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Expert Review

Contribution of Carrier-Mediated Transport Systems to the Blood–Brain Barrier as a Supporting and Protecting Interface for the Brain; Importance for CNS Drug Discovery and Development

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Abstract. The blood–brain barrier (BBB) forms an interface between the circulating blood and the brain and possesses various carrier-mediated transport systems for small molecules to support and protect CNS function. For example, the blood-to-brain influx transport systems supply nutrients, such as glucose and amino acids. Consequently, xenobiotic drugs recognized by influx transporters are expected to have high permeability across the BBB. On the other hand, efflux transporters, including ATP-binding cassette transporters such as P-glycoprotein located at the luminal membrane of endothelial cells, function as clearance systems for metabolites and neurotoxic compounds produced in the brain. Drugs recognized by these transporters are expected to show low BBB permeability and low distribution to the brain. Despite recent progress, the transport mechanisms at the BBB have not been fully clarified yet, especially in humans. However, an understanding of the human BBB transport system is critical, because species differences mean that it can be difficult to extrapolate data obtained in experimental animals during drug development to humans. Recent progress in methodologies is allowing us to address this issue. Positron emission tomography can be used to evaluate the activity of human BBB transport systems *in vivo*. Proteomic studies may also provide important insights into human BBB function. Construction of a human BBB transporter atlas would be a most important advance from the viewpoint of CNS drug discovery and drug delivery to the brain.

KEY WORDS: blood–brain barrier; brain capillary endothelial cells; carrier-mediated transport; efflux transport; influx transport; pharmacoproteomics.

INTRODUCTION

In clinical development, candidate CNS-acting drugs have the poorest success rate (1). Development of more than 98% of such candidates has had to be discontinued because of poor permeability across the blood–brain barrier (BBB), and this presents a major problem to the pharmaceutical industry (2,3). To overcome this, it is necessary to understand the molecular basis of transport functions at the BBB, and to utilize this knowledge during drug development.

To cross the BBB, molecules have to cross the brain capillary endothelial cells, so that permeation across the plasma membrane is the step that determines the permeability across the BBB. Simple diffusion of small molecules across the plasma membrane of brain capillary endothelial cells is dependent on their lipophilicity, so increasing the lipophilicity of such molecules is an efficient strategy to increase the BBB permeability, and has been applied to drug development in the pharmaceutical industry for a long time. However, recent progress in BBB research has revealed that multiple transporters are expressed at the BBB, and influence the BBB permeability of various small molecules that are their substrates. This has opened the door to a range of molecular-based strategies for drug development and drug targeting; that is, development of drugs that are well distributed to the brain can be achieved by incorporating structures that are recognized by the blood-to-brain influx transport systems, or are not recognized by the brain-to-blood efflux systems.

Fig. 1 illustrates three typical BBB transport modes which involve transporters expressed on the plasma membrane of brain capillary endothelial cells. One is the blood-to-brain influx transport system that supplies nutrients, including glucose, amino acids and nucleotides, to the brain. The second one is the drug efflux pump that prevents entry of xenobiotics into the brain by pumping them out into the circulating blood, and the third is the brain-to-blood efflux transport system that acts to eliminate metabolites and neurotoxic compounds from brain interstitial fluid. These systems function cooperatively as a CNS supporting and protecting system. In other words, the BBB is a barrier against many drugs, and recognition by the various BBB transport systems greatly influences drug distribution into the brain. Therefore, an understanding of the molecular mechanisms of BBB transport (Table I and Fig. 2) is critical for efficient drug delivery and drug targeting to the brain. In this review, we will highlight progress in studies on the molecular mechanisms of the carrier-mediated transport systems at the BBB in relation to their importance in influencing the BBB permeability of drugs.

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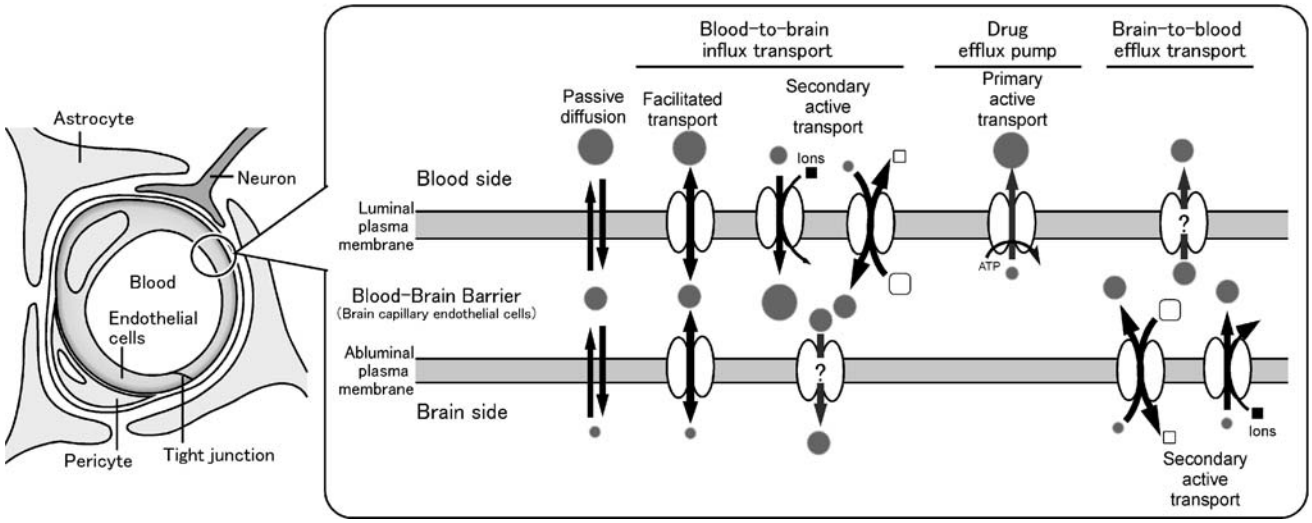

Fig. 1. Transport system at the BBB. The transport system at the BBB can be classified into three categories; blood-to-brain influx transport, drug efflux pump and brain-to-blood efflux transport. The abluminal transporter in the influx transport system and the luminal transporter in efflux transport, which are marked by *a question mark* are not well characterized.

BLOOD-TO-BRAIN INFLUX TRANSPORTERS AS DRUG DELIVERY PATHWAYS AND AS TARGET PROTEINS FOR CNS DRUG DISCOVERY

The blood-to-brain influx transporters supply hydrophilic nutrients and other essential molecules, such as glucose and amino acids, to the brain (Table I and Fig. 2a). Hence, the development of drugs that structurally mimic substrates of influx transport is an effective strategy to increase the BBB permeability. Table II summarizes the parameters of the blood-to-brain influx transport of various compounds. The influx permeability rates vary by up to 2,000-fold (3,700 μl min^{-1} g^{-1}

Table I. Transporters and Transport Systems Expressed at the BBB

Transporters	Substrates	Localization	Direction
Energy transport system			
GLUT1	D-Glucose	L, A	In
MCT1	L-Lactate, monocarboxylates	L, A	In
CRT	Creatine	L, A	In
Amino acid transport system			
LAT1/4F2hc[system L]	Large neutral amino acids	ND	In
CAT1 [system y^+]	Cationic amino acids	ND	In
EAAT1, 2, 3	Anionic amino acids	A	Ef
ASCT2	L-Asp, L-Glu	A	Ef
ATA2 [System A]	Small neutral amino acids	ND	Ef
xCT/4F2hc [System x_c^-]	L-Cystine/L-Glu	ND	In
TAUT [System β]	Taurine, β-alanine	ND	In, Ef
System ASC/system B^{0+}	L-Ala and others	ND	Ef
Neurotransmitter transport system			
GAT2/BGT1	γ-Aminobutyric acid	ND	Ef
SERT	Serotonin	L, A	ND
NET	Norepinephrine	A	ND
Organic anion transport system			
OAT3	PAH, HVA, indoxyl sulfate	A	Ef
oatp2	Digoxin, organic anions	L, A	In, Ef
oatp14	Thyroid hormones	ND	In
OCTN2	Carnitine	ND	In
Nucleoside transport system			
CNT2	Nucleosides	ND	In
ABC transporters			
ABCA1	Cholesterol	ND	ND
ABCB1/MDR1	Vincristine, cyclosporin A	L	Ef
ABCC4/MRP4	Topotecan	L	Ef
ABCG2/BCRP	Mitoxantron, topotecan	L	Ef

Localization; luminal side (L) and abluminal side (A) of brain capillary endothelial cells. Direction; blood-to-brain influx transport (In) and brain-to-blood efflux transport (Ef). *ND* Not determined. The table is taken from Ohtsuki *et al.* (105) with some modification.

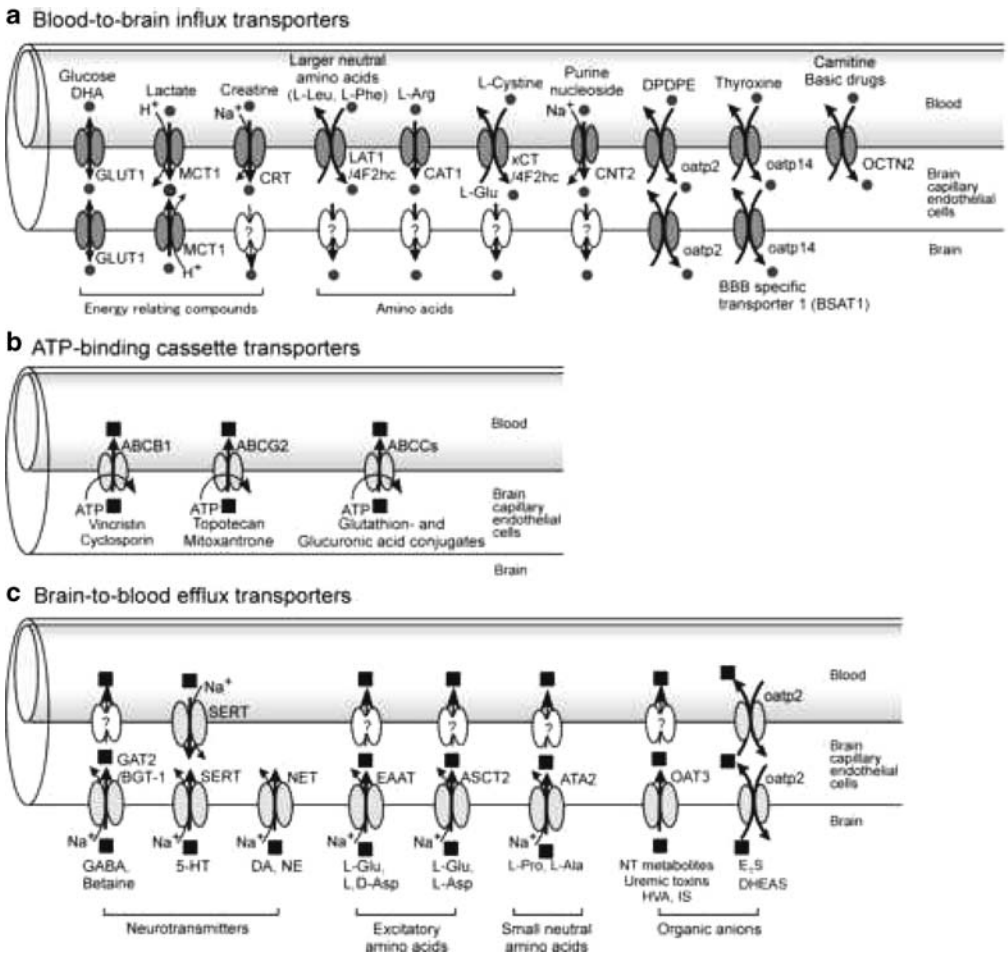

Fig. 2. Transporters at the BBB. **a** Transporters mediating the blood-to-brain influx transport. **b** ATP-binding cassette (ABC) transporters as the drug efflux pumps. **c** Transporters mediating the brain-to-blood efflux transport.

brain for L-phenylalanine vs 1.6 μl min^{-1} g^{-1} brain for creatine; Table II), depending on the transport system involved.

L-Tyrosine, L-tryptophan, and L-histidine are precursors of neurotransmitters, and are transported from the blood to the brain via a Na^+-independent neutral amino acid transporter (system L) at the BBB (Table II). System L also transports L-leucine, L-isoleucine, L-valine, L-methionine, L-threonine, and L-phenylalanine as substrates for protein synthesis in the brain. System L consists of LAT1 (SLC7A6) and the heavy chain of the 4F2 cell-surface antigen (4F2hc), and LAT1 is selectively expressed at the BBB (4,5). Several amino acid-mimetic drugs are transported across the BBB by system L, and the influx permeability rates of L-leucine and L-phenylalanine are greater than that of glucose (Table II). Therefore, system L is potentially important for drug delivery to the brain. L-Dopa is transported across the BBB by system L, and is readily biotransformed in the brain to dopamine (5,6). The alkylating agent melphalan (phenylalanine mustard), the antiepileptic drug gabapentin, and the muscle relaxant baclofen are substrates of system L, and are transported into the

Table II. Parameters of the Blood-to-Brain Influx Transport System at the BBB

	Plasma Concentration (μM)	Km (μM)	Vmax (nmol min^{-1} g^{-1} brain)	Influx Permeability Rate (μl min^{-1} g^{-1} brain)	Reference
L-Phenylalanine	80	11	41	3,700[a]	(106)
L-Leucine	180	26	64	2,500[a]	(107)
Mepyramine		4,400	7,120	1,620[a]	(20)
L-Arginine	120	56	24	429[a]	(108)
L-Dopa				190	(109)
Glucose	5,500	11,000	1,420	129[a]	(24)
Lactate	1,100	1,800	91	51[a]	(24)
Adenine	10	11	0.5	45[a]	(24)
Taurine				37	(36)
Creatine	50–200			1.6	(26)

[a] Influx permeability rate was determined by dividing Vmax by K_m, indicating maximum influx permeability rate.

brain (6,7). System L at the BBB is saturated by endogenous amino acids under normal conditions, since the half-saturation concentration (K_m) for system L are smaller than the plasma concentrations of neutral amino acids (Table II). Although substrate drugs are thought to be continuously transported from the blood to the brain, a high-protein diet reduces the concentration of these drugs in the brain due to competitive inhibition at the BBB. Indeed, it has been reported that a high-

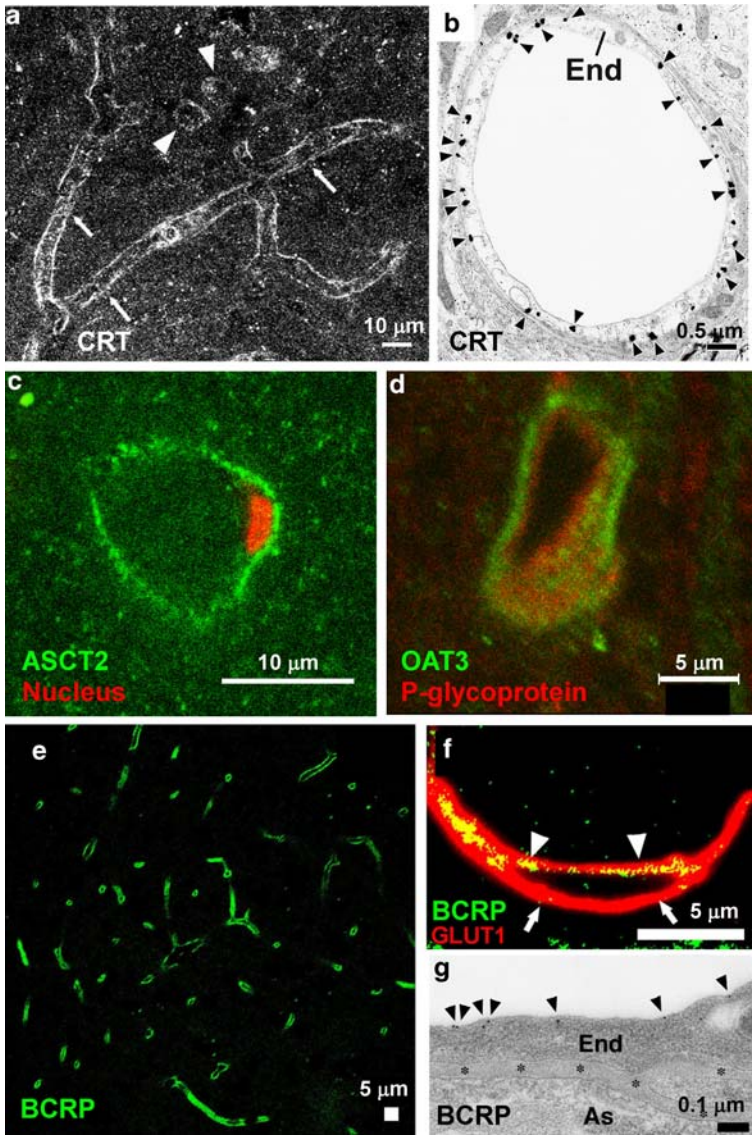

Fig. 3. Localization of transporters at brain capillary endothelial cells. **a**, **b** Localization of CRT (26,27). **a** Intense immunofluorescence of CRT at brain capillaries (*arrows*), with moderate staining in neuronal perikarya (*arrowheads*). **b** Immunogold labeling of CRT in a cortical capillary. Immunogold particles (*arrowheads*) are associated with both the luminal and abluminal cell membranes of mouse brain capillary endothelial cells (*End*). **c** Immunofluorescence of ASCT2 (*green*) was detected at the abluminal membrane of mouse brain capillary endothelial cells (56). Nuclei were stained with propidium iodide (*red*). **d** Double immunostaining for OAT3 (*green*) and P-glycoprotein (*red*) in rat brain. OAT3 and P-glycoprotein were detected at the abluminal and luminal membranes of brain capillary endothelial cells, respectively (71). **e**–**g** Localization of BCRP (54). **e** Intense immunofluorescence of BCRP was detected at brain capillaries. **f** Double immunostaining of BCRP (*green*) and GLUT1 (*red*). The overlap of BCRP staining and GLUT1 staining is shown in *yellow*. GLUT1 was detected at both luminal and abluminal membranes, while BCRP was found only at the abluminal membrane. **g** Post-embedding immunogold electron microscopy for BCRP in the adult cerebral cortex. BCRP was preferentially localized along the luminal cell membrane of capillary endothelial cells (*End*, *arrowheads*). *Asterisks* indicate basement membranes. *As* Astrocytic process.

protein diet increased the plasma levels of neutral amino acids and decreased the effect of L-dopa (8).

D-Glucose, which is the main energy source for the brain, is transported from the circulating blood to the brain via an Na^+-independent glucose transporter, GLUT1 (SLC2A1), at the BBB. GLUT1 is a facilitated transporter of hexoses and is localized on the luminal (blood) and abluminal (brain) sides of the BBB [Fig. 3f (9)]. It also transports L-dehydroascorbic acid, an oxidized form of L-ascorbic acid (vitamin C), to supply the brain with L-ascorbic acid (10). It is conceivable that, after having been transported into the brain, L-dehydroascorbic acid is reduced to L-ascorbic acid, which would be retained in the brain, since it cannot be transported through GLUT1. As a result, GLUT1 would contribute to maintaining the ten-fold greater concentration of L-ascorbic acid in the brain than that in the plasma (10,11).

L-Serinyl-β-D-glucoside analogues of Met^5enkephalin have been shown to have greater BBB permeability than the parent peptides (12). This suggests that GLUT1 is responsible for transporting glycosylated peptides. However, chemotherapeutic agents coupled with D-glucose (D-glucose-chlorambucil derivatives) have been shown to inhibit GLUT1-mediated transport, and so these derivatives are not transported (13). The substrate specificity of GLUT1 seems to be very strict, and so the choice of a carrier for drug delivery to the CNS needs to be considered very carefully.

Various other transport systems and transporters have been identified as blood-to-brain influx transporters at the BBB (Table I and Fig. 2a). MCT1 (SCL16A1) mediates influx transport of monocarboxlic acids, such as lactate and pyruvate (14). The expression of MCT1 in the brain and the brain uptake rate of lactate are increased during the suckling period (15,16). It is thought that the induction of a lactate uptake system in the brain allows the use of the lactate in milk. The BBB has a transport system for basic amino acids, such as L-lysine and L-arginine (Table II). CAT1 (SCL7A2) transports such basic amino acids, and its expression is concentrated in brain capillaries (17). CNT1 (SCL28A1) mediates transport of nucleosides and their analogues (18). Oatp2 (SLCO1B1) mediates transport of organic anions and opioids (19). These blood-to-brain influx transport systems are candidates for drug delivery pathway to the brain, so clarification of the requirements for substrate recognition by these transporters is important.

Some H_1-antagonists manifest their sedative action after having been distributed to the brain through the BBB. In vitro studies revealed that mepyramine, an H_1-antagonist, is transferred across the BBB via a carrier-mediated transport system (20–22). The uptake of mepyramine into primary-cultured bovine brain capillary endothelial cells is inhibited by other H_1-antagonists, such as azelastin, ketotifen, cyproheptadine, and emedastine (22). The uptake of mepyramine was also inhibited by imipramine, propranolol, and lidocaine (22). Furthermore, a trans-stimulatory effect was observed in mepyramine uptake into the brain capillary endothelial cells by preloading with mepyramine and the above inhibitors (21,22). These observations indicated that the compounds exhibiting inhibitory effects are also recognized by the blood-to-brain influx transport system for mepyramine. The influx permeability rate of mepyramine (1,620 μl $min^{-1} \cdot g^{-1}$ brain) is 13-fold greater than that of glucose [129 μl $min^{-1} \cdot g^{-1}$ brain (20,23,24)]. Therefore, this transport system is potentially very important for the delivery of cationic drugs to the brain. The uptake of mepyramine by immortalized rat brain capillary endothelial cells (RBEC1) was not inhibited by substrates of MCT1, OATs, oatps, OCTs and OCTNs (25), and the transporter(s) involved in the mepyramine transport at the BBB has not been identified yet.

Another possible drug target would be factors that regulate the influx transport system at the BBB. Creatine plays a key role in energy storage in the brain, as well as muscle. Brain creatine is supplied from the circulating blood by a BBB transport system involving CRT [SLC6A8 (26)]. As shown in Fig. 3a and b, CRT is localized at brain capillaries and neurons. Immuno-electronmicroscopy indicated that CRT is expressed at both luminal and abluminal membranes of brain capillary endothelial cells (27). CRT expressed at the luminal membrane would mediate creatine supply to the brain. The function of CRT at the abluminal membrane is not yet known. It has been reported that creatine has a neuroprotective effect (28,29), so that increase or maintenance of brain creatine levels is likely to protect neurons in various disease states. However, oral administration of 20 g creatine per day for 4 weeks produces only about a 9% increase in total creatine in the human brain (30). This may be because CRT at the BBB is almost saturated by plasma creatine. The plasma level of creatine is 50–100 μM in the human and 200 μM in the mouse (31), while K_m of creatine uptake by conditionally immortalized mouse brain capillary endothelial cells (TM-BBB cells) was found to be 16.2 μM (26). Therefore, induction of CRT at the BBB is a possible strategy to increase brain creatine levels and to prevent neurodegeneration. Growth hormone induced the myocardial expression of CRT, and co-expression of glucocorticoid-inducible kinase SGK1 or mammalian target of rapamycin (mTOR) stimulated electrogenic creatine transport of CRT in *Xenopus* oocytes (32–34). These proteins would be targets for induction of CRT at the BBB.

Taurine has a neuroprotective effect, like creatine. Taurine transport at the BBB is mediated by TAUT (SLC6A6), and expression of TAUT in brain capillary endothelial cells is induced by TNF-α (35,36). Oxidative stress induces xCT (SLC7A11) in brain capillary endothelial cells, leading to enhanced synthesis of glutathione, a radical scavenger, in the brain via an increase of cystine supply from the plasma across the BBB (37). Therefore, regulation of these transporters also plays a role in neuroprotection, and would be a potential target for new drugs.

ATP-BINDING CASSETTE (ABC) TRANSPORTERS AS A FUNCTIONAL BARRIER RESTRICTING DRUG DELIVERY TO THE BRAIN

A drug efflux pump system exists at the luminal membrane of brain capillary endothelial cells and prevents distribution of drugs to the brain by exporting them from endothelial cells to the circulating blood. The molecules involved are ATP-binding cassette (ABC) transporters (Table I and Fig. 2b), which mediate export of substrates from cells coupled with the hydrolysis of ATP. P-Glycoprotein (P-gp/MDR1/ABCB1), a well known ABC transporter of tumor cells, was detected immunohistochemically in the luminal membrane of brain

capillary endothelial cells in 1989 (38). The active efflux transport function of P-gp at the BBB was demonstrated by using cultured brain capillary endothelial cells (39). P-gp not only lowers the permeability rate of its substrates across the BBB, but also lowers the brain interstitial fluid-to-plasma concentration ratio of unbound drug (40,41). The pharmaceutical impact of P-gp was firstly demonstrated in mdr1a knockout mice, which lack the mouse homologue of P-gp. In mdr1a knockout mice, the brain-to-plasma concentration ratios of ivermectin and vinblastine were increased by 26- and 11-fold compared with those of wild-type mice (42). Many investigators have confirmed that P-gp expressed at the BBB plays a very important role in restricting the entry of xenobiotics from the circulating blood into the brain.

The ABC transporter superfamily consists of subfamilies A to G (ABCA-G), and 48 subtypes have been identified in

Table III. Substrates of MDR1, BCRP and MRP4

Transporter	Substrates
MDR1/ABCB1	Acebutolol[1], Actinomycin D[2], Amprenavir[2], Azidopine[3], Betamethasone[4], Calcein-AM[5], Cepharanthin[6], Cerivastatin[7], Chloroquine[2], Cimetidine[8], Clarithromycin[2], Colchicine[1], Cortisol[4], Cyclosporin A[9], Daunorubicin[9], Dexamethasone[9], Digitoxin[10], Digoxin[11], Dipyridamole[2], Docetaxel[12], Domperidone[13], Doxorubicin[1], Eletriptan[2], Emetine[2], Epinastine[14], Erythromycin[15], Estradiol-17β-D-glucuronide[16], Estrone[17], Ethynyl estradiol[17], Etoposide[1], Fexofenadine[18], Grepafloxacin[19], Hoechst 33342[20], Imatinib[21], Indinavir[11], Irinotecan, Ivermectin[22], Lansoprazole[23], Levofloxacin[19], Loperamide[2], Losartan[24], Lovastatin[25], Methylprednisolone[4], Mitoxantrone[2], Morphine[1], Neostigmine[2], Omeprazole[23], Pantoprazole[23], Prazosin[2], Prednisolone[4], Puromycin[2], Quinidine[2], Ramosetron[26], Ranitidine[1], Reserpine[2], Rhodamine 123[20], Ritonavir[11], Saquinavir[2], Somatostain[27], Sparfloxacin[19], Substance P[27], Talinolol[1], Taxol[1], Terfenadine[25], Trimethoprim[2], Vecuronium[28], Verapamil[9], Vinblastine[2], Vincristine[11]
BCRP/ABCG2	Azidodeoxythymidine, Bisantrene, Cerivastatin[7], Doxorubicin[29], Daunorubicin[29], Dehydroepiandrosterone-3-sulfate[30], Etoposide[31], Estrone-3-sulfate[30], Estradiol-17β-D-glucuronide[30], Folate[32], Flavopiridol[33], Hoechst 33342[34], Imatinib mesylate[35], Mitoxantrone[29], Methotrexate[30], Prazosin[29], Pantoprazole[36], Pravastatin[7], Rhodamine 123[29], SN-38[37], Topotecan[36]
MRP4/ABCC4	cAMP[38], cGMP[38], Dehydroepiandrosterone-3-sulfate[39], Estradiol-17β-D-glucuronide[38], Folate[40], Methotrexate[40], Prostaglandin E1[41], Prostaglandin E2[41]

Substrates were identified by means of vesicle uptake studies, transcellular transport studies, or intracellular accumulation studies.

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humans (43). Subfamilies A, B, C and G function at the plasma membrane. ABCA and ABCG include subtypes which function as sterol transporters, such as ABCA1 and ABCG1. ABCB1 (P-gp), the ABCC family and ABCG2 are considered to be the main active drug efflux transporters. The multidrug resistance-associated protein (MRP/ABCC) family and breast cancer resistance protein (BCRP/ABCG2/MXR/ABCP) have been reported to be expressed as active efflux drug transporters at the BBB, as well as P-gp.

The MRP family transports anionic compounds, such as glucuronic acid conjugates and glutathione conjugates. The expression of MRP subtypes at the BBB is still controversial, because brain capillary preparations are contaminated with brain parenchymal cells, which also express subtypes of the MRP family, and the expression of ABC transporters changes during *in vitro* cell culture, even in primary culture. In addition, there may be species differences in subtype expression. As shown in Table IV, we recently demonstrated that MRP1 and MRP4 mRNA are highly expressed in rat brain capillary endothelial cells freshly isolated by magnetic cell sorting with a capillary endothelium-specific antibody (anti-PECAM-1 antibody) (44). In contrast, MRP1 mRNA expression was not detected in primary-cultured porcine brain capillary endothelial cells (45). Nevertheless, MRP4 expression at the BBB is consistent among the previous reports, and its luminal expression has been demonstrated in human brain capillary endothelial cells (46). Moreover, in MRP4 knockout mice, the brain distribution of topotecan was increased compared with that in wild-type mice (47). These findings strongly suggest that MRP4 acts as an active drug efflux transporter at the BBB. MRP4 transports cyclic nucleotides [Tables III (48)], and MRP4 reduced cytotoxicity by nucleobase analogs, such as azidothymidine, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (48,49). The BBB possesses an efflux transport system for azidothymidine and 6-MP (50,51). Therefore, it is conceivable that MRP4 plays a role in efflux transport of these compounds and lowers the distribution of nucleobase analogues into the brain.

BCRP transports doxorubicin, etoposide, mitoxantrone, topotecan and other anionic compounds (Table III). Immunohistochemical analyses have indicated that BCRP is localized at the luminal membrane of human, mouse and rat brain capillary endothelial cells [Fig. 3e–g (52–54)]. Although BCRP knockout mice are available, the function of BCRP as an active drug efflux transporter at the BBB has not been fully clarified yet. It might be necessary to identify specific substrates of BCRP in order to examine the *in vivo* contribution of BCRP to the BBB efflux transport system. Our in vitro study demonstrated that BCRP is involved in the efflux of mitoxantrone and prazosin from conditionally immortalized rat brain capillary endothelial cells (53).

Table IV. mRNA Levels of Mrp and Mdr1a in Rat Brain Capillary Endothelial Cells Purified by Magnetic Cell Sorting

	Target mRNA levels ($\times 10^{-3}$ Target/β-Actin)	% Mdr1a
Mrp1	38.7±2.2	82.9
Mrp2	0.142±0.034	0.0304
Mrp3	2.94±0.91	6.30
Mrp4	25.0±4.8	53.5
Mrp5	4.12±0.45	8.82
Mrp6	0.493±0.077	1.06
Mdr1a	46.7±4.6	

Brain capillary endothelial cells were freshly isolated from rat brain by magnetic cell sorting with anti-PECAM-1 antibody. The expression levels of target mRNA were normalized with respect to that of β-actin. Values represent the mean±SEM of three separate preparations (n=6). The values were taken from Ohtsuki *et al.* (44).

As described above, the ABC transporters each have a different, though broad, substrate specificity, and function cooperatively at the BBB, forming an effective functional barrier against xenobiotics with various structures. Thus, to develop efficient CNS-active drugs, the expression profile and substrate specificities of the ABC transporter subtypes will need to be established.

BRAIN-TO-BLOOD EFFLUX TRANSPORTERS AS A PHYSIOLOGICAL AND PHARMACOLOGICAL CLEARANCE PATHWAY

The brain produces various metabolites and neurotoxic compounds, and their accumulation is believed to one of the causes of neurodegenerative diseases, so the efflux transport system is required to act as a clearance system to maintain CNS function (Table I and Fig. 2c).

Excitatory amino acid, such as L-glutamic acid and L-aspartic acid, undergo efflux from the brain across the BBB (55). This efflux transport is isomer-selective, and D-aspartic acid is not a substrate. ASCT2 (SLC1A5) is localized at the abluminal membrane of brain capillary endothelial cells and mediates isomer-selective efflux transport of excitatory amino acids [Fig. 3c (56)]. Subtypes of excitatory amino acid transporter (EAATs/SLC1A) have been reported to be expressed in isolated bovine brain capillary endothelial cells (57). Nevertheless, the expression of EAATs at the BBB does not explain the stereoselective BBB efflux transport of aspartic acid, since EAATs transport both isomers (58). The contribution of EAATs to the BBB transport system may small compared with that of ASCT2. The BBB has efflux transporters of neurotransmitters as well. It expresses GAT2 (SLC6A12), which is a different subtype from those expressed in neurons and astrocytes, and mediates the efflux transport of γ-aminobutyric acid (59). The BBB expresses SERT and NET as transporters of serotonin and norepinephrine, respectively (60). These efflux transporters are likely to play a role in maintaining normal neurotransduction.

Several neuromodulators also undergo efflux from the brain across the BBB. Dehydroepiandrosterone sulfate (DHEAS) is a neurosteroid which can interact with GABA type A receptors and sigma receptors to increase memory and learning ability, and to protect neurons against excitatory amino acid-induced neurotoxicity (61). Oatp2 expressed at the BBB plays a role in the BBB efflux transport of DHEAS (62). Oatp2 is localized on both the luminal and abluminal membranes of rat brain capillary endothelial cells (63). Interestingly, since oatp2 is a bidirectional transporter (64), it is also involved in the entry of a cyclic opioid pentapeptide, [D-penicillamine2,5]enkephalin (DPDPE), into the brain (65). In addition to oatp2, OATP-A (SLCO1A2), oatp3 (Slco1a5) and oatp14 (Slco1c1) are expressed at the human, mouse and rat BBB, respectively [Tables I and V (19,66,67)]. Oatp14 was

Table V. Substrates of the OATP (SLCO) Family

Transporter	Species	Substrates
Oatp1a4/Oatp2	Rodents	Biotin[1], BQ-123[2], Bromosulfophthalein[2], Cholate[3], Dehydroepiandrosterone-3-sulfate[2], Digoxin[3], Estradiol-17**β**-D-glucuronide[3], Estrone-3-sulfate[3], Fexofenadine[4], Ouabain[3], Pravastatin[5], Rocuronium[6], Taurochenodeoxycholate[7], Taurocholate[3], Tauroursodeoxycholate[7], Thyroxine[8], Triiodothyronine[8]
OATP1A2/OATP-A	Human	BQ-123[9], Bromosulfophthalein[10], Cholate[10], Dehydroepiandrosterone-3-sulfate11, Deltorphin II[12], Estradiol-17β-D-glucuronide[9], Estrone-3-sulfate[13], Fexofenadine[4], Glycocholate[9], *N*-methylquinidine[6], Ouabain[13], Prostaglandin E2[9], reverse Triiodothyronine[14], Rocuronium[6], Taurocholate[10], Tauroursodeoxycholate[10], Thyroxine[14], Triiodothyronine[14]
OATP1C1/OATP-F/Oatp14	Human, rodents	Bromosulfophthalein[15], Estradiol-17β-D-glucuronide[16], Estrone-3-sulfate[15], reverse Triiodothyronine[16], Thyroxine[16], Triiodothyronine[15]
Oatp1a5/Oatp3	Rodents	BQ-123[17], Bromosulfophthalein[17], Cholate[18], Dehydroepiandrosterone-3-sulfate[17], Estradiol-17β-D-glucuronide[17], Estrone-3-sulfate[17], Glycochenodeoxycholate[18], Glycocholate[18], Glycodeoxycholate[18], Glycoursodeoxycholate[18], Ouabain[17], Prostaglandin E2[17], Rocuronium, Taurochenodeoxycholate[18], Taurocholate[8], Taurodeoxycholate[18], Tauroursodeoxycholate[18], Thyroxine[8], Triiodothyronine[8]

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firstly identified as a brain-specific anion transporter (BSAT1) by means of a subtractive screening study between brain capillary, liver and kidney cDNA libraries (68), suggesting that oatp14 is selectively expressed at the BBB. More detailed studies to identify the subtypes of the oatp family expressed at the BBB and to clarify their contribution to the BBB transport system will help increase our understanding of BBB function and the potential for specific delivery of organic anionic drugs.

Metabolism of monoamine neurotransmitters is critical for their inactivation. Neurotransmitter metabolites should be eliminated from the brain, or they would affect the turnover of neurotransmitters. Homovanillic acid (HVA) is a major metabolite of dopamine. The HVA concentration in blood and urine is widely used as an indicator of dopaminergic neuronal activity in the brain. Previous studies have indicated that the HVA concentration in the brain is increased when probenecid or octanoic acid is administered peripherally (69,70). These results suggested that a probenecid-and octanoic acid-sensitive transporter(s) mediates the efflux transport of HVA at the BBB. We have clarified that OAT3 (SLC22A8) is the transporter responsible for HVA efflux at the BBB, and is localized at the abluminal membrane of rat brain capillary endothelial cells [Fig. 3d (71,72)]. HVA transport by OAT3 was inhibited by probenecid and octanoic acid. Furthermore, metabolites of neurotransmitters inhibited OAT3-mediated transport of HVA, while the neurotransmitters themselves had no inhibitory effect (71). This result indicates that OAT3 may mediate the BBB efflux transport of various neurotransmitter metabolites.

The BBB efflux transport mediated by OAT3 is a physiologically multi-functional transport system. We have reported that an endogenous uremic toxin (indoxyl sulfate (IS)) and some drugs (6-MP, 6-TG) are excreted from the rat brain via OAT3 (50,73). During maintenance chemotherapy for acute lymphoblastic leukemia, CNS relapses often occur due to penetration and proliferation of leukemic cells in the brain, because of the limited brain distribution of thiopurine nucleobase analogs, such as 6-MP and 6-TG (74). Microdialysis studies have shown that the brain-to-blood efflux of 6-MP across the BBB is 20-fold greater than the blood-to-brain influx (75). Thus, OAT3-mediated BBB efflux transport plays a crucial role in limiting the effects of thiopurine nucleobase analogs in the brain. OAT3 takes up drugs from the brain interstitial fluid into endothelial cells, and other efflux transporter(s) should mediate the transport out of endothelial cells into the circulating blood. ABC transporters, such as MRP4, are likely to function at luminal membrane cooperatively with OAT3.

Valproic acid (VPA), which is a broad-spectrum anticonvulsant drug, is known to exhibit restricted distribution to the brain (76). It has been reported that VPA uptake by the brain is relatively higher than that of phenytoin or phenobarbital,

and the limited VPA distribution into the brain is due to extensive brain-to-blood efflux transport at the BBB (77). Uptake of VPA into the brain was reduced in the presence of medium-chain fatty acids, such as hexanoate, octanoate and decanoate, but not by short-chain fatty acid, such as propionate or butyrate, suggesting that VPA is taken up into the brain via a transport system for medium-chain fatty acids (78). VPA undergoes efflux from the brain to the circulating blood with a half-life of 3.7 min, and this efflux is inhibited by an excess of VPA, suggesting the involvement of a carrier-mediated process (79). MCT1 (SLC16A1) is present at the BBB and mediates transport of monocarboxylic compounds (14). However, although MCT1 interacts with VPA, it may not mediate VPA transport (80). Alternatively, VPA was reported to enhance fluorescein accumulation in primary cultured bovine capillary endothelial cells as effectively as indomethacin and probenecid, so that MRPs may be involved in VPA efflux transport (81). However, another report noted that calcein accumulation in primary cultured bovine capillary endothelial cells was not increased in the presence of VPA at the same concentration (82). Further studies are needed to identify the efflux transporter for VPA at the BBB.

SPECIES DIFFERENCES IN CARRIER-MEDIATED TRANSPORT SYSTEMS AT THE BBB

One reason for the poor success rate in development of CNS-acting drugs is considered to be species differences in the BBB transport system, such as substrate specificities, expression profile of transporters and regulation of transporters, so that animal studies can not readily be extrapolated to humans. Katoh *et al.* (83) have reported species differences in MDR1-mediated transport of diltiazem, cyclosporin A and dexamethasone among human, monkey, dog, rat and mouse. The apparent K_m values of diltiazem showed a 12-fold range among these species. Yamazaki *et al.* (84) also reported that digoxin and indinavir showed a greater transport ratio in mouse mdr1a-expressing cells than in human MDR1-expressing cells by means of a transcellular transport study. Tahara *et al.* (85) compared the transport rate of OAT3 among human, monkey and rat, and found differences between primate and rat in the transport activities for indoxyl sulfate, 3-carboxyl-4-methyl-5-propyl-2-furanopropionate and estrone-3-sulfate. These observations imply that substrate screening should be done with human transporters during drug development.

The protein expression of transporters was analyzed by means of immunohistochemical studies using human brain slices and cultured human brain capillary endothelial cells. Although immunohistochemical studies provide important information about the localization of transporters, the specificity of each antibody should be carefully considered. For example, anti-P-gp antibody (C219) showed cross-reactivity with muscle protein, such as myosin heavy chain (86). Moreover, in human autopsy samples, the condition of the samples, such as pathological and fixation conditions, is also likely to affect the localization of transporters and antibody reactivity. Virgintino *et al.* (87) reported that P-gp was localized at the luminal membrane of human cerebral cortex microvessels using JSB-1 antibody in conjunction with confocal microscopy. Golden *et al.* (88) reported the colocalization of immunoreactivity of P-gp and glial fibrillary acidic protein, a marker of astrocyte foot processes, in isolated human brain capillaries using MRK16 antibody and confocal microscopy. The recent electron immunomicroscopy study by Bendayan *et al.* (89), using MRK16 antibody, showed that P-gp is expressed at the plasma membrane on human brain capillary endothelial cells, pericytes and astrocyte foot processes. Futhermore, they reported that P-gp immunoreactivity was predominantly detected at the abluminal membrane of capillary endothelial cells rather than the luminal membrane. Regarding other drug efflux transporters, MRP1, MRP4, MRP5 and BCRP were reported to be localized at the luminal membrane of human brain capillary endothelial cells (46,52). However, Mrp1 mRNA expression was not detected in isolated human brain microvessels by RT-PCR (90). Information about transporter localization at the human BBB is still limited and partially conflicting.

The immunohistochemical and mRNA expression studies have provided some information about the involvement of transporters at human BBB, but functional evidence is still necessary to understand the contributions of individual transporters to the human BBB transport system. Recent progress in positron emission tomography (PET) markers and imaging techniques has made it possible to evaluate the BBB transport function in humans in more detail. Ikoma *et al.* (91) quantified the transfer of ^{11}C-verapamil, a P-gp substrate, from plasma to brain by PET imaging analysis. Hsiao *et al.* (92) demonstrated that the brain-to-blood ratio of ^{11}C-verapamil was increased by 79% by cyclosporin A in humans, and that of ^{3}H-verapamil was increased by 75% at an equivalent cyclosporin A concentration in rats. The *in vivo* P-gp function at the BBB of rhesus monkey has also been investigated by comparing the brain uptake of ^{11}C-verapamil in the presence or absence of PSC833, a P-gp inhibitor (93). Using the same PET technique, Takano et al. (94) examined the effect of MDR1 polymorphism (C1236T, G2677T and C3435T) on the *in vivo* permeability rate of ^{11}C-verapamil in humans. Although no difference was observed between CGC and TTT haplotypes, this approach is important to evaluate the *in vivo* contribution of each transporter to the human BBB transport system. The brain uptake of *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine (L-FET) in pig was measured by PET imaging, and L-FET was found to inhibit the transport of tryptophan by human LAT1, suggesting that it may be a useful PET marker for evaluating system L function at the human BBB.

Functional changes in transporters, especially drug efflux transporters, at the human BBB are expected to have a great influence on individual drug response. Expressional changes in disease states and single nucleotide polymorphisms (SNPs) have been analyzed in the case of MDR1, but the impact on drug response is still unclear, since the contributions of individual transporters to the human BBB transport system are unknown. Induction of MDR1 mRNA and P-gp protein, as well as MRP1, MRP4, MRP5 and MRP6 mRNAs, was observed in primary-cultured brain capillary endothelial cells derived from an epileptic patient (95), leading to the proposal that the induction of these drug efflux transporters is one of the causes of anti-epileptic drug resistance. The influence of MDR1 SNPs on the drug response and distribution into the brain is still under investigation, though there are several reports demonstrating a positive relation between MDR1 polymorphism and CNS drug response. Highly significant

linkage disequilibrium was reported among C1236T (exon 12), G2677T (exon 21) and C3435T (exon 26), and epileptic patients with the CGC, TGC, and TTT haplotypes were reported to be more likely to be drug-resistant (96). There is also a report demonstrating a relation between MDR1 polymorphism and response to olanzapine treatment in schizophrenia (97). Regarding C3435T polymorphism, the T allele carrier group exhibited an increase of drug response associated with an increased plasma concentration of olanzapine, while such a relationship was not seen for the 3435CC group.

STRATEGIES TO INCREASE BBB PERMEABILITY BY UTILIZING COMPONENTS OF THE BBB TRANSPORT SYSTEM

By using blood-to-brain influx transporters, it should be possible to deliver hydrophilic compounds to the brain in a controlled manner. When the transporter is saturated, the transport rate of the compounds will become constant. However, the structures of suitable compounds are restricted by the narrow substrate specificity, and drug interaction arising from competitive inhibition at the transporter would have to be considered.

Drug efflux pumps and the brain-to-blood efflux transport system not only lower the BBB permeability rate, but also decrease the distribution to the brain of substrate drugs. The brain interstitial fluid-to-plasma concentration ratio of unbound drug is determined by the influx-to-efflux transport ratio. The brain interstitial concentration of quinolone antibiotics is lower than that in plasma due to the fact that the BBB efflux permeability rate is 10- to 100-fold higher than the BBB influx permeability rate (40). Therefore, lowering the efflux transport rate is an important strategy to enhance drug efficacy in the brain by increasing the drug concentration in the brain interstitial fluid.

Inhibition of a drug efflux pump or efflux transport system is likely to lead to an increase of drug distribution to the brain by lowering the efflux transport rate. However, the transporters are also expressed in peripheral tissues and inhibition might change the distribution of drugs in those tissues. Another possible strategy is to screen compounds that are not recognized by the drug efflux pump and efflux transport system at the BBB. This is currently considered as the most effective and important strategy for the development of CNS-acting drugs, and *in vitro* P-gp substrate screening assay is being introduced in the early developmental stage of drug development. Nevertheless, as described above, P-gp is not the only transporter that restricts drug distribution into the brain. Moreover, the function of drug efflux transporters at the human BBB has not been fully clarified.

To estimate BBB permeability, it is necessary to predict the influx and efflux transport rates. Cultured cells expressing exogenous transporter genes and membrane vesicles prepared from cells expressing transporters are useful tools for predicting transport rate across the BBB from individual transport studies. Many researchers have established cultured brain capillary endothelial cells, including primary-cultured brain capillary endothelial cells, as *in vitro* BBB models. However, in bovine primary-cultured BCEC, the expression of GLUT1 is suppressed by 150-fold compared with the *in vivo* level (98). Since the function of brain capillary endothelial cells in the BBB is regulated by the surrounding cells, including astrocytes and pericytes (53,99), the down-regulation is likely to be due to lack of cell–cell interaction. Berezowski *et al.* (100) and Cecchelli *et al.* (101) have reported that bovine brain capillary endothelial cells co-cultured with rat astrocytes formed rigid tight junctions and transcellular drug permeability showed a good correlation with *in vivo* BBB permeability.

Immortalized cell lines have the advantages of easy handling and good reproducibility. We have established conditionally immortalized mouse and rat brain capillary endothelial cell lines (TM-BBB and TR-BBB, respectively), and showed that some of their transport functions were well correlated with the *in vivo* BBB transport function (102). However, these cell lines have lower expression of mdr1a and some organic anion transporters than *in vivo* cells, and lack tight junctions and cell polarity. Introducing the gene for the tight junction protein claudin-5 partially restored the barrier function in TR-BBB cells (103). Recently, Weksler *et al.* have reported a new immortalized human brain capillary endothelial cell line (hCMEC/D3). These cells express tight junction proteins, such as claudin-5, JAM-A and ZO1, as well as efflux transporters, such as MDR1, MRPs and BCRP. They also reported a good correlation between *in vitro* and *in vivo* BBB permeability in D3 cells. Although further analysis is necessary, this cell line is likely to be a useful tool to clarify the molecular mechanisms of the human BBB transport system (104).

PERSPECTIVES; PHARMACOPROTEOMICS OF THE BBB TRANSPORTERS AS AN APPROACH TO DRUG TARGETING TO THE BRAIN

Many studies have demonstrated the importance of carrier-mediated transporters in the distribution of small molecules into the brain. However, we are still far from understanding the whole transport system at the BBB. Advances in proteomics technique using mass spectrometry make it possible to analyze the proteome, and the plasma, liver, kidney and brain preoteomes are currently being determined by the Human Proteome Organization. BBB proteomics should provide the overall expression profile of all transporters at the BBB, i.e., the transporter atlas of the BBB.

A human transporter atlas of the BBB would be the key to reconstructing the human BBB transport system and predicting drug permeability across the human BBB from in vitro transport data. The intrinsic transport activity of each human transporter protein could be determined by transport study with cell lines expressing the target transporter protein. Then, *in vivo* drug permeability across the human BBB could be predicted by integrating *in vitro* transport activities with the transporter atlas of the human BBB. The present proteomics technique still has issues to be solved, because transporters are glycosylated high-molecular membrane proteins that are expressed at low levels. Furthermore, quantitative information about transporter expression is necessary to evaluate the contribution of each transporter to the overall BBB transport system. Therefore, it is necessary to develop a novel method to identify and quantify transporter proteins at the plasma membrane comprehensively, to construct a transporter atlas. This would open a new field, pharmaco-

proteomics, in human BBB transporter research, allowing us to consider the influence of changes in disease status, aging, and individual differences. Construction of a human BBB transporter atlas will trigger significant progress in CNS drug discovery and delivery.

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