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Drug Transporters in the Central Nervous System: Brain Barriers and Brain Parenchyma Considerations

GLORIA LEE, SHANNON DALLAS, MEERA HONG, AND REINA BENDAYAN[1]

Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

***Abstract*——Drug transport in the central nervous system is highly regulated not only by the blood-brain and the blood-cerebrospinal fluid barriers but also in brain parenchyma. The novel localization of drug transporters in brain parenchyma cells, such as microglia and astrocytes, suggest a reconsideration of the present conceptualization of brain barriers as it relates to drug transport. That is, the cellular membranes of parenchyma cells act as a second "barrier" to drug permeability and express transporters whose properties appear similar to those localized at the conventional brain barriers. This review will focus on the molecular characteristics, localization, and substrate specificities of several classes of well known membrane drug transporters (i.e., the organic cation, organic anion, nucleoside, P-glycoprotein, and multidrug resistance proteins) in the brain. Comparisons to similar transporters localized within the peripheral system and clinical implications of the functional expression of specific drug transport families will be discussed when appropriate. Nutrient and neurotransmitter transporters, whose characteristics have been reviewed extensively elsewhere, will not be considered in this review.**

[1] Address for correspondence: Dr. Reina Bendayan, Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, ON M5S 2S2, Canada. E-mail: r.bendayan@utoronto.ca

I. Introduction

Disorders of the central nervous system (CNS[2]) remain difficult to treat pharmacologically due to poor drug permeability across brain barriers such as the blood-brain and blood-cerebrospinal fluid (CSF) barriers. Therapeutic agents can, however, cross these barriers by a variety of different mechanisms other than passive diffusion including transcytosis, receptor-mediated absorptive endocytosis, and/or facilitated/active transport systems. Once across these initial barriers, drug accumulation in the brain can be further restricted by a number of mechanisms including passive efflux in the bulk flow of the cerebrospinal fluid (sink effect), metabolic degradation, and active efflux transport including mechanisms dependent on P-glycoprotein (P-gp) and the multidrug resistance protein (MRP). Although the mechanisms of drug transfer in and out of the CNS have been fairly well characterized at the interfaces themselves, little information exists on the role of the brain parenchyma in the disposition of drugs. Recent studies demonstrating the existence of both influx and efflux transporters within glial cells such as astrocytes and microglia (Hong et al., 2000, 2001; Declèves et al., 2000; Dallas et al., 2001; Lee et al., 2001) highlight the complexity of drug distribution within the CNS.

The primary interfaces between the CNS and the peripheral circulation are the blood-brain barrier (BBB) and the blood-CSF barrier (Fig. 1, A and B) (Mooradian, 1994; Groothius and Levy, 1997). Tight junctions between the cerebral endothelial cells at the blood-brain interface and between the choroid plexus (CP) epithelial cells represent the structural basis of these barriers and permit the brain to function in a highly regulated and stable environment. The CNS contains two regulated fluid compartments, the interstitial fluid that surrounds the neurons and glia and the CSF that fills the ventricles and cushions the external surfaces of the brain. Due to its large surface area (i.e., 1000-fold larger than that of the CP), the BBB serves as the primary interface between the CNS and the peripheral circulation, whereas the blood-CSF barrier plays a less prominent role. Therefore, the subordinate role of the blood-CSF barrier to CNS drug delivery results from substrates present in the CSF having access only to the brain parenchyma directly neighboring the ventricles and CSF space. The BBB, on the other hand, interfaces with the entire interstitial fluid compartment of the CNS (Pardridge, 1997).

[2] Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; P-gp, P-glcoprotein; MRP, multidrug resistance protein; BBB, blood-brain barrier; CP, choroid plexus; AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type 1; RT-PCR, reverse transcription-polymerase chain reaction; PGT, prostaglandin transporter; OAT, organic anion transport; OCT, organic cation transport; NT, nucleotide transport; GSH, glutathione; ECF, extracellular fluid; MDR, multidrug resistance; ABC, ATP-binding cassette.

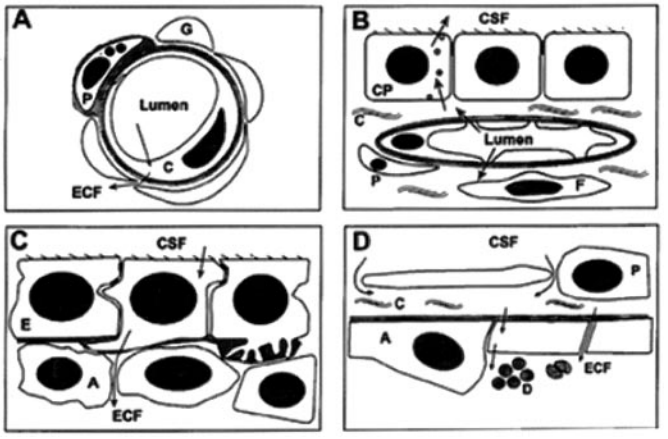

FIG. 1. Anatomy of blood-brain and blood-CSF interfaces. A, the BBB is formed by brain capillary (C) endothelial cells that are joined together by tight junctions. The brain endothelium and pericytes (P) are surrounded by a basement membrane and glial foot processes (G). Blood-borne solutes must cross from the lumen to the brain extracellular fluid (ECF). B, CP capillaries are fenestrated and surrounded by a basement membrane, which may also envelope pericytes (P). Solutes leaving the choroid capillary reside in the extracellular space containing fibroblasts (F) and collagen (C) and must cross the CP before entering the CSF. The CP has ciliated apices that are joined by tight junctions. C, ventricular surfaces are lined by ciliated ependymal cells (E), which are joined by tight junctions. A basement membrane is present between the ependymal cells and astrocytic processes. Solutes in the CSF may traverse across or between ependymal cells to enter brain ECF. D, lining of the brain surfaces. The CSF percolates through the subarachnoid space [containing collagen (C) and the pia matter (P)], and comes in contact with the glia limitans. The glia limitans, covered by a basement membrane, consists of flat astrocytic processes (A) with no intercellular junctions. CSF may exchange with brain ECF by traversing across or between glial processes. Reprinted with permission from Groothuis and Levy (1997).

In addition to the physical barriers provided by the tight junctions along the BBB and blood-CSF barrier, the presence of drug-metabolizing enzymes at the two interfaces provides an additional enzymatic barrier. Drug-metabolizing enzymes have been identified in the cerebral microvessels, choroid plexuses, leptomeninges, and in some circumventricular organs (Ghersi-Egea et al., 1995). These include the cytochrome P450 hemoproteins, several cytochrome P450-dependent monooxygenases, NADPH-cytochrome P450 reductases, UDP-glucuronosyltransferases, alkaline phosphatases, glutathione (GSH) peroxidases, and epoxide hydrolases (Ghersi-Egea et al., 1988, 1993, 1994; Meyer et al., 1990; Perrin et al., 1990). Degradation or biotransformation products are likely eliminated from the brain either by specific transport systems within the BBB or by diffusion from the parenchyma into the CSF by bulk flow (Ghersi-Egea et al., 1995).

The objective of this review is to discuss the location and functional expression of membrane drug transporters in brain barriers (i.e., BBB and CP) and in brain parenchyma (i.e., astrocytes and microglia).

II. The Blood-Brain Barrier and the Choroid Plexus

The BBB is composed of a monolayer of brain capillary endothelial cells that are fused together by tight junc-

tions. Under normal physiological conditions, these tight junctions form a continuous, almost impermeable, cellular barrier that prevents the passive influx of a variety of substances with the exception of the smallest, lipid-soluble molecules (Reese and Karnovsky, 1967). The absence of fenestrations, vesicular traffic, and pinocytosis in brain capillary endothelia further restrict free flow between brain interstitium and blood.

The endothelial cells of the BBB contain numerous membrane transporters involved in the influx/efflux of various essential substrates such as electrolytes, nucleosides, amino acids, and glucose (Fig. 2). Membrane permeation mechanisms can involve passive diffusion, carrier-mediated (facilitative), and/or ATP-dependent (active) processes and are similar to well characterized transport systems in other tissues (i.e., D-glucose, L-amino acid carrier systems, Na^+/K^+-ATPase), although the capacity and rate of transport can vary widely. There appears to be an asymmetric distribution of membrane-bound nutrient carriers across the BBB. One example of this asymmetry involves the facilitative glucose transporter, GLUT-1. This transporter is highly expressed by BBB microvessels, with higher levels of expression at the abluminal membranes compared with the luminal side (Pardridge and Boado, 1993). In general, Na^+/K^+-ATPase and the A-system amino acid transporters are primarily located on the abluminal side of cerebral endothelial cells (Sanchez del Pino et al., 1995) whereas Ca^{2+}-ATPases are expressed on both luminal and abluminal endothelial membranes and in the plasmalemmal vesicles of the endothelium (Vorbrodt, 1988).

In addition to carrier-mediated mechanisms, transcytosis of macromolecules in and out of the brain or CSF has also been reported (van Deurs, 1979; Broadwell, 1989). Furthermore, receptor-mediated and adsorptive endocytosis processes at the BBB exist for both hormones and plasma proteins (Abbott and Romero, 1996). Examples of these receptors include the endothelial barrier antigen (function undetermined), OX-47 (an integral plasma membrane glycoprotein that is involved in cell-to-cell recognition), and the endothelial glycocalyx (possible role in vascular permeability and surface charge) (Vorbrodt, 1988; Rippe and Haraldsson, 1994).

In addition to the presence of numerous receptors and transporters, the endothelial cells of the BBB also express metabolic enzymes such as alkaline phosphatase, peptidases, several cytochrome P450 isozymes (IIE1/IIB1/IIB2), UDP-glucuronosyltransferase, and GSH *S*-transferase. The enzyme alkaline phosphatase, which hydrolyzes phosphorylated metabolites, is present on both luminal and abluminal membranes of the endothelial cell. However, it is more heavily concentrated on the luminal side (Lawrenson et al., 1999). Cytochrome P450 IIE1 is expressed in most cerebral microvessels as well as in the astrocytic foot processes whereas cytochrome P450 IIB1/2 has been detected in both endothelial cells and neighboring pericytes (Volk et al., 1991). The conjugating enzyme UDP-glucuronsyltransferase is localized to rat brain capillaries (Ghersi-Egea et al., 1994) and one α-class GSH *S*-transferase has been detected in both cerebral capillaries and astrocytic foot processes (Johnson et al., 1993).

The blood-CSF barrier plays a vital role in the selectivity and permeability of the CP membrane to various nutrients and xenobiotics. The CP is a leaf-like highly vascular organ that protrudes into the ventricles. It is comprised of fenestrated capillaries that are surrounded by a monolayer of epithelial cells joined together by tight junctions (Fig. 1B) (Groothius and Levy, 1997; Segal, 2000). These tight junctions form the structural basis of the blood-CSF barrier and seal together adjacent polarized epithelial cells (also known as ependymal cells). Thus, once a solute has crossed the capillary wall, it must also penetrate the ependymal cells before entering the CSF (Fig. 1C).

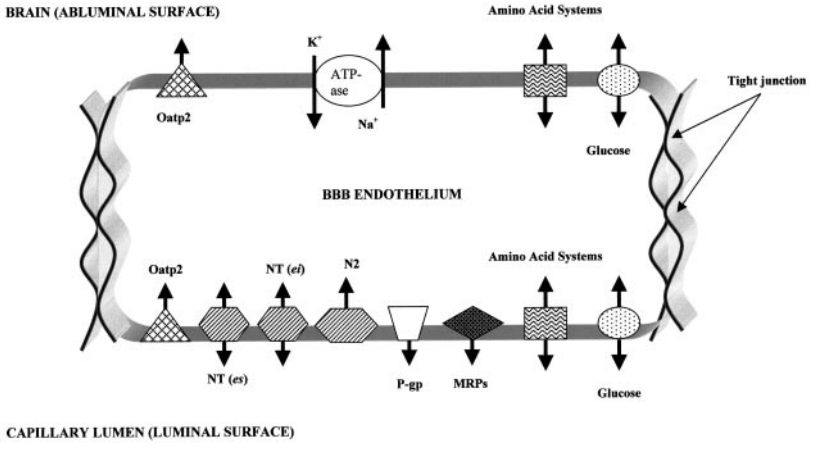

FIG. 2. Selected transport mechanisms along the BBB. A general depiction of the polarized expression of transporters for drugs and essential nutrients on a BBB endothelial cell. The arrows indicate the direction of transport. For a more descriptive representation of the major drug transport systems in the BBB [organic cation and anion transporters, nucleoside transporters (N2, *es*, and *ei*), and efflux systems (P-gp and MRP)], please refer to Figs. 4, 5, 6, and 7. Adapted from Betz et al., 1980; van Asperen et al., 1997.

The primary role of the CP is to produce and maintain the homeostatic composition of the CSF. The CP continuously secretes CSF, which is reabsorbed back into the circulation primarily by the arachnoid villi located in the superior sagittal sinus. The total volume of CSF (140 ml) is replaced 4 to 5 times daily (Enting et al., 1998). This continuous flow of CSF through the ventricular system into the subarachnoid space (Fig. 1D) and exiting into the venous system provides a "sink" that reduces the steady-state concentration of a molecule penetrating into the brain and CSF (Saunders et al., 1999). The sink effect is greater for large molecular weight and lipid-insoluble molecules. The CSF also contains approximately 0.3% plasma proteins, totaling 15 to 40 mg/ml, depending on sampling site (Felgenhauer, 1974). This is in contrast to the extracellular space of the normal adult brain, which contains no detectable plasma proteins (Azzi et al., 1990). An increase in CSF protein concentration has been observed under pathological circumstances, in some cases due to an increased permeability of the BBB (McAuthur et al., 1992).

As is the case with the BBB, the CP exhibits a polarized expression of receptors, enzymes, ion channels, and transport systems that regulate the CSF composition via processes of secretion and reabsorption (Spector and Johanson, 1989). The apical side expresses the Na^+/K^+-ATPase pump, ion channels for Cl^-, K^+, and Na^+/HCO_3^- cotransport carriers (Fig. 3). Studies have also revealed the expression of facilitated and sodium-dependent carriers for the transport of nonelectrolytes (Davson and Segal, 1970; Johanson et al., 1990; Garner and Brown, 1992). The basolateral side is lined with Na^+/H^+ antiporters, Cl^-/HCO_3^- antiporters, facilitated carriers for nonelectrolytes, and a carbonic anhydrase (Davson and Segal, 1970; Deng and Johanson, 1989; Johanson et al., 1990). In addition to various receptors and transporters, the CP expresses high levels of metabolic enzymes including UDP-glucuronosyltransferase and epoxide hydrolase, as well as cytochrome P450 IIB1/2, α- and μ-class GSH *S*-transferases, and GSH peroxidase (Tayarani et al., 1989; Volk et al., 1991; Johnson et al., 1993; Ghersi-Egea et al., 1994).

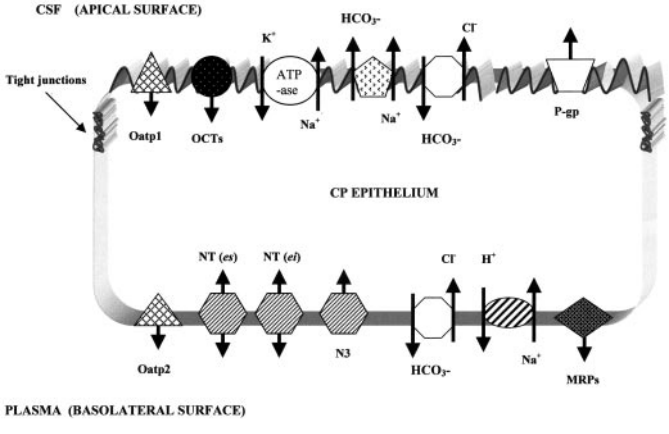

FIG. 3. Selected transport mechanisms along the blood-CSF barrier. A general description of the polarized expression of transporters for drugs and essential nutrients along a CP epithelial cell. The arrows indicate the direction of transport. For a more descriptive schema of the major drug transport systems in the CP [organic cation and anion transporters, nucleoside transporters (*es, ei* and N3), and efflux systems (P-g and MRP)], please refer to Figs. 4, 5, 6, and 7. Adapted from Johanson, 1988; Spector and Johanson, 1989.

The endothelial cells of the BBB and the epithelial cells of the CP thus provide more than a physical barrier between the brain and the peripheral circulation. The blood-brain and the blood-CSF barriers actively regulate the passage of solutes, regulatory proteins, metabolic fuels, neurotransmitter precursors, essential nutrients, and xenobiotics between the CNS and the blood. The presence of drug-metabolizing enzymes within the two brain compartments suggests an important role in the detoxification of potentially harmful xenobiotics and pharmacological agents.

III. Brain Parenchyma

The brain parenchyma is made up of neurons and the surrounding neuroglia cells. Neuroglia were originally thought to be passive cells that provided only structural support to the surrounding neurons (Compston et al., 1997; Araque et al., 1999). These cells were classified as neuroglia or "nerve glue" due to their spindle-shape and their "soft, medullary, fragile nature". This purely structural role for neuroglia has been abandoned since these cells are now known to have multiple functions in regulating an optimal interstitial environment.

There are two primary types of neuroglial cells that comprise the brain parenchyma, the macroglia, and microglia. The macroglia consist of astrocytes and oligodendrocytes, which like neurons possess an ectodermal origin and proliferate throughout life, particularly in response to injury (Peters et al., 1991). Microglia are smaller than macroglia and are considered to be the resident immune cells of the brain. Microglia are also capable of proliferating in response to injury. However, their origin, whether mesodermal or neuroectodermal, remains under debate (Schelper and Adrian, 1986; Boya et al., 1991).

A. Astrocytes

Astrocytes possess a star-shaped morphology and contain numerous cytoplasmic fibrils, of which the glial acidic fibrillary protein is the main constituent (Walz, 2000). There are two main types of astrocytes, fibrous (type-2) and protoplasmic (type-1), and they seem to differ in their location, cytoplasmic filament content, and antibody staining. Fibrous astrocytes are found mainly in the white matter of the brain, possess numerous filaments, and stain positive with the A2B5 antibody. Protoplasmic astrocytes are located primarily in the gray matter, contain less cytoplasmic filaments and stain negatively to the A2B5 antibody (Black et al., 1993).

Astrocytes are not only cytoskeletal support cells for neurons but possess numerous functions that aid in maintaining the normal homeostatic environment of the CNS. Kuffler et al. (1966) first demonstrated that astrocytes were nonexcitable cells with a large membrane potential that was sensitive to changes in extracellular K^+ concentrations. These results suggested that astrocytes were active participants in the homeostatic maintenance of the CNS by locally removing excess K^+ that had been released from active neurons (termed K^+ spatial buffering). Astrocytes are also involved in the initiation and regulation of immune and inflammatory events during injury and infection (Aschner, 1998). Secretion of cytokines such as interleukin-1 and -6, tumor necrosis factor-α, interferon-γ, and granulocyte colony-stimulating factor in response to infection and injury (Malipiero et al., 1990; Benveniste, 1993) may play an important role in the initiation and maintenance of neurotoxic immune responses within the injured CNS and further propagate CNS damage. For example, cytokine secretion by astrocytes and microglia is likely involved in the pathogenesis of human immunodeficiency virus-1 (HIV-1) dementia, a neurologic disorder characterized by destruction and dysfunction of neurons, that is observed in end-stage AIDS patients (Epstein and Gendelman, 1993; Rausch et al., 1999).

In addition to structural and immunological functions, astrocytes also maintain physiological extracellular neurotransmitter concentrations through their removal from the extracellular fluid (Fonnum, 1984; Anderson and Swanson, 2000). The importance of this excess removal is demonstrated by the removal of the excitatory neurotransmitter glutamate (Fonnum, 1984). Elevated brain levels of glutamate have been implicated in the pathogenesis of a variety of CNS disorders including amyotrophic lateral sclerosis, epilepsy, and cerebral infarctions (Anderson and Swanson, 2000).

Studies in both cultured and isolated astrocytes have shown that these cells express a wide variety of neurotransmitter receptors including glutamate, glycine, taurine, γ-aminobutyric acid, as well as several monoamines (Pearce et al., 1986; Usowicz et al., 1989; Shain and Martin, 1990; Kanner, 1993). The presence and perisynaptic location of these receptors on astrocyte foot processes suggest a signaling mechanism between neurons and astrocytes (Lieberman et al., 1989; Dani et al., 1992; Bruckner et al., 1993). Furthermore, astrocytes also appear to be intimately associated with neighboring glial and endothelial cells. Studies in brain endothelial-astrocyte and microglial-astrocyte cocultures suggest that astrocytes provide a variety of endogenous signals and diffusible factors that may serve to induce the formation of tight junctions, the expression of various proteins, maintain overall BBB integrity and promote differentiation and maturation of microglia (Debault and Cancilla, 1980; Tao-Cheng et al., 1987; Laterra and Goldstein, 1991; Minakawa et al., 1991; Tanaka and Maeda, 1996). In addition, astrocyte expression of various adhesion molecules (i.e., neural cell adhesion molecule, astrotactin, and L1) may guide immature nerves cells from their site of cell division to their final destination during brain maturation (Rakic, 1990). Recent evidence suggests that astrocytes possess a number of nutrient and drug transport proteins including several nucleoside transporters (Hosli and Hosli, 1988; Gu et al., 1996; Sinclair et al., 2000) as well as the ATP-dependent, membrane-bound, drug efflux transporters P-gp and MRP (Pardridge, 1997; Declèves et al., 2000).

B. Microglia

Microglia, first described by the Spanish neuroanatomist del Rio-Hortega (1932), represent 5 to 20% of the total glial population within the CNS (Lawson et al., 1990; Raivich et al., 1999). Although microglia appear to be ubiquitously distributed within the CNS, actual numbers vary according to region. For example, the basal ganglia and cerebellum have considerably greater amounts than the cerebral cortex (Dickson et al., 1991). The origin of microglia has been a long-standing and often controversial issue historically (Ling and Wong, 1993; Theele and Streit, 1993; Cuadros and Navascues, 1998) due in part to the lack of unique cell markers. Several studies support an ectodermal origin for microglia (Hao et al., 1991; Richardson et al., 1993; Fedoroff et al., 1997). However, with the discovery of various histological markers for microglia in the 1980s, most evidence supports a mesodermal origin, possibly through circulating monocytes that colonize the parenchyma following vascularization or via bone derived precursor cells that migrate during gestation (Jordan and Thomas, 1988; Perry and Gordon, 1991; Thomas, 1992; Theele and Streit, 1993). Several different modes of entry of microglial precursors into the developing CNS have been suggested including traversion of the pial surface of the meninges, crossing the endothelial cell wall of blood vessels of the CNS, and traversion of the epithelial cells lining the ventricles (Cuadros and Navascues, 1998; Navascues et al., 2000). Regardless of the specific area of invasion, following CNS entry, microglia precursors distribute throughout the CNS and differentiate into their mature (ramified) form.

Several morphologically distinct microglia have been identified including ramified (or resting), spheroid (or activated), and phagocytic types (Dickson et al., 1991). In normal adult brain, microglia are mostly found in a ramified or resting state and appear as small highly branched cells. Ultrastructural features of microglia, as determined by electron microscopy, include an irregular nucleus, clumped chromatin, and a sparsely occupied cytoplasm (Kitamura et al., 1977; Dickson et al., 1991). Following injury or infection, microglia become activated, which results in retraction of processes, proliferation, and up-regulation of several cell surface factors. The level of microglia activation appears to be graded

according to the type and severity of brain injury involved (Raivich et al., 1999). Streit et al. (1988) have demonstrated this phenomenon using rat-derived facial nerves. Following reversible axotomy (crushing of the nerve), microglia proliferate and surround the nerves while emitting several soluble trophic factors such as basic fibroblast growth factor and nerve growth factor (Heumann et al., 1987; Gomez-Pinilla et al., 1990; Araujo and Cotman, 1992). Increased expression of various integrins and major histocompatibility complex class I markers also occurs. Thus, the microglia appear to play a neuroprotective effect in the spheroid or activated stage, and aid in the recovery of reversibly damaged neurons. Conversely, ricin-induced degeneration of neurons (a irreversible and lethal event) results in microglia becoming fully activated phagocytes. This stage of activation is characterized by a significant increase in the expression of markers observed in the phagocytic stage including several integrins ($\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_M\beta_2$), and major histocompatibility complex class I and II antigens. Thus, microglia show remarkable "functional plasticity" depending on the severity of injury (Streit et al., 1988). A large body of evidence now exists which implicates excessive microglia activation and proliferation in the development of neuronal death in various pathological disease states. Examples include the Wernicke-Korsakoff syndrome, Parkinson's disease, Alzheimer's disease, ischemia, and several HIV-1 related pathologies in the CNS (Todd and Butterworth, 1999; McGeer and McGeer, 1998; Walton et al., 1999; Akiyama et al., 2000; Xiong et al., 2000). A mechanistic commonality observed in these various diseases is microglial production of a variety of neurotoxins in excess, including nitric oxide, tumor necrosis factor-α and reactive oxygen species such as peroxide. Excessive production of these factors leads to a cascade of effects including activation of astrocytes, further activation of microglia, and finally neuronal death.

Microglia express a wide variety of ion channels including multiple potassium, calcium, and sodium channels (Eder, 1998). The expression patterns of ion channels in microglia depend on the functional state of the cells and are involved in a variety of physiological functions including proliferation, ramification, maintenance of membrane potential, intracellular pH regulation, and cell volume regulation (Frelin et al., 1988; Faff et al., 1996; Klee et al., 1999). In addition, both ionotropic and metabotropic types of glutamate receptors also appear to be expressed in microglia (Ong et al., 1996; Gottlieb and Matute, 1997; Biber et al., 1999; Lopez-Redondo et al., 2000; Noda et al., 2000). Kainate (GluR5–7), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (GluR1–4), and *N*-methyl-D-aspartate (NR2A/B) ionotropic receptor subtypes have been identified, as well two metabotropic glutamate subtypes (mGlu5a and mGlu5b). Roles for these receptors in microglia are not fully known. However, one possible theory suggests increased production of cytotoxic cytokines, such as tumor necrosis factor-α, following ischemia and traumatic brain injuries (Noda et al., 2000). Less is known concerning the expression of the well characterized drug transporters within microglia. We have recently identified two nucleoside drug transporters within a continuous rat microglia cell culture system (Hong et al., 2000, 2001). As well, we have positively identified the existence of a functional form of P-gp within these rat-derived microglia (Lee et al., 2001).

C. Oligodendrocytes and Neurons

Several reviews summarize transport of ions, neurotransmitters, and nutrients within neurons and their myelinating cells oligodendrocytes (Vannucci et al., 1997; Seal and Amara, 1999; Verkhratsky and Steinhauser, 2000). However, there are limited studies documenting peripheral drug transporters localized to either the oligodendrocytes or neurons (Busch et al., 1998).

IV. Methods to Quantitate Drug Transport into/out of the Central Nervous System—In Vivo and In Vitro Methods

A. In Vivo Models to Study Drug Transport across the Blood-brain Barrier and the Choroid Plexus

In vivo and in vitro techniques utilized to examine drug transport in the brain will only be briefly discussed as a review of these methods is beyond the scope of this paper and can be found elsewhere (Fenstermacher et al., 1981). In vivo BBB models of drug transport can be broadly categorized according to methodological approach. Single passage techniques such as the indicator diffusion/dilution (Crone, 1963, 1965), brain uptake index (Oldendorf, 1970), and external registration (Raichle et al., 1974, 1976) measure the uptake of substances into the CNS following a single passage through the brain upon injection into the blood stream. A major disadvantage of the single passage techniques is that transport estimates of drugs or solutes with extremely slow uptake may be inaccurate due to the short solute exposure times (Enting et al., 1998). Multipassage techniques, then, can be used to allow the test substance longer circulation times. Intravenous administration (Enting et al., 1998) and microdialysis methods are examples of multipassage techniques (Parsons and Justice, 1994; Boschi et al., 1995; de Lange et al., 1995). These techniques are model-dependent, and the method of data analysis (i.e., two-compartment model, three-compartment model, etc.) is normally chosen prior to the experiment. Therefore, once chosen, the results are model specific and may not necessarily be indicative of the actual transport and metabolic processes within the tissue (Fenstermacher et al., 1981). Finally, perfusion techniques, such as the in situ perfusion method, expose the brain tissue to the test substance by perfusion with a physiological buffer (van Bree et al., 1992). This model

was developed to provide further control over the experimental conditions (pH, temperature, etc.) and to avoid metabolism of the test substance during transfer across the BBB. Compared with single or multipassage methods, permeability coefficients can be measured accurately over a 10^4-fold range (Takasato et al., 1984) making this method 100-fold more sensitive. Therefore, measurements of brain uptake of poorly penetrating compounds ($P = 10^{-8}$ to 10^{-7} cm $\cdot$ s^{-1}) or rapidly penetrating compounds ($P = 10^{-4}$ cm $\cdot$ s^{-1}) can be determined allowing for the characterization of carrier-mediated transport at the BBB (Smith et al., 1984). The involvement of complex surgery and the requirement of mathematical models are the main disadvantages of the perfusion models (Takasato et al., 1984; Enting et al., 1998)

Although a number of experimental approaches have been developed to quantify transport of compounds from the blood into the CSF, many of these methodologies have proven to be inaccurate or do not produce useful data. In addition, the experimental procedure is quite complex and requires a certain amount of surgical skill and experience. The more common methodologies include the CNS deconvolution technique (van Bree et al., 1989) and the in situ CP model (Ames et al., 1964). The CNS deconvolution technique is based on serial sampling of the CSF and numerical deconvolution of data to determine a transport profile of the drug in a single living animal. The in situ CP model replaces the endogenous CSF with oil such as ethyl iodophenylundecylate, which allows the CP to be easily visualized. The fluid droplets that are formed on the surface are collected and a steady-state clearance fraction of the drug can be determined (Ames et al., 1964).

B. In Vitro Models to Study Drug Transport in the Brain

In general, in vivo methodologies to study drug transport in the CNS are costly. Furthermore, it is often difficult to maintain control of environmental factors such as pH, temperature, osmotic pressure, oxygen, carbon dioxide, as well as physiological responses (metabolism, tissue distribution, excretion) that occur in the animal under normal and experimental conditions (Freshney, 1994). An alternative to in vivo studies of drug transport is in vitro cell and tissue culture systems. Tissue culture techniques were developed as a method for studying the behavior of a specific population of cells free of systemic variations that may arise in the animal both during normal homeostasis and under stress of an experiment (Harrison, 1907). The development of tissue culture transport systems has revolutionized the drug transport field and has resulted in an explosion of research over the last 50 years. Not only do cell cultures provide a level of control over the environment and various physiological responses, they also provide specific information on the type of transporter(s) involved and relative pharmacokinetic parameters such as carrier affinity and specificity. Nevertheless, these systems are limited in that many of the phenotypic and functional characteristics of the original tissue may be lost (i.e., tight junctions in brain endothelial cells, production of specific factors by cells, expression and activity of various transporters) due to culture conditions and the absence of endogenous factors and signals (Freshney, 1994). For example, gene expression of some drug transporters in the brain (i.e., P-gp and MRP) can be both up- and down-regulated in culture (Regina et al., 1998). This change in gene expression that sometimes occurs in culture may be a consequence of a variety of factors such as culture conditions (presence of serum in media and nature of substratum) and the absence of endogenous factors and signals that are present in vivo. Consequently, caution must be taken when extrapolating in vitro tissue culture data to either in vivo models or clinical practice.

A common method of studying in vitro drug transport of nonpolarized cells involves culture and growth of isolated cells on impermeable polystyrene strata (e.g., 24-well plates) and measurement of the cellular uptake/accumulation or efflux of a radiolabeled substrate or fluorescent probe. Specific transporter characteristics can then be examined utilizing known transporter inhibitors, metabolic inhibitors, etc., which are appropriate for the transporter of interest (Hunter et al., 1991; Hong et al., 2001). Polarized cells, such as epithelial and endothelial cells, can also be grown on porous filter membranes, which provide the option of examining both basal-to-apical and apical-to-basal transport of substrates. Recently, Miller et al. (2000) have developed a novel method to characterize transport properties of substrates in isolated brain capillaries using fluorescent substrates, confocal microscopy, and quantitative image analysis. This method minimizes the possibility of altered transporter expression and activity observed in cell culture systems (Regina et al., 1998; Gaillard et al., 2000) and provides direct evidence of transport in isolated capillaries, which can be masked by other efflux transporters (i.e., MRP) in conventional transport assays. Most importantly, this method provides spatial resolution where the substrate fluorescence can be distinguished from that in the endothelium and associated cells.

By far, the most extensively studied cells of the brain are the endothelial cells of the blood-brain barrier (van Bree et al., 1992). Growth of homogenous cultures of brain microvessel endothelial cells, both primary cultures or immortalized cultures, have been described from various mammalian species including rat, bovine, and human (Tsuji et al., 1992; Begley et al., 1996; Seetharaman et al., 1998). A variety of drug transporters have now been identified and characterized utilizing these methods and will be discussed in detail including organic cation transporters (Wu et al., 1998a), organic

anion transporters (Gao et al., 1999), nucleoside transporters (Thomas and Segal, 1996), P-gp (Tatsuta et al., 1992), and MRP (Regina et al., 1998).

The BBB is not an isolated tissue. Therefore, in an attempt to produce a more representative in vitro BBB model, the coculture BBB system was developed (Meyer et al., 1991). Cocultures of endothelial and astrocyte cells allow for greater cell differentiation and may express specific proteins that are not present in monocultures (Regina et al., 1998). For example, primary rat brain capillary endothelial cultures generally do not maintain tight junctions past the fourth cell passage. However, addition of astrocyte-conditioned culture media can re-establish these junctions (Tao-Cheng et al., 1987). Furthermore, compared with single cultures, BBB cocultures have a down-regulated expression of MRP (Regina et al., 1998) and up-regulated P-gp expression (Gaillard et al., 2000), which is reflective of the in vivo expression patterns. Thus, a coculture system provides a more physiologically accurate representation of the BBB and allows for more meaningful studies of drug transport, metabolism, and drug-drug interactions at the cellular level. The coculture system is achieved by growth of the endothelial cells and astrocytes either in the same culture dish or via growth on the opposite sides of a porous filter, which permits cell to cell contact between the astrocyte foot processes and the endothelium (Pardridge, 1999).

Drug transport across the epithelial cells of the CP has also been well characterized (Washington et al., 1996). A variety of mammalian cell culture systems have been described and produce an impermeable cell monolayer that displays many of the characteristics of the CP barrier in vivo (Zheng et al., 1998; Haselbach et al., 2001). Transporters identified to date in the CP include organic anion transporters (Gao and Meier, 2001), P-gp and MRP (Rao et al., 1999), nucleoside transporters (Wu et al., 1994), and organic cation transporters (Suzuki et al., 1986).

In contrast to the BBB and CP, primary cultures and continuous cell lines of astrocytes and microglia are not polarized. As a result, only unidirectional cellular accumulation or efflux can be measured (Hertz et al., 1998). Although the functional expression of nucleoside transporters P-gp and MRP has been characterized to some extent in cultures of primary astrocytes (Hosli and Hosli, 1988; Gu et al., 1996; Declèves et al., 2000), drug transport studies in microglia remain extremely limited. Recently, we have characterized a Na^+-dependent nucleoside transporter (Hong et al., 2000) and a novel electrogenic zidovudine/H^+-dependent transporter (Hong et al., 2001) in microglia, utilizing a continuous rat brain microglia cell line (MLS-9) developed by Schlichter et al. (1996). These studies provide evidence that microglia express membrane transporters that may be important for drug transport and distribution in brain parenchyma. More recently, we have characterized the functional expression of P-gp (Lee et al., 2001) and MRP (Dallas et al., 2001) within primary and continuous microglia cell cultures.

In general, isolated spheroid microglia cells rarely differentiate into their mature ramified form in the absence of astrocytes (Tanaka and Maeda, 1996). Indeed, the continuous microglia cell line (MLS-9) does not exhibit the morphology of ramified, process-bearing microglia in culture (Hong et al., 2000). At confluence these cells are more characteristic of spheroid microglia precursors or "activated" microglia, with short processes and large egg-shaped cell bodies (Hong et al., 2000). Tanaka and Maeda (1996) have demonstrated that microglia-astrocyte cocultures promote highly differentiated and ramified microglia. As with brain endothelial-astrocyte cocultures, it appears that astrocytes provide a variety of diffusible factors that are present in vivo to promote differentiation of microglia cells in vitro. The study of drug transport in these microglia-astrocyte cocultures certainly warrants future investigation.

V. Drug Transport Mechanisms in the Brain

It was originally believed that membrane carriers localized at the brain barriers were solely responsible for the transport of endogenous substances into and out of the brain and that drug transport across the brain barriers was largely dependent on the physicochemical characteristics of the drug such as lipophilicity, molecular weight, and ionic state (Spector, 1990; Tamai and Tsuji, 2000). Generally, small, nonionic, lipid-soluble molecules penetrate easily across the BBB whereas larger, water-soluble, and/or ionic molecules will less likely exhibit passive diffusional processes (Spector, 1977, 1990). For some drugs the rate of entry and distribution in the CNS cannot be explained by passive processes that depend on the physicochemical characteristics listed above (Spector, 1987, 1988; Takasawa et al., 1997b). Many drug transporters that have been well characterized in peripheral tissues and are known to be involved in the influx and efflux of drugs (i.e., the organic cation, organic anion, nucleoside, P-gp, and MRP transporters), have now been identified in the brain. It is now recognized that these drug transporters may influence many pharmacokinetic characteristics of drugs in the processes of absorption, distribution, and elimination.

A. Organic Cation Transport Systems

A diverse group of organic cations, including endogenous bioactive amines (i.e., acetylcholine, choline, dopamine, epinephrine, norepinephrine, guanidine, N^1-methylnicotinamide, thiamine), therapeutic drugs (i.e., cimetidine, amiloride, mepiperphenidol, morphine, quinine, quinidine, tetraethylammonium, verapamil, trimethoprim), and xenobiotics (i.e., paraquat), are actively transported by the OCT system primarily in the liver

and kidney (Rennick, 1981; Zhang et al., 1998). At physiological pH, the nitrogen moiety of these compounds (generally primary, secondary, tertiary, or quaternary amines) bears a transient or permanent net positive charge, which is determined by the compound's pK_a value. Two distinct classes of OCT systems have been defined: a potential-sensitive transporter usually involved in the influx of organic cations and an H^+ gradient-dependent transporter, mediating efflux (Ullrich, 1994). The concerted action of these two OCT subtypes results in the vectorial transfer of cationic compounds from the blood into the luminal fluid across the renal tubular cells (Hsyu and Giacomini, 1987; Dantzler et al., 1989; Bendayan et al., 1990, 1994; Escorbar et al., 1994) or from the blood into the bile across the hepatocyte, intestinal epithelium, and the placental syncytiotrophoblast (Ganapathy et al., 1988; Prasad et al., 1992; Iseki et al., 1993; Zevin et al., 1997; Laforenza et al., 1998). In the brain, the physiological role of the OCT systems includes transport of cationic neurotoxins and neurotransmitters (Murakami et al., 2000).

At present, the OCT family includes three potential-sensitive (i.e., OCT1, OCT2, OCT3) and two H^+-driven systems (i.e., OCTN1 and OCTN2) (Table 1). In general, OCTs contain 12 transmembrane domains with a large extracellular hydrophobic loop between the first and second domains and a large intracellular hydrophobic loop between the sixth and seventh domains (Koepsell, 1998). Although the extracellular loop contains several glycosylation sites, the intracellular loop has a number of potential phosphorylation sites (Fig. 4). The exact membrane topology of this system remains to be fully characterized.

OCT1 was originally cloned from rat kidney (Grundemann et al., 1994), followed by the isolation of the mouse, human, and rabbit homologs (Schweifer and Barlow, 1996; Gorboulev et al., 1997; Zhang et al., 1997; Terashita et al., 1998). Similarly, OCT2 was cloned from rat kidney by homolog screening (Okuda et al., 1996). Subsequently, the human and porcine homologs were isolated and characterized (Gorboulev et al., 1997; Grundemann et al., 1997). Northern blot analysis has shown that both OCT1 and OCT2 are expressed primarily in the kidney and liver and, to a smaller extent, in the intestine (Grundemann et al., 1994; Okuda et al., 1996; Gorboulev et al., 1997; Zhang et al., 1997). Both of these transporters recognize a variety of endogenous and exogenous organic cations as substrates and exhibit considerable overlap in substrate specificity. Several cationic neurotoxins and monoamine neurotransmitters are accepted as substrates by OCT1 and OCT2 (Martel et al., 1996; Gorboulev et al., 1997; Zhang et al., 1997; Busch et al., 1998). In particular, the polyspecific, electrogenic OCT2 present in human neurons has been reported to mediate the transport of monoamine neurotransmitters dopamine, norepinephrine, serotonin, histamine, and the antiparkinsonian drugs, amantadine and memantine (Busch et al., 1998). In voltage-clamp experiments with rOCT1-expressing *Xenopus* oocytes, tracer flux of dopamine, serotonin, noradrenaline, histamine, and acetylcholine induced saturable currents with K_m values ranging from 20 to 100 μM (Busch et al., 1996). Although not detectable by Northern analysis, RT-PCR studies indicate that OCT1 and OCT2 may be expressed in the brain, but at very low levels (Gorboulev et al., 1997; Grundemann et al., 1997). A third organic cation transporter, OCT3, was cloned more recently from rat placenta and appears to be ubiquitously expressed (Kekuda et al., 1998). Moreover, OCT3 appears to be expressed more abundantly in the mammalian brain than either OCT1 or OCT2. For example, in situ hybridization studies demonstrated that OCT3 is widely expressed in several different brain regions, including the hippocampus, cerebellum, and cerebral cortex (Wu et al., 1998a). Accumulation studies utilizing tetraethylammonium demonstrated a low affinity (K_m = 2.5 mM) saturable system in OCT3 cDNA transfected HeLa cells (Kekuda et al., 1998). OCT3-specific methyl-4-phenylpyridinium uptake activity (apparent K_m = 91 μM) in a human retinal pigment epithelial cell line indicates that various cationic neurotoxins and neurotransmitters are OCT3 substrates. The order of affinity was amphet-

TABLE 1
Organic cation transporter family

OCT Process	Cloning Source	Driving Force	Tissue Specificity
hOCTN1	Human fetal liver	H^+ gradient	Liver, kidney, trachea, bone marrow, skeletal muscle, prostate, lung, pancreas, placenta, heart, uterus, spleen, and spinal cord
hOCTN2	Human placenta	H^+ gradient	High in heart, placenta, kidney, and pancreas; low in brain, liver, and lung
pOCT2	LLC-PK_1 cells	H^+ gradient	Kidney and brain
rOCT1	Rat kidney	Electrogenic	High in kidney cortex, liver, intestine, and colon
rOCT1A	Rat kidney	N.D.	Kidney cortex and medulla, liver, intestine, and colon
hOCT1	Human liver	Electrogenic	High in liver; low in kidney and intestine
rbOCT1	Rabbit kidney	Electrogenic	High in liver; low in kidney and intestine
rOCT2	Rat kidney	Electrogenic	High in kidney medulla; low in brain
hOCT2	Human kidney	Electrogenic	Kidney, brain, placenta; low in small intestine and spleen
rOCT3	Rat placenta	Electrogenic[a]	High in placenta; moderate in intestine, heart, and brain; low in lung and kidney; undetectable in liver

N.D., not determined.
[a] The system rOCT3 is electrogenic when expressed in *Xenopus laevis* oocytes but H^+-dependent if expressed in HeLa cells. In general, the substrate specificity of the OCT transporters include endogenous and exogenous amines (e.g. tetraethylammonium, N^1-methylnicotinamide, choline, thymine, cimetidine). Adapted from Koepsell, 1998; Zhang et al., 1998.

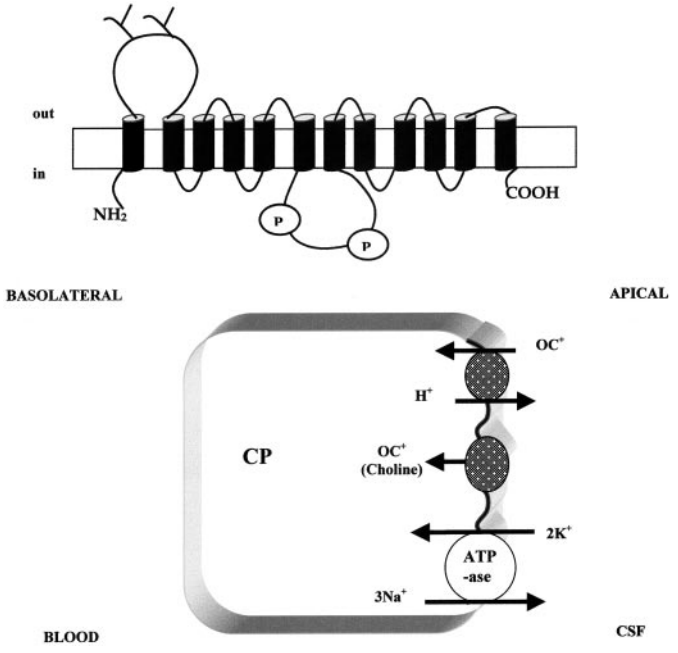

FIG. 4. Organic cation transport systems in the CP. The proposed topology for rat OCT1 is shown. P, phosphorylation sites. In the CP, several studies have reported the removal of organic cations (i.e., tetraethylammonium, choline) from the CSF into the blood by a variety of mechanisms (i.e., pH dependent, electrogenic). Only the OCT systems that have been experimentally localized to a specific side of the CP epithelium are presented. Arrows indicate the direction of transport. Adapted from Koepsell, 1998.

amine > desipramine > metamphetamine > dopamine > serotonin (IC_{50} range = 42–970 μM) (Wu et al., 1998a). The transport characteristics and steroid (i.e., β-estradiol) sensitivity provide strong evidence for the molecular identity of OCT3 as an extraneuronal monoamine transporter ($uptake_2$) (Wu et al., 1998a).

The H^+ driven organic cation transporters OCTN1 and OCTN2, were cloned originally from human fetal liver and placenta, respectively (Tamai et al., 1997; Wu et al., 1998b). OCTN1 is predominantly expressed in kidney, trachea, bone marrow, fetal liver, and in several human cancer cell lines but not in adult liver (Tamai et al., 1997). Northern blot analysis revealed that the expression of OCTN2 occurs mainly in heart, placenta, skeletal muscle, kidney and pancreas, with weak signals observed in brain, lung, and liver (Wu et al., 1998b). The regional brain distribution of this transporter remains to be established. When expressed in HeLa cells, OCTN2 mediates the transport of a number of classical organic cation transporter substrates including tetraethylammonium and methyl-4-phenylpyridinium (Wu et al., 1998b). Recently it has been shown that OCTN2 can transport carnitine in a Na^+-dependent manner in HEK293 cells (Tamai et al., 1998). Three other cDNA clones, NLT (cloned from rat liver) RST, and NKT (cloned from mouse kidney) exhibit significant sequence homology to the OCT transporter family. The transport functions of these three clones in the brain remains to be examined (Simonson et al., 1994; Lopez-Nieto et al., 1997; Mori et al., 1997).

Although OCT mechanisms in the kidney and liver are well characterized, experimental assessment of the transport mechanisms in the brain, such as via the CP, is limited by the small size, complex morphology, and anatomic inaccessibility of the CP epithelia. Some transport properties of the CP OCT have been obtained using in situ ventriculocisternal perfusion (Miller and Ross, 1976; Lanman and Schanker, 1980), preparations of isolated CP (Tochino and Schanker, 1965a,b), and apical membrane vesicles (Whittico et al., 1990). However, these techniques do not provide direct access to both interfaces of the intact CP epithelium and information on the energetics and polarities of the OCT carriers across the CSF-blood barrier remains incomplete. A notable tissue related difference between the OCT systems is that in the kidney, the transporter functions in the secretory direction (i.e., from blood to urine) whereas in the CP, the transporter functions in the absorptive direction (i.e., from CSF to blood). Carrier-mediated transepithelial absorption of both endogenous and xenobiotic organic cations (e.g., choline, N^1-methylnicotinamide, tetraethylammonium, cimetidine, serotonin, and norepinephrine) from CSF has been demonstrated both in vivo and in vitro using isolated CP tissue slices and ventriculocisternal perfusion techniques (Schanker et al., 1962; Tochino and Schanker, 1965b; Hug, 1967; Barany, 1976; Miller and Ross, 1976; Lanman and Schanker, 1980; Suzuki et al., 1985, 1986).

Apical tetraethylammonium uptake by rat cultured CP epithelial cells was a pH dependent saturable process with an apparent K_m of 315 μM (Villalobos et al., 1997) (Fig. 4). This affinity constant is similar to that reported for tetraethylammonium in both basolateral (160 μM, Ullrich et al., 1991; 280 μM, Brandle et al., 1992) and luminal (192 μM, Wright et al., 1995) membranes of the kidney. However, a K_m of 900 μM was estimated from tetraethylammonium inhibition of cimetidine transport by isolated CP in vitro (Suzuki et al., 1986). The apical tetraethylammonium uptake by cultured CP epithelium was markedly reduced (≥40%) by other organic cations, such as choline, mepiperphenidol, but not by the organic anion *p*-aminohippurate (Villalobos et al., 1997). This sensitivity of tetraethylammonium transport in cultured CP cells to quaternary ammonium compounds and its insensitivity to the organic anion *p*-aminohippurate corroborates previous studies of OCT transport across the intact CSF-blood barrier and in isolated CP (Schanker et al., 1962; Tochino and Schanker, 1965a; Hug, 1967; Miller and Ross, 1976; Lanman and Schanker, 1980).

Interestingly, transepithelial absorption of the organic cation cimetidine from rat CSF and CP uptake in vitro are inhibited by the organic anions benzylpenicillin and salicylic acid but not by tetraethylammonium and other quaternary ammonium compounds (Suzuki et al.,

1985, 1986, 1988). Furthermore, cimetidine inhibits transport of organic anions but poorly inhibits quaternary ammonium transport. The lipophilic organic bases quinidine and quinine are potent inhibitors of cimetidine transport in isolated rat plexus tissue and bovine ventricular brush-border membrane vesicles (Suzuki et al., 1986; Whittico et al., 1990). The transport kinetics of cimetidine, either by isolated rat CP, or by isolated bovine CP vesicles (proven to be pH driven in this system) were similar with apparent K_m values of 53 and 58 μM, respectively. These data suggest that cimetidine is transported across the CP apical membrane by a different mechanism than the brush-border membrane of the kidney, with a lower affinity and higher capacity (Gisclon et al., 1987). Electrogenic apical uptake of choline across the ventricular membrane of neonate rat CP has been demonstrated (Villalobos et al., 1999) (Fig. 4). Choline appears to be transported across the CSF-blood barrier (K_m = 16–50 μM) with greater affinity than by i) BBB (K_m = 225–445 μM); ii) apical membranes of the renal proximal tubule (K_m = 100 μM); and iii) the small intestine (K_m = 150 μM) (Aquilonius and Winbladh, 1972; Cornford et al., 1978; Lanman and Schanker, 1980; Saitoh et al., 1992; Wright et al., 1992).

There is also evidence for the transport of endogenous bioactive amines such as choline and thiamine across the BBB (Koepsell, 1998). An in vivo study showed that thiamine monophosphate is transported across the BBB into the brain by a saturable mechanism with a K_m of 2.6 to 4.8 μM, possibly by an organic cation transporter (Patrini et al., 1988). In cultured brain capillary endothelial cells, there is both saturable and nonsaturable uptake processes for choline (Sawada et al., 1999). The saturable process was energy-dependent (Galea and Estrada, 1992), sodium, and pH independent and could be inhibited by various OCT substrates and inhibitors (Sawada et al., 1999). Furthermore, in situ brain perfusion studies corroborate the in vitro data by demonstrating the presence of a sodium-independent transporter for choline uptake into the brain (K_m = 39–42 μM) (Murakami et al., 2000; Allen and Smith, 2001). These in vitro and in vivo results suggest that the choline transporter at the BBB is a member of the OCT family. The membrane location of these transporters remains to be elucidated.

B. Organic Anion Transport Systems

The liver and kidney are organs central to the elimination of endogenous and exogenous organic anions, many of which are harmful to the body (Pritchard and Miller, 1993; Ullrich and Rumrich, 1993; Meier, 1995; Muller and Jansen, 1997). Several families of multispecific organic anion transporters have been identified, of which the two main families, i.e., the organic anion transporter polypeptide (oatp), and the organic anion transporter OAT will be discussed (Sekine et al., 2000).

To date, seven isoforms [oatp1, oatp2, oatp3, OAT-K1, OAT-K2, OATP, prostaglandin transporter (PGT), and the liver-specific transporter-1 (LST-1)] have been identified in the oatp family (Sekine et al., 2000) (Table 2). In the liver, oatp1 and oatp2 are multispecific organic anion carriers that transport structurally unrelated anionic compounds in a sodium-independent manner (Meier, 1995; Muller and Jansen, 1997; Noe et al., 1997; Kakyo et al., 1999a). Both are expressed in the brain. Oatp1, a bidirectional organic anion/HCO_3^- and/or organic anion/glutathione exchanger, is expressed at the apical membrane of the CP (Angeletti et al., 1997; Li et al., 1998) in contrast to its basolateral localization in the hepatocyte (Bergwerk et al., 1996). It possesses a broad substrate specificity and mediates the transport of bile salts, steroid hormones, and a variety of organic anions and cations (Sekine et al., 2000). However, whether oatp1 is responsible for the uptake or efflux of organic anions across the CP remains to be elucidated (Angeletti et al., 1997). Oatp2, cloned from rat brain, is expressed in liver, kidney, brain capillaries, and the basolateral membrane of the CP (Noe et al., 1997; Gao et al., 1999).

TABLE 2
Organic anion transporter polypeptide (oatp) and organic anion transporter (OAT) families

OAT Processes	Cloning Source(s)	Substrate Specificity	Tissue Expression
Oatp family			
oatp1	Rat	Bile salts, steroid hormones, leukotriene C_4, bulky organic cations	Liver, CP, kidney
oatp2	Rat	Taurocholate, cholate, estrogen conjugates, ouabain, digoxin, DPDPE	Liver, kidney, BBB, CP
oatp3	Rat	Thyroxine, triiodothyronine, taurocholate	Kidney, retina
OAT-K1	Rat	Methotrexate, folate	Kidney
OAT-K2	Rat	Taurocholate, methotrexate, folate, prostaglandin E	Kidney
OATP (OATP-A)	Human	Bromosulfophthalein, cholate, taurocholate, glycocholate, taurochenodeoxycholate, tauroursodeoxycholate, opioid peptides (deltorphin II, DPDPE)	Liver, BBB, lung, kidney, testes
PGT	Rat	Unknown	Unknown
LST-1	Human, rat	Taurocholate	Liver
OAT family			
OAT1	Human, rat	PAH, dicarboxylates, cyclic nucleotides, prostaglandin E, urate, μ-lactam antibiotics, nonsteroidal anti-inflammatory drugs, diuretics	Kidney, brain
OAT2	Rat	PAH, salicylate, acetylsalicylate, prostaglandin E, dicarboxylates	Liver, kidney
OAT3	Human, rat	PAH, cimetidine, estrone sulfate	Liver, brain, kidney, eye
OAT4	Human	Estrone sulfate, DHEA sulfate	Kidney, placenta

DPDPE, [D-Pen(2), D-Pen(5)]enkephalin; PAH, *p*-aminohippurate; DHEA, dehydroepiandosterone (Sekine et al., 2000).

It mediates the uptake of bile acids taurocholate, cholate, estrogen conjugates, ouabain, and digoxin (Noe et al., 1997; Asaba et al., 2000). Oatp3, isolated from rat retina and expressed in kidney and retina, was shown to transport thyroid hormones and taurocholate (Abe et al., 1998). OAT-K1 and OAT-K2 are both localized to the luminal membrane of the renal proximal tubule (Masuda et al., 1997, 1999). OAT-K1 is involved in the transport of methotrexate and folate whereas OAT-K2 transports hydrophobic organic anions such as taurocholate, methotrexate, folate, and prostaglandin E2 (Masuda et al., 1997, 1999). OATP is the cloned human liver organic anion carrier that transports bromosulfophthalein, cholate, taurocholate, glycocholate, taurochenodeoxycholate, and tauroursodeoxycholate in a sodium-independent manner (Kullak-Ublick et al., 1995). It is expressed in human lung, kidney, and testes. Recently, OATP was shown to be expressed along the BBB in cultured human brain endothelial cells (Gao et al., 2000). This transporter was found to transport two opioid peptides, deltorphin II (K_m 330 μM) and the enkephalin analog, [D-Pen(2),D-Pen(5)]enkephalin (K_m ~202 μM), the latter also transported by rat oatp2 at the BBB (Kakyo et al., 1999). On the basis of sequence homology, PGT and LST-1 are believed to be oatp isoforms, of which the latter may be important for bile clearance (Kanai et al., 1995; Kakyo et al., 1999b).

The OAT family is primarily responsible for the elimination of organic anions from the kidney. While all four isoforms (OAT1, OAT2, OAT3, OAT4) are expressed in the kidney, a few are also expressed in the liver, brain, and placenta (Sekine et al., 2000). In general, these proteins possess 12 putative transmembrane domains, with large hydrophobic loops between the first and second, and the sixth and seventh domains (Sekine et al., 2000) (Fig. 5). *N*-Glycosylation sites are predicted on the hydrophobic loop between the first and second transmembrane domain (Kuze et al., 1999) as well as several phosphorylation sites on the loop between the sixth and seventh domains (Sekine et al., 2000).

Organic anion transporters are categorized into three classes depending on their energy requirements: sodium-dependent OATs, sodium-independent facilitators or exchangers, and active OATs that require ATP. The active and sodium-independent OATs possess broad substrate specificity and are primarily involved in the secretion of organic anions in both kidney and liver. The sodium-dependent OATs on the other hand, have a narrow substrate specificity and play a major role in the reabsorption of essential anionic substances into the proximal tubules of the kidney (Sekine et al., 2000) (Table 2).

In the kidney, it is well established that anionic drugs and other xenobiotics are actively transported from the blood to the urine (Pritchard and Miller, 1993). The basolateral step is indirectly coupled to the sodium gradient by Na^+/dicarboxylate cotransport, which maintains a large in > out gradient for the α-ketoglutarate/organic anion exchange (Shimada et al., 1987; Pritchard, 1988, 1990). This exchanger (OAT1) has recently been cloned in rat (Sekine et al., 1997; Sweet et al., 1997) and human (Cihlar et al., 1999; Hosoyamada et al., 1999; Lu et al., 1999). OAT1, specifically expressed in kidney, is a multispecific organic anion/dicarboxylate exchanger that interacts with a variety of organic anions, i.e., *p*-aminohippurate (K_m = 14.3 μM), dicarboxylates, cyclic nucleotides, prostaglandin E, urate, antibiotics, nonsteroidal anti-inflammatory drugs, and diuretics (Sekine et al., 1997). OAT2 is a liver-specific organic anion transporter and accepts *p*-aminohippurate, salicylate and

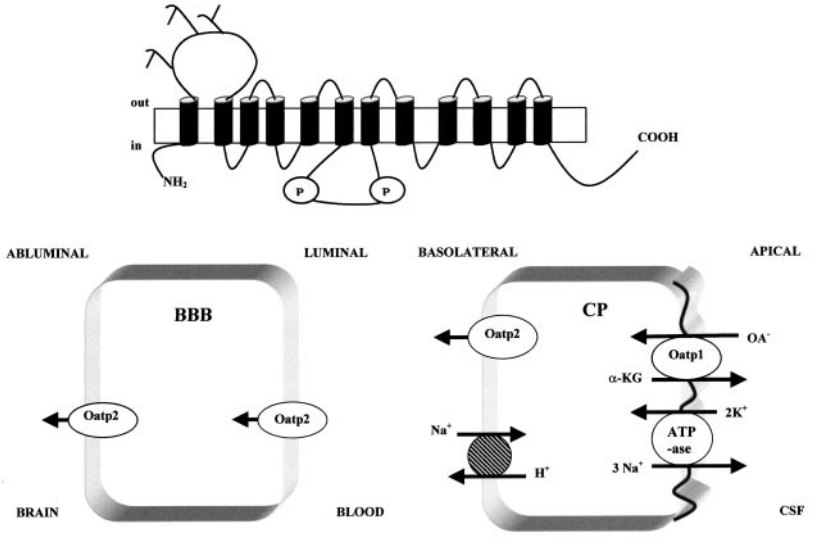

FIG. 5. Organic anion transport systems in the BBB and CP. The proposed topology for rat OAT1 is shown. P, phosphorylation sites. In addition to the transport of a variety of organic anions across the brain barriers, the active uptake of digoxin across the BBB and the secretion of estrogen conjugates and opioid peptides across the CP have been reported. Only the OAT systems that have been experimentally localized to a specific side of the BBB endothelium and CP epithelium are shown. Arrows indicate the direction of transport. Adapted from Sekine et al., 2000.

acetylsalicylate, prostaglandin E, and dicarboxylates as substrates (Sekine et al., 1998). Apical renal exit of organic anions is also carrier-mediated but is not well characterized and may involve either potential or exchange driven mechanisms (Pritchard and Miller, 1993).

In the brain, the expression of OAT1 is very low (Sekine et al., 1997). Recently, a new member, OAT3, was isolated from rat brain by RT-PCR cloning methods (Kusuhara et al., 1999). OAT3 mRNA is expressed in liver, brain, kidney, and eye. When expressed in *Xenopus* oocytes, it mediates the transport of *p*-aminohippurate (K_m = 65 μM) and cimetidine. Acidic metabolites of neurotransmitters (i.e., dopamine, epinephrine, norepinephrine, and serotonin) inhibited the uptake of estrone sulfate by OAT3 suggesting its role in the excretion/detoxification of endogenous anionic substrates from the brain (Kusuhara et al., 1999). OAT4, expressed in the placenta and kidney, is a novel member of the multispecific OAT family exhibiting approximately 38 to 44% amino acid sequence homology to the other members of the OAT family (Cha et al., 2000). It mediates the transport of estrone sulfate, dehydroepiandrosterone sulfate, and a variety of anionic compounds (i.e., bile salts, sulfobromophthalein, diuretics) in a sodium-independent manner.

Direct mechanistic information on organic anion systems along the blood-CSF barrier is sparse, owing in part to the small size and physical inaccessibility of the plexus and in part to gaps in our understanding of the mechanisms and driving forces mediating OAT processes (Pritchard and Miller, 1993). Although the molecular mechanisms responsible for CP transport are largely unexplored, one fundamental difference from excretory renal epithelia is evident: organic anions are transported into the blood, not extracted from it. Indeed, this reversal in function is reflected in other important ways, most notably in the unique apical distribution of Na^+,K^+-ATPase in CP, whereas it is basolateral in virtually all other epithelia (Quinton et al., 1973; Ernst et al., 1986; Villalobos et al., 1997).

In addition to the blood-CSF barrier, OAT systems have also been localized along the BBB. Studies show that P-gp-deficient mice [mdr1a(−/−)] exhibit significantly increased brain concentrations of a variety of drugs, including digoxin, a cardiac glycoside (Mayer et al., 1996; Schinkel et al., 1996; Kim et al., 1998). Interestingly, the digoxin concentration continued to increase in the brains of the mdr1a(−/−) over a long time period despite diminishing blood levels, suggesting a possible active uptake system for digoxin along the BBB (Mayer et al., 1996). It is now established that this system is the novel multispecific OAT (Oatp2) that possesses an extremely high affinity for digoxin (K_m = 0.24 μM) and has been cloned from rat brain. This transporter is localized along both the luminal and abluminal membranes of the BBB (Noe et al., 1997; Gao et al., 1999) (Fig. 5). Moreover, uptake studies in conditionally immortalized mouse brain capillary endothelial cells (TM-BBB4) that express Oatp2 show that dehydroepiandrosterone sulfate is a substrate for this transporter (K_m = 34.4 μM) (Asaba et al., 2000). Recently, the first human OATP (now called OATP-A), which was cloned from liver, was shown to be expressed along the BBB in cultured human brain endothelial cells (Gao et al., 2000). This transporter was found to transport two opioid peptides, deltorphin II (K_m = 330 μM) and [D-Pen(2),D-Pen(5)]enkephalin (K_m ~202 μM), the latter also transported by rat Oatp2 at the BBB (Kakyo et al., 1999).

As first documented by Pappenheimer et al. (1961), active transporters eliminate organic anions from the brain, thus preventing the buildup of potentially toxic compounds. In vivo and in vitro kinetic studies have suggested the presence of efflux transport pathways for organic anions in the BBB and blood-CSF barrier. CP was shown to mediate the removal of organic anions from the CSF into the blood for their subsequent elimination by liver or kidney. This has been established by the observations that organic anions are eliminated from CSF after intraventricular administration and/or during ventriculocisternal perfusion and that the respective organic anions are accumulated in the isolated CP (Suzuki et al., 1997). This includes neurotransmitter metabolites [i.e., 5-hydroxyindole acetic acid (from serotonin) and homovanillic acid (from dopamine) (Neef et al., 1967; Cserr and Van Dyke, 1971; Forn, 1972)] as well as various anionic compounds [i.e., 2,4-dichlorophenoxy acetic acid, methotrexate, salicylate, and benzylpenicillin (Rubin et al., 1968; Lorenzo and Spector, 1973; Pritchard, 1980; Suzuki et al., 1987)]. It has also been suggested that an apical Oatp1, an energy-dependent and probenecid-sensitive transport system, mediates the uptake of 17β-estradiol 17β-D-glucuronide (K_m = 3.4 μM) in isolated rat CP (Nishino et al., 1999). Similar kinetic constant for oatp1 transport of this substrate has been reported in cRNA-injected oocytes and cDNA-transfected mammalian cells (Meier et al., 1997). When microinjected into cerebral cortex, the rapid and saturable elimination of *p*-aminohippurate from the brain was observed (Kakee et al., 1997). These results suggest that these efflux transport properties are consistent with transport by the OAT family, especially OAT3. The uptake of the anion 2,4-dichlorophenoxy acid by bovine CP brush-border membrane vesicles and intact bovine and rat plexus tissues involved a glutarate/organic anion exchanger (Pritchard et al., 1999) (Fig. 5). These results are similar to those reported for *p*-aminohippurate uptake by renal basolateral membrane vesicles (Shimada et al., 1987; Pritchard, 1988). The apical localization of an organic anion/dicarboxylate exchanger was demonstrated by the expression of OAT1-green fluorescent protein constructs in rat CP (Pritchard et al., 1999), in direct contrast to the basolateral localization of this same construct in rat proximal tubules (Sweet et al.,

1999). In addition, the nucleoside analog didanosine, was shown to be transported from CSF into the blood by a probenecid-sensitive OAT system across the rat CP, whereas zidovudine is recognized, but not transported, by this system (Galinsky et al., 1990; Wong et al., 1993; Masereeuw et al., 1994; Takasawa et al., 1997a). Moreover, the presence of transporters for cellular extrusion on the basolateral membrane would account for the efficient transcellular transport of organic anions across the CP from CSF to the blood side. Recently, it has been established that MRP homologs (MRP1/MRP2) are responsible for the cellular extrusion of organic anions from various organs (Keppler and Konig, 1997; Muller and Jansen, 1997; Suzuki and Sugiyama, 1998) including the CNS via the BBB (Kusuhara et al., 1998) (see *Section V.D.*).

In summary, as described for the OCT systems, a vectorial transfer of organic anions is mediated by a concerted action of members of the OAT family and ATP-binding cassette (ABC) family of multispecific transporters. The polar localization of these transporters may complement their function in the transport of organic anions into and/or out of the CSF. Although localization and transport mechanisms are unclear in the brain, several multispecific OAT systems, predominantly OAT3, and to a lesser extent Oatp1, Oatp2, and OAT1 have been identified at the CP and BBB. Future immunohistochemical and transport analysis of these OAT systems, and those yet to be identified, will reveal the cellular localization and transport regulation of these carriers and elucidate more precisely their physiological role in the CNS.

C. Nucleoside Transport Systems

Purine and pyrimidine nucleosides and their metabolic products are the precursors of the nucleic acids, DNA and RNA, and participate in numerous biological brain processes. For example, the nucleoside adenosine modulates neuronal and cerebral vascular functions by interacting with specific receptors on brain cells and blood vessels (Bender et al., 1980; Thampy and Barnes, 1983). In general, nucleosides are synthesized endogenously via de novo synthetic pathways (Carver, 1999). However, a number of tissues including brain are deficient in de novo nucleotide synthetic pathways and rely on the salvage of exogenous nucleosides to maintain nucleoside pools and to meet their metabolic demands (Fox and Kelley, 1978). Therefore, the brain is dependent on a continuous and balanced supply of purine and pyrimidine nucleoside constituents from both synthesis in situ and the blood (Santos et al., 1968; Tremblay et al., 1976; Kraupp and Marz, 1995).

Nucleosides and their analogs form the basis of a wide variety of clinical agents that are used in the treatment of brain cancers, cardiac disorders, parasitic, and viral diseases (Paterson et al., 1981; Daval et al., 1991; Perigaud et al., 1994). The purine nucleoside, adenosine, exerts significant cardiac effects and is used clinically in the treatment of cardiac arrhythmias (Brady et al., 1996). Nucleoside analogs (i.e., zidovudine, lamivudine, didanosine, and abacavir) are currently used in the treatment of patients with HIV infection (Beach, 1998). Most nucleosides and their analogs exert their biological activity intracellularly, but due to their hydrophilic nature do not readily permeate the lipid bilayer. Therefore, the uptake or release of nucleosides and/or nucleoside analogs in mammalian cells is mediated by multiple distinct transporters (Cass, 1995; Cass et al., 1998). Currently, eight functionally distinct nucleoside transporters have been identified (Table 3). The classification of nucleoside transport proteins is based on functional and pharmacological characteristics including transport mechanism (*e* = equilibrative, *c* = concentrative), sensitivity to nitrobenzylmercaptopurine riboside (*s* = sensitive, *i* = insensitive), and selectivity sequence of permeant molecules.

Equilibrative processes are widely distributed among mammalian cells and tissues and exhibit broad permeant selectivity (Plagemann et al., 1988; Gati and Paterson, 1989; Belt et al., 1993; Griffiths et al., 1997a,b; Crawford et al., 1998). The two bidirectional, equilibrative systems, *es*/ENT1 and *ei*/ENT2, have similar kinetic properties, but differ markedly in their sensitivity to nitrobenzylmercaptopurine riboside (Plagemann and Wohlhueter, 1980; Belt, 1983; Belt and Noel, 1985). ENT1 and ENT2 have been isolated from both human and rat placenta (Griffiths et al., 1997b; Yao et al., 1997). Both equilibrative transporters are inhibited by low concentrations (0.1–100 nM) of dipyridamole and dilazep (Plagemann et al., 1988). The *es* transporter is inhibited at low concentrations (≤1 nM) (Belt, 1983) as a direct result of the interaction of nitrobenzylmercaptopurine riboside with high affinity binding sites (K_d = 0.1–1 nM) (Cass et al., 1974; Jarvis and Young, 1980) whereas the *ei* transporter is not affected by nitrobenzylmercaptopurine riboside up to >10 μM (Jarvis and Young, 1980; Belt, 1983; Belt and Noel, 1985).

Unlike the ubiquitous equilibrative systems, concentrative NT processes have only been identified in selected mammalian cells: macrophages (Plagemann and Aran, 1990), choroid plexus (Wu et al., 1992), microglia (Hong et al., 2000), leukemia cells (Crawford et al., 1990), splenocytes (Darnowski et al., 1987), intestinal cells (Vijayalakshmi and Belt, 1988), and renal brush-border membrane vesicles (Williams et al., 1989). These transporters, with the exception of *cgs*/N6 and *cs*/N5 systems, are insensitive to nitrobenzylmercaptopurine riboside and dipyridamole at concentrations up to 10 μM (Plagemann et al., 1988; Vijayalakshmi and Belt, 1988). Evidence suggests that six subclasses of concentrative transporters exist that display a complex pattern of overlapping substrate selectivity (Cass, 1995; Flanagan and Meckling-Gill, 1997). Due to overlapping broad substrate specificities, a complex phenotype can result

TABLE 3
The nucleoside transporter family

NT Process	Na^+ Dependence	Na^+:Nuc Stoichiometry	Sensitivity To NBMPR	Substrate Specificity	Tissue Specificity
Equilibrative transporters					
es	—		+	Pur and Pyr Nuc	Widely expressed in mammalian cells
ei	—		—	Pur and Pyr Nuc	Widely expressed in mammalian cells
Concentrative Transporters					
cif (N1)	+	1:1	—	Pur Nuc, Urd	Mouse splenocytes, rat enterocytes, macrophages, liver, leukemic cells
cit (N2)	+	1:1	—	Pyr Nuc, Ado	Mouse enterocytes, rat/rabbit kidney
cit (N4)	+	1:1	—	Pyr Nuc, Ado, Guo	Human brush border kidney cells
cib (N3)	+	2:1	—	Pur and Pyr Nuc	Human colorectal, leukemic cells, CP, microglia
cs (N5)	+	N.D.	+	Ado analogs	Human leukemic cells
csg (N6)	+	1:1	+	Guo	Acute promyelocytic leukemic cells

—, negative; +, positive; N.D., not determined; Pur, purine; Pyr, pyrimidine; Nuc, nucleosides; Urd, uridine; Ado, adenosine; Guo, guanosine. NT terminology: *es*, equilibrative, sensitive to nitrobenzylmercaptopurine riboside (NBMPR) inhibition; *ei*, equilibrative, insensitive to NBMPR; *cif* or N1, concentrative, NBMPR insensitive, accepts formycin B as a permeant; *cit* or N2, concentrative, NBMPR insensitive, common permeant, thymidine; *cib* or N3/N4, concentrative, NBMPR insensitive, broad specificity; *cs* or N5, concentrative, NBMPR sensitive; *csg* or N6, concentrative, NBMPR sensitive, accepts guanosine as a permeant. The corresponding cloned NT proteins both in humans and rats are: *es* = ENT1, *ei* = ENT2, *cif* (N1) = CNT2/SPNT, *cit* (N2) = CNT1. Adapted from Wang et al., 1997a; Cass et al., 1998.

whereby one nucleoside may be simultaneously transported by more than one process within one cell or tissue. The nucleosides are transported against a concentration gradient into the cell, coupled with the movement of Na^+ down a concentration gradient (Jarvis et al., 1989). These transporters use the physiological Na^+-gradient ($\sim$140 mM $_{out}$ > 5–10 mM $_{in}$) generated by the ubiquitous Na^+/K^+-ATPase (Cass, 1995). Thus, these processes are described as inwardly directed Na^+/nucleoside symporters (Lee et al., 1988).

Concentrative transporters mediate the nucleoside flux against their concentration gradient by Na^+ or K^+ cotransport mechanisms. The major types of Na^+ nucleoside cotransport systems can be classified based on functional studies, including primarily substrate selectivity. The N1 (or cif) system is selective for purines, with guanosine and formycin B being the commonly used substrates. The N2 (or cit) transporter is selective for pyrimidines, and thymidine is the usual test substrate. Both types have been cloned from humans and rats (hCNT1/rCNT and hSPNT1/SPNT for N2 and N1, respectively) and when heterologously expressed, they display a 1:1 Na^+/nucleoside-coupling ratio (Cass et al., 1998). The N3 (or cib) transporter has a 2:1 Na^+/nucleoside stoichiometry and is broadly selective for both purines and pyrimidines. The fourth type, N4, has similar N2 stoichiometry and selectivity but guanosine and adenosine are also transported. The less characterized N5 (or cs) transporter is nitrobenzylmercaptopurine riboside-sensitive and exhibits selectivity for adenosine and formycin B (Cass, 1995).

There are large differences in the functional expression of NT depending on cell type. For instance, erythrocytes express only a single NT system, *es* (Woffendin and Plagemann, 1987), whereas both *es* and *ei* are present in BeWo human choriocarcinoma cells (Boumah et al., 1992) and murine L1210 leukemia cells and IEC-6 intestinal epithelial cells, (Vijayalakshmi and Belt, 1988; Crawford et al., 1990). In addition, NT activities within the same cell type vary with changes in growth state (Jakobs et al., 1990; Meckling-Gill et al., 1993), differentiation (Sokoloski et al., 1991; Jones et al., 1994), and neoplastic transformation (Meckling-Gill and Cass, 1992). For example, induction of monocytic or neutrophilic differentiation of HL-60 human leukemia cells leads to a decrease in *es* NT activity and an increased Na^+-dependent NT activity (Lee et al., 1991; Sokoloski et al., 1991; Goh et al., 1993).

Several NT systems have been identified in the BBB and blood-CSF barrier (Fig. 6). Early in vivo studies revealed that at the BBB, purine ribonucleosides cross from blood to brain by facilitated diffusion, whereas pyrimidine deoxyribonucleosides did not show appreciable uptake into the brain (Cornford and Oldendorf, 1975; Spector and Berlinger, 1982; Spector and Huntoon, 1983). Further experiments using cultured endothelial cells and isolated brain capillaries demonstrated the expression of *cit*/N2, *es* and *ei* NT systems at the BBB (Kalaria and Harik, 1986; Thomas and Segal, 1997). The low BBB transport of deoxyribonucleosides suggests that the measured saturable entry of these solutes occur predominantly through the CP. In vitro studies using isolated rabbit CP have identified several NT systems including an *es*, *ei,* and a concentrative N3 type transporter mediating the transport of both ribonucleosides and deoxyribonucleosides at the CP (Spector, 1982; Kalaria and Harik, 1986; Wu et al., 1992, 1994). In vivo, both N3 and *ei* systems have been shown to transport nucleosides from blood to CSF (Spector, 1982). The N3 transporter identified in rabbit CP tissue slices is selective for naturally occurring purine and pyrimidine ribo- and deoxyribonucleosides (i.e., guanosine, inosine, formycin B, uridine, cytidine; IC_{50} range = 5–23 μM), base modified nucleoside analogs (i.e., 5-fluorouridine, 2-chloroadenosine; IC_{50} range = 12–24 μM), but not for synthetic nucleoside analogs (i.e., zidovudine, zalcitabine) substituted on the ribose ring (Wu et al., 1992, 1994). Similarly, Wu and colleagues (1992) have found that the N3 NT system in rabbit CP tissue slices transports both uridine (K_m = 18 μM) and thymidine (K_m = 13 μM).

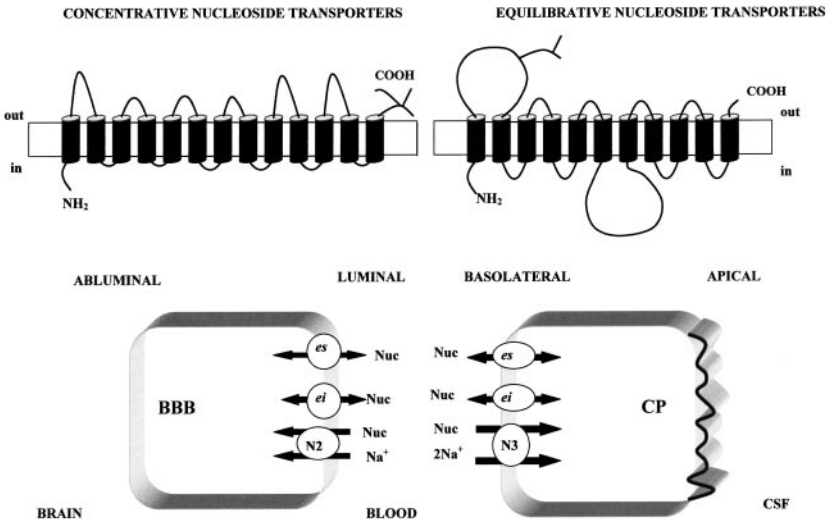

FIG. 6. Nucleoside transport systems in the BBB and CP. The predicted topologies for the equilibrative and concentrative nucleoside transport systems are shown. Although the specific localization of NT systems at the blood-brain and blood-CSF barriers has not been determined, the predicted distribution of those characterized nucleoside transporters involved in the salvage of essential nucleosides is presented. Arrows indicate the direction of transport. Adapted from Cass et al., 1998.

Sodium-stimulated hypoxanthine uptake also occurs in rabbit CP slices (Washington and Giacomini, 1995).

Studies using rat and guinea pig whole brain membrane preparations have demonstrated the presence of a nitrobenzylmercaptopurine riboside-sensitive NT system, which specifically recognizes adenosine as its preferred endogenous substrate (Geiger et al., 1985; Jarvis and Ng, 1985). The wide distribution of ENT1 or *es* systems in human and rat adult brains has been verified using RT-PCR, Northern blot, and in situ hybridization methods (Anderson et al., 1999a). Similarly, *ei* NT transcript expression has been recently detected in several regions of the rat brain suggesting that adenosine levels in brain is achieved by multiple transport processes (Anderson et al., 1999b). However, the actual membrane localization of these facilitative and active NT systems has not been investigated.

Preliminary evidence suggests that active NT systems are also present in brain parenchyma. In cultured human astrocytes, Gu et al. (1996) reported the presence of a concentrative uptake system for adenosine. Although these transporters have yet to be fully characterized, the NT inhibitors dipyridamole, nitrobenzylmercaptopurine riboside, and dilazep biphasically inhibited the inhibitor-sensitive component of adenosine transport (IC_{50} = 1.3, 0.7, and 3.3 nM, respectively). More recently, Sinclair et al. (2000) identified a nitrobenzylmercaptopurine riboside-insensitive transporter (rENT2) as the primary functional NT transporter in rat C6 glioma cells.

In addition to NTs that transport nucleosides into the brain, there also exists a saturable carrier system for the efflux of nucleosides out of the brain (Spector, 1980, 1985; Suleiman and Spector, 1982; Spector and Huntoon, 1983; Wu et al., 1993). An equilibrative NT system, sensitive to nitrobenzylmercaptopurine riboside, is responsible in part for the elimination of formycin B and uridine from the rat and rabbit CSF, respectively, in vitro (Wu et al., 1992 and 1993). Similarly, the efflux of thymidine out of the CSF in intraventricularly perfused rabbits was partially nitrobenzylmercaptopurine riboside-sensitive and saturable with a K_m of 17.8 μM (Thomas et al., 1997).

The involvement of NT systems in the transport of nucleoside analog drugs, such as zidovudine, stavudine, and didanosine has been demonstrated in several cell systems. This includes intestinal transport of stavudine by N3- and N2-type NT systems (Waclawaski and Sinko, 1996), Na^+-dependent hCNT2 transport of didanosine (Ritzel et al., 1998) and rCNT1 transport of zidovudine (Huang et al., 1994; Yao et al., 1996). Interestingly, nucleoside transporters identified at the BBB and blood-CSF barrier (i.e., Na^+-dependent NT system N3) are not involved in the transport of nucleoside analog drugs (Terasaki and Pardridge, 1988; Wu et al., 1994). Our laboratory has found evidence for NT-mediated transport within brain parenchyma. A sodium-dependent NT responsible for the uptake of thymidine was characterized in rat microglia using a continuous cell line (MLS-9 cells) (Hong et al., 2000). The Na^+/nucleoside-coupling stoichiometry was found to be 2:1 and the standard inhibitor for equilibrative NT, nitrobenzylmercaptopurine riboside, did not inhibit thymidine uptake. The K_m (44 μM) and V_{max} (273 pmol/mg/min) for thymidine uptake in this system were similar to Na^+-dependent NTs in bovine renal and brush-border membrane vesicles, respectively (Williams and Jarvis, 1991; Gutierrez and Giacomini, 1993). Furthermore, various purine and pyrimidine nucleosides inhibited thymidine uptake in a concentration-dependent (IC_{50} = 30–40 μM) and competitive manner (K_i = 38–45 μM). These properties are

remarkably similar to the broad substrate selectivity of a *cib* (N3) type of NT (Wang et al., 1997b). Zidovudine was found to be a potent noncompetitive inhibitor for thymidine uptake in microglia (IC_{50} = 0.6 μM) suggesting that it only acts as an inhibitor of the system and not as a substrate.

We have also characterized a novel electrogenic zidovudine/H^+-dependent transporter (Hong et al., 2001) in microglia. Kinetic analysis of zidovudine uptake by confluent monolayers of MLS-9 cells showed a saturable low-affinity system (K_m = 1 mM) sensitive to membrane potential and pH. Specificity studies revealed that zidovudine uptake was sensitive to organic cations (verapamil, mepiperphenidol, quinidine, cimetidine, and N^1-methylnicotinamide; IC_{50} values in the range of 156 to 200 μM) but was unable to transport the standard OCT substrate, tetraethylammonium. The system was also insensitive to classic organic anions (benzyl penicillin, salicylic acid, *p*-aminohippuric acid) and probenecid, a standard OAT inhibitor. Furthermore, nucleosides (thymidine, cytidine, guanosine, and adenosine), standard nucleoside analog inhibitors (dipyridamole, dilazep, and 6-(4-nitrobenzylthio-9-β-D-ribofuranosylpurine), and nucleoside analogs (lamivudine, abacavir, didanosine, and zalcitabine) did not inhibit zidovudine uptake by microglia. Although the zidovudine system in microglia has some specificity features of an organic cation transporter, it involves a carrier, distinct from other cloned OCT systems that is novel in its sensitivity to pH and membrane potential. This system may play a significant role in the transport of other weak organic cation substrates and metabolites in brain parenchyma (Hong et al., 2001).

D. Efflux Transport Systems

1. P-Glycoprotein. P-gp is a 170-kDa plasma membrane, energy-dependent efflux pump that belongs to the ABC superfamily of transporters (Ling, 1997). Originally discovered in Chinese hamster ovary cells selected for colchicine resistance, these cells exhibited broad cross-resistance to a number of naturally occurring structurally diverse antineoplastic agents including anthracyclines, vinca alkaloids, and taxanes (Juliano and Ling, 1976). Consequently, this phenomenon was termed multidrug resistance (MDR) (Biedler and Riehm, 1970; Kessel and Bosmann, 1970).

P-gp is a product of the MDR gene. In humans, two MDR genes, MDR1 and MDR2 (also called MDR3), have been cloned and sequenced (Chen et al., 1986; Roninson et al., 1986). Although the MDR1 protein is involved in the MDR phenotype (Gros et al., 1986; Ueda et al., 1987), the protein encoded by the human MDR2 gene functions as a phosphatidyl translocase in the liver (Ruetz and Gros, 1994). In addition to the human P-gp homologs, hamster pgp1, 2, and 3 (Gerlach et al., 1986) and mouse mdr1a, 1b, and 2 (Gros et al., 1986) also exist (Table 4). Similar to their human counterparts, murine P-gp encoded by mdr1a/b confers the MDR phenotype (Gros et al., 1986; Ueda et al., 1987; Schinkel et al., 1994, 1995) whereas the mdr2 gene product serves in the transport of hepatic phospholipid into the bile (Smit et al., 1993).

The primary sequence encoding the P-gp protein is organized in two tandem repeats, joined by a linker region. Each repeat is composed of an amino-terminal hydrophobic domain containing six potential transmembrane segments followed by a hydrophilic intracytoplasmic domain encoding an ATP-binding site (Fardel et al., 1996) (Fig. 7). Mutations in one or both nucleotide-binding consensus sequences results in failure to confer MDR, suggesting that both ATP-binding sites are required for substrate transport (Rothenberg and Ling, 1989). Three putative glycosylation sites are located within the first extracellular loop of the protein and do not appear to be involved in the transport of substrates. Studies with tunicamycin treatment, which blocks *N*-linked glycosylation, did not demonstrate altered drug sensitivity in human MDR cells (Beck and Cirtain, 1982). In addition, P-gp is phosphorylated at several sites (Hamada et al., 1987) by cAMP-dependent protein kinase A and by protein kinase C (Mellado and Horwitz, 1987; Chambers et al., 1992). Treatment with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, which stimulated P-gp phosphorylation, resulted in increased drug resistance and decreased drug accumulation in some multiresistant cell lines (Fine et al., 1988), whereas in the presence of protein kinase inhibitors, there appeared to be increased anticancer drug accumulation within some cells (Bates et al., 1993). These results suggest that P-gp activity may be modulated by phosphorylation.

P-gp substrates include a wide variety of naturally occurring antineoplastic agents such as anthracyclines (doxorubicin, daunorubicin, mitoxantrone), vinca alkaloids (vincristine, vinblastine), epipodophyllotoxins (etoposide, teniposide), and taxanes (taxol, taxotere), as well

TABLE 4
The P-glycoprotein family

P-glycoprotein	Human	Species		Tissue Specificity
		Mouse/Rat	Hamster	
Class I	MDR1	mdr1a	pgp1	Intestine, brain (BBB, astrocytes, microglia), heart, and kidney
Class II	MDR1	mdr1b	pgp2	Gravid mouse uterus, adrenal gland, kidney, heart, and brain (astrocytes, microglia)
Class III	MDR3 or MDR2	mdr2	pgp3	Liver, adrenal gland, spleen, heart, and muscle

pgp, P-glycoprotein. In general, class I and II substrates include organic cations and lipophilic compounds whereas class III substrates are primarily phospholipids (Ford, 1995; Schinkel, 1997).

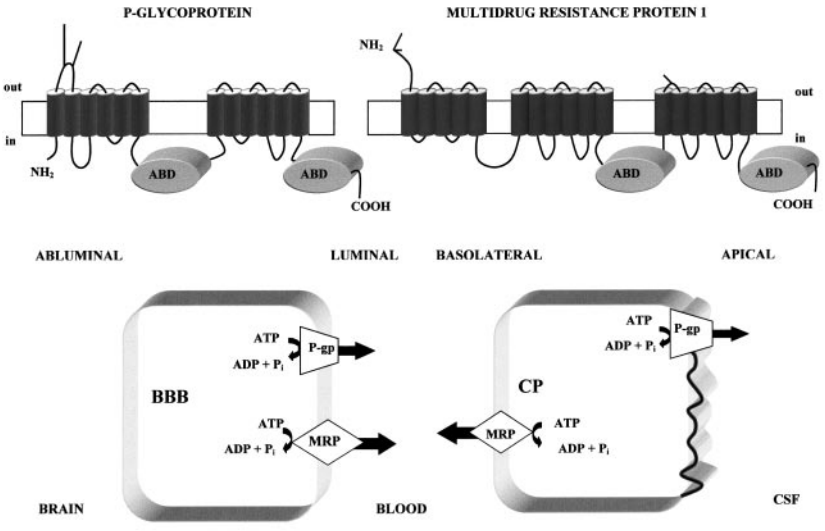

FIG. 7. P-gp and MRP efflux systems in the BBB and CP. The predicted membrane topology models for MRP1 and P-gp are provided. MRP2, -3, and -6 have structures similar to MRP1 and are characterized by an extra N-terminal extension (i.e., five transmembrane segments connected to a P-gp-like core by a cytoplasmic linker). Both MRP4 and -5 lack this additional transmembrane domain but contain the cytoplasmic linker in addition to the P-gp-like core. ABD, ATP-binding domain. Only the efflux systems that have been experimentally localized to a specific side of the BBB endothelium and CP epithelium are presented. Arrows indicate the direction of transport. Adapted from Ueda et al., 1997; Borst et al., 1999.

as immunosuppressive agents (cyclosporin A and its analog PSC833), cardiac glycosides (digoxin), antibiotics (rifampin), pesticides (ivermectin), and protease inhibitors (saquinavir, indinavir, ritonavir, nelfinavir). Currently identified P-gp transport inhibitors include calcium channel blockers (verapamil), calmodulin antagonists (trifluoperazine), quinolines (quinidine), cyclosporin A, and the protease inhibitors. Many of these compounds function as both P-gp substrates and inhibitors and may interact with P-gp at more than one binding site (Ford, 1995). Studies using mdr1a/b gene knockout mice show that P-gp deficient mice are viable, fertile, and healthy compared to wild type, but exhibit significantly elevated drug levels, particularly in the brain (Schinkel et al., 1994; Schinkel, 1997).

The exact mechanism by which P-gp extrudes substrates from the cell cytoplasm remains unresolved. Substrates may bind to a cytoplasmic region of P-gp, resulting in an energy-dependent conformational change that shuttles the drug to the outside of the plasma membrane. This "classical" model of a transporter resembles a typical substrate-enzyme interaction. Alternatively, recent evidence suggests that P-gp may act as a "flippase" where membrane-bound drug is relocated between inner and outer lipid leaflets of the plasma membrane (Higgins and Gottesman, 1992; Sharom, 1997). This model is consistent with the mdr2 protein, which acts as a flippase for phosphatylcholine (Romsicki and Sharom, 2001). Whether P-gp transports substrates by a combination of several different methods remains to be elucidated.

In the periphery, P-gp is found on the apical surface of intestinal and renal epithelia, pancreas, the secretory glands in the endometrium of pregnant mice, biliary canalicular membranes of hepatocytes, and on the luminal side of the blood-testis barrier (Fojo et al., 1987; Thiebaut et al., 1987, 1989; Cordon-Cardo et al., 1989; Croop et al., 1989; Borst et al., 1993; Schinkel, 1997). In the brain, P-gp expression has primarily been investigated at the barriers. Although P-gp is expressed on the apical side of the CP epithelia (Rao et al., 1999), the location of P-gp along the brain endothelial microvessels remains debatable. Immunohistochemistry studies and luminal membrane isolation have localized P-gp to the luminal surface of the brain endothelium (Sugawara et al., 1990; Jette et al., 1993; Beaulieu et al., 1997; Drion et al., 1997). However, P-gp has also been identified at the abluminal surface of endothelial cells on neighboring astrocyte foot processes (Golden and Pardridge, 1999). The primary P-gp isoform detected in brain microvessels is mdr1a whereas mdr1b mRNA is the main isoform detected in brain parenchyma (Regina et al., 1998). The level of P-gp and specific homologs expressed also varies depending on the isolation procedure. Regina et al. (1999) reported the variability in P-gp levels between immortalized endothelial cell lines and isolated brain microvessels. It also appears that a down-regulation of mdr1a, and an up-regulation of the P-gp isoform mdr1b occurs in rat brain endothelia cultures (Barrand et al., 1995; Regina et al., 1998). Finally, P-gp is expressed in primary cultures of rat brain astrocytes but at lower levels when compared with primary endothelial cultures (Declèves et al., 2000). This weak expression in astrocytes correlated with a weak functional activity as various P-gp modulators produced only a slight effect on the uptake of the P-gp-specific substrate colchicine. Thus, if expressed at the BBB on astrocyte foot processes, the higher expression of P-gp at the endothelial level would

be more important at limiting the entry of xenobiotics into the brain than at the astrocyte level (Declèves et al., 2000).

Data from our laboratory suggest that P-gp is also expressed and functional in brain microglia (Lee et al., 2001). Using a continuous rat brain microglia cell line (MLS-9), immunocytochemistry studies reveal the location of P-gp along the nuclear envelope and plasma membrane of microglia. In primary microglia cultures, RT-PCR analysis detected both mdr1a and mdr1b, whereas in the MLS-9 cells only mdr1b gene was expressed. This was corroborated by Western blot analysis; a single band with a molecular weight of 170 to 180 kDa, similar to those reported for P-gp in other cell lines, was detected (Doige and Sharom, 1992; Regina et al., 1998). Functional studies using the known P-gp substrate, digoxin, demonstrated that digoxin accumulation in microglia was significantly increased in the presence of various P-gp inhibitors (verapamil, quinidine, cyclosporin A, PSC 833), protease inhibitors (saquinavir, ritonavir, indinavir), and sodium azide, a metabolic inhibitor. Digoxin accumulation was not increased in the presence of standard MRP inhibitors such as sulfinpyrazone, indomethacin, and probenecid. These results provide the first evidence for the functional expression of P-gp in microglia and imply that entry of pharmacological agents may be prevented within the brain parenchyma as well as the BBB.

2. Multidrug Resistance Protein Family. A second efflux transport protein subfamily, which belongs to the ABC protein superfamily and can confer MDR, is the MRP. Thus far the mammalian MRP family consists of seven proteins ranging from 1325 to 1545 amino acids (Borst et al., 1999; Konig et al., 1999a). All MRPs contain two transmembrane domains of six α-helices each (P-gp-like core) connected to a cytoplasmic linker (L_o) region (Fig. 7). In addition MRP1, -2, -3, and -6 contain up to six additional membrane-spanning helices (TMD_o) at the NH_2 terminus (Lautier et al., 1996; Borst et al., 1999; Klein et al., 1999). Although this extra N-terminal domain is not required for drug transport, the linker region (Lo) is absolutely necessary to maintain the protein transport properties (Gao et al., 1996; Bakos et al., 1998). MRP1, -2, and -3 appear to have overlapping substrate specificities, but differ with respect to kinetic properties (Keppler et al., 1999). Most cells appear to express multiple MRP family members, with high levels of one MRP generally dominating (Keppler et al., 1999). While MRP2, -3, and -6 are found mainly in the liver and kidney, and MRP4 is found in high concentrations in the prostate, MRP1 and -5 appear to be ubiquitous, and both proteins are expressed in the brain (Klein et al., 1999). Within polarized cells (e.g., kidney and liver) MRP2 is the only homolog located in the apical membrane (similar to P-gp), MRP1, -3, and -5 are all routed to the basolateral membrane (Flens et al., 1996; Schaub et al., 1997; Konig et al., 1999b; Kool et al., 1999). The general features of the MRP homologs are summarized in Table 5.

MRP1, by far the most characterized MRP family member, is a 190-kDa plasma membrane-bound protein that has been implicated in resistance to a number of anticancer agents (i.e., multidrug resistance) including anthracyclines, epipodophyllotoxins, and several vinca alkaloids. It is both glycosylated and phosphorylated. Cloned in 1992 by Cole and colleagues, MRP has many functional similarities with P-gp (i.e., somewhat overlapping substrate specificities), even though they possess only a 15% amino acid homology. Physiologically, MRP1 appears to play an important role in the transport of several GSH, glucuronide, and sulfate conjugates, including conjugated leukotrienes (Lautier et al., 1996; Leier et al., 1996; Loe et al., 1996a), steroid glucuronides (Jedlitschky et al., 1996; Loe et al., 1996b), and GSH disulfide (Leier et al., 1996). Thus, MRP1 may also play a role in the regulation of intracellular redox potential, flux of ions (Jirsch et al., 1993; Rappa et al., 1999), inflammatory mediation, and elimination of potentially toxic endo- and xenobiotics (O'Brien and Tew, 1996; Deeley and Cole, 1997). Furthermore, MRP1 transport of heavy metal oxyanions such as sodium arsenate and antimony potassium tartrate suggests that MRP1 may play a protective role against environmental toxins.

Several MRP knockout mouse models have been developed recently (Lorico et al., 1997; Rappa et al., 1999). As murine MRPs possess greater than 85% homology with human MRPs, the transgenic mouse model is a powerful tool for further understanding the physiological role of these proteins. The absence of MRP does not appear to alter the viability, fertility, and/or biochemical

TABLE 5
The human multidrug resistance protein family

Isoform	Other Names	Year Cloned	Primary Site of Expression	Expression in Brain
MRP1	ABCC1	1992	Ubiquitous	BBB, CP, AST, WB, MG
MRP2	ABCC2; cMOAT	1994	Liver/kidney/gut	No
MRP3	ABCC3; MOAT-D	1996	Liver/kidney/adrenals	BBB[a]
MRP4	ABCC4; MOATB	1996	Prostate/lung/testis	BBB
MRP5	ABCC5; MOAT-C	1996	Ubiquitous	BBB
MRP6	ABCC6; MOAT-E	1998	Liver/kidney	BBB
MRP7	ABCC10	2001	Skin/stomach	WB

MOAT, multispecific organic anion transporter; WB, whole brain homogenate; AST, astrocytes; MG, microglia.
[a] Expression in blood brain barrier is controversial. Generally the substrate specificity of MRP includes glucuronide, sulfate, and glutathione-conjugated compounds, large hydrophobic anions, and various cationic and neutral compounds in the presence of physiological concentrations of glutathione (Borst et al., 1999, Borst et al., 2000).

profile of MRP knockout mice versus wild-type mice (Rappa et al., 1999). Some caution must be used however when utilizing murine models for characterization of substrate and for extrapolation to the human MRP protein. Differences have been identified for example in the transport of anthracyclines by murine and human MRP. On the other hand, these differences can be exploited by researchers to further characterize the specific MRP domains required for substrate transport (Stride et al., 1999).

MRP2 (cMOAT) appears to play an important role in the biliary excretion of various GSH and glucuronide conjugates including bilirubin glucuronide (Konig et al., 1999a). Furthermore, a splice mutation of the human MRP2 gene results in development of hyperbilirubinemia, also known as the Dubin-Johnson Syndrome (Paulusma et al., 1997; Kajihara et al., 1998; Wada et al., 1998; Toh et al., 1999). Conversely, MRP3, which is located in the basolateral membrane of hepatocytes, may be involved in bile acid uptake from the gut (Borst et al., 2000). The physiological functions of the remaining MRP family members is currently unknown, however recent studies demonstrating the ability of MRP4 and 5 to transport nucleotide analogs suggest these two MRP homologs may be involved in nucleotide/nucleoside transport in vivo (Schuetz et al., 1999; Jedlitschky et al., 2000; Wijnholds et al., 2000). Thus, the substrate specificity of MRP appears to be quite broad. Many MRP substrates are amphiphilic anions with at least one negatively charged group; however not all amphiphilic anions are transported (Jedlitschky et al., 1996). For example, the anthracyclines daunorubicin and doxorubicin cannot enhance the transport of LTC_4 and direct transport of these agents is unaffected in the presence of GSH (Loe et al., 1998).

To date, the highest affinity substrate of MRP1, as determined through membrane vesicle transport studies, has been identified as the GSH-conjugated leukotriene LTC_4, with a K_m of approximately 100 nM (Jedlitschky et al., 1996; Loe et al., 1996a). Other efficiently transported substrates of MRP1 include the anthracyclines doxorubicin and daunorubicin, the vinca alkaloids vincristine and vinblastine, and the epipodophyllotoxin etoposide. The anthracyclines and vinca alkaloids are not direct substrates for MRP (Jedlitschky et al., 1996) but rather are only transported efficiently in the presence of physiologically relevant concentrations of GSH. Two mechanisms for transport of MRP substrates have been suggested, i.e., anionic compounds are transported directly, whereas some cationic and neutral compounds may be cotransported in the presence of GSH. GSH itself does not appear to be a direct substrate for the MRP transporter. Studies characterizing the transport properties of MRP have been difficult in intact cells due to the concurrent expression of P-gp and OATP transporters in most cells. Although several potent inhibitors of P-gp exist (PSC833, cyclosporin A), identifying specific and potent inhibitors of MRP has been more difficult. One inhibitor identified to date is the leukotriene antagonist MK-571, a potent inhibitor (K_i = 0.6 μM) of LTC_4 transport in membrane vesicles (Leier et al., 1994). LY329146 (structural analog of the estrogen receptor modulator raloxifene) also inhibits LTD_4 transport into HL60/ADR cells with an IC_{50} = 0.8 μM (Norman, 1998). Both indomethacin and the isoflavanoid genestein alter intracellular concentrations of GSH and are therefore very useful as specific inhibitors of MRP1 in vitro (Draper et al., 1997). Finally, transport studies of MRP substrates have been further aided by the development of specific antibodies for MRP. The monoclonal antibodies QCRL2–4 recognize distinct intracellular epitopes specific to MRP1 and have been shown to inhibit transport of several MRP1 substrates including vincristine, daunorubucin, and aflatoxin-B1 in inside-out vesicles (Loe et al., 1996b, 1997, 1998). Unfortunately, specific inhibitors for each MRP homolog do not exist, therefore caution must be used in the interpretation of results when utilizing inhibitors in whole cell systems that contain multiple isoforms of MRP.

Although the exact mechanism by which GSH enhances MRP substrate transport is currently unknown, MRP may contain bipartite binding sites that would allow for direct binding of drug-GSH complexes or sequential binding of GSH and drug (Loe et al., 1996a). It has been suggested that one of these binding sites may have a high affinity for drug and low affinity for GSH, whereas the second binding site is of the opposite conformation (Borst et al., 1999). Whether GSH may elicit a conformational change in the MRP protein that might favor transport of selected compounds remains unknown (Loe et al., 1996a). GSH itself appears to be a poor substrate of MRP and is transported only marginally in the absence of a proper MRP substrate (Leier et al., 1996). Thus, it appears there are two mechanisms for transport of MRP substrates: anionic compounds are transported directly, whereas some cationic and neutral compounds require the presence of GSH, likely via cotransport.

Enhancement of substrate transport (aflatoxin B_1-GSH, and vincristine) by GSH does not appear to be a consequence of an alteration in the redox state of MRP since other reducing agents (2-mercaptoethanol, dithiothreitol, L-cysteine) have not been able to alter the transport of these substrates in a similar manner (Loe et al., 1997). Whether GSH (and possibly other unknown organic anions) and unconjugated substrates interact molecularly for the purpose of cotransport is presently unclear (Rappa et al., 1997). Data from studies undertaken in GSH-depleted cells provide further evidence for the role of GSH in the efflux of some unconjugated substrates. Rappa et al. (1997) demonstrate that intracellular etoposide accumulation in wild-type embryonic stem cells is increased significantly in the presence of DL-buthionine sulfoximine, an irreversible inhibitor of

γ-glutamylcysteine (Rappa et al., 1997). Within double knockout stem cells, BSO depletion had no effect. Furthermore, in double knockout mice, Lorico et al. (1997) demonstrated that levels of GSH were increased in all tissues, although only highly expressing cells displayed significance (i.e., lung, colon, and muscle). γ-Glutamylcysteine activity was unchanged in the knockout mice, thus making it unlikely that increased GSH synthesis, as opposed to increased GSH efflux, was responsible for the observed GSH increases. Within T14 HeLa cells, vincristine transport is increased markedly in the presence of GSH. However, the precursor dipeptides cysteinylglycine and γ-glutamylcysteine do not affect vincristine transport, thus it appears the tripeptide is required for stimulation of vincristine transport (Loe et al., 1998). Finally, overexpression of MRP leads to decreased intracellular GSH levels in some cell lines (Loe et al., 1998).

MRP expression and localization within the brain is only beginning to be understood. High levels of MRP1 have been detected in the CP using RT-PCR and Western blot techniques (Nishino et al., 1999). Protein and mRNA levels of MRP1 in CP were 5-fold higher than that observed in lung, a tissue known to express MRP1 in abundance (Nishino et al., 1999). Using a combination of fluorescent confocal and electron microscopy, Rao et al. (1999) determined the localization of MRP1 to be primarily basolateral within cultured CP. In addition, the transport of 17β-estradiol 17β-D-glucuronide, both an MRP1 and an OATP1 substrate, was probenecid-sensitive. Thus, transcellular transport of organic anions across the CP may be synergistically mediated via the combined effort of Oatp1 and MRP1 at the apical and basolateral membranes, respectively.

Whereas MRP1 and MRP5 expression has been observed in rat, in mouse and human brain endothelial cells (Kusuhara et al., 1998; Regina et al., 1998; Homma et al., 1999; Nishino et al., 1999; Zhang et al., 2000) similar to P-gp the level of expression is quite dependent on the isolation procedure and cell systems utilized (Seetharaman et al., 1998; Gutmann et al., 1999; Sugiyama et al., 1999). In rats, Regina et al. (1998) noted that MRP1 is overexpressed in rat brain endothelial cell cultures, with primary cultures having higher amounts compared with immortalized cell lines. Furthermore, MRP1 is expressed in higher amounts in brain homogenate, as opposed to isolated microvessels. This finding is not surprising since recently MRP1 expression was detected using RT-PCR in rat astrocyte cultures (Declèves et al., 2000). Corroborating the findings of Regina et al. (1998), Declèves et al. (2000) observed a higher expression of MRP1 in primary astrocytes, compared with primary brain endothelial cells. In this in vitro astrocyte model, indomethacin, probenecid, and sulfinpyrazone increased accumulation of the fluorescent probe fluorescein by 30 to 80% at concentrations of 10 μM, 1 mM, and 2 mM, respectively (Declèves et al., 2000). Sulfinpyrazone also increased the uptake of radiolabeled vincristine within astrocytes. In an in vitro bovine endothelial cell system, Gutmann et al. (1999) reported an up-regulated MRP protein expression following culture of these endothelial cells for a period of 10 days. More recently, studies using RT-PCR (Zhang et al., 2000) have found that in addition to MRP1, MRP4–6 are also expressed to a high degree within brain endothelial cells. Similarly, the newest member of the MRP family MRP7 was detected in whole brain homogenate using RT-PCR (Hopper et al., 2001). Recently we have characterized the functional expression of MRP within the MLS-9 microglia cell (Dallas et al., 2001). Using the MRP substrate vincristine, we observed increased substrate accumulation in the presence of various MRP inhibitors including MK571, genestein, and sulfinpyrazone. Vincristine uptake was both energy- and glutathione-dependent, which suggests a functional form or forms of MRP is/are located within the microglia cell line. Furthermore, while the protease inhibitors saquinavir and indinavir acted as potent inhibitors of vincristine transport, none of the currently available nucleoside analogs drugs showed an appreciable effect. Interestingly, RT-PCR studies have demonstrated the absence of MRP1 within the MLS-9 cell line, whereas MRP1 was present in primary microglia cells derived from the same species of rat (Lee et al., 2001). Studies are now underway in our laboratory to clarify the expression pattern of MRP within both the continuous cell line and primary microglia using both RT-PCR and Western blotting.

The clinical implications of MRP protein expression in the brain are apparent when one considers the prognosis of primary brain tumor patients. The 5-year survival rate of patients with astrocytomas and glioblastomas is extremely low, largely in part due to a high degree of MDR and therefore therapeutic failure of anticancer agents (Mousseau et al., 1993; Hosli et al., 1998). Furthermore, it now appears that MRP may also play a role in resistance to other classes of drugs. For example, accumulation of the MRP substrate calcein acetoxymethyl ester is increased significantly in the presence of the anti-HIV-1 protease inhibitors saquinavir, ritonavir, indinavir, and nelfinavir in the MRP1-overexpressing cell line VM1–5 (Srinivas et al., 1998). Recently Wijnholds et al. (2000) have characterized MRP5-related transport of the anti-HIV nucleotide analog 9-(2-phosphonylmethoxyethyl)adenine in polarized Madin-Darby canine kidney II (MDCKII) cells transfected with MRP5 cDNA constructs. Reduced accumulation and enhanced efflux of the MRP substrate *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) was observed in the MRP5-overexpressing cells in the presence of the MRP inhibitors. Whereas the MRP inhibitor sulfinpyrazone caused almost complete inhibition of DNP-GS efflux, probenecid, indomethacin, and dipyridamole had little effect. Sulfinpyrazone also decreased the efflux 9-(2-phosphonylmethoxyethyl) adenine at low concentrations ($IC_{50} < 0.5$

mM). The authors suggested that MRP5 may play a role in resistance of HIV-1 patients to nucleoside analog drugs. Whether antiretroviral drugs are transported by MRP1 and/or -5 within the brain to an appreciable, and clinically relevant extent, remains to be examined.

VI. Summary

The brain is a dynamic and highly regulated organ compartmentalized by the BBB (cerebral endothelial cells), and the blood-CSF barrier (CP epithelial cells). Brain parenchymal cells (i.e., neuroglia and neurons) exist within this highly regulated environment and function in intimate interplay with one another. Each brain compartment possesses a specific and selective set of metabolic enzymes, receptor proteins, and secretory factors that serve to maintain the homeostatic environment that is necessary for normal function within that compartment. In addition, the localization and expression of various putative drug transporters in these barriers play a critical role in the influx/efflux of numerous xenobiotics and has an important impact on the overall pharmacokinetic/pharmacodynamic profile of drugs in the brain (i.e., distribution, pharmacological response, drug-drug interactions). Novel localization and functional expression of standard transporters (i.e., NT) as well as the efflux transporters (P-gp and MRP) in brain parenchyma suggest a reconsideration of the present conceptualization of brain barriers as it relates to drug transport. The cellular membranes of parenchyma cells, such as microglia and astrocytes, also act as barriers to drug permeability and express transporters whose properties appear similar to those localized to the conventional brain barriers (i.e., cerebral endothelial cells and CP epithelial cells). Much work is needed to fully characterize the drug transporters at the parenchymal barrier site to fully appreciate its role in clinical practice.

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