

Contemporary issues in toxicology

Brain barrier systems: a new frontier in metal neurotoxicological research

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Abstract

The concept of brain barriers or a brain barrier system embraces the blood–brain interface, referred to as the blood–brain barrier, and the blood–cerebrospinal fluid (CSF) interface, referred to as the blood–CSF barrier. These brain barriers protect the CNS against chemical insults, by different complementary mechanisms. Toxic metal molecules can either bypass these mechanisms or be sequestered in and therefore potentially deleterious to brain barriers. Supportive evidence suggests that damage to blood–brain interfaces can lead to chemical-induced neurotoxicities. This review article examines the unique structure, specialization, and function of the brain barrier system, with particular emphasis on its toxicological implications. Typical examples of metal transport and toxicity at the barriers, such as lead (Pb), mercury (Hg), iron (Fe), and manganese (Mn), are discussed in detail with a special focus on the relevance to their toxic neurological consequences. Based on these discussions, the emerging research needs, such as construction of the new concept of blood–brain regional barriers, understanding of chemical effect on aged or immature barriers, and elucidation of the susceptibility of tight junctions to toxicants, are identified and addressed in this newly evolving field of neurotoxicology. They represent both clear challenges and fruitful research domains not only in neurotoxicology, but also in neurophysiology and pharmacology.

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The microenvironment of the brain parenchyma is separated from fluctuations in ion and metabolite concentrations in the blood by two barrier structures. The barrier that separates the systemic blood circulation from the interstitial fluid (ISF) is defined as the blood–brain barrier (BBB), while the one that separates the systemic circulation from the CSF compartment is known as the blood–CSF barrier (BCB) (Table 1). During the last few decades, the role of brain barrier systems in controlling CNS homeostasis has received substantial attention (Davson and Segal, 1996). In addition to their role in movement of materials between the systemic circulation and cerebral compartments, brain barriers, especially the choroid plexus, actively participate in various aspects of brain function, such as involvement in the early stages of brain development,

brain maturation, CNS homeostasis, and neuroendocrine regulation (Strazielle and Gherzi-Egea, 2000a, 2000b; Zheng, 2001a, 2001b). More recently, a great research effort has been devoted to the understanding of the pathophysiological influence of the barriers in neurological disorders, including barrier involvement in chemical-induced brain edema, aberrant brain development, and possibly the initiation of neurodegenerative diseases. This review examines recent progress in this research area, with particular emphasis on the role of brain barriers in metal-induced neurotoxicities. In addition, we discuss research needs and potential new directions for improved understanding of toxicological aspects of blood–brain barrier function, both in health and diseases.

Brain barriers in neurotoxicity: structural and functional basis

The BBB is a specialized structure that maintains the neuronal microenvironment, playing a pivotal role in CNS

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Table 1
Structural comparison of the BBB and the BCB

	Blood–brain barrier	Blood–CSF barrier
Anatomy	<ul style="list-style-type: none"> ● Endothelial cells of cerebral capillary ● Basement membrane ● Perivascular feet of astrocytes ● Interstitial fluid (ISF) 	<ul style="list-style-type: none"> ● Endothelial cells of choroidal capillary ● Basement membrane ● Choroidal epithelium ● Cerebrospinal fluid (CSF)
Tight junctions	Between endothelia	Between epithelia
Location	Whole brain	Choroid plexus in lateral, 3rd, and 4th ventricles
Surface area	155 cm ²	75 cm ²
Blood flow rate	0.9–1.8 ml/min/g	4–6 ml/min/g
Efficiency as a barrier	Highly efficient	Efficient, but functionally leaky

homeostasis, fibrinolysis and coagulation, vasotonus regulation, and blood cell activation and migration during physiological and pathological processes. Regulation of blood–brain tissue exchange is accomplished by individual endothelial cells, which are continuously linked by tight junctions (referred to as *ZONULAE OCCLUDENTES*), thus isolating the brain from the blood and preventing oncotic and osmotic forces to govern blood–tissue exchange. A pivotal function of the endothelial cells is to regulate the selective transport and metabolism of substances from blood to brain as well as in the opposite direction from the parenchyma back to the systemic circulation.

For most solutes and macromolecules, permeability across the BBB is dependent upon their lipophilicity and size. Small water-soluble nutrients and macromolecules needed for proper brain functioning can cross the barrier through specific carrier mechanisms or facilitated diffusion. Some of these carriers are symmetrically distributed both on the luminal and abluminal membranes of the endothelial cells, while others have an asymmetric distribution (Davson and Segal, 1996). For example, the abluminal membrane contains a greater abundance of the enzyme Na⁺,K⁺-AT-Pase than the luminal membrane. This enzyme forms the basis of a pump that simultaneously transports Na⁺ out of the endothelium into the brain, and K⁺ out of the brain into the endothelium. K⁺ has a potent effect on the transmission of nerve impulses and neuron firing; the asymmetric distribution of this ATPase, thus, participates in maintaining low K⁺ concentration in the extracellular fluid of the CNS.

The high degree of tightness of the junctions that link brain endothelial cells virtually prevents any paracellular passage from occurring in physiological conditions. In addition, several transport proteins can prevent the brain entry, or increase the brain-to-blood efflux, of various compounds. P-glycoproteins (i.e., multidrug resistance proteins, MDR) are ATP-binding cassette (ABC) transporters that belong to the ABCC carrier protein family and are expressed in cerebral capillaries. Both in vivo and in vitro experiments have shown that they are responsible for the efflux of a wide

range of structurally unrelated compounds from the endothelial compartment back into the blood. As a result, the net brain influx of hydrophobic drugs, such as vinca alkaloid antineoplastic drugs, ivermectin, cyclosporin A, digoxin, loperamide, or antiviral protease inhibitors, is relatively low (Schinkel et al., 1996; Kim et al., 1998). In addition, other, yet uncharacterized organic anion transport protein(s) seem to be involved in the efflux of more hydrophilic compounds, such as organic anions cefodizime, valproic acid, azidothymidine, and baclofen, as their efflux has been shown to be saturable in vivo (Suzuki et al., 1997). Significant activities and/or expression of several phase I and phase II drug metabolizing enzymes as well as certain antioxidant enzymes have been reported in rat cerebral microvessels. Thus, the cerebral capillaries appear to form an enzymatic barrier between the blood and the brain for various xenobiotics (reviewed in Strazielle and Ghersi-Egea, 2000a).

It is noteworthy, however, that the tightness of the interepithelial junctions of the BCB at the choroid plexus is not as complete as for the cerebral capillary endothelial cells. As a result, very hydrophilic drugs and toxicants may, to a limited extent, get access to the brain via the choroid plexus. The influence of choroid plexuses on the brain bioavailability of drugs is a result of both morphological and biochemical properties characterizing the choroidal epithelium. First, the exchanges taking place at the choroid plexus are favored by their unusual location and histological organization: the choroidal epithelium forms a monolayer at the interface between two circulating fluids, the choroidal blood, whose flow rate is relatively high (Szmydynger-Chodobska et al., 1994) and the CSF, whose flow is brisk in the ventricles (Ghersi-Egea et al., 1996). In addition, the surface area available for exchange between these two fluids is significantly enhanced by the apical microvilli and the basolateral infoldings of this epithelium (Strazielle and Ghersi-Egea, 2000b). Second, this tissue has a high capacity for drug metabolism. The enzymatic activity of several drug-metabolizing enzymes can reach hepatic levels, as demonstrated for several conjugation enzymes and an epoxide hydrolase isoform (Strazielle and Ghersi-Egea, 2000a). The activity of antioxidant enzymes such as glutathione peroxidase also reaches high levels in the choroid plexuses (Tarayani et al., 1989). Third, numerous organic anion and cation transporters are expressed at high level in the choroidal epithelium. Among them, members of the SLC21 family (oatp) are distributed on either the basolateral or the apical membrane, whereas members of the SLC22 family, i.e., organic anion transporters (OATs) and organic cation transporters (OCTs), all appear to be located strictly on the apical membrane. The multidrug resistance protein MRP1 is located at the basolateral membrane of the choroidal cells (reviewed by Ghersi-Egea and Strazielle, 2002). These different transporters, alone or coupled to intracellular drug metabolism, either form an efficient functional barrier to different blood-borne toxic compounds and drugs or accelerate the elimination of toxic endogenous metabo-

lites, as well as xenobiotics or drugs out of the CSF, hence influencing their overall cerebral bioavailability (Suzuki et al., 1997; Strazielle and Ghersi-Egea, 1999; Ghersi-Egea and Strazielle, 2002).

Metal transport and toxicity to the brain barriers

Many metals (calcium, copper, magnesium, manganese, iron, zinc, cobalt, and molybdenum) are essential and required for optimal CNS function. They play important roles in brain function as catalysts, second messengers, and gene expression regulators. Being essential cofactors for functional expressions of many proteins, trace elements are needed to activate and stabilize enzymes, such as superoxide dismutase, metalloproteases, protein kinases, and transcriptional factors containing zinc finger proteins. Clearly, metals must be supplied to the CNS at an optimal level, because both deficiency and excess can result in aberrant CNS function. Thus, transport of metal ions across the BBB is the first step in regulating their CNS levels. A series of active or receptor-mediated transport systems inherent to the BBB vasculature serve to control the transport of these metals into the brain, maintaining their optimal concentrations. Other metals that are nonessential (i.e., have no known functional attributes), such as mercury (Hg) and lead (Pb), can also readily gain access to the CNS.

At least nine metals have been found to accumulate in the BBB and BCB (Zheng, 2001a). Clinically, poisoning with Pb, Hg, and arsenic (As) have been demonstrated to induce vascular destruction and cerebral hemorrhage (discussion follows). The damage to the endothelial structure of the BBB is a fundamental cause of leakage of blood-borne materials to surrounding brain parenchyma.

Lead

The BBB has long been known to be a target for Pb toxicity. One of the explicit clinical manifestations in acute Pb poisoning is brain swelling, accompanied with herniation, ventricular compression, and petechial hemorrhages (Pentschew, 1965; Smith et al., 1960; Struzynska et al., 1997). An increased incidence of cerebral hemorrhage, thrombosis, and arteriosclerosis has been described in a group of British battery workers exposed to extremely high levels of Pb (Dingwall-Fordyce and Lane, 1963). Under these exposure conditions, Pb-induced microvascular damage is prevalent with leaky microvessels, as evident by a characteristic opening of the interendothelial tight junctions and by enhanced pinocytotic activity. Certain macromolecules such as horseradish peroxidase, trypan blue, and albumin have been noted to extravasate into the surrounding neuronal parenchyma in proximity to the microvessels with altered permeability (Goldstein et al., 1974; Struzynska et al., 1997).

Chronic exposure to high concentrations of Pb also leads to cerebral vascular damage in young animals. Developing rats that develop Pb encephalopathy exhibit an extravasation of albumin, fibrinogen, and fibronectin, as seen in extensive staining in the cerebellar cortex, with diffuse spread to the white matter of the corresponding folium (Sundstrom et al., 1985). Pb appears to have a unique affinity to cerebral endothelial cells, where it accumulates in much greater concentrations than in other brain cell types (Struzynska et al., 1997; Toews et al., 1978). The changes in endothelial bud (or angioblast) may actually precede those involving neurons and glia. Thus, some have suggested that Pb encephalopathy probably results from the death of many of these buds (Press, 1977).

Pb is known to accumulate in the choroid plexus to a greater extent than in brain endothelial cells. Friedheim et al. (1983) found that Pb levels in human choroid plexus increased significantly with age, while Pb in the brain parenchyma did not. Furthermore, Manton et al. (1984) reported a 100-fold increase in Pb levels in human choroid plexus compared with that in the brain cortex. Animal studies corroborated these findings (Zheng et al., 1991; O'Tuama et al., 1976). A significant aspect of these findings is that an age-related accumulation of Pb in a particular tissue within the human brain is most likely associated with environmental exposures.

Once inside the cells, Pb ions may interfere with Ca^{2+} regulatory processes, alter protein phosphorylation cascades, and hinder BBB development. X-ray analysis has shown that Pb is preferentially sequestered in mitochondria of capillary endothelial cells (Silbergeld et al., 1980). Pb appears to accumulate in the same intramitochondrial compartment as Ca^{2+} ; thus it has been linked to disruptions in intracellular Ca^{2+} metabolism as well as alterations in trans-epithelial transport processes.

Pb may also interfere with cellular phosphorylation by acting on the protein kinase system. In both endothelial cells of BBB and epithelial cells of BCB, Pb potently activates protein kinase C (PKC) (Markovac and Goldstein, 1988; Zhao et al., 1998). PKC is activated as a consequence of receptor-dependent increases in intracellular Ca^{2+} concentrations and diacylglycerol (DAG). Activation leads to the translocation of the enzyme from the soluble fraction to membrane-associated particulate component of the cells. Studies with specific PKC activators have shown that PKC activation is closely associated with loss of epithelial barrier function, increase in transendothelial permeability, and inhibition of astroglia-induced endothelial differentiation (Gainer, 1985; Lattera et al., 1992). Using isolated brain microvessels, it has been demonstrated that picomolar concentrations of Pb activate PKC (Markovac and Goldstein, 1988). In cultured choroidal epithelial cells, incubation with Pb in culture medium increases the membrane-bound PKC activities by 5.2-fold (Zhao et al., 1998).

Pb may interact with astrocytes to indirectly affect BBB

functions. Several lines of evidence support a critical role for astrocytes in the modulating the functions of CNS capillaries (Rubin and Staddon, 1999). For example, astrocytes can induce angiogenesis and BBB properties in endothelial cells of nonneural origin (Janzer and Raff, 1987). Bressler and Goldstein (1991) examined the effects of Pb on an *in vitro* model of cerebral endothelial differentiation. Under normal conditions, coculture of capillary endothelia with astrocytes prompts endothelial cells to form capillary-like structures. This phenomenon, however, was inhibited by incubation with Pb in culture medium.

Pb accumulation in the choroid plexus can selectively alter certain functions of the BCB. Of the proteins in the CSF, transthyretin (TTR or prealbumin) is exclusively produced and secreted by the choroid plexus (Herbert et al., 1986). In humans, TTR serves as a major thyroid hormone binding protein in the CNS and conveys about 60–80% of CSF thyroxine (Schreiber et al., 2001; Thomas et al., 1989). It is noteworthy that the TTR gene of the choroid plexus is expressed early in fetal development, a phenomenon consistent with the importance of thyroid hormones in embryonic brain development (Cavallaro et al., 1993; Thomas et al., 1989).

Chronic Pb exposure selectively reduces TTR levels in the CSF (Zheng et al., 1996). The alteration of TTR is directly correlated with Pb accumulation in the choroid plexus Pb. A pulse–chase study tracing newly synthesized TTR molecules labeled with [³⁵S]methionine in cultured cells suggested that Pb treatment inhibited the synthesis of total [³⁵S]TTR. Moreover, Pb exposure impeded the trans-epithelial transport of [¹²⁵I]T₄ both *in vivo* and *in vitro* (Zheng et al., 1999a, 2003).

Thyroid hormones have striking effects on the CNS, particularly during the developmental period. Deprivation of thyroid hormones in children causes irreversible mental retardation (Dussault and Ruel, 1987). It is reasonable to suggest that Pb exposure, by depressing TTR production and secretion by the choroid plexus, may impair the transport of thyroid hormones from the blood to the cerebral compartment, which might account for the loss of cognitive abilities observed in Pb-poisoned children.

It is generally assumed that the transported species of Pb is either free Pb²⁺ or a low-molecular-weight complex of Pb. Free Pb²⁺ in human serum approximates 1/5000 of total Pb (Al-Modhefer et al., 1991); under “normal” exposure to Pb, which would be roughly equal to 1×10^{-12} M free Pb, Pb has no appreciable affinity and binding to transferrin, ruling out transferrin-mediated transport across the BBB. Deane and Bradbury (1990) suggest that Pb transport across the BBB is by a passive diffusion of Pb²⁺ or a simple inorganic complex, such as PbOH⁺. The same authors have also proposed that Pb was pumped out of brain endothelial cells back to blood via a Ca²⁺–ATPase that is localized to both the luminal and abluminal membranes of CNS capillaries; this mechanism has also been reported to be opera-

tive in Pb efflux from human red blood cells (Simons, 1993).

Mercury

Methylmercury (MeHg) can easily cross the barriers at the blood–brain interface and at the placenta. Consumption of high MeHg levels during pregnancy may lead to encephalopathy in the offspring (Davis et al., 1994). There are distinct differences in the distribution of pathological changes in young compared to adult brain upon MeHg exposure (Takeuchi et al., 1979). CNS damage following MeHg exposure in adults is primarily in specific areas, such as the granule layer of cerebellum and the visual cortex of cerebrum. When exposures to MeHg occur *in utero* or at an early age, the damage to the CNS is ubiquitous. Generally, the earlier the exposure, the more generalized the damage (Choi, 1989). It has, thus, been hypothesized that, in addition to a higher susceptibility of immature or differentiating neural cells, these differences are also caused by an immature BBB, leading to a more generalized distribution of MeHg in the developing brain. After adult exposure, however, MeHg is found throughout the brain, and the localization does not correlate with pathological changes, suggesting distinct vulnerability of various regions to this metal.

Following exposure, MeHg accumulates in the BBB and BCB. Autopsy data from a Minamata Bay accident victim show that total Hg remained high in the brain as long as 26 years after exposure. Hg deposition was histochemically localized to microglial cells and Bergmann’s glial cells, in neurons of specific brain areas, and in epithelial cells of the choroid plexus (Takeuchi et al., 1989). Studies in Hg-treated animals also demonstrate significant cerebral edema, vacuolar change, spongy degeneration, and the loss of parenchyma (Choi et al., 1988).

Because of its high lipophilicity, MeHg by itself is capable of diffusing through the cell membrane without necessitating any specific carrier system; however, given its reactivity toward sulfhydryl groups, the amount of free MeHg in biological fluids at any given time is likely to be infinitesimal. An active process at the BBB has been proposed to transport MeHg into the brain. In blood, this highly neurotoxic metal is bound almost exclusively to proteins and low-molecular-weight sulfhydryl-containing compounds, such as cysteine. The MeHg–cysteine complex acts as an amino acid analog, similar in structure to methionine, and is transported by the L system carrier for neutral amino acids across the BBB (Aschner and Clarkson, 1988). The increased uptake of MeHg following its conjugation with L-cysteine is inhibited by coinjection of other large neutral amino acids (such as methionine), but not by injection of acidic amino acids (such as L-aspartic acid). These characteristics are consistent with L system-mediated transport. Thus, a MeHg complex is formed that mimics the behavior of normal endogenous substrates, utilizing transport systems inherent to the BBB to gain access to the CNS.

In general, inorganic mercurial salts are less neurotoxic than organic mercurial compounds, most likely because their rate of transport into the CNS parenchyma is lower than organic mercurials. Yet some inorganic Hg, such as mercuric chloride (HgCl_2), can act as direct barrier toxicants. In a cat study by Peterson and Cardoso (1983), an intracarotid injection of HgCl_2 induced BBB damage, as manifested by fluorescent display of Evans blue staining in brain parenchyma. Direct application of Hg containing tissue fixative to the brain tissue corroborated diffusion of intravenously administered Evans blue into brain parenchyma, suggesting an immediate breakdown of the BBB (Marlin et al., 1980). By tracing the ultrastructural localization of cerebral alkaline phosphatase, a cerebrovascular marker primarily located on the luminal side of cerebral endothelial cells, Albrecht et al. (1994) observed a disappearance of alkaline phosphatase reactivity from luminal endothelial cell membranes in HgCl_2 -exposed animals. This was accompanied by ultrastructural changes typical of the formation of “leaky” microvessels.

Iron

Among the processes involved in brain regulation of Fe, the entrance of Fe to the cerebral compartment represents a critical step in controlling cerebral iron homeostasis. Receptor-mediated endocytosis of Fe–transferrin is the most prevalent and important transport for physiological delivery of Fe into the brain. In the plasma, Fe circulates predominantly as the trivalent ion (Fe^{3+}) complexed with transferrin, a glycoprotein with an approximate molecular weight of 80 kDa. Transport of Fe across the luminal membrane of the capillary endothelium occurs by receptor-mediated endocytosis of ferric transferrin. The receptor is a disulfide-linked, integral membrane glycoprotein with a molecular weight of approximately 180 kDa. Its affinity for apotransferrin is approximately two orders of magnitude lower than its affinity for diferric transferrin (K_d 2–7 nM) (Smith et al., 1997). It has been generally accepted that transferrin-bound Fe gains access to brain via transferrin receptor (TfR) on brain capillary endothelial cells (Jefferies et al., 1984; Klausner et al., 1993).

Additional mechanisms for Fe delivery into the CNS may occur via a nonspecific metal transporter, divalent metal transporter 1 (DMT1). DMT1 has been cloned from the rat and belongs to the Nramp2 (natural-resistance-associated macrophage protein) family. Its cDNA encodes 562 amino acids with 12 putative membrane domains. As a proton-coupled metal transporter originally discovered in duodenal enterocytes, DMT1 mediates the active transport of many divalent metal ions other than Fe, including zinc, manganese, copper, cobalt, cadmium, and nickel (Bannon et al., 2002; Garrick et al., 2003). A defective DMT1 allele encodes a protein that is rapidly degraded and has little or no activity in Fe uptake assays (Su et al., 1998). In the microcytic anemia mouse and the phenotypically similar

Belgrade rat, orthologous mutations (glycine 185 to arginine) in the DMT1 gene result in significantly reduced dietary Fe absorption as well as brain Fe levels (Fleming et al., 1997, 1998). Recent studies using GFAP-IL6 transgenic mice with a constitutive BBB defect reveals a 40% increase in total Fe level in the brain. This is followed by a consequential lipid peroxidation in the neocortex and in the cerebellum of symptomatic animals (Castelnau et al., 1998).

Fe accumulates in both BBB and BCB, but its presence in the barriers does not appear to produce any known harmful effects. In Fe-enriched conditions, there is an increased rate of TfR internalization and a decreased rate of TfR externalization in cerebral endothelial cells. The BBB endothelial cells are able to accumulate more Fe intracellularly in the presence of excess extracellular Fe. When the storage of Fe exceeds its capacity, the rate of Fe transport across the BBB is proportional to systemic Fe concentrations (van Gelder et al., 1998).

Fe is also selectively taken up by the rat choroid plexus following an intravenous injection (Morris et al., 1992). With histochemical staining, a substantial amount of Fe can be seen in neuroglial cells and in the choroid plexus epithelial cells (Moos and Mollgard, 1993). In human subjects with a calcified choroid plexus, Fe concentration in the choroid plexus is about five times greater than in other brain tissues (Michotte et al., 1977).

The choroid plexus and the oligodendrocytes are the only two cell types in the CNS capable of producing transferrin. Thus, a role of the choroid plexus in brain Fe regulation has been suggested (Crowe and Morgan, 1992; Zheng et al., 1999b). Although some studies with antibody against TfR failed to localize the receptors to the choroid plexus (Kissel et al., 1998), others have confirmed their presence (Moos, 1996; Zheng et al., 1999b). Thus, it appears likely that the choroid plexus plays a pivotal role in coregulating cerebral Fe homeostasis. The degree to which the BCB contributes to overall brain Fe regulation, as compared to the role of the BBB, has yet to be established.

Manganese

Mn toxicity in humans is a well-recognized occupational hazard for people who inhale highly concentrated Mn dust. Though a variety of organs are affected, Mn-induced neurotoxicity (manganism) is considered to be the most sensitive endpoint (Aschner et al., 1999; Pal et al., 1999). Manganism is associated with elevated brain levels of Mn, primarily in those areas known to contain high concentrations of nonheme iron, especially the caudate-putamen, globus pallidus, substantia nigra, and subthalamic nuclei. There is no experimental evidence in support of Mn-induced direct damage to cerebral microvascular structure nor is much known about structural injury as a direct result of Mn toxicity in choroidal epithelial cells. Mn, however, readily accumulates in the choroid plexus (Michotte et al., 1977; Valois and Webster, 1989; Zheng et al., 1998). The influx of

Mn to the choroid plexus is about 150 and 1000 times greater than that of cerebral cortex and CSF, respectively, when the plasma concentration of the infused Mn is maintained constantly (Murphy et al., 1991).

The homeostasis of Mn in the CSF appears to be directly influenced by plasma Mn concentrations (Zheng et al., 1998). Several investigators have shown that the oxidative state of the Mn ion determines the transport properties of Mn at the brain barrier systems. Mn^{3+} , a major form of Mn ions in circulation, enters the brain via a transferrin receptor-mediated mechanism, while Mn^{2+} is readily taken up into the CNS, most likely as a free ion species or as a nonspecific protein-bound species (Aschner and Gannon, 1994; Rabin et al., 1993). More recently, studies using transferrin knock-out mice indicate that deficiency in circulating transferrin has no apparent effect on tissue distribution of Mn^{2+} (Dickinson et al., 1996). The role of the defective DMT1 allele in the transport of manganese across the BBB has been recently studied in homozygous Belgrade (b/b) rats, suggesting that DMT1 might serve as a putative transporter for Mn into the CNS (Chua and Morgan, 1997).

Recent studies by Yokel and his colleagues (2003) have probed the uptake and efflux of Mn to and from the CNS with an in situ brain perfusion technique. Brain K_{in} values for any one of the three Mn species studied, i.e., $^{54}Mn^{2+}$, ^{54}Mn citrate, and ^{54}Mn transferrin (^{54}Mn -Tf), generally did not significantly differ among brain regions and the choroid plexus. However, the brain K_{in} for Mn citrate was greater than those of Mn^{2+} and Mn–transferrin in a number of brain regions. Furthermore, ^{55}Mn citrate inhibited ^{54}Mn citrate uptake, and $^{55}Mn^{2+}$ inhibited $^{54}Mn^{2+}$ uptake, supporting the concept of carrier-mediated brain Mn influx. These findings have led the authors to hypothesize that Mn citrate may be a major Mn species entering the CNS (Crossgrove et al., 2003). Additional efflux studies by the same authors have also suggested a lack of active transport of Mn from the CNS to the systemic circulation (Yokel et al., 2003).

Whereas Mn poisoning does not directly produce histopathological impairment to the BBB and BCB, it may selectively alter the barrier's ability to regulate cerebral Fe homeostasis. Chronic Mn exposure in rats resulted in a 32% decrease in plasma iron. Surprisingly, the Fe concentration in the CSF of the same rats markedly increased by threefold, reflecting an influx of Fe from the systemic circulation to the cerebral compartment upon Mn exposure (Zheng et al., 1999b).

Under normal physiological conditions, the brain regulates Fe balance by controlling the influx of Fe into the brain (a TfR-mediated process), the storage of Fe (dependent upon availability of ferritin), and the efflux of Fe whose rate is controlled by bulk CSF flow (Connor and Benkovic, 1992; Jefferies et al., 1984). The posttranslational modulation of TfR and ferritin synthesis is regulated by a [4Fe-4S]-containing protein known as cytoplasmic aconitase (ACO1) or iron regulatory protein-I (IRP-I). In the absence of Fe, ACO1 exhibits a unique affinity to mRNAs that

possess a Fe responsive element (IRE) stem-loop structure, i.e., to the mRNAs of the major proteins in Fe metabolism including ferritin and TfR (Beinert and Kennedy, 1993; Klausner et al., 1993). Evidence has shown that Mn can alter aconitase enzymatic activity, presumably by competing with Fe for the fourth, highly labile Fe binding site of the [4Fe-4S] cube in the enzyme's active center (Zheng et al., 1998). Recent results further confirm that Mn^{3+} is evidently more effective than Mn^{2+} in enzyme inhibition (Chen et al., 2001). Such an action, while suppressing the enzyme's catalytic function, may increase its binding affinity to the mRNAs encoding ferritin and TfR, as demonstrated by an increased expression of TfR mRNA. This theory was independently verified by Smith and his colleagues, who further demonstrated that Mn increases the binding affinity of IRP1 to TfR mRNA in vitro (Kwik-Urbe et al., 2003).

Abnormal metabolism of Fe in systemic and cerebral compartments is reportedly associated with the etiology of a number of neurodegenerative diseases, including idiopathic Parkinson's disease (Connor and Benkovic, 1992; Youdim et al., 1993). Cellular Fe overload in the basal ganglia, particularly the substantia nigra, may catalyze the generation of reactive oxygen species and enhance lipid peroxidation. Such Fe-mediated oxidative stress may ultimately lead to the degeneration of nigrostriatal dopamine neurons in idiopathic Parkinson's disease patients.

Perspectives and research needs

Research during the past several decades clearly points to a critical role for the brain barrier systems in chemical-induced neurotoxicities. However, the linkage between barrier dysfunction and the etiology of various neurological disorders remains unclear, owing to the lack of rigorous research effort in this area. In the ensuing discussion, we propose several specific research needs, deemed critical for improved understanding of certain metal-induced neurotoxicities and the general functions of barrier systems both in health and disease.

The need to understand the concept of blood–brain regional barrier

Unlike other tissues and organs in the body, normal brain function relies on the delicate coordination of neuronal activity. Minor changes in brain chemistry can have a profound impact on learning, memory, and behavior. Furthermore, chemical-induced brain damage is highly selective to some brain areas, but not to others. For example, neurons in the basal ganglia, particularly in the substantia nigra, are selectively affected by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The inorganic forms of Pb and Mn accumulate distinctly in the hippocampus and basal ganglia, respectively. The intrinsic, unique biochemical pathways in vari-

ous brain regions may partially explain these discrepancies. It is likely also that the selective chemical disposition in the CNS, in which the brain barriers play a determining role, may contribute to the unique patterns of brain regional sensitivity to these neurotoxicants. Thus, future research effort should be devoted to establishing and characterizing the concept of blood–brain regional barrier, for example, the blood–striatum barrier or blood–hippocampal barrier (Zheng, 2001b). Regional differences in blood flow, cellular composition, morphological variation, protein expression patterns, functions of tight junctions, as well as the effect of toxicants on regional barriers, may hold important clues in understanding region-specific targeting by neurotoxicants.

The need to understand the pathophysiological changes of brain barriers in aging, the relationship of aged barriers with cerebral vascular diseases, and the role of chemical exposure in the aging process of brain barriers

The cerebral vasculature is prone to pathological changes over the life time. Clinical evidence has already shown that cerebral vascular disease is a major component in the aging brain. Thus, damaged barriers or altered permeability of the barriers to xenobiotics may represent a risk factor to development of neurotoxic consequences in the aged population. It is also essential to recognize that the aging process occurring at the barriers is likely governed by interaction between genetics and environmental factors.

The need to understand how chemical exposure disrupts the early brain development by affecting brain barrier functions

During early development, the rapid growth of the CNS might necessitate a functionally “leaky” structure of the BBB and BCB to accommodate the high demand of blood-borne nutrients for brain growth (Johanson, 1989). However, the properties of the barrier in early life, which are different from those of the adult, render the brain highly susceptible to insults from exposure to toxic metals. A prime example is the vulnerability of the developing brain to Pb neurotoxicity in children. It is also important to note that the brain barriers per se may become more sensitive to Pb toxicity at a very young age. As mentioned above, the BCB, by producing and secreting TTR, may participate in brain development. Taking into account the selective toxicity of Pb on TTR in the BCB, the cognitive defects in Pb-poisoned children may reflect, to a certain extent, a persistent Pb-induced disorder in brain barrier function.

The need to explore the susceptibility of barrier structure (tight junctions) to toxicants

The most fundamental issue in brain barrier physiology and pathophysiology is the integrity of tight junctions. The

permeability of brain barriers to any chemical is directly associated with the integrity of the endo/epithelial tight junctions and the proteins that construct them, e.g., claudins, occludin, junctional adhesion molecule, and membrane associate guanylate kinase proteins such as ZO-1, 2, and 3. Thus, a priority should be given to mechanistic studies on the modalities by which chemicals may alter genes, gene families, or proteins whose expression may interfere with optimal brain barrier functions.

The need to understand biotransformation at the brain barriers and how the bioactivation and deactivation contribute to chemical-induced neurotoxicities

Drug metabolism represents a major detoxification pathway but in some cases can lead to the oxidative or reductive bioactivation of a compound. The BBB is composed of endothelia and astrocytes and the BCB consists of epithelia. Drug metabolism activities have been associated with all three cell types, and examples of bioactivation at the BBB have been reported (Romero et al., 1996; Ghersi-Egea et al., 1998; Teissier et al., 1998). It is critical to ascertain the identity and to establish the relative expression of the different drug metabolizing enzyme isoforms present at the barriers. Whether metabolic activation can lead to alteration of the barrier functions and how the cells forming the barrier protect themselves against toxic metabolic products (see below) are deemed important topics for future research.

The need to understand the role of brain barriers in cleansing neurotoxic materials so as to maintain a stable homeostasis of the CNS

The efflux of a toxic compound or metabolite from the brain occurs via the CSF and by passive diffusion across the barriers. The efficiency of efflux may be greatly increased by an inwardly directed transport process at the abluminal (BBB) and apical (BCB) membranes and/or by an outwardly directed transport at the luminal (BBB) and basolateral (BCF) membranes. The SLC21, SLC22, ABCC, and ABCB transport protein families are of crucial importance in these processes at brain barriers. The molecular identity and polarized distribution of these different proteins need therefore to be fully elucidated at the BBB and BCB, and their relative contribution in the elimination of a given toxicant needs to be investigated.

How the transporters present at the brain-facing and blood-facing membranes work in concert to achieve an efficient protection of the brain against toxic insults also needs to be more clearly understood. Finally, determining whether these mechanisms might be altered by metals, thus increasing the susceptibility of brain cells to toxic events, constitutes an additional fruitful area of study.

The need to understand the interaction between cerebral endothelial cells and astrocytes and how toxicants may affect this interaction

Astrocytes may contribute to the integrity of the BBB in three ways: (1) by inducing the formation of BBB in early development, (2) by maintaining the intactness of BBB structure, and (3) by participating in the transport of substances across the BBB. Since the astrocytic–endothelial interaction is essential to BBB function, the consequences of the interruption of this intimate cooperation are quite obvious. Chemicals that directly act on astrocytic–endothelial interactions can compromise the BBB integrity. The mitochondrial poison 3-nitropropionic acid selectively damaged striatal astrocytes within a few hours of *in vivo* exposure. This is followed by extensive BBB breakdown and striatal lesions (Nishino et al., 1997). Referring to metal toxicity, chronic postnatal Pb exposure in rats induces astroglial hypertrophy in hippocampus (Selvin-Testa et al., 1997) and alters the timing of astrocyte differentiation and maturation in the cortex and hippocampus (Harry et al., 1996). Both Fe and Mn preferentially deposit in astrocytes (Aschner et al., 1999). Hg accumulates in astrocytes and also induces the expression of astrocytic metallothionein (MT; Aschner, 1996). Cd, among other tested metals such as MeHg, Pb, and Zn, is the most potent inducer of MT in astrocytes (Kramer et al., 1996). The consequence of MT induction by metals in astrocytes remains uncertain *vis-à-vis* its implications for barrier function. It is possible that toxic metals, once they escape the endothelial barrier, are immediately sequestered by astrocytic foot processes as part of endogenous brain defense mechanisms. Sequestration of metal ions in astrocytes not only limits the metal ions from further diffusing to other parts of brain, but it may also protect the endothelial cells themselves from metal insults.

The need to understand molecular defense mechanism whereby barrier cells exert unique ability to protect themselves against blood-borne insults

The sensitivity, as well as the tolerance of barrier cells to metal toxicity requires additional investigation. The capillary endothelia and choroidal epithelia are often the first and the most frequent ones to encounter blood-borne toxic metals. How, then do the barrier cells survive the toxic attack? Two mechanisms may confer resistance against damage to brain barriers that is induced by blood-derived toxic agents. First, as mentioned above, astrocytes as a part of the BBB may respond effectively to metal insults, removing the metal from the endothelia and sequestering it within a sub-cellular location. Second, as stated before, both types of barrier cells possess active defense systems. For example, the activities of superoxide dismutase, glutathione peroxidase, and catalase are significantly higher in isolated brain capillaries and choroid plexus than in parenchymal cells derived from the cerebrum and cerebellum (Tayarani et al.,

1989). Thus, the presence of active protective enzymes may effectively defend the barriers against free radical-initiated oxidative stress.

The need to understand the unique blood-to-brain transport systems at the barriers, such as DMT1, and other transporters, and how chemical exposure may alter the normal functions of these transporters

Both BBB and BCB are actively involved in many transport processes. Cerebral Fe regulation, for instance, may be achieved by cooperation of both barriers through a TfR-mediated mechanism. Mn exposure, by promoting overexpression of TfR at the barriers, may disrupt the balance of influx of Fe from the systemic circulation to the cerebral compartment.

With respect to the BCB, the choroid plexus regulates bidirectional transport and provides a highly selective pathway for materials to communicate between the blood and CSF. The choroid plexus synthesizes proteins and releases them into the CSF. TTR, transferrin, and ceruloplasmin, for example, are synthesized in the choroidal epithelial cells. The inhibitory effect of Pb on TTR production and secretion by the choroid plexus represents an example whereby a toxic metal induces neurotoxicity, at least in part, as a result of its effect on the BCB. In addition, there are many channels, transporters, carriers, and receptors on the BBB and BCB, and there is a lack of understanding on how toxic metals interact with them and to what degree these interactions contribute to neurological disorders.

The need to search for biomarkers representing the integrity of BBB and BCB

Cerebral endothelial cells and choroidal epithelial cells are unique cell types in the brain. While TTR has been suggested as a biomarker of choroidal epithelial cells and thus for the function of BCB (Zheng et al., 1996), its application for such a purpose has never been verified. No specific marker proteins or polypeptides have been sought or identified for the functional integrity of BBB. With the advance of proteomics, it would seem a technically feasible goal to define specific biomarkers in body fluids for clinical diagnosis of altered blood–brain barrier function.

In conclusion, the blood–brain interfaces protect brain tissues against organic and inorganic chemicals by means of different complementary mechanisms. Nevertheless, toxic metals could bypass these mechanisms and inflict damage to the brain parenchyma. The effectiveness of the barrier systems may also be compromised either in pathological situations or following toxic insults by compounds that target the blood–brain interfaces. Cumulative evidence has revealed that the brain barriers, the gatekeepers of the cerebral compartment, are subject to toxic insults from heavy metal exposure. Because of the special roles of brain barriers in

overall brain development and function, it is reasonable to postulate that injury to the barriers may contribute to metal-induced neurotoxicities. Our knowledge about this aspect remains embryonic. It is, therefore, imperative that future investigations address how brain barriers sequester toxic metals, whether toxic metals alter barrier functions, and what neurological consequences may ensue.

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