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Review

Recent advances in the brain-to-blood efflux transport across the blood–brain barrier

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Abstract

Elucidating the details of the blood–brain barrier (BBB) transport mechanism is a very important step towards successful drug targeting to the brain and understanding what happens in the brain. Although several brain uptake methods have been developed to characterize transport at the BBB, these are mainly useful for investigating influx transport across the BBB. In 1992, *P*-glycoprotein was found to act as an efflux pump for anti-cancer drugs at the BBB using primary cultured bovine brain endothelial cells. In order to determine the direct efflux transport from the brain to the circulating blood of exogenous compounds in vivo, the Brain Efflux Index method was developed to characterize several BBB efflux transport systems. Recently, we have established conditionally immortalized rat (TR-BBB) and mouse (TM-BBB) brain capillary endothelial cell lines from transgenic rats and mice harboring temperature-sensitive simian virus 40 large T-antigen gene to characterize the transport mechanisms at the BBB in vitro. TR-BBB and TM-BBB cells possess certain in vivo transport functions and express mRNAs for the BBB. Using a combination of newly developed in vivo and in vitro methods, we have elucidated the efflux transport mechanism at the BBB for neurosteroids, excitatory neurotransmitters, suppressive neurotransmitters, amino acids, and other organic anions to understand the physiological role played by the BBB as a detoxifying organ for the brain.

Keywords: Blood–brain barrier; Brain Efflux Index method; Conditionally immortalized brain capillary endothelial cell line; Neurotransmitter transport; Organic anion transporter; Organic anion transporting polypeptide

1. Introduction

It has been almost a 100 years since the physiological concept of the blood–brain barrier (BBB) was initially proposed (Goldmann, 1913). The BBB, which is formed by complex tight-junctions of the brain capillary endothelial cells,

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segregates the circulating blood from interstitial fluid in the brain (Reese and Karnovsky, 1967). An illustration of mouse brain capillary using FITC-conjugated wheat germ agglutinin is shown in Fig. 1A. However, the cellular volume of the brain capillaries is only 0.1–0.2% of the entire brain (Boado and Pardridge, 1991), and the brain capillaries are ramified, like the network in the cerebrum at intervals of about 40 μm. Although the BBB was thought to act as a static wall protecting the brain, application of recent advanced methodologies to study the BBB has led to the new concept that the BBB acts as a dynamic regulatory interface (Cornford, 1985). In vivo experimental methods, brain uptake methods, such as the brain uptake index (Oldendorf, 1971) and brain perfusion (Takasato et al., 1984), have been developed and used to elucidate the influx transport systems for nutrients such as amino acids, D-glucose, monocarboxylic acids, and nucleosides at the BBB. In vitro experimental methods, the brain capillary isolation method (Betz and Goldstein, 1978) and primary cultured bovine brain capillary endothelial cells (Audus and Borchardt, 1986) have been established and used to characterize the transport functions for nutrients and drugs. Using primary cultured bovine brain capillary endothelial cells, Tsuji et al. (1992) found that *P*-glycoprotein (ABCB1) acts as an efflux pump for the anti-cancer drug, vincristine, at the BBB. Schinkel et al. (1994) have developed the *mdr 1a* gene knock-out mouse and proved that *P*-glycoprotein, i.e., *mdr 1a* gene products, plays a key role in restricting the apparent cerebral distribution of vinblastine, a substrate of *P*-glycoprotein, across the BBB. Fig. 1B shows immunostaining of mouse brain capillary using anti-*P*-glycoprotein antibody (C219). The green signal (*P*-glycoprotein) is localized inside the capillary endothelium nuclei (red signal), suggesting that *P*-glycoprotein is located on the luminal (blood) side of the BBB. *P*-glycoprotein acts as an efflux transporter for relatively lipophilic substrates such as anti-cancer drugs and cyclosporin A at the BBB (Tsuji and Tamai, 1997). However, several hydrophilic substrates such as metabolites of cerebral neurotransmitters are present in the brain. We believe that these hydrophilic metabolites undergo efflux across the BBB to reduce the cerebral concentration and, so, it could play an important role in central nervous system (CNS) detoxification (Terasaki and Hosoya, 1999). Otherwise, they accumulate in the brain since hydrophilic metabolites are transported very slowly across the BBB by passive diffusion. Although primary cultured bovine brain capillary endothelial cells have led to a number of important findings about BBB transport, brain endothelial cell lines are required to study transport mechanisms. A brain endothelial cell line could replace the need for large numbers of animals because the cellular volume of the brain capillaries is only 0.1–0.2% of the entire brain (Boado and Pardridge, 1991). Moreover, there is marked down-regulation of the activity of D-glucose and neutral amino acid transporters in the in vitro BBB model. Using Northern blotting, it has been shown that the mRNA expression of GLUT1 (SLC2A1) in isolated brain capillaries is over 100-fold greater than that in primary cultured bovine brain capillary endothelial cells (Pardridge, 1999a). This down-regulation of mRNA expression in culture cells is a disadvantage for characterizing the transport functions and molecular cloning of transporters at the BBB. Therefore, the brain endothelial cell lines need to maintain in vivo transport functions and mRNA expression to allow the investigation of transport mechanisms and identification of the corresponding genes.

In this review, we shall focus on brain-to-blood efflux transport systems of endogenous substrates in the brain across the BBB to identify the detoxifying system for the brain by using newly developed in vivo and in vitro experimental systems.

2. Brain Efflux Index method as a novel in vivo method to evaluate BBB efflux transport

Although the mechanism of brain-to-blood efflux transport is important for understanding the physiological and/or pathophysiological roles of the BBB, it has not yet been fully investigated. Therefore, the Brain Efflux Index (BEI) method has been developed to evaluate direct in vivo efflux

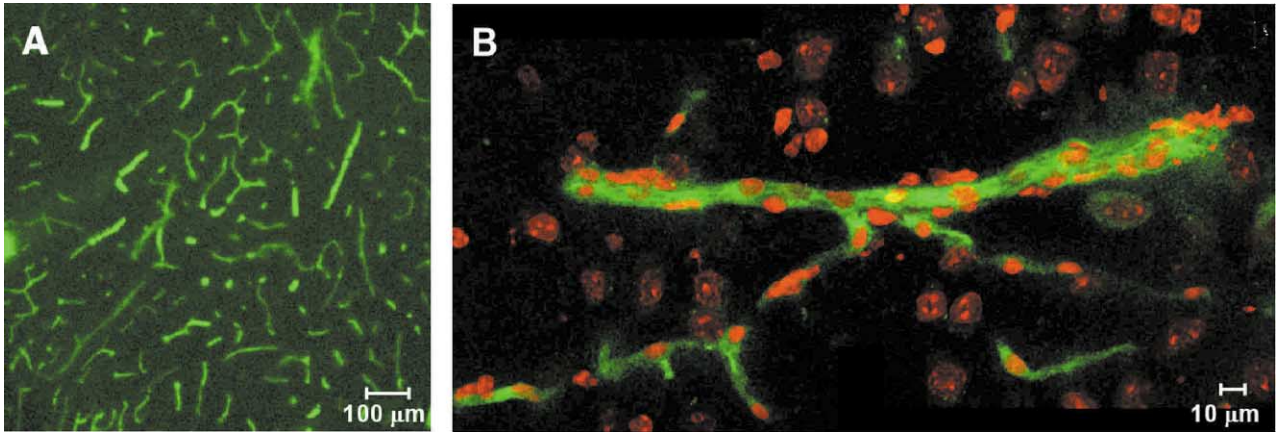

Fig. 1. Fluorescence images of brain capillary. (A) Fluorescence of FITC-conjugated wheat germ agglutinin (green). Mouse brain was excised 15 s after i.v. administration of FITC-conjugated wheat germ agglutinin. Cryosection (20 μm) of mouse brain was visualized by confocal laser microscopy. (B) Immunostaining by anti-*P*-glycoprotein antibody (C219; Green) in mouse brain. Nuclei were visualized by staining with propidium iodide (red).

transport across the BBB (Kakee et al., 1996). This method can directly determine the elimination rate of the radiolabeled substrate from the brain, which is compared with that of a BBB impermeable reference, [^{14}C]inulin or [^{3}H]D-mannitol, after intracerebral microinjection into the parietal cortex area 2 (Par2) region of rats (Fig. 2). The Par2 region was selected to administer a test substrate because only less than 3% of [^{14}C]inulin was found in the contralateral cerebrum, cerebellum, and cerebrospinal fluid (CSF) 20 min after microinjection, indicating that diffusion into the rest of the CNS from the injection site was limited. Therefore, the apparent elimination of test substrate reflects the efflux process across the BBB (Kakee et al., 1996). [^{3}H]3-*O*-Methyl-D-glucose (3-OMG), which is the non-metabolizable form of D-glucose and a substrate of GLUT1, undergoes efflux from the brain across the BBB with an apparent efflux constant (K_{eff}) of 0.129 min^{-1}, while [^{3}H]L-glucose, which is the stereoisomer of D-glucose, is not eliminated as well as [^{14}C]inulin (Kakee et al., 1996). GLUT1 is known to be a bi-directional facilitated transporter and specifically expressed at the BBB in the brain (Pardridge et al., 1990). The brain-to-blood transport direction for the stereoselective carrier-mediated transport system at the BBB can be characterized by using the BEI method. Moreover, the brain-to-blood efflux transport rate of 3-OMG is the same as that of blood-to-brain influx transport rate, suggesting that efflux transport rate measured by BEI method is not overestimated (Kakee et al., 1996). This is an event of significance as a positive control to characterize carrier-mediated transport in the brain-to-blood transport direction. Antiviral drugs such as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (DDI) are known to restrict distribution to the brain (Terasaki and Pardridge, 1988). Using the BEI method, these drugs were found to undergo efflux from brain to the circulating blood across the BBB via a probenecid-sensitive carrier-mediated transport system. This is different from the nucleoside transport system since thymidine and inosine failed to inhibit AZT and DDI efflux transport, respectively (Takasawa et al., 1997). Recently, Zhang and Pardridge (2001a,b) used the BEI method to show that

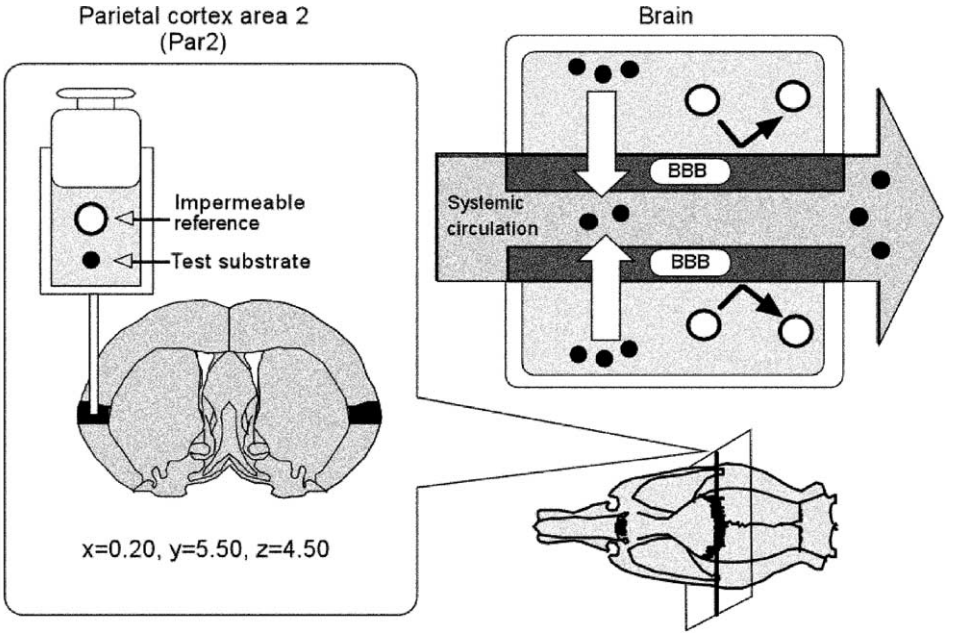

Fig. 2. Principle of the BEI method. The BEI value was estimated from the percentage of substrate remaining in the ipsilateral cerebrum (100-BEI) at the designated time after microinjection of the test and impermeable reference mixture (0.1–0.2 μl) into rat Par2 region.

immunoglobulin G and apo-transferrin, which are plasma proteins, undergo efflux from brain to the circulating blood. Although insulin and holo-transferrin are known to be transported from blood to brain via transcytosis of the BBB (Pardridge, 1999b), this is the first evidence using the BEI method to prove that brain-to-blood efflux transport of proteins operates at the BBB.

3. Conditionally immortalized cell lines as a novel in vitro BBB model

A transgenic mouse (Tg mouse) and rat (Tg rat) harboring the temperature-sensitive simian virus 40 (tsA58 SV 40) large T-antigen have been developed as a source of conditionally immortalized cell lines (Obinata, 1997; Takahashi et al., 1999). Several conditionally immortalized cell lines with differentiated functions, such as hepatocytes, gastric surface mucous cells and retinal pigmented epithelial cells, have been developed from the Tg mouse (Obinata, 1997). These transgenic animals have several advantages as far as establishing immortalized cell lines are concerned (see other review, Terasaki and Hosoya, 2001). As illustrated in Fig. 3, the tsA58 SV 40 large T-antigen gene is stably expressed in all tissues and cultured cells can be easily immortalized by activation of the SV 40 large T-antigen at 33 °C. The activated large T-antigen is thought to induce cell proliferation by interacting with the retinoblastoma gene products (Rb) and p53, which normally regulate cell proliferation, and inactivate the growth-suppressive function of these two proteins (Obinata, 1997). We established conditionally immortalized brain endothelial cell lines from the Tg mouse (TM-BBB) (Hosoya et al., 2000b) and Tg rat (TR-BBB) (Hosoya et al., 2000c). TM-BBB and TR-BBB cells express endothelial markers and possess transporters and transport functions for compounds like GLUT1 and *P*-glycoprotein. TM-BBB and TR-BBB cells express GLUT1 at 55 kDa and transport 3-OMG with a Michaelis–Menten constant (K_m) of 6.59 and 9.86 mM, respectively. These values are very similar to the in vivo ones. Moreover, the extrapolated in vivo transport rate of 3-OMG from in vitro data was 1/8 and 1/4 compared with the reported in vivo values, respectively (Hosoya et al., 2000b,c). TM-BBB and TR-BBB cells express *mdr 1a* and *1b* mRNA and *P*-glycoprotein at 170 kDa. TM-BBB cells exhibit efflux transport activity for cyclosporin A since cyclosporin A cell accumulation in TM-BBB cells is enhanced in the presence of verapamil and vincristine, typical *P*-glycoprotein inhibitors (Hosoya et al., 2000b,c). TR-BBB cells also express MRP1 (ABCC1) and LAT1 (SLC7A6) mRNA. Thus, these cell lines maintain certain in vivo transport functions and mRNAs and are a suitable in vitro model for the BBB (Terasaki and Hosoya, 2001).

4. Co-culture system of conditionally immortalized rat brain capillary endothelial, astrocyte and pericyte cell lines

The brain capillary endothelium is surrounded by the pericyte and astrocyte foot process as shown in Fig. 4. The overall brain microvascular biology is a function of the paracrine interaction between endothelial cells and two other cells (Pardridge, 1999a). Indeed, astrocytes and pericytes modulate brain endothelial cell tight-junctions (Janzer and Raff, 1987) and growth (Yamagishi et al., 1993), respectively. However, these investigations were carried out using different species, of different ages and genetic backgrounds (Yamagishi et al., 1993; Ceballos and Rubio, 1995; Raub, 1996). They were also carried out using endothelial cells and astrocytes or pericytes, but not a combination of the three cells. Therefore, these may not reflect the in vivo situation. In addition to TR-BBB cells, we have established astrocyte (TR-AST) (Tetsuka et al., 2001a) and pericyte cell lines (TR-PCT) (Asashima et al., 2002) from Tg rats to investigate the paracrine interaction between brain endothelial cells and two different cells involving the in vivo BBB (Fig. 4). Another advantage is that these cells are from the same genetic background, because they were established from the same line of Tg rats. TR-AST cells exhibit expression of glial fibrillary acidic protein and positive immunostaining for anti-A2B5 antibody, characteristics asso-

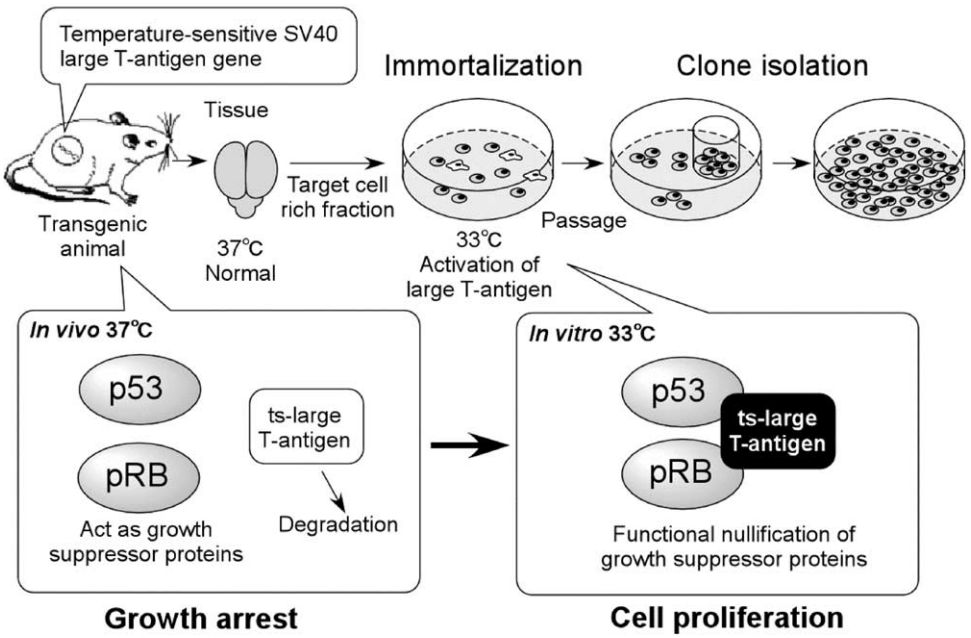

Fig. 3. Establishment of conditionally immortalized cell lines and characteristics of transgenic animals harboring tsA58 SV 40 large T-antigen gene.

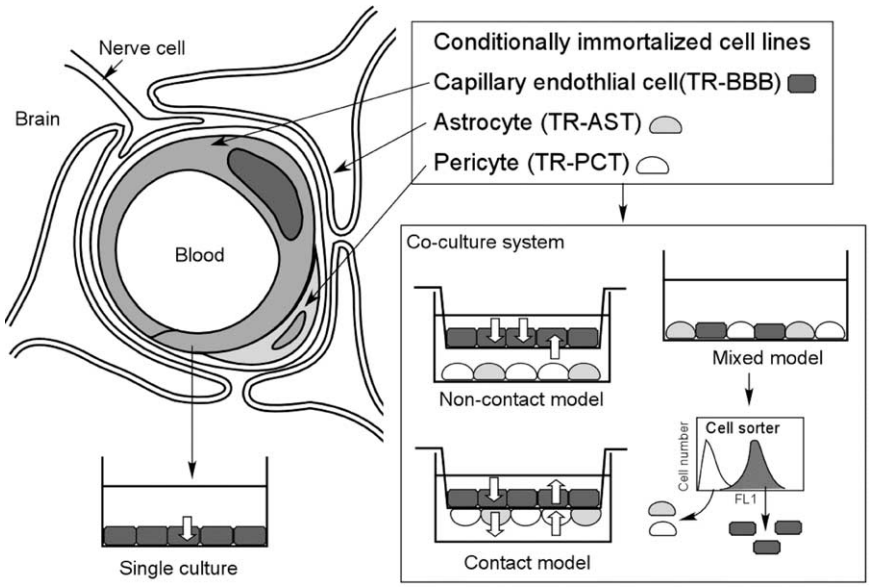

Fig. 4. Co-culture systems using conditionally immortalized cell lines for development of the in vitro BBB system. The white arrows indicate the direction of substrate transport.

ciated with type 2 astrocytes. Of the two types of astrocytes, type 1 appears at the embryonic stage, while the number of type 2 increases with the development of the CNS (Miller et al., 1985). Therefore, TR-AST cells are most likely to reflect the adult stage. TR-PCT cells are stained by von Kossa reagent, exhibit negation of contact inhibition, expression of platelet-derived growth factor receptor-β and angiopoietin-1 mRNA. It is possible to observe a paracrine interaction between TR-BBB cells and TR-AST cells and/or TR-PCT cells as co-culture systems: (1) conditioned medium method (non-contact model), (2) contact co-culture system (contact model) using Transwell®, and (3) mixed co-culture system (mixed model) (Fig. 4). The mixed model involves TR-BBB cells being cultured with TR-AST and/or TR-PCT cells in the same dish and then the TR-BBB cells are separated from other cells using a cell sorter system. The mRNA levels of *mdr 1a* and occludin, a tight-junction protein, in TR-BBB cells were up-regulated when TR-BBB cells were co-cultured with TR-AST cells using both non-contact and contact models. However, *mdr 1b*, MRP1, and GLUT1 mRNA levels were not affected. Although *mdr 1a* was not changed, occludin mRNA in TR-BBB cells was up-regulated by TR-PCT cells using the non-contact model (Hori et al., 2002). These results suggest that soluble factors secreted from astrocytes and/or pericytes play an important role in regulating *mdr 1a* and occludin mRNA at the BBB. These systems are useful models for investigating BBB functions in vitro and cell-to-cell interactions.

5. Blood–brain barrier transport as a CNS detoxifying system for endogenous substrates and drugs

5.1. Organic anion transporting polypeptide (oatp)

The OATP2 (LST-1; OATP-C; SLC21A6) in human liver and oatp1 (Slc21a1) and oatp2 (Slc21a5) in rat liver is located in the sinusoidal membrane and is involved in the uptake of organic anions (Reichel et al., 1999) while OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), OAT4, OCTN1 (SLC22A4) and OCTN2 (SLC22A5) have been identified as organic anion transporters (OATs) and are mainly present in the kidney (Sekine et al., 2000; Tamai et al., 1998). From the standpoint of detoxification in the brain, hydrophilic end metabolites undergo efflux from brain across the BBB. If these compounds are transported across the BBB via passive diffusion, this process is very slow for hydrophilic compounds and, therefore, they accumulate in the brain. Although other possible elimination pathways are thought to be a removal by CSF bulk outflow and efflux transport at the blood–CSF barrier, the diffusion process from the brain parenchymal tissue to the circumventricular organs is slower than the brain-to-blood carrier-mediated efflux transport process across the BBB when the test substrate is microinjected into the Par2 region (Kakee et al., 1996; Hosoya et al., 2000a). Several organic anions are present as endogenous metabolites and undergo brain-to-blood efflux transport via 'renal- and liver-like' OATs at the BBB.

Dehydroepiandrosteron sulfate (DHEAS) is a neurosteroid which can interact with γ-aminobutyric acid (GABA) type A receptors and sigma receptors to increase memory and learning ability and protect neurons against excitatory amino acid-induced neurotoxicity (Wolf and Kirschbaum, 1999). The oatp2 in rat liver and OATP2 in human liver mediates DHEAS uptake (Reichel et al., 1999). Using the BEI method, [^{3}H]DHEAS has been shown to be eliminated with a K_{eff} of 2.68×10^{-2} min^{-1} from the brain to the circulating blood across the BBB (Asaba et al., 2000). This efflux transport is a saturable process with a K_m of 33 μM and is inhibited by common rat oatp substrates such as taurocholate (TC), cholate, sulfobromophthalein (BSP), and estrone-3-sulfate (E_1S). In the case of oatp2, in particular, specific inhibitors like digoxin inhibit this process, suggesting that DHEAS undergoes efflux transport across the BBB via oatp2 which is present in rat brain capillary endothelial cells (Gao et al., 1999). [^{3}H]DHEAS uptake by TM-BBB cells is a saturable process with a K_m of 34 μM. Although there is an interspecies difference between mice and rats, the K_m value estimated for DHEAS uptake by

TM-BBB cells is identical to that determined by the in vivo BEI method. DHEAS uptake is significantly inhibited by common oatp inhibitors and digoxin. Conversely, [^{3}H]digoxin uptake by TM-BBB cells is significantly inhibited by DHEAS. RT-PCR and sequence analysis suggests that oatp2 is expressed in TM-BBB cells. These results suggest that oatp2 at the BBB is involved in the efflux transport of DHEAS from brain to the circulating blood. E_1S is also a neurosteroid and one of the substrates of oatp2 (Reichel et al., 1999). Using the BEI method, E_1S and estrone are eliminated from brain to the circulating blood across the BBB (Hosoya et al., 2000a) (Fig. 5). E_1S efflux transport is a saturable process with a K_m of 96 μM and is inhibited by common oatp substrates. Mutual inhibition with DHEAS was also observed, suggesting that oatp2 mediates E_1S as well as DHEAS (Fig. 5). Gao et al. (2000) found that human OATP-A (SLC21A3) and oatp2 transport cyclic opioid pentapeptide, [D-penicillamine^{2,5}]enkephalin (DPDPE) and deltorphin II as substrates in a study using *Xenopus laevis* oocyte expressed OATP-A or oatp-2 cRNA. They hypothesize that OATP-A and oatp2 mediate the influx transport of these peptides across the BBB. Dagenais et al. (2001) found that the brain uptake of [^{3}H]DPDPE by *mdr 1a* knock-out mice was ~ 12-fold greater than that in normal mice. This blood-to-brain influx transport was a saturable process with a K_m of 24 μM and it could be inhibited by digoxin. This in vivo evidence supports the hypothesis that oatp2 plays a role in blood-to-brain influx transport. Although it appears contradictory to argue that oatp2 is involved in both influx and efflux transport at the BBB, oatp2 seems to be an exchange transporter that mediates bi-directional transport (Li et al., 2000). Moreover, cyclic pentapeptide BQ-123, which is an endothelin receptor antagonist, has been shown to undergo efflux from brain to the circulating blood using the BEI method (Kitazawa et al., 1998). This

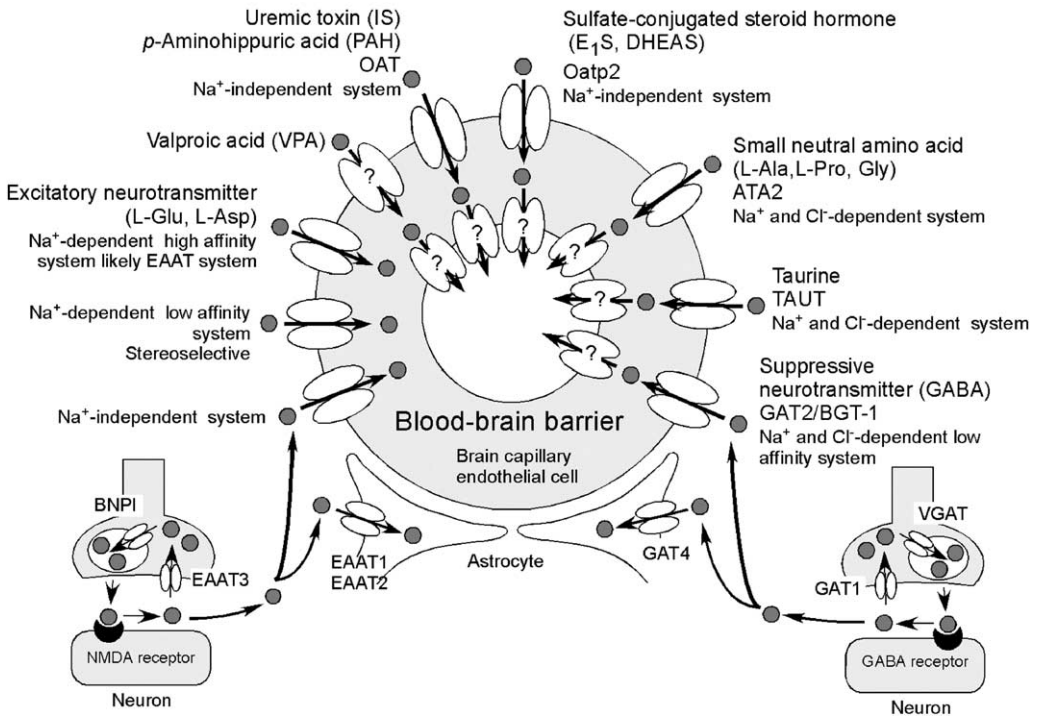

Fig. 5. Putative localization of brain-to-blood efflux transporters expressed at the BBB and the differential expression of neurotransmitter transporters among neurons, astrocytes and capillary endothelial cells. Although these transporters are known to be present at the BBB, the BEI study is thought to characterize transporters on the abluminal side of the BBB.

brain-to-blood efflux transport process is saturable and inhibited by TC. This supports the hypothesis that oatp2 is involved in the efflux transport of BQ-123 at the BBB since BQ-123 is a better substrate of oatp2 than oatp1 in the liver (Reichel et al., 1999). These findings suggest that oatp2 at the BBB acts as if it is a 'liver-like' organic anion efflux transport system exhibiting a detoxifying action in the brain.

5.2. Organic anion transporter

p-Aminohippuric acid (PAH) is known to be excreted in the urine via the OAT (Sekine et al., 2000). PAH was used as a model substrate for identifying whether a 'renal-like' organic anion efflux transport system is present at the BBB using the BEI method. PAH undergoes efflux from brain to the circulating blood across the BBB with a K_{eff} of 5.87×10^{-2} min^{-1} (Kakee et al., 1997). Brain-to-blood efflux transport of PAH is a saturable process with a K_m of 400 μM. This suggests that a carrier-mediated organic anion transport process for PAH is present at the BBB. OAT1–4 have been cloned and characterized as transporters for organic anions (Sekine et al., 2000). OAT1 and 3 are present in kidney and mediate PAH as a substrate. Moreover, Northern blot analysis suggests that OAT3 is present in the brain (Kusuhara et al., 1999). Indoxyl sulfate (IS), a uremic toxin, is excreted in the kidney and accumulated in uremic patients who have a reduced renal clearance (Niwa, 1996). IS, which is an endogenous organic anion, interacts with PAH transport in the renal tubules (Niwa and Ise, 1994). Using the kidney uptake index method, IS uptake was inhibited by probenecid, PAH, and cimetidine, suggesting that IS transport is involved in OAT in the kidney. IS uptake by rat OAT3 (rOAT3)-expressing *Xenopus laevis* oocytes is saturable with a K_m of 158 μM (Fig. 6). This process is inhibited by PAH, benzylpenicillin and cimetidine, which are substrates for rOAT3, but not by salicylic acid and indomethacin, a substrate of rOAT2 and a potent inhibitor of rOAT1, respectively (Deguchi et al., 2002). These results show that IS is a substrate of rOAT3 although OAT1 cannot be ruled out at the present time and this suggests that IS is a good

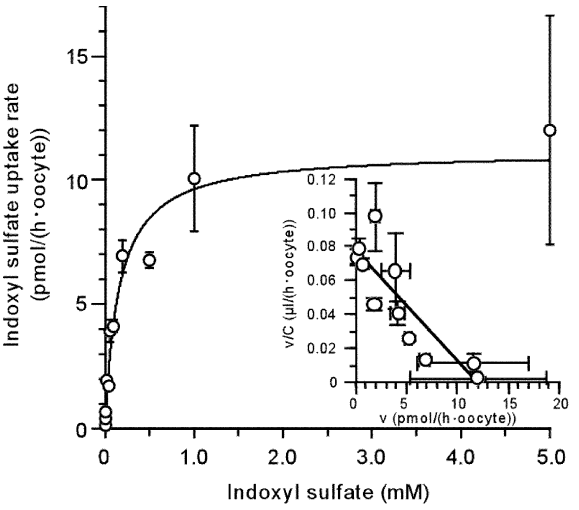

Fig. 6. [^{3}H]Indoxyl sulfate (IS) uptake by *Xenopus laevis* oocytes expressing rOAT3. Concentration-dependence of IS uptake by *Xenopus laevis* oocytes expressing rOAT3. Inset: the Eadie–Scatchard plot of the same data is shown. The terms v and C represent the IS uptake rate and concentration of IS, respectively. Each point represents the mean ± S.E.M. of 8–10 oocyte determinations. *Cited from:* Deguchi et al. (2002) with permission.

model substrate to identify whether the 'renal-like' organic anion efflux transport system is present at the BBB. Using the BEI method, IS undergoes efflux from brain to the circulating blood with a K_{eff} of 1.08×10^{-2} min^{-1} and this process is saturable with a K_m of 298 μM (Ohtsuki et al., in press) (Fig. 5). Further studies will reveal whether OATs are involved in the 'renal-like' organic anion efflux transport system at the BBB.

Valproic acid (VPA), which is a broad-spectrum anticonvulsant drug, is known to exhibit restricted distribution to the brain (Painter et al., 1981). The BEI method has been used to investigate VPA efflux transport across the BBB. [^{3}H]VPA undergoes efflux from brain to the circulating blood with a K_{eff} of 0.188 min^{-1} and this efflux is inhibited by an excess of VPA, suggesting a carrier-mediated process (Kakee et al., 2002). Monocarboxylic acid transporter (MCT)1 (SLC16A1) is present at the BBB and mediates transport of monocarboxylic compounds (Takanaga et al., 1995). Although, MCT1 interacts with VPA, it may not mediate VPA transport (Tamai et

al., 1995). Further studies are needed to identify the efflux transporter for VPA at the BBB.

5.3. Brain-to-blood efflux transport of acidic amino acids

L-Glutamic acid (L-Glu) and L-aspartic acid (L-Asp) are excitatory neurotransmitters in the brain and L-Glu also participates in cerebral energy metabolism and the synthesis of GABA and glutathione. As illustrated in Fig. 5, the excitatory amino acid transporters (EAAT1–4) are present in neuronal and glial cells, maintaining the extracellular L-Glu and L-Asp concentrations at subneurotoxic levels (Kanai, 1997). Using the BEI method, L-Glu and L-Asp have been shown to undergo efflux from brain to the circulating blood with a K_{eff} of 3.45×10^{-2} min^{-1} and 0.210 min^{-1}, respectively while D-Asp remained over a 20 min period after microinjection (Fig. 5). Interestingly, the K_{eff} value for L-Asp was 7-fold greater than that for L-Glu (Hosoya et al., 1999). O'Kane et al. (1999) reported that EAAT1 (GLAST; SLC1A3), EAAT2 (GLT1; SLC1A2), and EAAT3 (EAAC1; SLC1A1) are present on the abluminal (brain) side of the BBB. However, these transporters accept D-Asp as a substrate as well as L-Glu and L-Asp (Kanai, 1997). Therefore, it is possible that these transporters do not make a significant contribution to the brain-to-blood efflux transport of acidic amino acids in vivo. Using TM-BBB cells, an Na^+-dependent low affinity transporter is likely involved in selecting L- and D-Asp efflux transport at the BBB (Tetsuka et al., 2001b) (Fig. 5).

5.4. Brain-to-blood efflux transport of GABA

GABA, which is the main suppressive neurotransmitter, is widely distributed throughout the CNS. The GABA transporters (GATs) in neuronal and glial cells play a key role in maintaining the extracellular GABA concentration (Amara and Arriza, 1993) and abnormalities in regulating the GABA concentration in the CNS are involved in disorders such as Parkinson's disease, Alzheimer's disease, Huntington's chorea, encephalitis, and epilepsy. Using the BEI method, it has been shown that [^{3}H]GABA is eliminated from brain with a K_{eff} of 4.15×10^{-2} min^{-1}. The K_{eff} of [^{3}H]GABA increased by 173% in the presence of 3.3 mM nipecotic acid, which is a specific inhibitor of neuronal and glial GABA transporters, i.e., GAT-1 (SLC6A1) and GAT-3 (SLC6A11) (Fig. 5). Moreover, the efflux clearance of [^{3}H]GABA was 16-fold greater than its influx clearance (Kakee et al., 2001). Betain/GABA transporter-1 (BGT-1; SLC6A12), which corresponds to GAT2 in the mouse, is involved in this efflux transport at the BBB (Takanaga et al., 2001). [^{3}H]GABA uptake by TM-BBB cells is Na^+-, Cl^--, and concentration-dependent with a K_m of 679 μM. GAT2/BGT-1 expression in TM-BBB cells and mouse brain capillaries has been shown by RT-PCR and Western blot analysis. Moreover, confocal immunofluoresent microscopy of dual-labeled mouse brain sections has demonstrated the co-localization of GAT2/BGT-1 and *P*-glycoprotein, a BBB-specific marker (Fig. 1B), on brain capillaries labeled with anti-GAT2/BGT-1 antibody and anti-*P*-glycoprotein antibody, respectively. These results suggest that GAT2/BGT-1 mainly acts as a brain-to-blood efflux transporter of GABA when inactivation of GABA is not possible in neuronal and glial cells (Fig. 5).

Immunohistochemical analysis using the confocal immunofluoresent microscopy revealed that other neurotransmitter transporters, such as norepinephrine transporter (SLC6A2) and serotonin transporter (SLC6A4), are localized at the abluminal side and both the luminal and abluminal sides of the BBB, respectively, suggesting that these transporters may be involved in the inactivation of monoamines released from neurons around the BBB (Wakayama et al., in press).

5.5. Inducible amino acid transporter for osmoregulation at the BBB

The GAT2/BGT-1 gene has been found in the kidney as a betaine and GABA transporter to concentrate urine (Yamauchi et al., 1992) and a tonicity-responsive enhancer element (TonE) in its 5′-flanking region has been identified (Kaneko et al., 1997). Betaine is one of the major compatible osmolytes in the kidney (Yamauchi et al., 1992)

and is produced by choline oxidation in the brain. Moreover, GAT2/BGT-1 may not be able to act efficiently by terminating GABA release from presynaptic terminals, at least under normal conditions, since the affinity of GAT2/BGT-1 for GABA is lower than that of other GAT isoforms (Borden, 1996). Under hypertonic conditions, the concentrations of small organic solutes, such as taurine, myo-inositol, and betaine, are increased in the brain. Concomitantly, the corresponding transporters undergo up-regulation (Bitoun and Tappaz, 2000). Osmolarity regulation in the brain is very important for maintaining a constant milieu in the CNS since hypertonicity causes brain edema. TR-BBB cells express TAUT (SLC6A6) mRNA and regulate Na^+- and Cl^--dependent taurine transport. In salt-loaded rats, the expression of BGT-1 is more marked than that of TAUT in the whole brain (Bitoun and Tappaz, 2000).

System A is a transporter of small neutral amino acids that accepts L-alanine (L-Ala), L-proline (L-Pro), glycine (Gly), and α-methylaminoisobutyric acid (MeAIB) as substrates. It has been suggested that system A is present on the abluminal side of the BBB (Fig. 5) since MeAIB, which is a specific substrate for system A, has been shown to be taken up in an Na^+-dependent manner from the brain side using isolated rat brain capillaries (Betz and Goldstein, 1978) and isolated abluminal membrane vesicles from bovine brain endothelial cells (Sanchez del Pino et al., 1995). Recently, three Na^+-dependent small neutral amino acid transporters have been identified as system A isoforms, namely, ATA1 (GlnT; SAT1; SLC38A1) (Varoqui et al., 2000), ATA2 (SAT2; SA1; SLC38A2) (Sugawara et al., 2000b), and ATA3 (SLC38A4) (Sugawara et al., 2000a). Using the BEI method, [^{3}H]L-Pro and [^{3}H]Gly have been shown to undergo efflux from the brain over a period of 40 min, whereas [^{3}H]MeAIB did not (Takanaga et al., 2002). The efflux transporter of MeAIB may not be present on the luminal side of the BBB and, therefore, MeAIB would not undergo brain-to-blood efflux transport even though MeAIB is taken up on the abluminal side. [^{3}H]L-Pro uptake by TR-BBB cells is an Na^+-dependent process with high and low affinity saturable components. The corresponding K_m values are 425 μM and 10.8 mM. The manner of inhibition of amino acids for [^{3}H]L-Pro uptake is consistent with system A. Although all three isoforms of system A have been found to be expressed in the rat brain capillary-rich fraction and TR-BBB cells, ATA2 mRNA was present in 93-fold and 2140-fold greater concentrations than ATA1 and ATA3 mRNA in TR-BBB cells, respectively. As shown in Fig. 7, under hypertonic conditions (450 mOsm/kg), ATA2 mRNA in TR-BBB cells is induced by up to 373% as well as activating [^{3}H]MeAIB uptake (Takanaga et al., 2002). ATA2 mRNA is predominantly expressed and mainly responsible for the osmo-regulated system A at the BBB. GAT2/BGT-1, TAUT, and system A play a pivotal role in osmo-regulation at the BBB to maintain water and salt homeostasis in the brain.

6. Conclusion

Several efflux transport systems at the BBB have been characterized and identified by using a combination of the in vivo BEI method and in vitro cultured brain capillary endothelial cells, TM- and TR-BBB cells. These efflux transport systems at the BBB play an important role in protecting the brain from potential toxins. The Human Genome Project has now been completed and all the human genes have been identified (The Human Genome Project has reported the draft sequences of the entire human genome). At least 533 gene-related transporters have been identified (Venter et al., 2001) and the cDNA cloning of BBB transporters has recently started. Pardridge and his group have used a gene microarray approach to identify BBB-specific anion transporter 1 (BSAT1) using suppression subtractive hybridization between BBB cDNA and cDNA produced from mRNA isolated from liver and kidney (Li et al., 2001). Current molecular biological techniques like DNA microchip analysis will allow the identification of BBB-specific transporter genes in the near future. The combination between these techniques and co-culture systems among TR-BBB, TR-AST, and TR-PCT could be useful for identifying inducible genes for paracrine interaction between endothelial cells and other cells. The

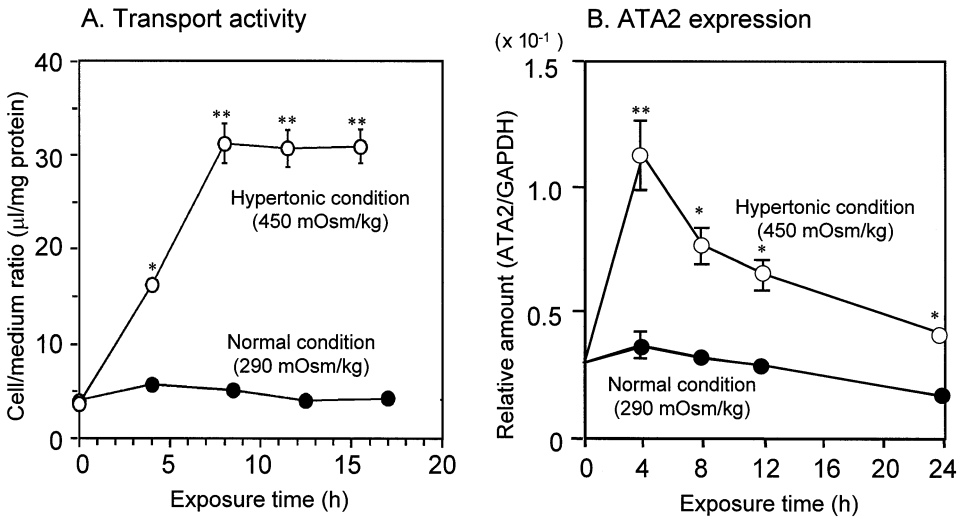

Fig. 7. Osmotic-regulation of system A at the BBB. (A) The hypertonic activation of [^{3}H]MeAIB uptake by TR-BBB cells. [^{3}H]MeAIB uptake was performed for 5 min after incubation in normal (290 mOsm/kg, close circles) and hypertonic (450 mOsm/kg, open circles) culture medium. (B) The hypertonic effect on the expression of ATA2 mRNA in TR-BBB cells. TR-BBB cells were incubated in normal (290 mOsm/kg, close symbols) and hypertonic (450 mOsm/kg, open symbols) culture medium over 24 h. Each point represents the mean ± S.E.M. ($n = 3$–8). *$P < 0.01$, **$P < 0.001$, significantly different from normal culture medium incubation (control). *Cited*

in vivo and in vitro BBB experimental systems are important tools for investigating BBB-specific transport functions and identifying the corresponding genes.

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