DDT Vol. 6, No. 3 February 2001

Efflux transport systems for drugs at the blood–brain barrier and blood–cerebrospinal fluid barrier (Part 1)

Hiroyuki Kusuhara and Yuichi Sugiyama

Penetration through the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCSFB) is necessary if a drug is to achieve the required concentration for a desired pharmacological effect. Efflux transport systems at the BBB and BCSFB provide a protective barrier function by removing drugs from the brain or cerebrospinal fluid and transferring them to the systemic circulation, respectively; several transporters at the BBB and BCSFB have been identified. Efflux transport should be taken into consideration during drug development to improve brain penetration and to avoid drug–drug interactions involving these transporters and subsequent side effects.

Hiroyuki Kusuhara and *Yuichi Sugiyama
Department of Biopharmaceutics
Graduate School of Pharmaceutical Sciences
University of Tokyo, Tokyo
Japan
*tel: +81 3 5841 4770
fax: +81 3 5800 6949
e-mail: sugiyama@mol.f.u-tokyo.ac.jp

▼ Brain capillary endothelial cells (BCEC) and the choroid plexus, also known as the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCSFB), are interfaces that separate the brain parenchyma and cerebrospinal fluid (CSF) from the systemic circulation, respectively. These barriers restrict the penetration of drugs and toxic substances from the circulating blood into the CNS. As a consequence, some drugs cannot achieve a concentration sufficient to exert their desired pharmacological effects.

The surface area of the BBB has been estimated to be 5000-fold greater than that of the BCSFB, and therefore the BBB is considered to be the main route for the uptake of endogenous and exogenous ligands into the brain parenchyma[1–4]. The brain capillaries are characterized by tight junctions and the paucity of fenestra and pinocytotic vesicles (Fig. 1)[1–4]. By contrast, the capillary in the choroid plexus does not have tight junctions and is more leaky[1–4], whereas choroid epithelial cells are connected to each other by tight junctions (Fig. 1)[1–4]. These anatomical features minimize the non-specific permeation of xenobiotics via the paracellular route, such that BCECs and choroid epithelial cells act as static walls. Compounds circulating in the blood must penetrate the brain via the transcellular route and, therefore, molecules with a low intrinsic permeability across the lipid bilayer, either because of having a large MW or high hydrophilicity, exhibit poor brain penetration.

Levin demonstrated that there is a positive correlation between the lipophilicity of 27 selected compounds and their ability to cross the BBB (Fig. 2)[5]. However, there are drugs that exhibit poor brain penetration despite their high lipophilicity, for example, the antineoplasmics doxorubicin and vincristine (Fig. 2)[5]. The poor brain distribution of these compounds was difficult to explain by the static wall concept alone, and this led to the proposal that efflux transport systems exist at the BBB and BCSFB barriers and actively eliminate drugs from the brain. As a result, the total uptake of drug into the brain parenchyma and CSF is reduced. Because transporter-mediated efflux is a saturable process, non-linearity in the penetration of drugs into the CNS is likely to occur. Drug–drug interactions are also possible through the inhibition of efflux transporters. Brain penetration of drugs that pharmacologically target the CNS could therefore be improved either by co-administering suitable inhibitors or by modifying the

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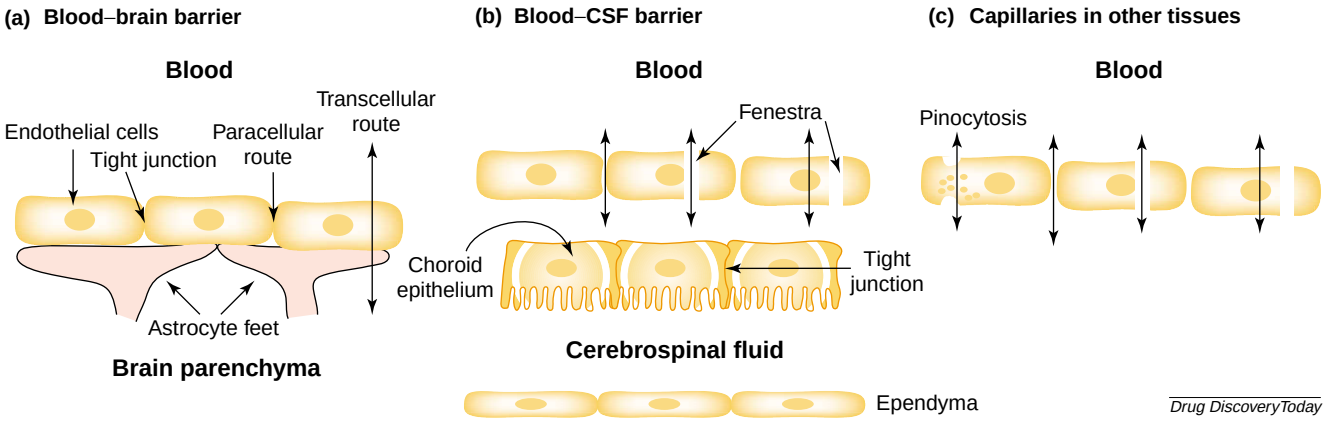

Figure 1. Schematic diagrams of the blood–brain barrier [BBB, (a)] and blood–cerebrospinal fluid barrier [BCSFB (b)]. The tight junction is a specific feature of brain capillary endothelial cells and choroid epithelial cells. It minimizes the penetration of drugs from circulating blood into the brain parenchyma and cerebrospinal fluid via the paracellular route. Therefore, drugs have to penetrate the CNS transcellularly. These barriers restrict the penetration of drugs that have low intrinsic permeability across the lipid bilayer because of their high MW and/or high hydrophilicity. This leads to poor drug distribution in the brain. Both brain capillary endothelial cells and choroid epithelial cells provide a barrier function to protect the CNS and are referred to as the blood–brain barrier and the blood–cerebrospinal fluid barrier, respectively. Transporters play an important role in restricting the penetration of drugs into the CNS by removing them into the circulating blood. (c) shows the mechanisms of transport that occur in the blood capillaries of other tissues where cell–cell junctions are not as tight as in the BBB and BCSFB. Abbreviation: CSF, cerebrospinal fluid.

drug so that it is not recognized by the efflux transporter. Whether poor brain penetration of a drug is attributed to poor membrane permeability or active efflux should be investigated further to be understood during drug development.

This review focuses on the role of transport systems at the BBB and BCSFB barriers. Other reviews dealing with similar topics have also been published[1–4,6,7].

Brain efflux index (BEI) method: evaluating efflux transport

An intracerebral microinjection technique that measures the fraction of drug remaining in the brain is a simple method to evaluate efflux transport. Two studies have characterized the efflux transport of 1-naphthyl-17β-glucuronide (N17βG) and a cyclic peptide, RC160 (a somatostatin analogue), following their microinjection into the cerebral cortex of rats and mice, respectively, and measurement of the radioactivity of N17βG or RC160 remaining in the brain[8,9]. The injection site was examined (parietal cortex area 2, hippocampal fissure, entorhinal cortex, field CA2 of Ammon's Horn and frontal cortex) and a volume of 0.2–2 μl/rat of radioactive compound was microinjected into the cerebral cortex[10]. To correct for any inter-individual differences in the quantity of injected drug, a reference compound, [^{14}C]carboxy-inulin, [^{3}H]inulin or [^{14}C]inulin, was co-administered with the test compound[8,10]. Depending on the injection site, the radioactivity of [^{14}C]carboxy-inulin associated with CSF specimens ranged from 2% to 60% of the total injected radioactivity of [^{14}C]carboxy-inulin[10]. Increasing the volume of the injection reduces the recovery of [^{14}C]carboxy-inulin in the brain, from 70% to 50%[10]. Parietal cortex area 2 and 0.2 μl appeared to be the optimal injection site and injection volume, respectively[10]. No significant reduction in recovery of [^{14}C]carboxy-inulin and [^{3}H]mannitol in the ipsilateral cerebrum was observed following microinjection, suggesting that leakage is only a minor contribution to the total efflux from the brain[10].

p-Aminohippurate (PAH) is a low-MW, hydrophilic organic anion. The efflux of [^{3}H]PAH from the brain is rapid after injection; the elimination half-life being 20 min (Fig. 3a)[11]. The elimination is saturated at high concentrations and an apparent K_m (Michaelis–Menten constant) value for the efflux of PAH (determined by using concentrations of non-radiolabeled PAH in the injectate), was 2.4 nmol/0.2 μl injectate (Fig. 3b)[11]. Administration of unlabeled PAH to the CSF did not affect the elimination of [^{3}H]PAH from the brain[11]. These results support the hypothesis that PAH elimination after microinjection into the cerebral cortex occurs mainly via the BBB in a carrier-mediated manner, and that the BEI method is a good approach for evaluating efflux via the BBB[11]. The efflux transport of the cyclic peptide BQ123 (an endothelin-receptor antagonist), taurocholate (TCA, a bile acid), quinidine (an anti-arrhythmic drug), HSR903 (a quinolone antibiotic), azidodeoxythymidine (AZT) and dideoxyinosin (DDI) (both antiviral agents) has been characterized by this method[12–15].

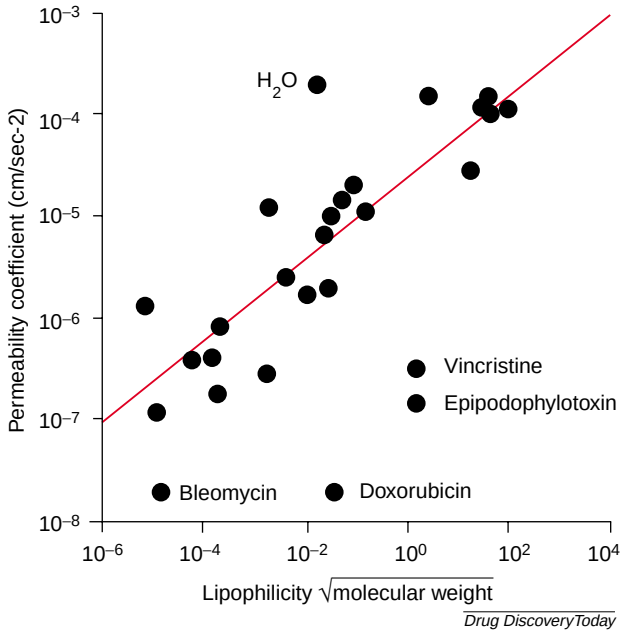

Figure 2. The positive correlation between the lipophilicity (octanol–water partition coefficient /√molecular weight) and the permeability coefficient across the blood–brain barrier (BBB) is shown. The permeability coefficients of 27 compounds were evaluated *in vivo* using the single time point method. The drugs were administered intravenously to rats, and plasma concentrations measured between 0 and 6 min. The permeability coefficient was then obtained from the concentration of drug in the brain, the area under the plasma concentration–time curve and the surface area of the BBB. Based on these data, a strategy that increases a compound's lipophilicity has been adopted in drug development to improve brain penetration. However, as shown, doxorubicin, epipodophylotoxin, vincristine and bleomycin did not fit this correlation. This led to the hypothesis that transporter-mediated efflux transport across the BBB might occur. Reproduced, with permission, from Ref. 5.

Multispecific transporters expressed at the CNS barriers

Efflux transporters: P-glycoprotein

There are two isoforms of human P-glycoprotein (P-gp) that are referred to as MDR1 and MDR2 (also called MDR3). There are three isoforms in mouse: Mdr1a (Mdr3), Mdr1b (Mdr1) and Mdr2[6,16–18]. Human MDR1 and mouse Mdr1a and Mdr1b are primary active transporters that have broad substrate specificity for compounds such as vinca alkaloids and anthracyclines, and as a result they confer multidrug resistance to tumor cells (Table 1)[6,16–18]. Human MDR2 and mouse Mdr2 are flippases for phosphatidylcholine on the canalicular membrane of the liver and these do not confer multidrug resistance[6,16–18]. Although P-gp is an efflux transporter, it decreases not only the steady-state accumulation but also the uptake of colchicine, vinblastine, etoposide and daunorubicin at an early phase[19,20]. This phenomenon is also observed *in vivo*. In mdr1a-deficient CF–1 mice, the brain uptake determined at an early phase using the brain perfusion technique was significantly increased, which is consistent with a previous observation *in vitro*[21]. The precise molecular mechanism for this has not yet been elucidated; it has been proposed that P-gp might act as a 'hydrophobic vacuum cleaner', in that the substrates are recognized and extruded directly from the plasma membrane before entering the cytoplasm[6,16–18].

P-gp is also expressed in organs associated with elimination processes such as the liver and the kidney[16,18,22], and with barriers restricting the penetration of xenobiotics into cells in the small intestine, testes and placenta[23–26]. Immunohistochemical studies have revealed that P-gp is expressed on the brain capillary and localized on the luminal membrane[23,27–30], which is consistent with the localization of P-gp on primary cultured BCECs or immortalized BCECs[28,31]. However, Pardridge and colleagues have demonstrated overlapping expression of P-gp with glial fibril acidic protein (GFAP, a marker protein for astrocytes), but not with GLUT-1 (a glucose transporter expressed on BCECs), in human brain tissues. This suggests that P-gp is expressed on the astrocyte, but not on the BCEC[32]. The role of P-gp on the BBB has been investigated *in vitro* and *in vivo* and is discussed in Part 2 of this review.

Western blot analysis demonstrated the expression of P-gp on the choroid plexus, and the band corresponding to P-gp was not detected in Mdr1a/Mdr1b double knockout mice[33,34]. In contrast to the BBB, there is no staining in capillaries of the human choroid plexus[23]. P-gp is localized sub-apically on primary cultured rat choroid epithelial cells, but the localization of P-gp *in vivo* remains to be identified[33]. Considering the direction of P-gp mediated transport, substrates of P-gp might be taken up into the CSF. After intravenous administration of doxorubicin and 99mTc-sestamibi (a radiolabelled pharmaceutical) to non-human primates and rats, respectively, the CSF concentration was low compared with the concentration in blood[33,34]. These results could be attributed to the active efflux mediated by multidrug resistance-associated protein-1 (MRP1) on the basolateral membrane, because these compounds are also substrates of MRP1[35]. The physiological relevance of P-gp in choroid epithelial cells therefore remains unclear.

Efflux transporters: multidrug resistance-associated protein 1

The multidrug resistance-associated protein-1 (MRP1) is a primary active transporter that transports conjugated metabolites such as glutathione- and glucuronide-conjugates (Table 1)[18,36,37]. MRP1 confers multidrug resistance to anticancer drugs, such as doxorubicin, daunorubicin, epirubicin, vincristine, vinblastine and etoposide[18,36,37]. In

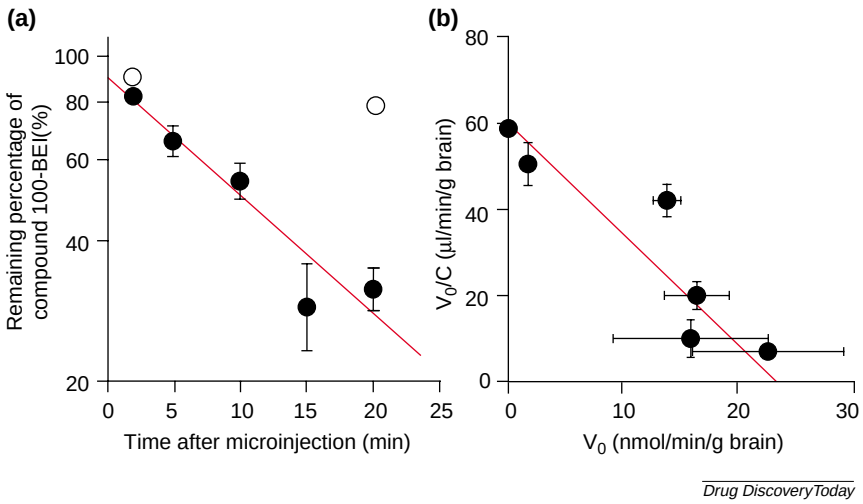

Figure 3. (a) The time-profile of the remaining fraction of [^{3}H]PAH in the brain after microinjection into the parietal cortex area 2 region is shown. 100-BEI (%) represents the recovery of [^{3}H]PAH corrected by that of a marker compound, [^{14}C] carboxy inulin. The elimination half-life of [^{3}H]PAH from the brain was ~20 min. (b) Concentration-dependence for the efflux of [^{3}H]PAH after microinjection is shown. The efflux was evaluated in the presence of 0.2, 2, 5, 10 or 20 nm/0.2µl injectate non-radiolabeled PAH in the injectate. The apparent K_m value was determined to be 2.4nmol/0.2µl injectate by fitting to the Michaelis–Menten equation. Because the efflux of [^{3}H]PAH from the brain exhibited saturation, the involvement of an efflux transport system is suggested. Reproduced, with permission, from Ref. 11. Abbreviation: V_0, velocity of efflux.

contrast to conjugated metabolites, the reduced form of glutathione is required for any ATP-dependent uptake of vincristine in membrane vesicles prepared from cells expressing MRP1[36,38]. Correspondingly, reduced-glutathione transport via MRP1 is also stimulated by vincristine and verapamil, suggesting that MRP1 co-transports vincristine and reduced-glutathione[39].

MRP1 is expressed and localized on the basal membrane of testis Sertoli cells and the basolateral membrane of lung epithelial cells[40,41]. Western blot analysis has demonstrated that MRP1 is expressed in the enriched membrane fraction of human and rat choroid plexus and whole cell lysate of mouse choroid plexus, and Mrp1 has been localized to the basal membrane of primary cultured rat choroid epithelial cells[33]. The presence of expression of MRP1 on the BBB is debatable. Western blot analysis and RT-PCR suggest that MRP1 is expressed on isolated rat brain capillaries, primary cultured bovine BCECs and immortalized mouse BCECs[42–44]. However, Seetharaman and coworkers report that no expression of MRP1 was detected in isolated human brain capillary by immunohistochemical staining[29], whereas expression of P-gp and platelet-endothelial cell adhesion molecule-1 (PECAM-1; an endothelial marker) was clearly detected[29]. In addition, no significant immunohistochemical staining was reported in mouse brain[45]. The expression of MRP1 is greater in bovine brain homogenate and bovine primary cultured BCECs than in brain capillary[43]. This contradictory evidence for the expression of MRP1 on BCECs could be because of contamination of the capillary fraction with parenchymal cells, the level of which might vary between laboratories.

Table 1. Substrates of primary active transporters at the blood–brain and blood–cerebrospinal fluid barriers

Primary active transporter	Species	Substrate examples	Refs
Mdr1a	Mouse	Asimadoline, cyclosporin A, digoxin, glucocorticoids, etoposide, vinblastine, indinavir, ivermectin, loperamide, morphine, phenytoin, verapamil, vecuronium, anti-emetics, anthracyclines	14, 15, 21,
MDR1	Human	Anthracyclines, β-adrenoceptor blockers, aldosterone, cyclosporin A, glucocorticoids, vinca alkaloids, digoxin, diltiazem, glucuronide conjugates, etoposide, ivermectin, loperamide, methotrexate, morphine, anti-emetics, calcium-channel blockers, phenytoin, ranitidine, rapamycin, rhodamine-123, HIV protease inhibitors	6, 16 ,18
MRP1	Human	Glucuronide and glutathione conjugates[a], 3-α-sulfatolitho-cholyltaurine, vincristine	18, 36–39
MRP5	Human	cAMP, cGMP, glutathione conjugates, fluorochrome	47, 48

[a]In the presence of glutathione.

Table 2. Substrates of secondary and tertiary active transporters for organic anions at the blood–brain and blood–cerebrospinal fluid barriers

Secondary/tertiary active transporter (organic anions)	Species	Substrate examples	Refs
Oatp1	Rat	Opioid agonists, glucuronide conjugates, estrone sulfate, ochratoxin A, ouabain, pravastatin, *N*-(4,4-azo-*n*-pentyl)-21-deoxyajmalinium, rocuronium	49, 50
Oatp2	Rat	Biotin, bile acids, dehydroepiandrosterone and estrone sulfates, digoxin, opioid agonists, ouabain, pravastatin[a], *N*-(4,4-azo-n-pentyl)-21-deoxyajmalinium, rocuronium	49, 50
OATP/OATP A	Human	Bile acids, sulfobromophthalein, estrone sulfate, deltorphin II, *N*-(4,4-azo-n-pentyl)-21-deoxyajmalinium, [D-pen 2,5]enkephalin, *N*-methyl-quinidine, *N*-methyl-quinine, rocuronium	49, 50
Oat1	Rat	Cidofovir, dideoxynucleotides, glutarate, urate, indomethacin, methotrexate, ochratoxin A, salicylates, *p*-aminohippurate, prostaglandin E_2	54–56
OAT1	Human	*p*-aminohippurate	
Oat3	Rat	Cimetidine, estrone sulfate, ochratoxin A, *p*-aminohippurate	54–56

[a]Only in oocytes.

Zhang examined the expression of MRP1–MRP6 on primary cultured bovine BCECs and the capillary enriched fraction, and the expression of MRP1, MRP4, MRP5 and MRP6 was demonstrated both in the primary cultured cells and in the capillary enriched fraction[46]. Recently, MRP5 was shown to accept glutathione conjugates and cyclic nucleotides as substrates[47,48]. However, further studies are required to confirm their expression at the BBB.

Uptake transporters: organic anion transporting polypeptide

The organic anion transporting polypeptide (Oatp) family has been characterized as a multispecific organic anion transporter (Table 2). Generally, members of the Oatp family accept amphipathic organic anions such as TCA and estradiol-17β-glucuronide ($E_2$17βG) as substrates[49,50]. Rat Oatp1 and Oatp2, and human OATP A, have been shown to be expressed at the BBB and BCSFB[51–53]. In the rat choroid plexus, immunohistochemical staining localized Oatp1 to the brush-border membrane of the choroid plexus, and Oatp2 to the basolateral

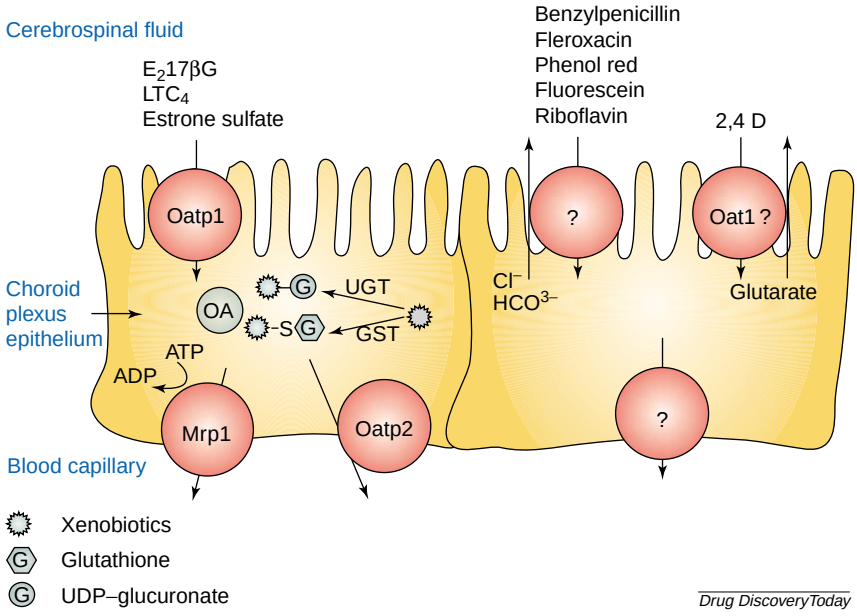

Figure 4. A possible scheme for efflux transporter systems for organic anions at the blood–cerebrospinal fluid barrier of the rat is shown. Oatp1 and Oatp2 are expressed on the brush-border and basolateral membrane, respectively. MRP1 expressed on the basolateral membrane is considered to actively eliminate glutathione conjugates and glucuronides that are formed in the choroid epithelial cells. They are considered to be responsible for the efflux transport of estradiol-17β-glucuronide (E17βG) and 1-naphthyl-17β-glucuronide. Although the molecular mechanisms have not yet been identified, the uptake systems for benzylpenicillin and 2,4 D are suggested to be different from Oatp1. Abbreviations: OA, organic anion; Oatp1, organic anion transporting polypeptide 1; Oatp2, organic anion transporting polypeptide 2; Oat, organic anion transporter 1; Mrp1, multidrug resistance associated protein 1; UGT, UDP-glucuronosyl transferase; GST, glutathione transferase; LTC_4, leukotriene C_4; 2,4D, 2,4-dichlorophenoxyacetate.

membrane[51,52] (Fig. 4). Using P-gp and GFAP as markers for the luminal membrane of the BCEC and the interface between the abluminal membrane of the BCEC and the astrocyte foot, respectively, double immunofluorescence staining revealed that Oatp2 is expressed on both the luminal and abluminal membrane of the BCEC in rat brain[52]. In humans, immunohistochemical staining of brain tissue suggested that OATP A is expressed in the brain capillaries, although its localization has not been confirmed[53]. Because Oatp2 can mediate bi-directional transport, involvement in both uptake and efflux is possible. Localization of Oatp2 at the luminal membrane of the BBB suggests that substrates of Oatp2 are absorbed by the brain from systemic blood. However, the distribution volume of TCA, determined using the *in situ* brain perfusion technique, is close to the capillary volume and adherent water volume, that is, the volume of a non-brain-permeable marker, in the rat[13]. Further studies are therefore required to reveal the mechanism of Oatp2 transport.

Uptake transporters: organic anion transporter

The organic anion transporter 1 (OAT1) is a multispecific transporter that accepts small, hydrophilic, organic anions such as PAH (Table 2). Northern blot analysis has shown that both OAT1 and OAT3 (Table 2) are expressed in the rat brain, although their localization in the brain has not been determined[54–56]. A transport feature of OAT1 is its transstimulation by dicarboxylate, which was also observed in the brush-border membrane vesicles of the choroid plexus[54–57]. It is therefore hypothesized that OAT1 is localized to the brush–border membrane of the choroid plexus and is responsible for the efflux transport of 2,4-dichlorophenoxyacetate (2,4 D) from the CSF[57]. According to *in vivo* studies using the BEI technique, there is an efflux transport system for PAH at the BBB[11]. Because PAH is a typical substrate for members of the OAT family[54–56], OAT1 and/or its related protein(s), such as OAT3, are expected to be responsible for the uptake of PAH from the brain to the BCEC.

In Part Two of this article we present an overview of the *in vitro* and *in vivo* studies that have been used to examine transports at the BBB and BCSFB, and refer to the transporter that we have described here.

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