Pharmaceutical Research, Vol. 12, No. 10, 1995

Review

Cerebrovascular Permeability to Peptides: Manipulations of Transport Systems at the Blood-Brain Barrier

Berislav V. Zlokovic[1,2]

Received April 6, 1995; accepted May 24, 1995

The study of peptide transport across the blood-brain barrier (BBB) is a field fraught with conflicting interpretations. This review presents a fairly strong case that peptides can be differentially transported at the BBB. However, minimal transport of peptides could have important impact on central nervous system (CNS) functions since only small amounts are needed for physiologic, pharmacologic and/or pathologic effects. Several BBB peptide transport mechanisms (i.e., receptor-mediated, absorptive-mediated, carrier-mediated and non-specific passive diffusion), as well as non-transport processes (i.e., endocytosis without transcytosis, aborption and metabolism) are discussed. It is emphasized that peptide transport systems at the BBB could be important targets for both therapeutic delivery of peptides and the development of certain brain pathologies. Strategies to manipulate peptide BBB transport processes have been discussed including lipidization, chemical modifications of the N-terminal end, coupling of transport with post-BBB metabolism and formation of potent neuroactive peptides, up-regulation of putative peptide transporters, use of chimeric peptides in which non-transportable peptide is chemically linked to a transportable peptide, use of monoclonal antibodies against peptide receptors, and binding of circulating peptides to apolipoproteins. It is suggested that future directions should be directed towards development of molecular strategies to up-regulate specific BBB peptide transporters to enhance brain delivery of peptide neuropharmaceuticals, or to down-regulate transport of peptides with potential role in cerebral pathogenesis.

THE BLOOD-BRAIN BARRIER

During the last two decades, the anatomic concept of an impermeable blood-brain barrier (BBB) has been seriously challenged. It has become gradually accepted that what is true for the behavior of some vital dyes and horse radish peroxidase in front of the BBB, may not hold for many biologically active molecules and/or for the cells of the immune system (1). Recent transport kinetic, metabolic, cellular and molecular studies have given a major impetus to a better understanding of how the BBB function, and what advantages can be possibly taken from the therapeutic point of view. It has been demonstrated that several classes of metabolic substrates, neuroactive and regulatory peptides, plasma-derived proteins, and various groups of centrally active pharmacotherapeutics are able to utilize specialized "shuttle" services at the BBB. The current concept of a BBB states that the microvascular endothelium in concert with astrocytes, pericytes and possibly microglia, regulates the homeostasis of the neural milieu, rather than simply impeding solute exchanges between blood and brain, as recently reviewed (1–3).

[1] Departments of Neurological Surgery, Physiology and Biophysics and Division of Neurosurgery, Children's Hospital Los Angeles, Univeristy of Southern California School of Medicine, Los Angeles, California 90033.

[2] To whom correspondence should be addressed.

Cell Biology and Anatomy

The cell biology of the BBB reveals the close proximity and paracrine type of interactions between cerebral endothelial cells, pericytes, astrocytes, microglia and neurons (1–3). Figure 1 illustrates the anatomic relationship between different cells involved functionally into the BBB complex. The BBB is a continuous zipper-like tight-junctioned endothelial cellular layer. The microvascular endothelium shares a common basement membrane with astrocytes and pericytes. Astrocytes send their foot processes to invest more than 90% of endothelial capillaries, while in the capillaries the ratio between pericytes and endothelial cells is approximately 1:2. Neuronal endings may directly innervate the endothelium, while microglia seem to be located in the vicinity of pericytes.

The endothelial layer needs to be viewed as two separate membranes, one on the inside of the vessels (luminal) and that of the outside (abluminal) separated by 300 to 500 nm tick cytoplasm. The surface area of cerebral capillary endothelium is about 100 cm^2 per gram of brain tissue. In the adult human brain the total surface area of microvasculature is 12 m^2, the total length of capillaries 650 km, the diameter of a capillary lumen is about 6 μm, and the capillaries are 40 μm apart.

The endothelial cells express numerous functional proteins that are involved in various transport, receptor-signal transduction and cell-mediated mechanisms in the brain. It

0724-8741/95/1000-1395$07.50/0 © 1995 Plenum Publishing Corporation

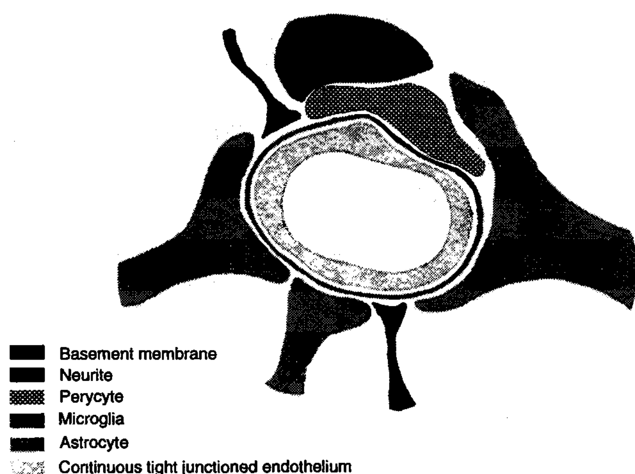

Fig. 1. A five-cell paradigm of the cell biology of the blood-brain barrier. The anatomic relationship between microvascular endothelium, astrocytes, pericytes, microglia and neuronal endings.

appears that different regulatory molecules, many of those being peptides, are involved in the control of BBB specific mechanisms. They may be secreted by astrocytes and possibly some neural endings, acting in a paracrine fashion on the abluminal side of the BBB, and/or alternatively may originate from circulating blood acting on the luminal side of the BBB.

Transport Biology

Circulating molecules use a number of different mechanisms available at the BBB for their transport (1). These include: 1) lipid-mediated transport of many small highly lipophilic drugs; 2) carrier-mediated transport for hydrophilic nutrients and their drug analogs; 3) plasma-protein mediated transport of acidic drugs, peptides and highly lipophilic drugs; 4) carrier-, receptor-, and/or absorptive-mediated transcytosis of peptides including proteins; and 5) bulk flow transcytosis, i.e., pinocytosis and tubulocanalicular transport irrespective of the molecular size, that is minimal under physiologic conditions, but may become significant under certain pathologic circumstances. There is also evidence that some viruses express antigens that have affinity for various receptor- or absorptive-mediated mechanisms at the BBB, while the expression of specific adhesion molecules on both the leukocytes and brain endothelial cells are required for the cellular interactions to occur (1).

Lipophilicity

Lipophilic compounds with molecular weight less than 600 are generally assumed to readily enter the brain via diffusion into the lipid phase of the endothelial plasma membrane. The lipid solubility can be assessed by determining the olive oil/water and/or octanol/Ringer partition coefficient (PC), as well as by computing the number of hydrogen bonds in the molecule. It has been shown that the increased lipid solubility and a smaller number of hydrogen bonds usually result in better penetrability of the drug. Classical examples are morphine-codeine-heroin model (5), and the steroid hormone model (6). However, there are exceptions from this rule including several highly lipophilic peptides. Effects of peptide lipophilicity and lipidization on BBB transport is discussed below.

Transport Systems for Metabolic Substrates

Most metabolic substrates are hydrophilic, and they traverse the BBB by specific carrier-mediated transport systems expressed both at the luminal and abluminal sides of the endothelial cells (4). It is believed that these carriers are enzyme-like proteins that are under genetic regulation, and operate bidirectionally. Several systems have been described in brain capillaries including these specific for: 1) hexoses; 2) monocarboxylic acids; 3) neutral amino acids; 4) basic acids; 5) acidic amino acids; 6) amines 7) nucleosides; 8) purine bases; and more recently 9) pyrimidine bases. These transport systems have been characterized mainly by physiologic techniques. The molecular nature of most BBB nutrient and ion transport systems is still relatively poorly understood, except for the hexose carrier (7), and more recently the BBB sodium pump (8).

PEPTIDES

Peptides are defined as anywhere in size from two amino acids (e.g., carnosine) to proteins of a molecular weight of 100,000 or more. Multiple biologic actions of peptides in brain suggest that these agents may be utilized as neuropharmaceuticals in the treatment of various disorders of the central nervous system (CNS) (reviewed in 9 and 10). It has been suggested that the role of peptides in the CNS is complex underlying the basic physiologic phenomena of the brain such as: 1) neurotransmission and/or neuromodulation; 2) regulation of the neuroendocrine axis; 3) regulation of cerebral blood flow; 4) regulation of CSF secretion; 5) mediation of the integrity of the BBB; 6) modulation of the BBB permeability to nutrients; 7) regulation of water and electrolyte content of the brain; 8) regulation of the expression of specific proteins at the BBB, and in other cells of the CNS. Therefore peptides exert important behavioral effects, effects on higher integrative functions of the brain and on the vegetative functions of the CNS. There are several disorders of the brain in which peptides are implicated in pathogenesis, and therefore several peptide agonists and/or antagonists can be used as potential neuropharmaceuticals. To enumerate a few: Alzheimer's Disease (AD), depression, mania, schizophrenia, amnesia, migraine-headache, stroke, insomnia, alcohol abuse, anxiety, obsessive-compulsive disorder, cerebral acquired immune deficiency syndrome (AIDS), chronic pain and etc. (9,10) As with many potential neuropharmaceuticals, peptides must be able to undergo transport from blood to brain in order to be therapeutically effective.

BBB Peptide Enigma: History and Present Era

It has been generally accepted for a number of years that peptides do not cross the BBB because they are highly polar compounds. This view has been supported by the results of short-term (5 to 15) kinetic in vivo transport experiments in rats, when brain extraction of several peptides was found to be practically indistinguishable and/or just barely above the uptake of metabolically inert polar molecules used as markers of the cerebrovascular space (11–16) (Table I).

Table I. Brain Uptake of Radiolabeled Peptides and Inert Polar Molecules Following Intracarotid Injection. Data Taken from Refs. 11–16

Peptide	Numbers of residues	Extraction, %
Carnosine	2	1.1
Gly-Phe	2	0.9
Gly-Leu	2	0.5
Glutathione	3	0.6
		12.0–18.0
Thyrotropin-releasing hormone	3	1.0
		0.15
Leucine enkephalin	5	2.4
		none
[a]DADLE	5	2.0
Methionine enkephalin	5	15.0
		2.4
β-casomorphin-5	5	1.9
[b]DGAVP	8	1.2
Cholecystokinin 8	8	none
Arginine vasopressin	9	2.4
Lysine vasopressin	9	1.3
Oxytocin	9	1.3
Delta sleep inducing peptide	9	measurable
Gonadotrophin-releasing hormone	10	1.1
		1.3
Substance P	11	0.5
Somatostatin	14	0.01
β-endorphin	31	1.9
Inert polar molecules	*Molecular weight*	*BUI(%)*[c]
D-mannitol	167	1.8
		2.1
Sucrose	342	2.3
Inulin	5000	1.4
Dextran	70,000	0.9

[a] DADLE, D-Alanine[2]-D-Leucine[5]-enkephalin.
[b] DGAVP, des-Gly-NH_2 arginine vasopressin.
[c] BUI, brain uptake index values.

These initial brain uptake index (BUI) studies suggested that peptide distribution in the brain does not exceed the volume of brain vascular space, with an exception of the tripeptide glutathione (GSH), as demonstrated by recent studies in which the precautions were taken to keep GSH in the reduced form (17–19). An earlier study with methionine-enkephalin indicated a significant BBB uptake of this opioid peptide (20), but later work did not confirm these findings (11). Recent studies, however, by employing more sensitive techniques such as long-term brain perfusion method and intravenous injection method, have demonstrated that some circulating peptides with important physiologic functions and possible pathologic implications are transported into brain capillaries, and through the BBB, via specific transporters and/or receptors. Molecular characterization at the protein and/or mRNA level for most of these peptide specific BBB transport systems is still incomplete. Few exceptions include insulin and transferrin receptors (reviewed in 21), and preliminary evidence for the expression of BBB GSH transporters (22), as described below. This review will focus primarily on work from our laboratory and data obtained with arterial infusions of a) small neuroactive peptides; b) antioxidant agent GSH; and c) soluble Alzheimer's amyloid-β peptide (sAβ) free and/or complexed with apolipoproteins J (apoJ) and E (apoE).

Brain Perfusion Model

A long-term vascular brain perfusion (VBP) model in the guinea-pig has been used extensively to investigate transport of peptides at the BBB in vivo. Details of this technique have been described elsewhere (23,24). The method allows measurements of the cerebrovascular permeability surface area (PS) product of test-peptide over periods of up to 30 min. This represented a considerable extension of the exposure time to the BBB in comparison to 15s BUI method (11–15), or 1 min. brain arterial saline infusion technique in rats (25), that has been later adapted to perfusions as long as 10 min. (26). It has been suggested that 10 min of VBP is 600 times more sensitive than the BUI method in which a transit time of test-molecules through the cerebral circulation is about 1s (10). To ensure during brain perfusion the conditions close to the physiologic in vivo situation, brain is perfused with mock plasma (e.g., glucose, electrolytes) containing red blood-cells as an oxygen carrier, and dextran to maintain the colloid osmotic pressure (23). The perfusion pressure and flow are continuously monitored to approximate cerebral blood flow of about 1 ml/min/g. Physiologic parameters are continuously monitored including respiration, heart rate, arterial blood pressure, and in some experiments electroencephalogram. The biochemical parameters in perfused brain including water and electrolyte content, Na/K ratio, ATP and lactate content remain normal during perfusion, indicating no swelling and/or oxidant stress in brain tissue (23,24). This has been confirmed by ultrastructural studies showing normal cellular relationships within the neuropil between dendrites and neuronal soma, normal content of neurotransmitters in axonal endings, as well as the intactness of brain capillaries and preserved relationship between endothelial cells and pericytes (27). The intactness of the BBB has been also demonstrated to several different size vascular markers.

Methodologic Principles

The brain volume of distribution (V_D) for radiolabeled peptides and inert polar molecules (V_o) can be calculated as (23,24): $V_D = C_{BR}/C_{PL}$ (Eq. 1), where C_{BR} and C_{PL} are DPM or CMP/g of brain, and DPM or CPM/ml of perfusate. During perfusion, the concentration of peptide test tracers in the arterial inflow, C_{PL}, is constant in the VBP model. The following guidelines and logic might be considered when characterizing peptide transport systems at the BBB:

(i) The existence of a transport system is inferred from the observation that the brain V_D for peptide exceeds the plasma volume (V_o) for a molecule such as sucrose or albumin.

(ii) The finding that $V_D > V_o$ may infer transport or it may infer one of the following processes: 1) simple absorption to the brain microvascular endothelium; 2) endocytosis without transcytosis at the endothelium; 3) metabolism of the peptide at the endothelial surface by ecto-enzymes followed by brain uptake of radiolabeled metabolites; thus

there are at least three other physiologic processes other than actual transport that may yield a $V_D > V_o$ for a given peptide.

(iii) A technique other than vascular perfusion must also be performed in order to differentiate these four processes, i.e., actual transport, absorption, endocytosis, metabolism. The capillary depletion technique was developed to help differentiate these processes (26). When brain tissue was subjected to the capillary depletion step, V_D values for microvessels and capillary-depleted brains were calculated by Eq. 1, and DPM or CPM for tracers were expressed per unit mass of microvascular and capillary-depleted brain tissue, respectively. By adding a capillary depletion step following the VBP a fraction of test-peptide that is tightly bound and/or sequestered by brain capillaries can be determined. However, recent studies with the stable μ—opioid peptide agonist [D-Arg^2,Lys^4]-dermorphin analogue have shown that the capillary depletion technique cannot be used for peptides that are non-specifically bound to the microvasculature or which are bound with relatively low affinity, such that the peptide dissociates from the vascular pellet during homogenization of brain (28). High-performance liquid chromatographic (HPLC) analysis of brain tissue and microvascular pellet may help to differentiate the metabolism of peptide in the brain parenchyma and/or at the BBB (28–30). The autoradiographic analysis of perfused brain tissue (31) or immunocytochemical analysis (32) can be additionally used as an independent confirmation of the transcytosis of test-peptides and proteins. This is in particular important when a loosely bound microvascular fraction of peptide may interfere with measurements in post-capillary supernatant giving a picture of an apparent uptake of peptide into brain parenchyma.

(iv) As indicated in Table II, several methods (e.g., capillary depletion, autoradiography, immunocytochemistry) were combined with the brain perfusion to show the existence of an actual transport system for a given peptide as opposed to one of the non-transport processes (e.g., endocytosis, absorption, metabolism). It is noteworthy that transcytosis of an intact peptide may co-exist with its metabolism, as shown for example with AVP (30). Also, peptide transport system may be operative at the luminal side of the BBB, as demonstrated for Leu-Enk (33,34), but its metabolism at the abluminal side and/or in brain parenchyma may be so rapid to preclude the transcytosis of intact opioid peptides (29).

(v) The designation of a peptide transport system is provisional until there is biochemical and/or molecular confirmation of the existence of the putative transport system. Such biochemical confirmation typically comes in the form of affinity cross-linking of ligands to saturable binding sites that are detected by SDS-PAGE and autoradiography. For example this has been done for the BBB insulin and insulin-like growth factor (IGF) receptors (21), and is a classical approach for biochemical characterization of receptor/transport systems. Characterization of the putative BBB peptide transporters can also be studied by expressing peptide transport in Xenopus laevis oocytes, as recently done

Table II. Cerebrovascular Permeability to Radiolabeled Peptides and Inert Polar Molecules Determined by the Vascular Brain Perfusion Model. Data taken from Refs. 24, 26, 29, 31, 33, 34, 41–46.

Transport mechanism	Peptide	PS (ml/g/s × 10^6)
I. Receptor-mediated transcytosis	s$A\beta_{1-40}$-apolipoprotein J[a]	172.7
	Apolipoprotein J	72.3
	Arginine vasopressin	46.3
	s$A\beta_{1-40}$	44.7
	Insulin	17.3
II. Absorptive-mediated transcytosis	Cationized IgG[b]	50
	Cationized BSA[c]	33.3
	Cationized BSA-D-[ala^2]β-endorphin	33
	IgG (native, species homologous)	9.6
III. Carrier-mediated transport at the luminal side	Leucine enkephalin	60.3
	Delta sleep inducing peptide	15.5
IV. Non-specific transport	Cyclosporin A	20.5
	Thyrotropin releasing hormone	20.3
V. Non-transport process (e.g., endocytosis, absorption, metabolism)	Apolipoprotein E3	11.2
	Apolipoprotein E	10.8
	Apolipoprotein E4	6.0
	s$A\beta_{1-40}$-apolipoprotein E3	3.1
	s$A\beta_{1-40}$-apolipoprotein E	1.5
	D-[ala^2]β-endorphin	unmeasurable
	β-endorphin	unmeasurable
	Inert polar molecules	
	Sucrose	4.6
	Mannitol	4.0
	Dextran	0.8

[a] s$A\beta_{1-40}$, soluble amyloid β(1–40).

[b] IgG, immunoglobulin G.

[c] BSA, bovine serum albumin. Capillary depletion technique was used to discriminate between transcytosis (I and II) vs. non-transport process (V). Immunocytochemistry was additionally used for s$A\beta_{1-40}$ and IgG, and autoradiography for insulin. Metabolism of vasopressin and opioid peptides was demonstrated by HPLC analysis.

for GSH (22), and fractionation of brain capillary poly $(A)^+$ RNA could be used to determine size fractions that express peptide transport, that is standard molecular approach to establish the presence of specific transcripts.

Neuropeptides

By definition, brain peptides or neuropeptides are peptides which have been shown to exist within the cells, axons and terminals of the CNS (9). Besides being present in the CNS most of these peptides have been originally identified in various peripheral tissues. Mammalian neuropeptide families can be classified as gastrointestinal peptides, growth factors, hypophyseal peptides, hypothalamic-hypophyseotropic hormones, neurohypophyseal hormones, opioid peptides and others.

Early Evidence for and Against BBB Transport

Earlier studies have shown that several systemically administered peptides such as enkephalins and endorphins, thyrotropin-releasing hormone (TRH), and cholecystokinin (CCK) are capable of eliciting changes in CNS functions (35–37). It has been even suggested that an important characteristic for most peptides is that they can have CNS actions after systemic administration, providing they are given in sufficiently high pharmacologic dose (38). This implies that they can reach the brain by way of the general circulation, assuming that the primary site of action is not localized in the periphery. On the other hand, some centrally administered peptides such as arginine-vasopressin (AVP) and biologically active fragments of CCK were found in significant quantities in the urine and plasma of laboratory animals (39,40), suggesting transport from the cerebrospinal fluid (CSF) to blood, and/or CSF to brain to blood. In contrast to these observations, BUI studies indicated the absence of a significant brain uptake of enkephalins and their more stable synthetic analogs, AVP, delta sleep inducing peptide (DSIP), carnosine, hypothalamic releasing factors, bombesin, growth factors and etc. (Table I).

Evidence for Significant BBB Permeability

During last ten years the VBP method has been applied to study a number of neuropeptides including insulin, AVP, leucine-enkephalin (Leu-Enk), DSIP and thyrotropin-releasing hormone (TRH) (30,31,33,34,41,42). The VBP model was also used to study highly lipophilic immunosuppressive peptide cyclopsorin A (43), soluble Alzheimer's amyloid β ($sA\beta_{1-40}$) (44), as well as proteins such as homologous immunoglobulin G (IgG) (45), apolipoproteins J and E (46), cationized albumin and cationized IgG (26), and chimeric peptides such as cationized albumin-D-[ala^2]β-endorphin (29), as discussed below. The cerbrovascular permeability surface area or PS product for studied peptides is the same as the unidirectional blood-brain transport rate constant, K_{IN}, since the PS $\ll$ cerebral blood flow. When radiolabeled test-peptide is studied with varying concentrations of unlabeled peptide, PS for a given peptide is related to kinetic parameters as (24): $PS = V_{max}/(K_m + C_{PL})$ (Eq. 3), where V_{max} is the maximal transport capacity, K_m is the half-saturation constant. Unidirectional influx, J_{in}, of circulating peptide into brain can be calculated as (24): $J_{in} = PS\ C_{pl}$ (Eq. 4). In the presence of potential inhibitor and/or competitor at concentration C_i, the inhibitory constant K_i, may be calculated as: $K_i = J_i K_m C_i/(J_{in} - J_i)(K_m + C_{PL})$ (Eq. 5), where J_i is a saturable peptide flux in the presence of potential inhibitor and/or competitor.

As shown in Table II the PS values for Leu-Enk, AVP, TRH, insulin and DSIP were 13.1 to 3.4 fold higher than the PS product of cerebrovascular space marker sucrose. After 10 min of VBP, brain uptake of various neuropeptides expressed as the uptake of the label ranged between 1.5% and 6.5% of their respective concentrations in the arterial inflow. As discussed below, not all of this dose in brain could account for the intact peptide, and the amount of the intact peptide after 10 min. varied greatly from > 95% in a case of GSH, DSIP, TRH, and insulin (19,41,42,47), to about 12% for AVP (30), and almost 0% for opioid peptides (29). However, none of these peptides were significantly degraded at the luminal side the BBB, and possible locations for hydrolysis are likely to be on the abluminal side and//or in brain parenchyma. It is noteworthy that very small amounts of peptides over extremely short periods of time are capable to induce physiologic and/or pharmacologic responses, so even minimal transport could have important impact on CNS functions.

The PS products estimated by the intravenous injection technique for AVP (48), insulin (47) and sucrose (Table III) produced comparable values. When PS products for these peptides were compared with their olive oil-water PC coefficients and/or reciprocal values of the square root of the molecular weight, no correlation was found with either (Figure 2). The same was true for $sA\beta_{1-40}$, apo J, apo E, and complexes between $sA\beta_{1-40}$ and apolipoproteins. This analysis has confirmed our initial hypothesis that uptake of several peptides at the BBB may not be governed primarily by physicochemical factors such as lipophilicity and simple diffusion across the BBB. An important question to be answered next was whether significantly higher PS products of peptides vs. metabolically inert molecules are due to the presence of specific transport systems for peptides at the BBB, and/or alternatively they may reflect degradation of peptides followed by the uptake of peptide metabolites. As indicated in Tables II and III, specific transport systems have been demonstrated at the luminal side of the BBB for insulin and IGF's (21), Leu-Enk (33,34), AVP (49), DSIP (41), luteinizing hormone releasing hormone (LHRH) (50) and [D-ala^1]-peptide T-amide (50). Specific transport mechanisms were also described for cationized proteins (26), chimeric peptides (29), $sA\beta_{1-40}$ (44), apo J (46), transferrin (21) and monoclonal antibodies against transferrin receptors (51). For several peptides and proteins, including GSH, DSIP, TRH, LHRH, insulin, transferrin, IgG and etc., the BBB uptake was > 95% in the form of intact peptide (19,41,42,47,50). For others, such as opioid peptides (29,52) and AVP (30), significant hydrolysis ranging from total degradation (29) and 50 –88% degradation (30), has been suggested to occur during passage across the BBB, and/or in brain parenchyma following BBB transport (30). The findings of specific transport mechanisms for peptides at the luminal side of the BBB led us and other laboratories to perform a series of studies to determine whether these pep-

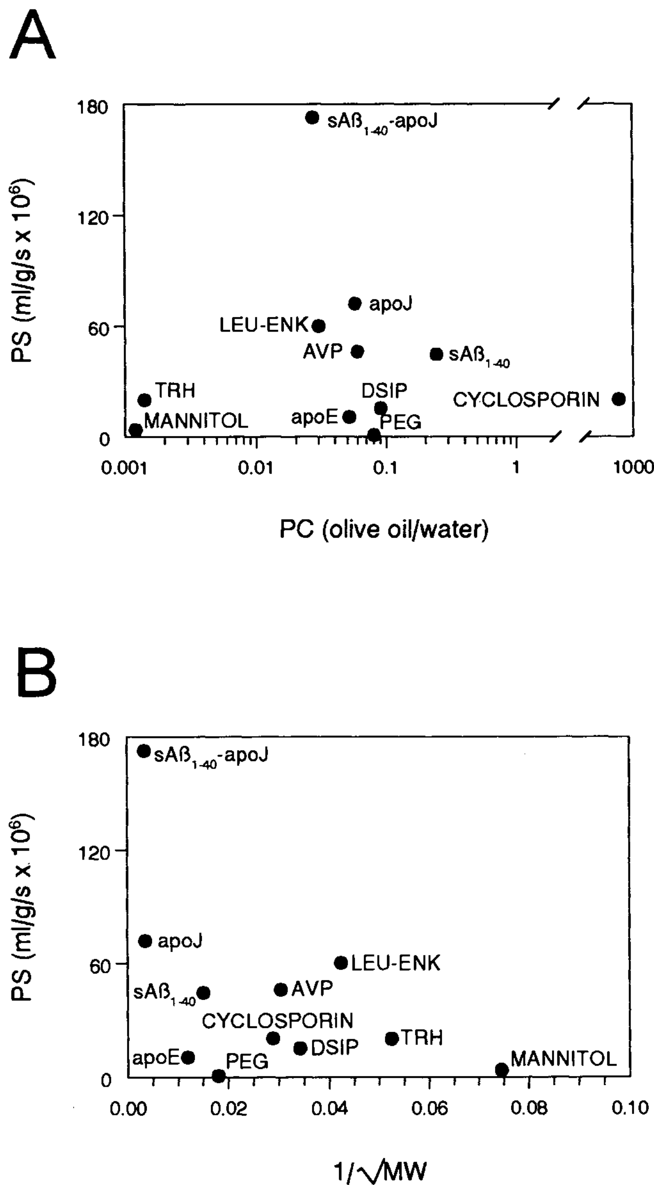

Fig. 2. Cerebrovascular permeability surface area (PS) product for radiolabeled peptides and inert polar molecules determined by vascular brain perfusion in guinea-pigs is plotted against A: olive oil/water partition coefficient (PC), and B: reciprocal value of the square root of the molecular weight of test-molecules. PS values taken from Table I.

tide transport systems can be utilized to manipulate the level of peptides in brain.

Opioid Peptides

Endogenous opioid peptides, enkephalins and endorphins, as well as endogenous opiate alkaloids, morphine and codeine, are present in brain and CSF in comparable concentrations, and it is believed that they jointly govern the opiate function of the brain (9). Due to their potent analgesic effects, transport of opioid peptides across the BBB has generated considerable interest. In a case of Leu-Enk, it has been shown that hydrolysis, the L-amino acid transporter, and μ and δ opioid receptors do not play a significant role

Table III. Cerebrovascular Permeability to Radiolabeled Peptides and Inert Polar Molecules Determined by the Intravenous Injection Method. Data taken from Refs. 43, 47, 48, 50, 51, 64

Peptide	PS (ml/g/s × 10^6)	Transport system
Luteinizing hormone-releasing hormone	208.3	yes
Cyclosporin A	44.8 to 54.3	none
Arginine vasopressin	41.2	yes
[D-Ala[1]]-peptide T-amide	21.6 to 41.6	yes
Insulin	15.8 to 22.6	yes
OX26 antibody	10.8	yes
Transferrin	1.8 to 3.8	yes
Nerve growth factor	1.08 to 2.6	not determined
Somatostatin analogs	0.66 to 3.4	none
Cyclo(His-Pro)	2.0	none
Inert polar molecules		
Sucrose	6.00 to 7.10	
Albumin (native)	0.16 to 1.08	
	0.06 to 0.15	
[a]IgG (native, species heterologous)	0.03 to 0.10	
Inulin	0.02 to 0.04	

[a] IgG, immunoglobulin G.

during its interaction with specific binding sites at the luminal endothelial membrane (33,34). The aminopeptidase inhibitors bestatin and bacitracin, fully saturated L-amino acid transporter, naloxone, specific μ-receptor agonist, Tyr-D-Ala-Gly-Me-Phe-NH(CH_2)$_2$OH, and δ-receptor antagonist allyl[2]-Tyr-AIB-Phe-OH, were without effect on Leu-Enk BBB uptake. A dose-dependent self-inhibition of Leu-Enk BBB uptake has indicated the presence of a low affinity but specific luminal transporter for the pentapeptide (34). However, Leu-Enk is degraded by abluminal aminopeptidases at the BBB, as shown by in vitro studies with isolated capillaries (52), and could be additionally hydrolyzed by brain aminopeptidases that act normally to terminate the actions of endogenous enkephalins (27). Similarly, the proteolysis of β-endorphin was demonstrated in capillary-depleted brain following the VBP (29). These results are in contrast with earlier observations of relatively high BBB permeability to four synthetic biologically potent analogs of endorphins and enkephalins (53). In previous studies the degree of brain and BBB degradation of these relatively stable analogs of opioid peptides has not been determined (53). Thus, it may be that reported PS values for blood-brain transport of intact opioid peptides were overestimated in previous studies (33,53), since significant proteolysis has been suggested by more recent VBP studies (29). Regarding earlier reported CNS effects of peripherally administered enkephalins and endorphins (35), it is possible that following administration of high pharmacologic doses, certain amount of intact opioid peptides may reach their brain targets before being degraded in plasma, at the BBB and/or by brain aminopeptidases, and therefore may cause the observed central effects.

Work with Leu-Enk has demonstrated that chemical modifications of peptide may influence significantly its carrier-mediated transport at the luminal side of the BBB. For this specific transport system, the N-terminal tyrosine resi-

due of Leu-Enk appears to be critical, since removal of the N-terminal tyrosine results in tetrapeptide that is unable to inhibit the BBB uptake of Leu-Enk (33,34). The observations that N-terminal alterations affect Leu-Enk transport to a greater extent than C-terminal modifications, and that naloxone and other opioid receptor antagonists were unable to inhibit peptide transport at the BBB (33,34), suggest that modifications of peptides may be made which affect transport, but have minimal effect on the biologic activity of the peptide in the CNS. The lipidization of [D-ala^2]Leu-Enk with adamantane (54) increased by 10 times its PC coefficient, but high concentrations of [D-ala^2]Leu-Enk-adamantane conjugates were still needed to induce analgesia, and it was not clear whether the lipidization resulted in substantial increase in BBB transport (54). The importance of the N-terminal tyrosine residue has also been demonstrated for selective and stereospecific transport of Tyr-MIF-1, methionine enkephalin and somatostatin in direction CSF-blood (55), but the clinical relevance of such modifications is limited due to the absence of these transport systems from the luminal side of the BBB. The strategy of using chimeric peptides to deliver more stable endorphin analogs to the brain is discussed below.

Vasopressin

AVP is a hormone/neuropeptide that regulates a number of peripheral (e.g., antidiuresis, glycogenolysis) and central (e.g., memory, learning) functions (9). A potential therapeutic application of AVP is that of memory disorders caused by brain trauma, AD and senile dementia. By using the VBP model, we have demonstrated that brain AVP levels can be influenced by plasma AVP and/or AVP receptor antagonists (30,49,56,57). Although the capillary sequestration rate of AVP was somewhat higher than for sucrose, its contribution to the overall brain uptake of AVP was minor, suggesting that most of the peptide has crossed the BBB (30). Bestatin-resistant saturation kinetics has been demonstrated at different BBB and non-BBB regions (49,56,57). The choroid plexus exhibited the highest affinity for circulating AVP. With respect to low nanomolar concentrations of AVP in peripheral blood, it has been suggested that the BBB AVP transport system may function to direct in almost linear fashion AVP into brain parenchyma. Thus, its physiologic relevance might be to continuously transport blood-borne AVP across the BBB, which in turn may represent an important source of brain AVP. A significant competitive inhibition of AVP transport across the BBB was obtained with the antagonist of vasopressinergic V_1-receptors, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid)-O-methyl-Tyr^2]AVP (TMeAVP). The regional K_i constants suggested that affinity of TMeAVP at the BBB was 1.7 to 2.5 fold less than of AVP itself (49). This indicates that the V_1-receptors may participate in the initial binding of AVP at the luminal side of the BBB, and/or possibly could mediate AVP transport across the BBB. It has been also demonstrated that AVP influx into the brain was not altered by peptide fragments AVP-(1–8), pressinoic acid, [pGlu^4 Cyt^6]-AVP-(4–9), the L-amino acid transport system substrates, or the agonist of vasopressinergic V_2-receptors, 1-desamino-8-D-AVP. Recent work has shown that AVP BBB transport system can be up-regulated by nicotine, and increases in blood-brain transport and capillary sequestration rates have been demonstrated following chronic nicotine treatment (58).

The HPLC analysis of capillary-depleted brain tissue indicated that the intact [^{3}H]-AVP-(1–9) progressively declined with time from 49% at 1 min. to 11.9% at 10 min. (30) (Figure 3). Concomitantly, the major detectable metabolite, [^{3}H]-phenylalanine, accumulated reaching about 50% at 10 min. The small radioactivity peaks were associated with different AVP fragments including [Cyt^6]-AVP-(3–9), AVP-(3–9) and AVP-(2–9). The hydrolytic pattern of AVP degradation suggested cleavage of the peptide bond from N-terminus and the formation of highly neuroactive fragment [pGlu^4 Cyt^6]-AVP-(4–9). Thus, it has been proposed that metabolism of circulating AVP after its transport across the BBB may not necessarily result in its functional inactivation. Since no changes in AVP BBB transport were observed in the presence of potent aminopeptidase inhibitor bestatin, it has been speculated that enzymatic proteolysis may take place at the abluminal side of the BBB, or in pericytes, and/or in brain parenchyma itself, as shown by local AVP microinjection studies (30).

Passive Diffusion

Passive diffusion has been proposed to explain blood-brain transport of α-melanotropin-stimulating hormone (MSH) (59) and TRH (42). In contrast to in vivo studies, the in vitro work with bovine brain microvascular endothelial layers suggested only passive diffusion for both AVP and DSIP in luminal to antiluminal direction (60). However, it is open to question whether specific BBB transport systems for peptides detected in vivo remain expressed under in vitro conditions. Studies on peptide transport across the blood-CSF barrier confirmed specific saturable mechanisms for DSIP (61) and Leu-Enk (62).

Peptide Lipidization and BBB Transport

Studies with cyclosporin A have provided an independent important conformation that high lipophilicity, i.e., its octanol/Ringer PC coefficient is close to 1,000, may not be

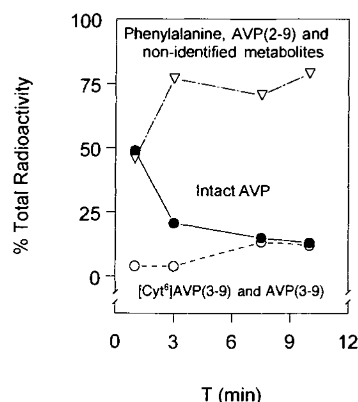

Fig. 3. Time course of degradation of 3 nM radiolabeled AVP determined in capillary-depleted brain following vascular brain perfusion in the guinea-pig. The hydrolytic pattern suggests formation of highly neuroactive fragment, [pGlu^4,Cyt^6]-AVP-(4–9). Modified from Ref. 30.

taken for granted as a predictor for high rate of BBB transport (43,63). Although the VBP studies demonstrated low to moderate permeability of free cyclosporin A (43), in the presence of blood, the extraction of cyclosporin at the BBB becomes almost insignificant due to avid binding of the peptide to blood components. It has been estimated that 10% of circulating cyclosporin is free, while 90% is bound to red blood cells, white cells and lipoproteins. It has been suggested that binding of cyclosporin A to lipoproteins may significantly retard its transport in comparison to free peptide, in spite of the fact that low density lipoproteins (LDL) exhibit low, but measurable transfer at the BBB. However, it has been also stressed that the holes formed in the BBB membrane that allow lipid-mediated transport are smaller than the molecular size of cyclosporin peptide, so that steric hindrances may impede its transport through the membrane (63). It has been also suggested that high lipophilicity may not be favorable for release of cyclosporin from the lipid phase of the BBB membranes into the water phase of brain extracellular fluid, that in turn may further impede the transport (43). Similar to cyclosporin, a highly lipophilic somatostatin analog (M.W. 1,019) with PC of 1.3, had barely detectable BBB uptake (63). Some studies, however, suggested that cyclization of small peptides (M.W. < 600) and formation of enzymatically stable diketopiperazines may result in significant BBB permeability, as shown with cyclo(Leu-Gly) in contrast to linear Leu-Gly dipeptide that exhibits minimal permeability (13). However, recent study with cyclo(His-Pro), a highly lipophilic dipeptide with PC coefficient of 0.81, has indicated very low BBB permeability (64) (Table III). Therefore, based on these studies we suggest that lipidization of peptides in general, may not be a strategy of choice to enhance peptide delivery across the BBB. Besides this, it has been noted that molecular modifications of the peptide backbone that are needed to increase the lipophilicity, are likely to result in substantial losses in peptide receptor binding affinity as indicated by in vitro assays, and possibly biologic activity.

Cationized Proteins

It has been well documented that cationization of proteins generally enhances cellular uptake. This strategy has been used to deliver cationized antibodies, i.e. IgG, as well as albumin across the BBB, at rates that were significantly higher than for native form of proteins (26) (Tables II and III). Importantly, cationization results in retention of 85–90% of antigen binding properties, and potential use of monoclonal antibodies as neuroimaging and neurodiagnostic agents in AD, brain tumors, stroke, brain injury and multiple sclerosis has been suggested. The proposed mechanism of BBB uptake is absorptive-mediated transcytosis, that exhibits typically saturation at higher concentrations in comparison to receptor-mediated transcytosis. The likelihood that IgG's with high pI may undergo preferential saturable transport across the BBB has been confirmed with homologous guinea-pig IgG (45).

Chimeric Peptides

According to this concept the nontransportable peptide can be delivered to the brain if attached to a peptide that normally undergoes the BBB transport. As shown with cationized albumin-D-[ala^2]β-endorphin, the cerebrovascular permeability to this chimeric peptide is almost identical to cationized albumin, while BBB transport of D-[ala^2]β-endorphin alone was barely detectable (29). Disulfide linkage strategy used to generate this chimeric peptide results in S-S linkage that is following the transport into brain parenchyma reduced by disulfate reductase releasing intact D-[ala^2]β-endorphin to reach the neuronal receptor targets. Alternative linker strategy uses avidin-biotin approach, since avidin due to its cationic nature is avidly taken up into brain capillaries in vitro, and across the BBB in vivo (65). Chimeric peptides may represent a valuable non-invasive approach to enhance delivery of peptides to the brain, and studies should be encouraged to evaluate the CNS effects of peptides transported across the BBB in a chimeric form.

Antibodies Against Peptide Receptors

Specific peptide receptors mediating transcytosis of insulin, IGF's and transferrin across the BBB have been characterized (reviewed in 21,51). It has been suggested that receptor-mediated transcytosis of large peptides and proteins involves receptor-mediated endocytosis at the luminal side, movement of the receptor-peptide complex across the endothelial cytoplasm, and receptor-mediated exocytosis at the abluminal side. Transferrin receptor was recently used to deliver anti-transferrin receptor OX26 antibodies from blood-to-brain (reviewed in 51). The BBB permeability to OX26 was higher than for transferrin itself, that exhibits in fact very low BBB permeability since its PS ptoduct has been measured in the presence of circulating plasma concentration of unlabeled transferrin of 25 μM that competes with the label for transport across the BBB (Table III). It has been also shown that nerve growth factor (NGF) conjugated to the OX26 antibody could be delivered to the brain, and its activity on growth of cholinergic cells has been documented in the fetal medial septal nucleus implanted into the anterior chamber of the eye (66). Vector-mediated delivery of monobiotynilated vasoactive intestinal peptide (VIP) analogue conjugated to avidin and OX26 resulted in an in vivo CNS effect of VIP, i.e., significant increase in cerebral blood flow, while non-conjugated biotynilated VIP analogue was incapable to produce such effect (67). The strategy to deliver specific monoclonal antibodies via insulin receptor deserves to be explored.

Glutathione

GSH participates in enzymatic elimination of hydrogen peroxide, organic peroxides, detoxification of foreign chemicals, maintenance of the thiol-disufide status of cells and storage and transfer of cysteine (9,10). It has been shown that inherited disorders in GSH metabolism cause severe neurologic defects, and that GSH may act as neuromodulator. GSH is present in astrocytes and endothelium in millimolar levels, but surprisingly little GSH is found in neurons. We recently obtained evidence that GSH transport is present at the BBB in rats and guinea-pigs, which is developmentally regulated (17–19). The HPLC analysis of brain parenchyma following 10 min arterial infusions of reduced [^{35}S]- GSH in the absence (Figure 4A) and [^{3}H]-GSH in the presence of the

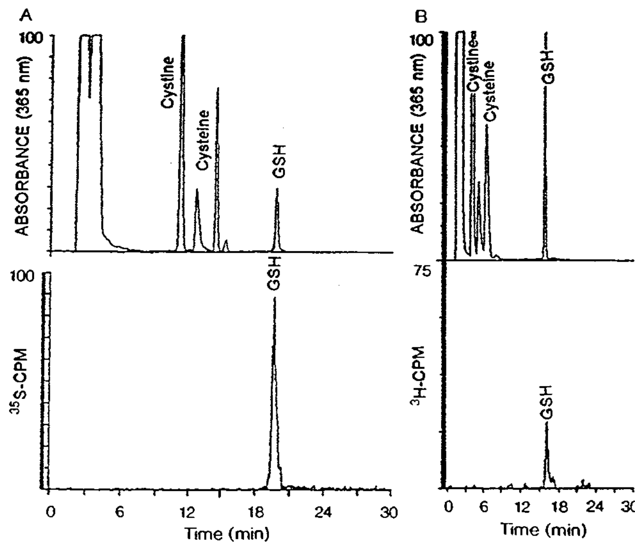

Fig. 4. The HPLC analysis of brain neocortical parenchymal tissue after 10 min of vascular brain perfusion with 40 nM [^{35}S]-cysteine-GSH under conditions of uninhibited GGT activity (A) and 40 nM [^{3}H]-glycine-GSH under conditions of inhibited GGT activity (B). A GGT inhibitor, serine borate, was infused 5 min before [^{3}H]-GSH and then continosly for 10 min wuth [^{3}H]-GSH. The difference in retention times in A and B is due to use of different columns. Modified from Ref. 19.

inhibitor of γ-glutamyl transpeptidase (GGT) activity (Figure 4B) indicated that in either case more than 96% of the radiolabel remains in its original form of GSH (19). Simultaneous VBP with two labels of GSH, i.e., [^{35} S-cysteine]-GSH and [^{3}H-glycine]-GSH resulted in no change in [^{35}S]/[^{3}H] ratio during passage of tracers from plasma into brain microvessels, and from there into capillary-depleted brain, providing an independent confirmation of transport of intact GSH (19). Considering abundance of GGT at the BBB, the explanation of this seemingly surprising result remains to be fully defined.

Preliminary work on characterization of the putative GSH transporter(s) established that rat and guinea-pig brain express transcript for the rat hepatic canalicular GSH transporter, RcGshT, which has been recently cloned (68). In addition using poly $(A)^+$ RNA from guinea-pig brain capillaries we have demonstrated expression of GSH transport in Xenopus laevis oocytes (22). On-going work with fractionation of brain capillary poly $(A)^+$ RNA is focused to determine size fractions that express GSH transport, and to determine whether in addition to RcGshT there is also a Na^+-dependent GSH transporter at the BBB, as indicated by VBP studies. Cloning of GSH transporters from brain capillaries should lead to better understanding of the regulation of GSH transport at the BBB and in brain, and may generate important information to develop therapeutic strategies to increase brain GSH levels in different types of cerebral oxidant injury.

Alzheimer's Amyloid-ß and Apolipoproteins

Amyloid beta (Aß), a 39–44 residue peptide, is the major constituent of amyloid fibrils deposited into senile plaques and cerebral blood vessels of patients with AD and related disorders (reviewed in 69). Aß is also identified as a soluble peptide (sAß) normally present in body fluids. The origin of Aß deposited in brain and cerebral blood vessels of patients with AD is uncertain, and whether or not sAß is the direct precursor of amyloid is not known. The current concept states that a key question in amyloidogenesis is to determine what factors are responsible for the conformational alteration of sAß into its fibrillar form. It has been shown that sAß specifically binds to apolipoprotein J (apo J) and different isoforms of apolipoprotein E (E3 and E4). The putative role of apo J and apo E in controlling levels of sAß in the extracellular and intracellular fluids of brain and other tissues, as well as their influence on Aß conformational state and fibril formation has been considered (69).

We have recently examined the BBB permeability and sequestration of a synthetic peptide homologous to major form of circulating Alzheimer's amyloid ß, $sAß_{1-40}$, in relation to peptide binding to apo E and apo J, using the VBP model (44,46). The BBB permeability and sequestration of both $sAß_{1-40}$ and apo J were significant due to the presence of specific transport mechanism(s). In contrast, apo E3 and apo E4 had minimal transport into the capillaries and across the barrier (Table I). Cerebrovascular permeability of $sAß_{1-40}$-apoJ complex was 3.8-fold higher than of $sAß_{1-40}$, while $sAß_{1-40}$-apo E3 and $sAß_{1-40}$-apo E4 exhibited 14.4 and 3-fold lower permeability in comparison to the peptide, respectively (70). A similar picture was obtained with the capillary sequestration; $sAß_{1-40}$ binding to apo J resulted in 3.2-fold enhancement, while its association with apo E3 and apo E4 diminished the capillary uptake by 4.9 and 9.6-fold (Figure 5). The quality and biologic activity of human recombinant apo E3, apo E4 and apo J, as well as of $sAß_{1-40}$, were tested by circular dichroism, solid-phase binding studies and competitive inhibition assays. Solid phase binding experiments have confirmed that the affinity of apo J to bind $sAß_{1-40}$ is 10 to 16-fold higher than of apo E3 and apo E4, respectively. In plasma, sAß is preferentially transported as a complex with apo J incorporated within high and very high density lipoproteins (71). As indicated by the in vitro binding data the apo E3 and apo E4 at physiological plasma concentrations cannot dissociate $sAß_{1-40}$-apo J complex. There-

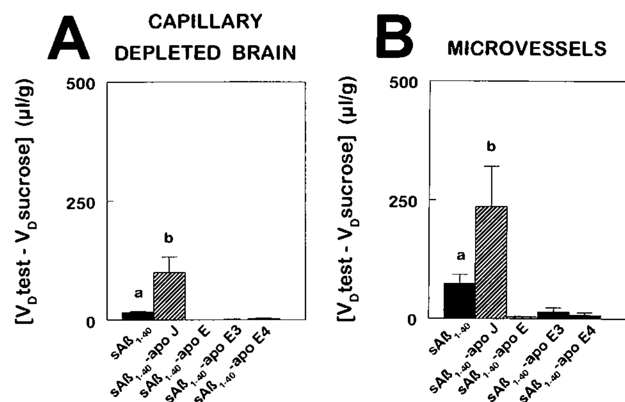

Fig. 5. Compartmental brain distribution of radioiodinated $sAß_{1-40}$ and $sAß_{1-40}$ complexed to apo J, apo E (as a mixture of isoforms with predominance in apo E3), apo E3 and apo E4, following 10 min of vascular brain perfusion in the guinea-pig. Modified from Refs. 44,46 and 70.

fore, in addition to negligible or low BBB transport and binding of the respective $sAß_{1-40}$-apo E3 and $sAß_{1-40}$-apo E4 complexes, the existence of these circulating complexes under normal conditions may be minimal and possibly of little physiologic significance (72). These studies suggest that capillary endothelium may play an important role in cerebrovascular and brain amyloidogenesis by regulating the capillary sequestration and transport of sAß free or complexed to apoJ, presumably through specific receptors at the luminal side of the BBB.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent studies from our and other laboratories have clearly indicated that if a given peptide/protein has a transport system at the luminal side of the BBB, and/or could be modified to use the existing transport mechanism, its levels in the brain and/or cerebral microvessels could be manipulated by arterial infusions and/or by intravenous administration. An exception from this rule are peptides that are extremely rapidly metabolized and inactivated by the BBB and/or in brain parenchyma, whose degradation rates exceed the transport rates. The enthusiasm for strategies such as peptide lipidization and/or enhanced passive diffusion due to increased lipophilicity, has been declined in recent years. New strategies based primarily on utilization of specific transport systems at the BBB have been designed, and several experimental peptide models have been developed. Work with Leu-Enk has demonstrated that chemical modifications of the N-terminal amino acid residue can influence significantly the carrier-mediated transport at the luminal side of the BBB, without affecting the biologic activity of the peptide in the CNS. The AVP model indicated that specific peptidergic receptors at the BBB may function to continuously deliver blood-borne peptide to the brain, while the post-BBB metabolism could be associated with formation of very potent neuroactive fragments. With GSH it has been shown that transport across the BBB allows more than 96% of the peptide to cross in the intact form, so the development of molecular strategies to up-regulate putative BBB GSH transporters would result in enhanced delivery and increased levels of GSH in brain. The use of chimeric peptides in which non-transportable peptide is chemically linked to a transportable peptide, i.e., to a cationized protein and/or peptide that exhibits a specific transport at the BBB, should be given serious considerations. An extension of this strategy represents the use of monoclonal antibodies against peptide receptors as vehicles for delivery of non-transportable peptides to the brain. Recently, we have demonstrated that binding of circulating peptide to transportable or non-transportable apolipoprotein from the circulation could be used as a strategy to modify its behavior in front of the BBB, as shown by $>$ 100-fold difference in the cerebrovascular permeability between sAβ-apoJ and sAβ-apoE complexes. The function of highly transportable apoJ as a carrier for sAβ across the BBB has been suggested, while non-transportable apoE3 and apoE4 could be utilized to abolish and/or reduce the peptide rate of entry. The strategy to reduce peptide entry from the circulation may also be important under certain pathologic settings, such as for example the Alzheimer's sAβ. Namely, it has been hypothesized that circulating sAβ may be implicated in vascular and/or parenchymal amyloidogenesis, so the desirable therapeutic strategy would be to prevent this accumulation.

To conclude, current work points out that peptide transport systems at the BBB are important therapeutic targets for peptide drug delivery to the CNS. They also could play a critical role in the development of brain pathologies such as cerebrovascular and brain amyloidogenesis in AD. A better understanding at the molecular level of the regulation of expression of putative BBB peptide transport systems may have a tremendous impact on the development of strategies to up-regulate specific transporters and enhance delivery of peptide neuropharmaceuticals, and/or to down-regulate transport of peptides with potential role in cerebral pathogenesis. In parallel, other ways that have already shown promising results should be explored in greater details, as for example delivery via monoclonal antibodies against insulin receptor, and regulation of peptide transport by apo J and apo E isoforms.

ACKNOWLEDGMENTS

This work was supported by NIH grants EY 09399, AG08051, NS31945, TRDRP 0071, and the Hoover Foundation.

REFERENCES

1. J.G. McComb and B. V. Zlokovic. Cerebrospinal fluid and the blood-brain interface. In W. R. Cheek, A. E. Marlin, D. G. McLone, D. H. Reigel, and M. L. Walker (eds), *Pediatric Neurosurgery,* W. B. Saunders Co., Philadelphia, 1994, pp. 167–185.
2. P. A. Cancilla, J. Bready, and J. Berliner. Brain endothelial-astrocyte interactions. In W. M. Pardridge (eds), *The Blood-Brain Barrier, Cellular and Molecular Biology,* Raven Press, New York, 1993, pp. 25–46.
3. I. M. Herman. Microvascular pericytes in development and disease. In W. M. Pardridge (eds), *The Blood-Brain Barrier, Cellular and Molecular Biology,* Raven Press, New York, 1993, pp. 127–136.
4. H. Davson, B. V. Zlokovic, L. Rakic, and M. B. Segal. *Introduction to the Blood-Brain Barrier,* Macmillan Press, London, 1993, pp. 146–293.
5. W. H. Oldendorf. Some relationships between addiction and drug delivery to the brain. In J. Frankenheim and R. M. Brown (eds), *Bioavailability of Drugs to the Brain and the Blood-Brain Barrier,* NIDA Research Monographs, Washington D.C., Government Publication, 1992, pp. 13–25.
6. W. M. Pardridge. Plasma protein-mediated transport of steroid and thyroid hormones, *Am. J. Physiol.* 251: E204–E208 (1987).
7. W. M. Pardridge and R. J. Boado. Molecular cloning and regulation of gene expression of blood-brain barrier glucose transporter. In W. M. Pardridge (eds), *The Blood-Brain Barrier, Cellular and Molecular Biology,* Raven Press, New York, 1993, pp. 395–440.
8. B. V. Zlokovic, J. B. Mackic, L. Wang, J. G. McComb, and A. A. McDonough. Differential expression of Na,K-ATPase α and β subunit isoforms at the blood-brain barrier and the choroid plexus, *J. Biol. Chem.* 268: 8019–8025 (1993).
9. M. B. Segal and B. V. Zlokovic. *Blood-Brain Barrier: Amino Acids and Peptides,* Kluwer Academic Publishers, Lancaster & Boston (1990), pp. 47–123.
10. W. M. Pardridge. Peptide Drug Delivery to the Brain, Raven Press, New York, 1991, pp. 23–52.
11. E. M. Cornford, L. D. Braun, P. D. Crane, and W. H. Oldendorf. Blood-brain barrier restriction of peptides and the low uptake of enkephalins, *Endocrinology* 103: 1297–1303 (1978).

12. W. H. Oldendorf. Blood-brain barrier permeability to peptides, pitfalls in measurements, *Peptides* 2: 109–111 (1981).
13. B. V. Zlokovic, D. J. Begley, and D. G. Chain. Blood-brain permeability to di-peptides and their constituent amino acids, *Brain Res.* 271: 66–71 (1983).
14. B. V. Zlokovic, M. B. Segal, D. J. Begley, H. Davson and L. Rakic. Permeability of the blood-cerebrospinal fluid and blood-brain barriers to thyrotropin releasing hormone, *Brain Res.* 358: 191–199 (1985).
15. B. V. Zlokovic, D. J. Begley, and D. G. Chain-Eliash. Blood-brain barrier permeability to leucine-enkephalin, D-alanine^2-D-leucine^5-enkephalin and their N-terminal amino acid (tyrosine), *Brain Res.* 336: 125–132 (1985).
16. A. Ermisch, H. J. Ruhle, R. Landgraf, and J. Hess. Blood-brain barrier and peptides, *J. Cereb. Blood Flow Metab.* 5: 350–358 (1985).
17. R. Kannan, J. F. Kuhlenkamp, E. Jeandidier, H. Trinh, M. Ookhtens, and N. Kaplowitz. Evidence for carrier-mediated transport of glutathione across the blood-brain barrier in the rat, *J. Clin. Invest.* 85: 2009–2113 (1990).
18. R. Kannan, J. F. Kuhlenkamp, M. Ookhtens, and N. Kaplowitz. Transport of glutathione at the blood-brain barrier of the rat: inhibition by glutathione analogs and age-dependence, *J. Pharmacol. Exp. Ther.* 263: 964–970 (1992).
19. B. V. Zlokovic, J. B. Mackic, J. G. McComb, M. H. Weiss, N. Kaplowitz, and R. Kannan. Evidence for transcapillary transport of reduced glutathione in vascular perfused guinea-pig brain, *Biochem. Biophys. Res. Commun.* 201: 402–408 (1994).
20. A. J. Lastin, C. Nissan, A. V. Schaly, and D. H. Coy. Blood-brain barrier, half-time disappearance and brain distribution for labeled enkephalin and a potent analog, *Brain Res. Bull.* 1: 583–589 (1976).
21. W. M. Pardridge. Recent advances in blood-brain transport, *Ann. Neurol. Pharmacol. Toxicol.* 28: 25–39 (1988).
22. R. Kannan, J-R. Yi, Y. Li, D. Tang, B. V. Zlokovic, and N. Kaplowitz. Expression of brain capillary GSH transport in Xenopus Laevis oocytes. *Soc. Neurosci. Abstr.* (1995, in press)
23. B. V. Zlokovic, D. J. Begley, B. M. Duricic, and D. M. Mitrovic. Measurement of solute transport across the blood-brain barrier in the perfused guinea-pig brain, method and application to N-methyl-α-aminoisobutyric acid, *J. Neurochem.* 46: 1444–1451 (1986).
24. B. V. Zlokovic. *In vivo* approaches for studying peptide interactions at the blood-brain barrier, *J. Control Release* 13: 185–202 (1990).
25. Y. Takasato, S. I. Rapoport, and Q. R. Smith. An *in situ* brain perfusion technique to study cerebrovascular transport in the rat, *Am. J. Physiol.* 247: H484–H493 (1984).
26. D. Triguero, J. B. Buciak, W. M. Pardridge. Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins, *J. Neurochem.* 54: 1882–1888 (1990).
27. B. V. Zlokovic, J. G. McComb, L. Perlmutter, M. H. Weiss, and H. Davson. Neuroactive peptides and amino acids at the blood-brain barrier: possible implications to drug abuse. In J. Frankenheim and R. Brown (eds), *Bioavailability of Drugs to the Brain and the Blood-Brain Barrier,* NIDA Research Monographs, Washington D.C., Government Publication, 1992, pp. 26–42.
28. A. Sam II, U. Bickel, U. Stroth, and W. M. Pardridge. Blood-brain barrier transport of neuropeptides: analysis with metabolically stable dermorphin analogue, *Am. J. Physiol.* 267: E124–E131 (1994).
29. W. M. Pardridge, D. Triguero, and J. L. Buciak. β-endorphin chimeric peptides: transport through the blood-brain barrier in vivo and cleavage of disulfide linkage by brain, *Endocrinology* 126: 977–984 (1990).
30. B. V. Zlokovic, W. A. Banks, H. ElKadi, J. Erchegyu, J. B. Mackic, J. G. McComb, and A. Kastin. Transport, uptake, and metabolism of blood-borne vasopressin by the blood-brain barrier, *Brain Res.* 590: 213–218 (1992).
31. K. R. Duffy, and W. M. Pardridge. Blood-brain barrier transcytosis of insulin in developing rabbits, *Brain Res.* 420: 32–38 (1987).
32. R. D. Broadwell, B. J. Balin, and M. Salcman. Transcytotic pathway for blood-borne protein through the blood-brain barrier, *Proc. Natl Acad. Sci.* 85: 632–636 (1988).
33. B. V. Zlokovic, M. N. Lipovac, D. J. Begley, H. Davson, and L. M. Rakic. Transport of leucine-enkephalin across the blood-brain barrier in the perfused guinea-pig brain, *J. Neurochem.* 49: 310–315 (1987).
34. B. V. Zlokovic, J. B. Mackic, B. Djuricic, and H. Davson. Kinetic analysis of leucine-enkephalin cellular uptake at the luminal side of the blood-brain barrier of an *in situ* perfused guinea-pig brain, *J. Neurochem* 53: 1333–1340 (1989).
35. A. J. Kastin, R. D. Olson, A. V. Schally, and D. H. Coy. CNS effects of peripherally administered brain peptides, *Life Sci.* 25: 401–414 (1979).
36. I. Bueno and J. P. Ferre. Central regulation of intestinal motility by somatostatin and cholecystokinin octapeptide, *Science* 216: 1427–1429 (1982).
37. W. A. Banks and A. J. Kastin. Aluminum alters the permeability of the blood-brain barrier to some non-peptides, *Neuropharmacology* 24: 407–412 (1985).
38. J. E. Zadina, W. A. Banks, and A. J. Kastin. Central nervous system effects of peptides. 1980–1985, a cross-listing of peptides and their central actions from the first six years of the journal *Peptides, Peptides* 7: 497–537 (1986).
39. R. G. Clark, P. M. Jones, and I. C. A. F. Robinson. Clearance of vasopressin from cerebrospinal fluid to blood in chronically cannulated Brattleboro rats, *Neuroendocrinology* 37: 242–247 (1983).
40. E. Passaro, H. Debas, W. Oldendorf, and T. Yamada. Rapid appearance of intraventricularly administered neuropeptides in the peripheral circulation, *Brain Res.* 241: 335–340 (1982).
41. B. V. Zlokovic, V. Susic, H. Davson, D. J. Begley, R. M. Jankov, D. M. Mitrovic, and M. N. Lipovac. Saturable mechanisms for delta sleep inducing peptide uptake at the blood-brain barrier of vascularly perfused guinea-pig brain, *Peptides* 10: 249–259 (1989).
42. B. V. Zlokovic, M. N. Lipovac, D. J. Begley, H. Davson and L. M. Rakic. Slow penetration of thyrotropin-releasing hormone across the blood-brain barrier of an *in situ* perfused guinea-pig brain, *J. Neurochem.* 51: 252–257 (1988).
43. D. J. Begley, L. K. Squires, B. V. Zlokovic, D. M. Mitrovic, C. W. Hughes, P. A. Revest, and J. Greenwood. Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin, *J. Neurochem.* 55: 1222–1230 (1990).
44. B. V. Zlokovic, J. Ghiso, J. B. Mackic, J. G. McComb, M. H. Weiss, and B. Frangione. Blood-brain barrier transport of circulating Alzheimer's amyloid β, *Biochem. Biophys. Res. Commun.* 197: 1034–1040 (1993).
45. B. V. Zlokovic, D. S. Skundric, M. B. Segal, M. N. Lipovac, J. B. Mackic, and H. Davson. A saturable mechanism for transport of immunoglobulin G across the blood-brain barrier of the guinea-pig, *Exp. Neurol* 107: 263–270 (1990).
46. B. V. Zlokovic, C. L. Martel, J. B. Mackic, E. Matsubara, T. Wisniewski, J. G. McComb, B. Frangione, and J. Ghiso. Brain uptake of circulating apolipoproteins J and E complexed to Alzheimer's amyloid β, *Biochem. Biophys. Res. Commun.* 205: 1431–1437 (1994).
47. J. F. Poduslo, G. L. Curran, and C. Berg. Macromolecular permeability across the blood-nerve and blood-brain barriers, *Proc. Natl. Acad. Sci.* USA 91: 5705–5709 (1994).
48. W. A. Banks, A. J. Horvath, and E. A. Michals. Carrier-mediated transport of vasopressin across the blood-brain barrier of the mouse, *J. Neurosci. Res.* 18: 326–332 (1987).
49. B. V. Zlokovic, S. Hyman, J. G. McComb, M. N. Lipovac, G. Tang, and H. Davson. Kinetics of vasopressin-arginine uptake at the blood-brain barrier, *Biochim. Biophys. Acta* 1025: 191–198 (1990).
50. W. A. Banks, A. J. Kastin, and C. M. Barrera. Delivering peptides to the central nervous system: dilemmas and strategies, *Pharmac. Res.* 8: 1345–1350 (1991).
51. P. M. Friden. Receptor-mediated transport of therapeutics across the blood-brain barrier, *Neurosurgery* 35: 294–298 (1994).
52. W. M. Pardridge and L. J. Mietus. Enkephalin and blood-brain

barrier: studies of binding and degradation in isolated brain microvessels, *Endocrinology* 109: 1138–1143 (1981).
53. S. I. Rapoport, W. A. Klee, K. D. Pettigrew, and K. Ohno. Entry of opioid peptides into the central nervous system, *Science* 207: 84–86 (1980).
54. N. Tsuzuki, T. Hama, T. Hibi, R. Konishi, S. Futaki, and K. Kitagawa. Adamantane as a brain-directed drug carrier for poorly absorbed drug: anti-nociceptive effects of [D-Ala^2] leu-enkephalin derivatives conjugated with I-adamantane moiety, *Biochem. Pharmacol.* 41: R5–R8 (1991).
55. W. A. Banks, A. J. Kastin, H. M. Sam, V. T. Cao, B. King, L. M. Maness, and A. V. Schally. Saturable efflux of the peptides RC-160 and Tyr-MIF-1 by different parts of the blood-brain barrier, *Brain Res. Bull.* 35: 179–182 (1994).
56. B. V. Zlokovic, M. B. Segal, J. G. McComb, S. Hyman, M. H. Weiss, and H. Davson. Kinetics of circulating vasopressin uptake by the choroid plexus, *Am. J. Physiol.* (Renal Fluid Electrolyte Physiol.) 260: F216–F224 (1991).
57. B. V. Zlokovic, S. Hyman, J. G. McComb, G. Tang, A. R. Rezai, and M. H. Weiss. Kinetics of vasopressin-arginine uptake by the hypothalamo-pituitary axis and pineal glands in guinea-pigs, *Am. J. Physiol. (Endocrinol. Metab.)* 260: E633–E640 (1991).
58. M. N. Lipovac, E. Barron, L. Perlmutter, M. H. Weiss, J. G. McComb, and B. V. Zlokovic. Chronic nicotine treatment increases blood-brain transport and capillary sequestration of vasopressin in nicotine-treated guinea-pigs, *Soc. Neurosci. Abstr.* 18: 1492 (1992).
59. J. F. Wilson. Low permeability of the blood-brain barrier to nanomolar concentrations of immunoreactive alpha-melanotropin, *Psychopharmacology* 96: 262–266 (1988).
60. K. L. Audus, P. J. Chickhale, D. W. Miller, S. E. Thompson, and R. T. Borchardt. Brain uptake of drugs: the influence of chemical and biological factors, *Adv. Drug Res.* 23: 1–64 (1992).
61. B. V. Zlokovic, M. B. Segal, H. Davson, and R. M. Jankov. Passage of delta sleep-inducing peptide (DSIP) across the blood-cerebrospinal fluid barrier, *Peptides* 9: 533–538 (1988).
62. B. V. Zlokovic, M. B. Segal, H. Davson, and D. M. Mitrovic. Unidirectional uptake of enkephalins at the blood-tissue interface of the blood-cerebrospinal fluid barrier: a saturable mechanism, *Regul. Peptides* 20: 33–44 (1988).
63. W. M. Pardridge, D. Triguero, J. Yang, and P. A. Cancilla. Comparison of *in vitro* and *in vivo* models of drug transcytosis through the blood-brain barrier, *J. Pharmacol. Exp. Ther.* 253: 884–891 (1990).
64. W. A. Banks, A. J. Kastin, V. Akerstrom, and J. B. Jaspan. Radioactively iodinated cyclo(His-Pro) crosses the blood-brain barrier and reverses ethanol-induced narcosis, *Am. J. Physiol. (Endocrinol. Metab.)* 264: E723–E729 (1993).
65. Y.-S. Kang and W. M. Pardridge. Use of neutral-avidin improves pharmacokinetics and brain delivery of biotin bound to an avidin-monoclonal antibody conjugate, *J. Pharmacol. Exp. Ther.* 269: 344–350 (1994).
66. P. M. Friden, L. R. Walus, P. Watson, S. R. Doctrow, J. W. Kozarich, C. Backman, H. Bergman, B. Hoffer, F. Bloom, and A.-C. Granholm. Blood-brain barrier penetration and *in vivo* activity of an NGF conjugate, *Science* 259: 373–377 (1993).
67. U. Bickel, T. Yoshikawa, E. M. Landaw, K. F. Faull, and W. M. Pardridge. Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery, *Proc. Natl. Acad. Sci.* 90: 2618–2622 (1993).
68. J.-R. Yi, S. Lu, J. Fernandez-Checa, and N. Kaplowitz. Expression cloning of a rat hepatic reduced glutathione transporter with canalicular characteristics, *J. Clin. Invest.* 93: 1841–1845 (1994).
69. J. Ghiso, T. Wisniewski, and B. Frangione. Unifying features of systemic and cerebral amyloidosis. *Mol. Neurobiol.* 8: 49–64 (1994).
70. B. V. Zlokovic, C. L. Martel, J. B. Mackic, E. Matsubara, T. Wisniewski, J. G. McComb, B. Frangione, and J. Ghiso. Blood-brain barrier transport and cerebral capillary sequestration of Alzheimer's amyloid ß: modifications by apolipoproteins J, E3 and E4, *Cereb. Vasc. Biol. Conference,* Paris, 10–12 July (1995).
71. A. Koudinov, E. Matsubara, B. Frangione, and J. Ghiso. The soluble form of Alzheimer's amyloid beta protein is complexed to high density lipoprotein 3 and very high density lipoprotein in normal human plasma, *Biochem. Biophys. Res. Commun.* 205: 1164–1171 (1994).
72. E. Matsubara, B. Frangione, and J. Ghiso. Characterization of apolipoprotein J-Alzheimer's amyloid beta interaction, *J. Biol. Chem.* 270: 7563–7567 (1995).