen2,D-Pen5] enkephalin and deltorphin II.[17,18,20–22] The driving force for rOatp1a4-mediated uptake remains unknown. Li et al.[23] demonstrated that rOatp1a4-mediated transport exhibited *trans*-stimulation. Taurocholate uptake by oocytes expressing rOatp1a4 was increased in the presence of an outward concentration gradient of taurocholate, glutathione and its conjugates, and thus, glutathione is one candidate for the driving force. Conversely, the efflux of dinitropheny-glutathione was increased in oocytes expressing Oatp1a4 compared with that in control

TABLE 1. *Members of SLCO/SLC21A and SLC22A Families Expressed in the BBB and BCSFB*

Name	Gene Symbc	Species	LocusID	OMIM	Tissue Distribution	Membrane Localization	Transport Mechanism
SLCO/SLC21A family							
Oatp1a4	*Slco1a4*	Rat	170698		li, b, r, bc, cp	SM(li), LM/AM(bc), BLM(cp)	ND
Oatp1a5	*Slco1a5*	Rat	80900		k, r, b, bc, lu, si, cp	BBM (si, cp)	ND
OATP1A2	*SLCO1A2*	Human	6579	602883	b, bc, Low: k, li, lu, t	LM/AM(bc)	ND
Oatp1c1	*Slco1c1*	Rat	84511		b, bc, cp	LM/AM(bc), BLM (cp)	ND
OATP1C1	*SLCO1C1*	Human	53919		b, t	ND	ND
SLC22A family							
Oat1	*Slc22a6*	Rat	29509		k	BLM(k)	OA/dicarboxylate antiport
OAT1	*SLC22A6*	Human	9356	607582	k, cp	BLM(k)	ND
Oat3	*Slc22a8*	Mouse	19879		k	ND	ND
Oat3	*Slc22a8*	Rat	83500		li (male), k, b, e, cp	BLM(k), AM (bc), BBM(cp)	OA/dicarboxylate antiport
OAT3	*SLC22A8*	Human	9376	607581	k, cp	BLM(k)	OA/dicarboxylate antiport
Octn2	*Slc22a3*	Mouse	20520		Ubiquitously	ND	Na^+ symport (carnitine) H^+ antiport (OC)
Octn2/CT1	*Slc22a3*	Rat	29504		Ubiquitously	ND	Na^+ symport (carnitine) H^+ antiport (OC)
OCTN2	*SLC22A3*	Human	6581	603377	Ubiquitously	ND	Na^+ symport (carnitine) H^+ antiport (OC)
RST	*Slc22a12*	Mouse			k, bc, cp	BBM (k)	facilitative (OA), exchanger (urate)
URAT1	*SLC22A12*	Human	116085	607096	k	BBM (k)	exchanger (urate)

The old nomenclature of SLCO/SLC21 listed in this table is as follows: Oatp1a4, Oatp2; Oatp1a5, Oatp3; OATP1A2, OATP or OATP-A; Oatp1c1, Oatp14; OATP1C1, OATP-F.

li = liver; b = brain; r = retina; bc = brain capillary; cp = choroid plexus; k = kidney; lu = lung; si = small intestine; t = testis; e = eye; SM = sinusoidal membrane; lm = luminal membrane; AM = abluminal membrane; BLM = basolateral membrane; BBM = brush border membrane; ND = not determined; OA = organic anion; OC = organic cation.

oocytes or oocytes expressing Oatp1, suggesting the possibility that Oatp1a4 mediates efflux as well as uptake across the plasma membrane.[23]

Oatp1a5 was cloned from the rat retina cDNA library, the cDNA of which consist of 670 amino acids with a molecular mass of 80 kDa.[17] An RNase protection assay revealed its expression in the brain, small intestine, lung, and retina.[24] Li et al.[25] quantified the Oatp1a5 mRNA expression and found abundant expression in the lung, cerebellum, and female cerebral cortex and, to a lesser extent, in the intestine. RT-PCR analyses have shown that Oatp1a5 is expressed in the blood-brain barrier, and choroid plexus.[26,27] Its membrane localization in the brain capillaries remains unclear although immunofluorescence was found to be associated with brain capillaries.[27] Oatp1a5 is localized on the brush border membrane of the choroid plexus epithelial cells and responsible for the uptake of amphipathic organic anions from the CSF.[26] Functional expression studies of Oatp1a5 revealed its broad substrate specificity for amphipathic organic anions, such as bile acids and steroid conjugates, and thyroid hormones.[17,24,26,28]

There is only one human isoform (OATP1A2) that shows relatively high homology to rodent isoforms such as Oatp1a4 and Oatp1a5. OATP1A2 is predominantly expressed in the brain where it is ubiquitously distributed, and to lesser extent, in the lung, liver, kidney, and testis.[29] Immunofluorescence studies have suggested its expression at human brain capillary endothelial cells, although the membrane localization of OATP1A2 at the human BCEC remains unclear.[21] The substrate specificity of hOATP1A2 is broad and includes amphipathic organic anions, type II organic cations, and peptides such as [D-penicillamine2,5]enkephalin and deltorphin II.[21,30,31]

The efflux transport of amphipathic organic anions (Oatp substrates) across the BBB

Leininger et al.[32,33] have demonstrated a saturable efflux of 1-naphtyl 17β-glucuronide and a cyclic peptide, RC-160 (a somatostatin analog) by measuring the re-

TABLE 2. *Uptake and Efflux Clearance across the BBB*

	Efflux				Uptake		Transporters	
Compound	k_{eff} min^{-1}	Vd ml/g brain	CL_{eff} μl/min/g brain	K_m* μM	CL_{up} μl/min/g brain	K_m μM	K_m (rodents) μM	
Organic anions								
Taurocholate[34]	0.0233			65.3	Below limit of detection		Oatp1a4	35[17], 187[38]
							Oatp1a5	20[17], 30[24]
Homovalinic acid[52]	0.0169			298			Oat3	274[52]
PAH[51]	0.0587	0.8	47.0	396	12.1		Oat3	65[50], 400[55]
							Oat3	65[50], 400[55]
PAH[63]	0.039			168				
Benzylpenicilin[51]	0.043	0.8		29	9.1[64]		Oat3	85[55]
Glucuronide-conjugates								
Naphtol 17β-glucuronide[32]	0.0231							
E217βG[37]	0.037						Oatp1a4	3[18], 17[37]
							Oatp1a5	1.2[50], 40[28]
							Oatp1c1	10[44]
							Oat3	8,4[37]
Sulfate-conjugates								
Estrone sulfate[36]	0.063	1.1	69.3	96			Oatp1a4	11[18]
							Oatp1a5	268[28]
							Oat3	2[50], 5.3[61]
DHEAS[35]	0.0268	4.7	126	33	11.4		Oatp1a4	17[20]
							Oatp1a5	162[28]
							Oat3	12[61]
Indoxyl sulfate[53]	0.0108	0.95	10.3	298			Oat3	158[53]
Peptides								
RC-160[33]	0.0546							
BQ-123[34]	0.00783			482			Oatp1a4	30[20]
							Oatp1a5	420[28]
DPDPE[41]					0.5 (6)†	24	Oatp1a4	19[21]
							Oatp1a5	140[28]
Thyroid hormone (thyroxine)[45]					600	1	Oatp1a4	6.5[17]
							Oatp1a5	5[17]
							Oatp1c1	0.18 (rat)[44], 0.34 (mouse)[45]

*Parameters were obtained from *in vivo* Km values corrected by a dilution factor. †The uptake clearance determined in P-gp knockout mice. DPDPE = [D-penicillamine(2,5)]-enkephalin; GSH = glutathione.

maining radioactivity in the brain after microinjection into the cerebral cortex (Table 2). In addition, the brain efflux index method has been employed to investigate the efflux transport across the BBB of taurocholate, estrone sulfate, dehydroepiandrosterone sulfate, and E217βG (Table 2), which was characterized by saturation, except E217βG due to its low solubility in the buffer, and inhibition by simultaneously administered probenecid.[34–37] Inhibition studies were carried out to investigate the contribution of transporters to the efflux transport of E217βG after microinjection (FIG. 2).[37] Simultaneous injection of digoxin, a high affinity substrate of Oatp1a4, caused a 40% inhibition of the elimination of E217βG from the brain, whereas probenecid and taurocholate inhibited the efflux of E217βG almost completely. The degree of inhibition by digoxin includes the contribution of Oatp1a4, and thus, Oatp1a4 account for the elimination partly. Because the effect of *p*-aminohippurate (PAH), a typical substrate/inhibitor of Oat/OAT (see below), was minimal, but significant (~20% inhibition), a PAH-sensitive efflux mechanism, presumably Oat3 described later, is involved in this efflux partly. Another unidentified taurocholate-sensitive transporter makes a relatively similar contribution to Oatp1a4 (~40%).[37]

Kitazawa et al.[34] found that the efflux of taurocholate across the BBB after microinjection was inhibited by cyclic peptides such as BQ-123 and occtreotide as well as probenecid and cholate. Conversely, the efflux of BQ-123 across the BBB was inhibited by taurocholate. Mu-

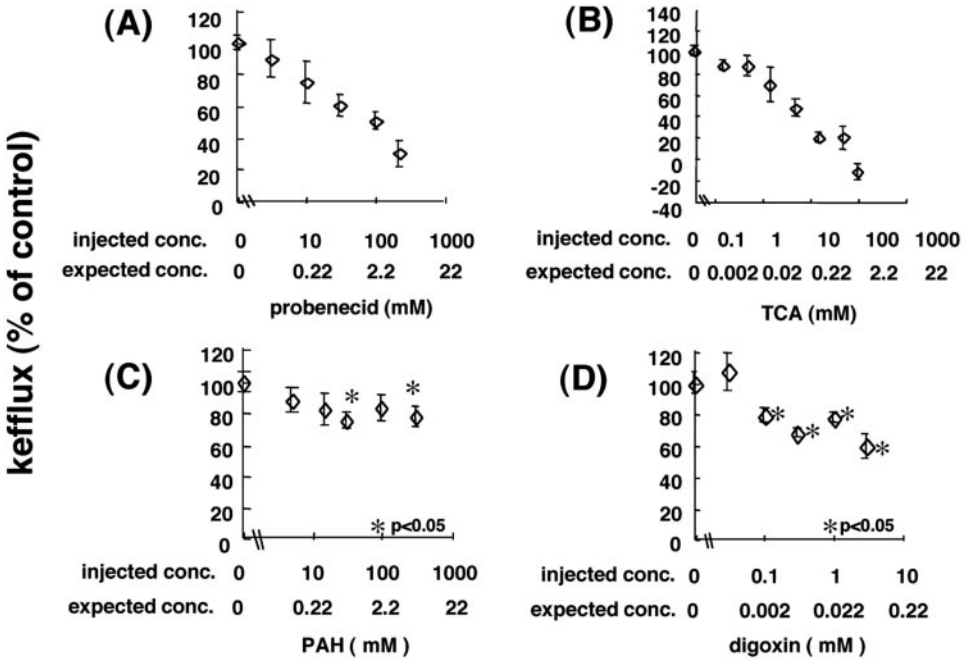

FIG. 2. Effect of probenecid, taurocholate, digoxin, and *p*-aminohippurate on the elimination of E217βG after intracerebral microinjection. E217βG is eliminated from the brain at a rate constant of 0.037 ± 0.001 min^{-1}. Probenecid (A), taurocholate (B), *p*-aminohippurate (C), and digoxin (D) were simultaneously microinjected into the cerebrum, and their effect on the elimination of E217βG from the cerebrum was examined. Each value of expected concentration is estimated by the concentration in the injectate divided by the dilution factor. Results are given as a ratio with respect to the elimination rate constant determined in the absence of unlabeled inhibitors. Each points represent mean ± SEM (n = 3). *, Significant different from the control ($p < 0.05$). Reproduced with permission from Sugiyama et al. Characterization of the efflux transport of 17β-estradiol-D-17β-glucuronide from the brain across the blood-brain barrier. *J Pharmacol Exp Ther* 298:316–322. Copyright© The American Society for Pharmacology and Experimental Therapeutics, 2001. All rights reserved.[37]

tual inhibition studies involving BQ-123 and taurocholate were carried out. Although the Michaelis constant (K_m) and IC_{50} values of taurocholate were not greatly different (0.4 *vs* 0.12 nmol/0.2 μl injectate, respectively), the corresponding parameters of BQ-123 showed a marked difference (2.9 and 0.074 nmol/0.2 μl injectate of BQ-123, respectively).[34] Thus, it is possible that taurocholate and BQ-123 are eliminated from the brain by different transporters. Their *in vivo* K_m values were roughly corrected by introducing a dilution factor to allow a comparison with the K_m values for Oatp1a4 (65 and 30 μM, respectively). The *in vivo* K_m value of taurocholate is within a range of the K_m value of Oatp1a4 (Table 2).[17,38] However, the *in vivo* K_m value of BQ-123 is much greater than that of Oatp1a4, whereas the IC_{50} value of BQ-123 for the efflux of taurocholate across the BBB is similar (Table 2). Taking these findings into consideration, it is likely that the efflux across the BBB is mediated by other transporter, although BQ-123 is a substrate of Oatp1a4.

Sulfoconjugates of steroids, such as estrone sulfate and dehydroepiandrosterone sulfate (DHEAS), undergo efflux across the BBB in intact form.[35,36] Especially, DHEAS is a so-called neurosteroid, which is synthesized from cholesterol locally in the neuron, and modulates neurotransmission,[39,40] and the efflux system for DHEAS has been considered to be one of inactivation mechanism.[35] The efflux clearance of DHEAS, estimated from the elimination rate constant and the uptake by brain slices, was markedly greater than the *in vivo* uptake clearance determined using the *in situ* brain perfusion technique (125 *vs* 11 μl/min · g brain) (Table 2).[35] The efflux of DHEAS was saturable with an *in vivo* K_m value similar to that of Oatp1a4 (Table 2) and was inhibited by probenecid, bile acids (taurocholate and cholate), and other sulfo-steroids (estrone sulfate and estradiol sulfate), whereas PAH had no effect. Estrone sulfate is another sulfate-conjugate whose efflux across the BBB was examined. Estrone sulfate also undergoes efflux across the BBB by a saturable mechanism and is inhibited by probenecid, bile acids, and DHEAS.[36] However, the *in vivo* K_m value of estrone sulfate was nine-fold greater than that for Oatp1a4 (Table 2), and thus, it is possible that the transporter responsible for the efflux transport of estrone sulfate is different from that of DHEAS.

In addition to efflux across the BBB, the luminal localization of Oatp1a4 at the BBB suggests its involvement in the uptake process. Dagenais et al.[41] demonstrated that the brain uptake of [D-penicillamine-2,5]-enkephalin was greater in Mdr1a-Pgp knockout mice (FIG. 3). The increased brain uptake in Mdr1a-P-gp

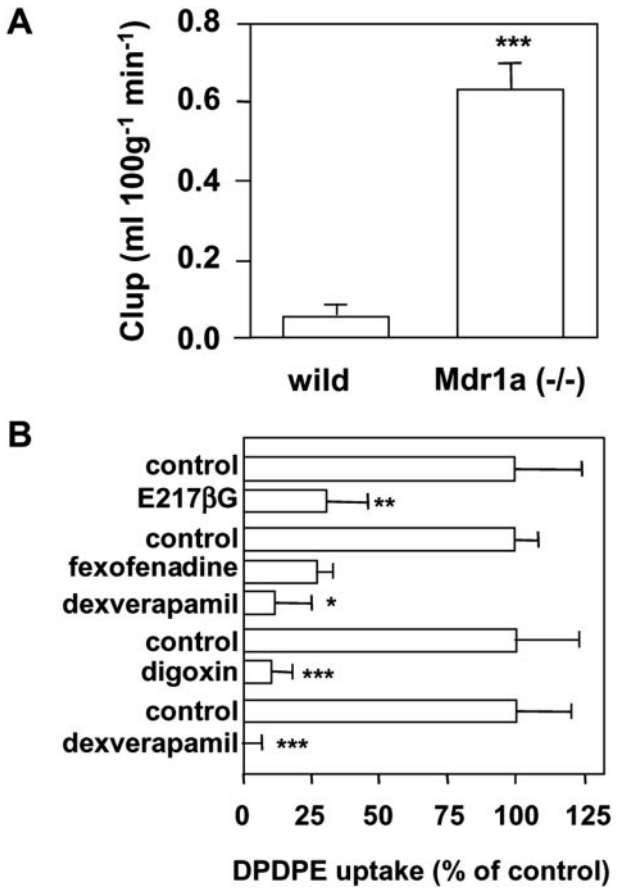

FIG. 3. Limitation of Oatp1a4-mediated uptake of [D-penicillamine-2,5]-enkephalin by P-gp at the BBB. A: The initial uptake clearance of [D-penicillamine-2,5]-enkephalin in the right cerebral hemisphere was determined in wild-type and Mdr1a P-gp knockout mice after 180 s of perfusion. Data are presented as mean ± SD (n = 4). ***, $p < 0.001$. B: The effect of inhibitors was examined for the brain uptake of [D-penicillamine-2,5]-enkephalin in Mdr1a P-gp knockout mice after 120 s of perfusion. The concentration used in this experiment is as follows: E217βG, 200 μM; fexofenadine, 150 μM; dexverapamil, 300 μM; digoxin 25 μM. Data are presented as mean ± SD (n = 4). ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$. Reproduced with permission from Dagenais et al. Uptake and efflux of the peptidic δ-opioid receptor agonist. *Neurosci Lett* 301:155-158. Copyright © 2001, Elsevier. All rights reserved. [41]

knockout mice was saturable with a K_m value of 24 μM, which is comparable with that of Oatp1a4 (19 μM[21]), and the involvement of Oatpla4 was supported by inhibition by Oatp1a4 substrates E217βG and digoxin (FIG. 3). Thus, it is likely that Oatp1a4 accounts for the luminal uptake of [D-penicillamine2,5]-enkephalin, although it undergoes active efflux by P-gp at the BBB and shows limited brain distribution. The brain uptake of another Oatp1a4 substrate, taurocholate, determined by the *in situ* brain perfusion method in rats did not show any significant uptake during the infusion period although the initial distribution volume was greater than that of inulin.[34] It may be possible that luminal efflux mechanism may limit the brain uptake of taurocholate at the BBB as in the case of [D-penicillamine2,5]-enkephalin, and knockout of the efflux transporter makes it possible to detect Oatp1a4-mediate uptake of taurocholate.

As *in vitro* model of the BBB, Hosoya et al.[42] have developed a conditionally immortalized cell line from a transgenic rat harboring the temperature-sensitive simian virus 40 large T-antigen. These cell lines express active large T-antigen and grow well at 33°C. They also grow at 37°C but not at 39°C; however their growth is restored when the temperature of the culture was lowered to 33°C. RT-PCR analysis showed that Oatp1a4 and Oatp1a5 are expressed in the cell line, at least at an mRNA level.[27,35] Significant uptake of DHEAS and digoxin was detected in TM-BBB4. The uptake of DHEAS by TM-BBB4 was saturable with a K_m value similar to that for Oatp1a4, and inhibited by amphipathic organic anions and digoxin, but not by PAH.[35] Therefore, it is likely that Oatp1a4 function is maintained in the cell line.

The molecular characteristics of Oatp1c1

Oatp1c1 was originally referred to as BBB-specific anion transporter 1 (BSAT1), which was isolated using gene microarray techniques by comparing the gene-expression profile of cDNA from the brain capillaries with that from the liver and kidney.[43] Oatp1c1 cDNA consists of 2148 bp that encode a 716-amino acid residue protein with 12 putative membrane-spanning domains. Oatp1c1 shows relatively low homology to Oatp1a4 (46% identity in amino acid levels). Northern blot analysis revealed its predominant expression in the brain,[43,44] and Oatp1c1-protein (90 kDa) was enriched in the brain capillary enriched fraction compared with brain homogenate.[44] Furthermore, immunohistochemical staining has demonstrated that Oatp1c1 is localized both on the luminal and abluminal membrane of the rat/mouse brain capillaries,[44] and the basolateral membrane of mouse choroid plexus.[45] Functional characterization using HEK293 cell expressing Oatp1c1 revealed that the substrates include organic anions, such as E217βG, cerivastatin and troglitazone sulfate, thyroxine, and its inactive metabolite [3,3′,5′triiodothyronine (reverse T3)].[44] Among these substrates, thyroxine and reverse T3 show the highest transport activity.[44]

Because the expression of Oatp1c1 has been reported to be regulated by plasma thyroid condition, it is suggested that Oatp1c1 plays an important role in the thyroxine transport at the BBB. Tohyama et al.[45] investigated the uptake mechanism of thyroxine by the brain using the *in situ* brain perfusion technique in mice, and found that thyroxine uptake by the brain is saturable. According to *in vitro* inhibition study using cDNA transfectant, taurocholate, estrone sulfate, and probenecid are inhibitors for Oatp1c1, but digoxin, PAH or benzylpenicillin are not. Thyroxin uptake by the brain was inhib-

ited by taurocholate completely, but estrone sulfate has partial effect. Therefore, Oatp1c1 accounts for the thyroxine uptake partly. Because digoxin and neutral amino acids had no effect, the contribution of Oatp1a4 and neutral amino acid transporter is minimal or none. OATP1C1 (SLCO1C1, OATP1C1), the human ortholog of Oatp1c1, is expressed in the brain where it is widely expressed except in the cerebellum, and in the testis where it is expressed in the Leydig cells.[46] Thyroxine and reverse T3 are substrates exhibiting high transport activities by OATP1C1 as well.[46] Whether OATP1C1 shows the same membrane localization at the BBB and choroid plexus remains to be elucidated.

SLC22

The SLC22 family includes organic cation transporters (Oct/OCT, *SLC22A1~3*), organic cation/carnitine transporters (Octn/OCTN, *SLC22A4, -5*) organic anion transporters (Oat/OAT, *SLC22A6~8, -10, -11*), and their related transporters such as URAT1/renal specific transporter (RST) (*SLC22A12*) and CT2 (*SLC22A16*). CT2 and URAT1/RST are transporters of carnitine and urate, respectively.[47,48] Among the members of the SLC22, OAT3, OCTN2, and RST have been shown to be expressed in the BBB (Table 1). This section focuses on the molecular characteristics of OAT3, OCTN2, RST, and briefly Oct/OCT family. Review articles about the Oat/OAT family are available.[49]

The molecular characteristics of Oat3/OAT3

Oat3 (*Slc22a8*) was cloned from rat brain cDNA library as homolog of organic anion transporter 1.[50] The cDNA consists of 536 amino acids with an apparent molecular mass of 92 kDa, and hydropathy plot analysis predicted 12 putative transmembrane domains. Oat3 shows the highest expression in the liver, and to lesser extent in the kidney, brain, and eye.[50] RT-PCR analysis using primers designed for three rat Oat mRNAs revealed the expression of Oat3 in the brain capillary enriched fraction, but the mRNA expression of other Oat isoforms, such as Oat1 and Oat2, was below the limit of detection.[51] Western blot analysis revealed the protein expression of Oat3 in the brain capillary enriched fraction with greater molecular mass than in the kidney.[51,52] Because the molecular mass is similar in the brain capillary and kidney after peptide *N*-glycarase F treatment, the difference in the apparent molecular mass is ascribed to the difference in glycosylation.[51] Immunochemical staining revealed abluminal localization of Oat3 in the brain capillaries,[51–54] however, faint signals were also reported on the luminal membrane of rat brain capillaries.[51] In addition, mRNA expression of Oat3 was detected in a immortalized rat BCEC (TR-BBB), although its protein expression remains unknown.[53] rOat3 is also expressed in brush border membrane of the choroid plexus,[55] whereas both OAT1 and OAT3 have been shown to be expressed in the human choroid plexus by immunohistochemical staining, although their membrane localization remains to be clarified.[56]

Functional expression in *X. laevis* oocytes and mammalian cells has revealed that Oat3 has a broad substrate specificity including amphipathic organic anions, such as E217βG, estrone sulfate, and dehydroepiandrosterone sulfate, hydrophilic organic anions, such as PAH and benzylpenicillin, and the organic cations, cimetidine and ranitidine.[50,55,57] Estrone sulfate uptake and efflux via Oat3 are not trans-stimulated by ochratoxin A, PAH, or estrone sulfate in Oat3-cRNA injected X. oocytes,[50] whereas Sweet et al.[58] demonstrated that estrone sulfate and PAH uptake by Oat3-expressing oocytes was stimulated by an outward concentration gradient of glutarate formed by coexpression of the sodium-dicarboxylate cotransporter (NaDC-1). The efflux of glutarate from inside the cells was greater in OAT3-expressing oocytes than in control oocytes and was stimulated by extracellular OAT3 substrates, such as α-ketoglutarate, glutarate, PAH, cimetidine, and urate. Therefore, it is likely that Oat3/OAT3 is an exchanger and an outward concentration gradient of dicarboxylates, such as α-ketoglutarate, formed by the NaDC and the tricarboxylate cycle, may drive Oat3/OAT3-mediated transport.

The Oat3 knockout mouse was established by Sweet et al.,[59] and this allows more direct insight into its role in the kidney and CP. This mouse strain is healthy and exhibits no significant physiological abnormalities compared with the corresponding wild-type mouse. Most of the uptake of amphipathic organic anions, such as taurocholate and estrone sulfate, is markedly reduced in kidney slices from the Oat3 knockout mouse compared with that from the wild-type mouse.[59] In addition, the accumulation of fluorescein is markedly reduced in the isolated CP from the Oat3-knockout mouse. In contrast, knockout of Oat3 gene did not affect the uptake of fluorescence-labeled methotrexate by isolated brain capillaries; however, the effect of knockout of Oat3 on the efflux transport of other Oat3 substrates remains to be elucidated. The microinjection technique that introduces test compounds into the cerebral cortex has been carried out in mice.[33,60] Comparing the elimination curve in wild-type and Oat3 knockout animals will reveal any involvement of Oat3 in efflux across the BBB.

The efflux transport of Oat3/OAT3 substrates across the BBB

It has been suggested that Oat3 plays a major role in the efflux transport of other Oat3 substrates, such as PAH, benzylpenicillin, indoxylsulfate, and homovanillic acid,[51–53] but only a limited role in the efflux transport of amphipathic organic anions such as estradiol 17β-gluco-

ronide (E217βG), DHEAS, and estrone sulfate, although it plays major role in the renal uptake of these amphipathic organic anions.[59,61,62]

PAH was found to be eliminated in a saturable manner from the cerebral cortex after microinjection.[63] The efflux clearance of PAH across the BBB (47 μl/min/g brain) was estimated by multiplying the elimination rate constant (0.059 min^{-1}) and the steady state uptake by brain slices (0.8 ml/g brain). The uptake clearance of PAH across the BBB was evaluated by the *in vivo* carotid artery injection technique and found to be below the uptake clearance of inulin (12 *vs* 17 μl/min/g brain, respectively) (Table 2). Thus, the efflux clearance of PAH across the BBB is greater than the uptake clearance. The efflux was saturable with a K_m value, corrected by a dilution factor, very close to its K_m value by the isolated rat choroid plexus (396 *vs* 354 μM) in which PAH uptake is suggested to be by Oat3 (Table 2); this is based on a comparison of kinetic parameters including the Ki values of inhibitors of the uptake by the isolated choroid plexus and Oat3.[55]

Benzylpenicillin is eliminated from the brain with similar elimination rate constant to that of PAH via a saturable mechanism.[51] Mutual inhibition studies were carried out to examine whether the efflux of PAH across the BBB shares the same transporter as benzylpenicillin.[51] The K_m value of PAH was comparable with its K_i for the efflux transport of benzylpenicillin, and vice versa. In addition, cimetidine and pravastatin, inhibitors of Oat3, significantly inhibited the efflux both of PAH and benzylpenicillin to a similar degree. Thus, this suggests that PAH and benzylpenicillin efflux is mediated by the same transporter at the BBB. The uptake of benzylpenicillin by the brain was evaluated in rat using the *in situ* brain perfusion technique (9 μl/min/g brain), and found to be a saturable mechanism accounting for most of the uptake.[64] If the distribution volume of benzylpenicillin in the brain is assumed to be similar to that of PAH, the efflux clearance across the BBB will be greater than the uptake clearance.

PAH, benzylpenicillin, and cimetidine significantly inhibit the efflux transport of homovanillic acid and indoxylsulfate across the BBB after microinjection into the cerebral cortex, and it is likely that the efflux transport of these compounds is mediated by the same transporter of PAH and benzylpenicillin, presumably Oat3. Homovanillic acid is one of the metabolites of dopamine. In the *X. laevis* oocyte expression system, the metabolites of dopamine and serotonin have been suggested to be substrates of Oat3 because they inhibit the uptake of estrone sulfate by rOat3.[50] Oat3 may be involved in the elimination of these inactivated metabolites from the brain.

The molecular characteristics of RST

RST was originally isolated from mouse kidney using the signal sequence trap method without any functional report[65] and is a mouse homolog of human urate transporter (URAT1) (74% identity at an amino acid level). Although Northern blot analysis demonstrated its predominant expression in the kidney, RT-PCR and Western blot analyses detected its expression in the brain capillary-enriched fraction and choroid plexus.[66] Thus, it is likely that the expression of RST is restricted to the brain capillaries and choroid plexus where its membrane localization remains to be elucidated. mRST-mediated uptake of organic anions was increased by substituting K^+ for Na^+ or by oubain- and Ba^{2+} treatments.[66] Because these treatments cause a depolarization of membrane voltage, RST has been hypothesized to be a facilitative transporter. Furthermore, introduction of RST cDNA to the LLC-PK1, an epithelial cell line from percine kidney, expressing Oat3 at the basal membrane increased the basal-to-apical transport of benzylpenicillin and urate (FIG. 4),[66] suggesting that it can mediate the efflux. In the BBB and BCSFB, RST may be involved in the efflux transport of organic anions across the BBB and BCSFB by coordination of Oat3. Using confocal microscopy, Breen[67] suggested that the excretion of fluorescein across the basolateral membrane of choroid plexus epithelial cells is membrane-voltage dependent. It is possible that RST accounts for this excretion in the choroid plexus, and coordination of Oat3 and RST allows vectorial transport across the BBB and BCSFB.

The molecular characteristics of the Octn/OCTN family

Three isoforms (Octn1~Octn3, *Slc22a4, -5*) have been characterized in rodents, whereas two isoforms (OCTN1 and OCTN2) have been characterized in humans. The Octn/OCTN family has been reviewed recently.[49]

Octn2 expression has been detected in primary cultured rat BCEC as well as whole brain.[68] Octn2/OCTN2 has been characterized as a sodium-dependent carnitine transporter,[69,70] although it also transports tetraethylammonium (TEA) with lower transport activity compared with that of carnitine.[71] Octn2 is hereditarily deficient in the juvenile visceral steatosis (*jvs*) mouse,[72] an animal model of primary systemic carnitine deficiency (OMIM 212140) caused by a single nucleotide mutation of Octn2 gene.[72] The functional involvement of Octn2 in the brain uptake of carnitine was investigated in normal and *jvs* mouse,[68,73,74] and it was found that functional loss of Octn2 is associated with decreased brain concentration of acetyl-carnitine.[73] Therefore, luminal localization at the BBB has been suggested, although its membrane localization in the brain capillaries has not been determined. Octn2 has been suggested to be involved in the renal elimination of organic cations because the renal

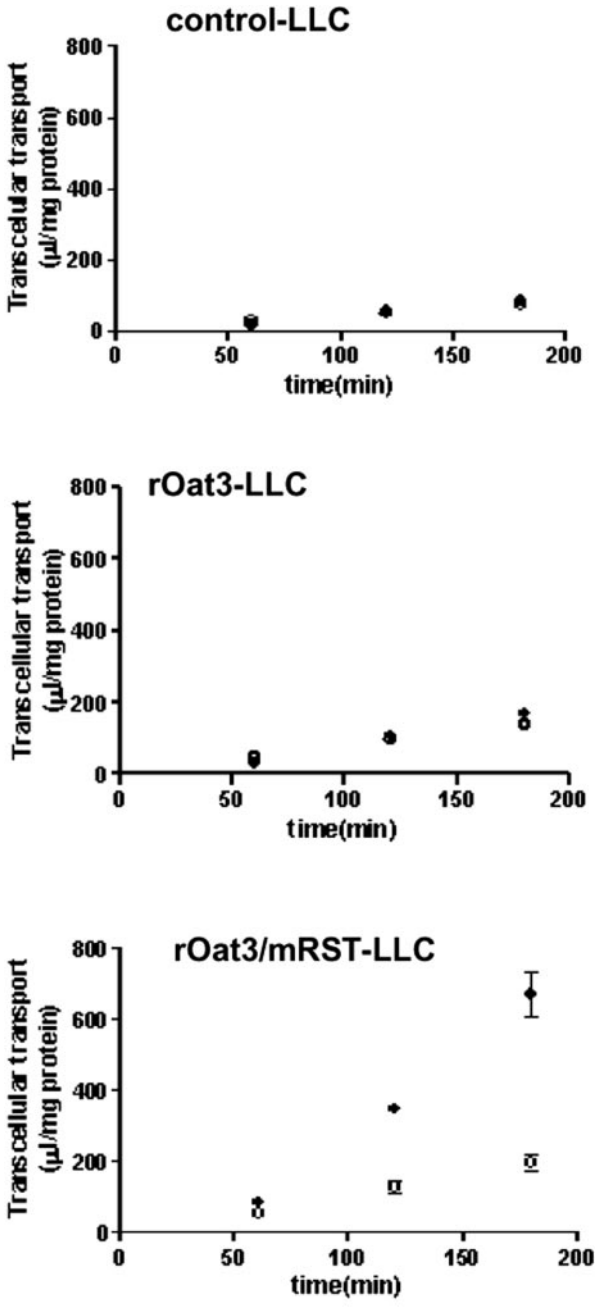

FIG. 4. Vectorial transport of benzylpenicillin in the double transfectant of rat Oat3 and mouse RST in LLC-PK1. Transcellular transport of benzylpenicillin (0.5 μM) across LLC-PK1 monolayers expressing rOat3 and both rOat3 and mRST (rOat3/mRST: double transfectant) was compared with that across the control LLC-PK1 monolayer. *Open* and *closed circles* represent the transcellular transport in the apical-to-basal and basal-to-apical directions, respectively. Each *point* represents the mean ± SEM (n = 3). Reproduced with permission from Imaoka et al. The renal-specific transporter mediates facilitative transport of organic anions at the brush border membrane of mouse renal tubules. *J Am Soc Nephrol* 15:2012-2022. Copyright © 2004, Lippincott Williams & Wilkins. All rights reserved. [66]

clearance of TEA was significantly decreased in *jvs* mouse compared with that in normal mice.[75] Luminal localization of Octn2 may play a role in the efflux of organic cations in an exchange of plasma carnitine and/or acethyl-carnitine because carnitine and TEA exhibit mutual trans-stimulation.[75]

The molecular characteristics of the Oct/OCT family

The OCT-mediated uptake is facilitative transport characterized by its membrane voltage dependence, and the isoforms of the Oct/OCT family show broad substrate specificity to small and hydrophilic organic cations, such as TEA and N-methylnicotineamide.[49,76] The expression of the Oct/OCT isoforms at the BBB remains unknown, whereas they are expressed in the choroid plexus, although the isoform expressed in the choroid plexus is controversial. RT-PCR analysis by Sweet et al.[77] detected the expression of Oct2 (*Slc22a2*) and Oct3 (*Slc22a3*) mRNA, but not Oct1 (*Slc22a1*) mRNA; however, mRNA quantification by Choudhuri et al.[78] revealed low-level expression of Oct1 and Oct3 in the rat CP, and the expression level of Oct2 mRNA was below the limit of detection. Oct1-, Oct2-, and Oct3-, as well as Oct1/Oct2 double knockout mice, have been developed, and these will be a useful animal model for examining the involvement of Oct1, Oct2, and Oct3 in the efflux transport of organic cations across the BBB.[79–81]

Overlapped substrate specificity between the members of SLC families and ABC transporters

ABC transporters have been characterized by one or two cytoplasmically located nucleotide binding domains acting as a catalytic domain for nucleotide hydrolysis. Some ABC transporters, which show broad substrate specificity, have been identified on the luminal membrane of the BCEC including P-glycoprotein, multidrug resistance-associated protein (MRP) -1, -2, -4, and -5 (ABCC1/ABCC2/ABCC4/ABCC5),[60,82,83,83a] and breast cancer resistant protein (BCRP/*ABCG2*).[84–86] The molecular characteristics of these ABC transporters have been reviewed.[87–90] This section focuses on the overlap of substrate specificity between Oatps/Oats and ABC transporters.

There is coordination of the members of SLC family and ABC transporters in the hepatobiliary transport,[12,90,91] and the renal secretion[92,93] of organic anions. Sasaki et al.[94] and Cui et al.[95] clearly demonstrated this coordination *in vitro* by introducing both OATP1B1/OATP1B3 and MRP2 to a polarized cell line (MDCK-II cells), and Mita et al.[96] have succeeded in establishing at cell line expressing Ntcp and Bsep in LLC-PK1 cells for hepatobiliary transport of bile acids.

Studies involving the BBB have shown that such coordination of ABC and SLC transporters also occurs in the BBB. As described previously, it has been suggested that the luminal uptake of [D-penicillamine-2,5]-enkephalin is accounted for by Oatp1a4, and it also undergoes efflux by P-gp at the BBB resulting a small brain uptake (FIG. 3).[41] In addition, fexofenadine, antihista-

TABLE 3. *Substrates of ABC Transporters Expressed in the BBB*

ABC Transporters	Localization	Substrates
P-gp/*ABCB4*	Luminal	Lipophilic neutral and cationic compounds E217βG, methotrexate, fexofenadine, DPDPE
MRP1/*ABCC1*	Luminal	glutathione- and glucuronide-conjugates estrone sulfate (+GSH)
MRP2/*ABCC2*	Luminal	Glutathione- and glucuronide-conjugates non conjugated amphipathic organic anions cyclic peptide (BQ-123)
MRP4/*ABCC4*	Luminal/abluminal	Steroids conjugated with sulfate and glucuronide (DHEAS and E217βG) prostaglandins, methotrexate, and folate nucleotides and nucleoside analogus (adfovir and 6-mercaptopurine)
MRP5/*ABCC5*	Luminal	cAMP, cGMP
BCRP/*ABCG2*	Luminal	Anticancer drugs (doxorubicin, mitoxantrone, topotecan and SN-38) glucuronide conjugates (E217βG, 4-methylumbelliferone glucuronide and E3040 glucuronide) sulfate conjugates (estrone sulfate, estradiol sulfate, DHEAS, 4-methylumbelliferone sulfate, and E3040 sulfate)

GSH = glutathione.

mine drug, is a substrate of P-gp and Oatp1a4,[97] and its brain uptake is increased in Mdr1a P-gp knockout mice.[97] It is possible that the luminal uptake of fexofenadine is mediated by Oatp1a4. In addition to P-gp, Sugiyama et al.[60] have suggested an involvement of Mrp1 in the luminal efflux of amphipathic organic anions by comparing the concentration time profile of E217βG after microinjection into the cerebral cortex between wild-type and Mrp1 knockout mice. It has been suggested that the uptake process at the abluminal membrane is accounted for by multiple transporters as described in the previous section, and thus, a coordination of uptake transporters and Mrp1 will account for the vectorial transport from the brain-to- blood side, at least partially together with P-gp. Mrp1 substrates include glucuronide and glutathione-conjugates, and estrone sulfate in the presence of glutathione, which are shared by the members of the SLCO/SLC21A and SLC22A families.

For other ABC transporters, there is no *in vivo* evidence supporting their involvement in efflux transport across the BBB (see *Note Added in Proof*), although there is conflicting evidence for the expression of Mrp2 in the BBB.[60,82] The substrate specificity of MRP2, MRP4, MRP5, and BCRP is summarized in Table 3. Because the substrate specificities show an overlap, they could be involved in the efflux transport at the luminal membrane of the BBB. Knockout animals have been established for Bcrp[98] and Mrp4,[99] which will allow us to investigate their contribution to the total efflux process.

CONCLUDING REMARKS

This review has summarized the recent progress in understanding the efflux transport systems at the BBB. Multiple transporters have been identified on the brain capillaries, and operate as detoxification system in the brain for a variety of natural and synthetic organic chemicals. They prevent drugs designed to treat CNS diseases from achieving clinically significant concentration in the brain. Overcoming the efflux systems at the BBB is one of the important topics for future research. Transporters on the luminal membrane, such as Oatp1a4 and Oatp1c1, may be available to deliver drugs to the CNS, and as in the case of [D-penicillamine-2,5]-enkephalin, and other P-gp substrates, overcoming efflux transporters at the luminal membrane will improve the pharmacological effect. Adachi et al.[100] have shown a correlation between transepithelial transport across a monolayer of LLC-PK1 expressing P-gp, and the ratio of the brain concentration between wild-type and Mdr1a P-gp knockout mice, which will allow us to quantitatively evaluate the contribution of efflux transporter at the BBB using *in vitro* studies. Such and *in vitro* system, including transfectants, will be very useful for other MRPs and BCRP as well as examining *in vitro*/*in vivo* correlations.

Interindividual difference in the efflux transport activity at the BBB due to genetic polymorphisms may give us a clue to understanding interindividual difference in drug response in addition to the interindividual difference in pharmacokinetics. Hoffmeyer et al.[101] demonstrated that there is a genetic polymorphism associated with P-gp expression in the intestine, and subsequent reports have demonstrated that this is true in other organs[102]. Whether it affects P-gp expression in the BBB remains to be elucidated, but it may be that such a genetic polymorphism may account for the interindividual difference in P-gp function at the BBB leading to interindividual difference in drug response. Whether genetic polymorphisms are associated with the function of transporters remains to be elucidated.

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Note added in proof: During preparation of this article, Mrp4 was shown to be involved in the active efflux at the BBB using Mrp4 knockout mice in which the elimination of topotecan from the brain has been significantly delayed.[83a]

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