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The Blood-brain Barrier: Principles for Targeting Peptides and Drugs to the Central Nervous System

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Abstract

The presence of the blood-brain barrier (BBB), reduces the brain uptake of many drugs, peptides and other solutes from blood. Strategies for increasing the uptake of drugs and peptide-based drugs include; structural modifications to increase plasma half-life; improving passive penetration of the BBB by increasing the lipophilicity of the molecule; designing drugs which react with transporters present in the BBB; and reducing turnover and efflux from the central nervous system (CNS).

Introduction

The blood-brain barrier (BBB) is a vital element in the regulation of the constancy of the internal environment of the brain. The composition of the extracellular fluid of the brain is controlled within very precise limits, largely independently of the composition of the circulating blood, to provide a stable environment in which the integrative neuronal functions of the brain can optimally take place. The blood-brain barrier is formed at the level of the endothelial cells of the cerebral capillaries. These cells are characterised by having tight continuous circumferential junctions between the cells of the capillaries thus abolishing any aqueous paracellular pathways between the cells (Brightman 1992). The endothelium is thus characterised by exhibiting a high transendothelial electrical resistance in the region of 1500–2000 Ω cm^2 (Butt et al 1990). The presence of the tight junctions and the lack of aqueous pathways between cells greatly restricts the movement of polar solutes across the cerebral endothelium.

Some regions within the central nervous system (CNS) lack a BBB and the capillaries are fenestrated allowing the free movement of solutes between the blood and the surrounding interstitial fluid. These areas are collectively termed the circumventricular organs (CVOs) and comprise the choroid plexus, the median eminence, the neurohypophysis, the pineal gland, the organum vasculosum of the lamina terminalis, the subfornical organ, the subcommisural organ and the area postrema. Some of these structures, the median eminence, the neurohypophysis and the pineal are neurohaemal organs specialised for the release of neuroendocrine secretion into the bloodstream. The other areas may be regarded as windows of the brain where a limited number of neurones within the immediate vicinity of the circumventricular organ have an unrestricted access to blood solutes. This access enables the brain to monitor closely the composition of the blood and to react accordingly. The ependymal cells surrounding the circumventricular organs have what appear to be tight junctions between them presumably to enclose a volume of brain extracellular fluid (ECF) surrounding the CVO to prevent diffusion of interstitial solutes away from the region of the circumventricular organ (Brightman 1992). The relative surface area of the permeable fenestrated capillaries of the circumventricular organs compared to the tight BBB capillaries is 1 : 5000. Given these considerations there is little possibility of these high permeability areas being able to influence the composition of the bulk of the brain extracellular fluid and the CVOs do not form a realistic route for drug entry into the brain.

The presence of the BBB is easily demonstrated by studying the distribution of the inert hydrophilic marker inulin (Begley 1992a, 1994) (Table 1). The percentage distribution spaces in brain regions with a BBB correspond to the plasma volume of the brain area. In the choroid plexus and the anterior and posterior pituitary where the capillaries are fenestrated the inulin space corresponds to the extracellular space of the tissue.

Because of the presence of the BBB a number of specific transport mechanisms are required to be present in the cerebral endothelial cells to ensure that the brain receives an adequate supply of nutrients. These are illustrated and described in Fig. 1.

Passive diffusion may occur across the endothelium, either through the cells themselves or through the tight junctions between cells. Movement across the BBB by passive mechanisms will be determined by considerations such as molecular weight and lipophilicity as discussed later. The entry of glucose into the brain is via a facilitated carrier GLUT-1 present in the endothelial cells. GLUT-1 is insulin insensitive and always shows a relatively high level of expression. It will however upregulate in chronic hypoglycaemia and downregulate in chronic hyperglycaemia.

Amino acids are transported across the BBB on a variety of transporters. Large neutral amino acids, such as tyrosine, phenylalanine, leucine, isoleucine, valine, histidine and methionine are transported into the brain by an energy-dependent transporter termed system-L which is present on the luminal and abluminal membranes and is principally directed from blood to endothelial cell and from endothelial cell into brain. System-ASC which transports as model substrates alanine, serine and cystine, plus a number of neutral amino acids such as threonine and asparagine is also expressed to a lesser extent and with the same directional properties as system-L. There is an obvious

Table 1. Percent inulin spaces in various regions of the guinea-pig brain determined 10 min after intravenous bolus injection.

Brain region	Inulin space (%)
Whole brain	1·77 ± 0·14
Olfactory bulb	2·30 ± 0·37
Hippocampus	1·45 ± 0·22
Caudate nucleus	1·26 ± 0·18
Parietal cortex	1·50 ± 0·18
Hypothalamus	2·45 ± 0·30
Choroid plexus	20·80 ± 1·10
Pituitary (anterior & posterior)	26·70 ± 2·90

The differences between brain area possessing a blood-brain barrier and areas where the capillaries are fenestrated may be illustrated by injecting experimental animals with radiolabelled inulin (5000 Da). Where there is a blood-brain barrier the inulin occupies the plasma space of the tissue which is between 2.45 and 1.26% depending on the region. Where the capillaries are fenestrated the inulin occupies the plasma and the extracellular space of the tissue giving spaces of approximately 25%. The inulin spaces were determined after an intravenous bolus injection of 50mCi [^{3}H]inulin in anaesthetised guinea-pigs. After 10 min, blood samples were taken by cardiac puncture and the animals were decapitated. Brain samples were then dissected out and the inulin space calculated as: inulin space = $C_{plasma}/C_{brain} \times 100$. Mean ± s.e.m.

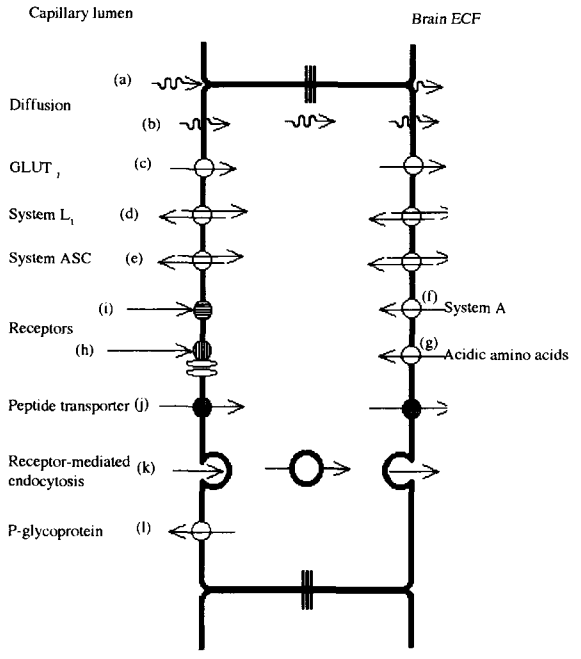


FIG. 1. Interactions of solutes with the blood-brain barrier. Solutes both in blood and in the brain extracellular fluid may interact with the BBB in a variety of ways. Solute which traverse the BBB by diffusion may either diffuse to a limited extent through the zona occludens (a) or directly across the endothelium (b). Polar solutes, for example glucose, employ a facilitated carrier Glut-1 (c), in the cell membrane and neutral amino acids employ Systems-L and ASC (d and e). Glut-1, system-L and System ASC are represented in the same orientation in both the luminal and the abluminal membranes of the cerebral capillary endothelial cells. System-A (f) transports glycine and a system for the acidic amino acids (g) transports glutamic acid and aspartic acid from the brain extracellular fluid into the cerebral endothelial cells. There are also receptors for blood-borne solutes on the luminal surface of the endothelium (i and h). These receptors may be linked to intracellular messengers or control the activity of other channels in the luminal membrane and thus alter the activity of the endothelial cells. Specific peptide transporters have been demonstrated in the luminal endothelial membrane (j) transporting peptides into the endothelium. If these peptides are to reach the brain extracellular fluid by this route there must be equivalent transporters in the abluminal membrane. Larger peptides and proteins will be internalized by an endocytic mechanism (k). This may be receptor-mediated or non-specific in nature. The level of endocytic activity in the cerebral capillary endothelium is much lower than in other tissues endothelia and transcytosis remains a controversial topic. Multidrug resistance protein or P-glycoprotein is expressed constitutively in the luminal membrane of the cerebral endothelial cells (l) and transports a variety of structurally unrelated substrates out of the endothelium. Many of these are lipophilic and potentially neurotoxic and would otherwise enter the brain to a greater extent.

overlap in the substrates acceptable to system-L and ASC. System-A, transporting principally glycine and proline is present on the abluminal membrane of the endothelial cells and is directed out of the brain (Betz & Goldstein 1978). A transporter for the dicarboxylic amino acids, glutamic and aspartic acid, is also present in the BBB and is directed out of the brain. Both system-A and system-ASC are sodium- as well as energy-dependent. A number of specific receptors for solutes also exists both on the luminal and abluminal surfaces of the endothelial cells. These receptors may be linked to second messengers such as cAMP or may modulate the activity of channels or transporters in the BBB. There may also be transporters in the cell membrane for solutes such as small peptides.

The level of endocytic activity in the BBB, compared with other endothelia, is minimal. However transcytosis for certain macromolecules may occur and form a low capacity transport mechanism for these solutes. Receptor-mediated endocytosis may be solute-specific and inducible by that solute.

The BBB also has a physiological and biochemical dimension in that the endothelial cells have a high density of mitochondria compared with other endothelia presumably reflecting a high level of oxidative ATP production. In addition the BBB is the site of a high level of enzyme activity directed towards the inactivation of centrally active blood-borne solutes and toxins (Audus et al 1992; Grieg 1992). The high enzyme activities present in or on the cerebral endothelial cells include monoamine oxidase (MAO) types A and B, L-amino acid decarboxylase (AAD), catechol-*O*-methyl transferase (COMT), butyryl-cholinesterase (BChE) and 4-aminobutyrate aminotransferase. Levels of gamma-glutamyl transpeptidase activity are high in cerebral endothelial cells and are thought to be related to amino acid transport phenomena. Also membrane-bound epoxidehydrolase (mEH), UDP-glucuronosyl-transferase (UGT), benzoxyresorufin-*O*-de-ethylase, NADPH cytochrome P450 reductase and glutathione-*S*-transferase activities are high in brain microvessel preparations and in the choroid plexus. These enzymes are thought to play a part in the metabolism of drugs and xenobiotic compounds.

The intrinsic membrane protein P-glycoprotein sometimes referred to as multidrug resistance protein (mdr-protein) is also present in the luminal plasma membrane of the endothelial cells constituting blood-brain barrier. This is an efflux pump which appears to be a constituent part of the BBB and transports a wide range of structurally unrelated substances out of cells. Pgp is an ATP-dependent pump and is a member of a family of intrinsic membrane proteins which are normally expressed at the BBB, the intestine, the liver and the kidney (Cordon-Cardo et al

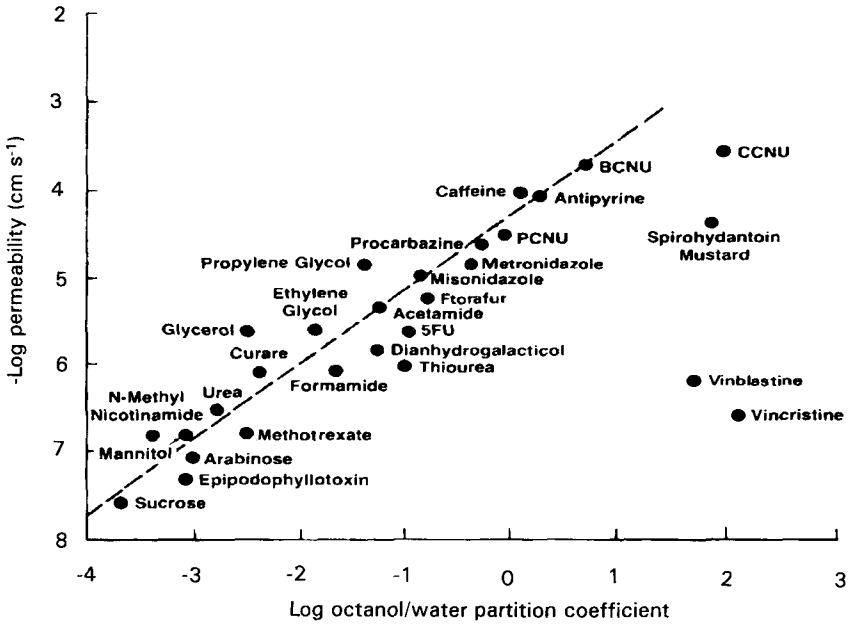


FIG. 2. The relationship of the octanol/water partition coefficient to blood-brain barrier permeability for some selected drugs. Substances falling on the dotted line would have a direct relationship between their brain uptake and their lipid solubility. Note that there is a wide scatter of points around the line. Vinblastine and vincristine are known substrates for the efflux pump P-glycoprotein expressed at the blood brain barrier and this limits their penetration into brain. Other substances which also lie well away from the line but are not shown on the plot are those which have carrier-mediated uptake mechanisms such as glucose and amino acids, which are fairly polar with partition coefficients between 1×10^{-2} and 1×10^{-4} but with high BBB permeabilities.

BCNU = 1.3-bis-chloro(2-chloroethyl)-1-nitrosourea; CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; PCNU = 1-(2-chloroethyl)3-(2,6,-dioxo-3-piperidyl)-nitrosourea; 5FU = 5-fluorouracil. Adapted from Greig (1992).

1989; Ruez & Gros 1994). The role of Pgp therefore appears to be a protective one transporting a range of potentially toxic substances out of the body. The expression of Pgp occurs in many tissues including malignant tissue after exposure to cytotoxic drugs where it confers resistance to a wide range of cytotoxic agents. Hence its alternative name multidrug resistance protein. In this situation its acts to maintain the intracellular levels of cytotoxic drugs below a toxic level and thus frustrates repeated cancer chemotherapy. Its constitutive role in the normal BBB is inferred to be a protective one reducing the entry of lipophilic and neurotoxic substances into the CNS.

Improving passive permeation of the BBB

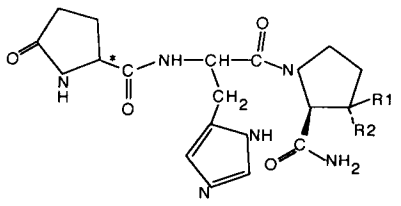
For many drugs that enter the brain by passive diffusion their brain-uptake and lipid solubility are well correlated (Fig. 2). Lipid solubility is most often determined as a partition coefficient between buffer and octanol and is quoted as the logarithm of the partition, $\log P_{oct}$. Many small solutes entering the brain do so by dissolving in the lipid of the cell membrane. In-vitro studies using cultured cerebral endothelial cells have suggested that in this system the relationship between lipid solubility and brain uptake might be a sigmoidal one (Oldendorf 1974; Van Bree et al 1988). With substances having a logP between −2.00 and 0 (partition coefficients of 0·01 and 1·00) lying on the more linear portion of the curve. Very hydrophobic substances show little further increase in brain uptake and hydrophilic substances which are polar and highly ionized at physiological pH also have a limited brain uptake.

It has been suggested by Levin (1980) that large lipid-soluble substances with a molecular weight above 500 Da become physically impeded from crossing the cell membrane and thus exhibit a molecular weight cut-off. This has been used to explain the low brain-uptake of substances such as vinblastine, vincristine and cyclosporin, which lie well off the regression line suggested in Fig. 2. However it is now well-established that these cytotoxic agents are substrates of P-glycoprotein (Tsuji et al 1992, 1993) which is actively extruding them from the cerebral endothelial cells. Surprisingly an increase in molecular volume may actually enhance brain uptake (Abraham et al 1994).

It has been suggested that delta logP, defined as the $\log P_{octanol/buffer}$ minus the $\log P_{cyclohexane/buffer}$ is a better predictor of brain uptake (Young et al 1988). Delta logP reflects the hydrogen-bonding capacity of a substance and this property in turn influences brain uptake. In general in a series of related substances those with a lower-hydrogen bonding potential enter the brain the most readily. The hydrogen-bonding potential determines the activation energy necessary to pluck a molecule out of the aqueous phase and into the lipid phase of a membrane. Various physical factors influencing brain uptake have been incorporated into a predictive equation for brain uptake (Abraham et al 1994).

To maximize the brain uptake and the bioavailability of a substance to the brain a number of molecular manipulations may be carried out to alter the properties of a substance; these may be summarized as:

1. Increasing the plasma stability and hence plasma half life.
2. Improving lipid solubility.



(a) pyroglutamyl-histidyl-prolinamide (TRH)
R1 = H, R2 =H

(b) pyroglutamyl-histidyl-3 methylprolinamide (Pyr-His-Mep-NH_2, RX 74355)
R1 = H, R2 = CH_3

(c) pyroglutamyl-histidyl-3,3 dimethylprolinamide (Pyr-His-Dmp-NH_2, RX 77368)
R1 = CH_3, R2 = CH_3

FIG. 3. Structure of TRH and related analogues RX 74355 and RX 77368. The substitution of the methyl groups into the proline residue increases the plasma half-lives and the central effectiveness of the molecules.

3. Enhancing or maintaining reactivity with existing BBB transport mechanisms.
4. Retaining central nervous activity.
5. Increasing the stability in brain extracellular fluid and reducing reactivity with efflux transport mechanisms in the CNS.

A number of chemical modifications can be carried out to improve the CNS activity of drugs. For example the peptide thyrotropin releasing hormone (TRH—pyroglutamyl-histidyl-prolinamide) has a short plasma half life due to circulating peptidases. To extend the half-life, methyl groups may be added to the prolinamide residue to produce a 3-methylprolinamide (RX74355) and a 3,3-dimethylprolinamide (RX77368) (Brewster et al 1981, 1983) (Fig. 3). These methyl substitutions increase the lipid solubility of the molecule and also the molecular volume. The substitutions also increase the first order half-lives in human plasma from 33 (TRH) to 210 (RX74355) and 1080 min (RX77368) (Table 2). The central nervous activity of the TRH analogues is also enhanced (Brewster et al 1981; Brewster 1983), possibly as the result of increased brain penetration although an enhanced central potency should also be considered.

Other simple modifications to peptides that may be made are to amidate the C-terminus and acetylate the N-terminus both of which confer an increased resistance to exopeptidases. In addition acetylation of the N-terminus also increases lipid solubility significantly.

An excellent example of increasing brain uptake by enhancing lipid solubility and reducing hydrogen bonding capacity is the chemical conversion of morphine to heroin (diacetyl-morphine). Substitution of the two hydroxyl groups of morphine by acetyl groups in heroin increases the brain uptake over twenty-fivefold (Oldendorf 1974). As a general rule for each pair of hydrogen bonds removed from a molecule there is a log order increase in BBB permeabilty. Once within the brain, heroin is rapidly converted to monoacetyl morphine and more slowly to morphine. Thus the rapid brain entry of heroin in comparison with morphine makes it a favoured drug of abuse and presumably enhances its addictive potential.

In the case of many peptides the introduction of D-isomers of the naturally occurring amino acids in to the peptide sequence can greatly enhance the plasma half life of the peptide. Again the reactivity with plasma peptidases is greatly reduced. For example octreotide (D-Phe-Cys-Phe-D-Tyr-Lys-Thr-Cys-Thr-OH : sandostatin : SMS 201-995) is an octapeptide analogue of somatostatin Ala-Gly Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH). The attenuated peptide with the D substitutions, D-Phe at position two of somatostatin and D-Trp at position five, extends the first order half-life of somatostatin from 2-3 min in human plasma to 113 min (Lamberts 1987). The substitution of D-Phe at position four also increases the growth hormone inhibiting potency of sandostatin compared with somatostatin by some 45 times. D-Amino acid substitutions also have marked effects on the plasma half-lives of encephalin analogues. Examples are two analogues of leucine encephalin (Tyr-Gly-Gly-Phe-Leu), namely DADLE

Table 2. First-order half-lives (min) of TRH, RX74355, and RX77368. There are significant species differences in the rates at which plasma and a brain homogenate will break down TRH and its analogues.

	TRH	RX74355 (Methylproline)	RX77368 (Dimethylproline)
Rat			
Plasma	22	114	390
Brain homogenate	9	54	190
Dog			
Plasma	>1500	>1500	>1500
Brain homogenate	11	90	174
Man			
Plasma	33	210	1080
Brain homogenate	18	66	168
Mouse			
Plasma	215	>1500	>1500
Brain homogenate	12	28	150

From Brewster (1983).

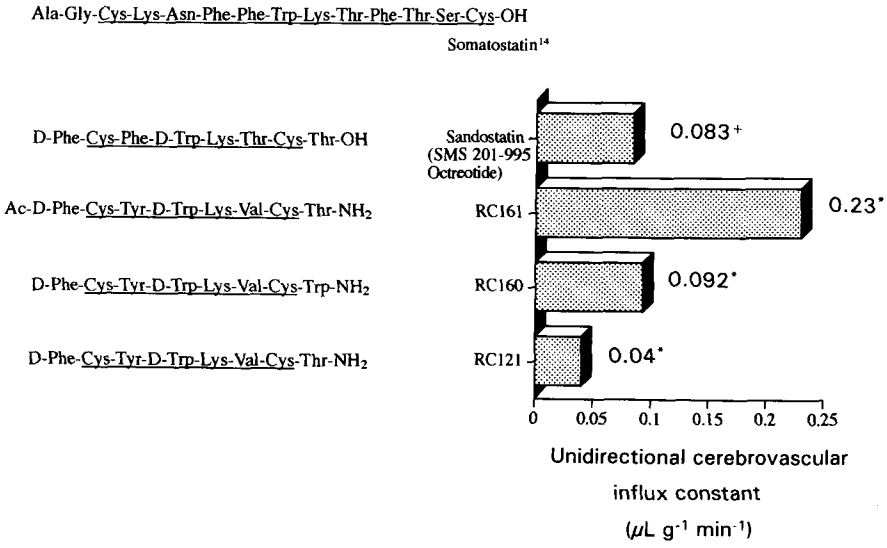


FIG. 4. Unidirectional cerebrovascular permeability constants (K_{in}) of some somatostatin analogues. The brain uptakes are determined by intravenous bolus injection techniques. The structure of the analogues is given with that of somatostatin for comparison. Unfortunately the brain uptake of somatostatin cannot be determined by a comparable method as it is very unstable in plasma. Data from Begley et al (1992b) and Banks et al (1990).

(D-Ala^2, D-Leu^5-encephalin) and dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg) both of which have greatly extended half-lives in plasma compared with the parent peptide.

A number of somatostatin analogues exist where the plasma half-life is extended and the lipid solubility is altered thus modifying two factors which influence brain uptake, plasma half life and biological potency of the molecules. Intravenous bolus injection studies have been carried out to measure the brain uptake of these peptides, (Banks et al 1990; Begley 1992b, 1994). These somatostatin analogues are amphiphilic and their brain uptakes are non-saturable suggesting that their penetration into the brain will be passive. However changes in the molecular structure of the analogues produce significant changes in brain uptake. Fig. 4 illustrates the brain uptakes of four octapeptide analogues of somatostatin, determined by an intravenous bolus injection technique (Banks et al 1990; Begley 1992b, 1994) which have D-Phe and D-Trp substitutions in the molecule. Compared with RC 121 the substitution of a Trp residue at position 8 compared with RC 160, thus introducing an additional aromatic side chain into the molecule, approximately doubles the cerebrovascular permeability constant. Acetylating the N-terminus of RC 121 as in RC 161, and therefore increasing the lipid solubility, increases the permeability constant almost sixfold. The peptide RC 160 has marked and prolonged analgesic actions after intravenous administration (Eschalier et al 1990).

Using synthetic peptide sequences which have hydrophobic properties may provide a mechanism for inserting a peptide into the cell membrane (Begley 1994). For example a number of naturally-occurring peptides have hydrophobic regions which demonstrate a natural affinity for cell membranes. Mellitin, a component of bee venom (Kaiser & Kezdy 1987), is a 26-amino acid peptide (Fig. 5). The N-terminal amino acids 1–20 contain two α-helical regions 1–10 and 13–20 which are hydrophobic and insert the molecule into the cell membrane, the lysine residues 21–26 are highly cationic and are thought to open up pores thus permeabilizing the membrane (Kaiser & Kezdy 1987).

Signal peptides are similar hydrophobic regions in a pre-pro-peptide or protein which enable the entire molecule to be inserted through the unit membrane of the endoplasmic reticulum. The signal amino acid sequence is not conserved between pre-pro-proteins and thus the common feature is the hydrophobicity (lipohilicity) of the region (Engelman & Steitz 1981; Emr & Silhavey 1983; Begley 1994). The

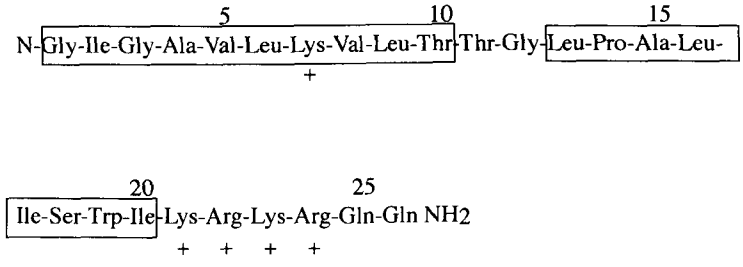


FIG. 5. Amino acid sequence of mellitin. The hydrophobic areas which insert into the cell membrane are boxed. Positively-charged side chains which disrupt the cell membrane are indicated with +.

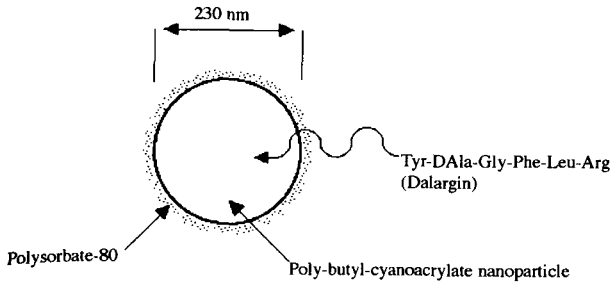


FIG. 6. Schematic diagram of a nanoparticle. The dalargin is absorbed onto the surface of the nanoparticle which is then coated with polysorbate-80.

function of the signal sequence is thus to enable a large polar protein or peptide to traverse a unit membrane without damage to either the pre-pro-protein or the membrane.

Indeed passive permeation of the BBB may not be limited to small molecules. Colloidal polymer particles (nanoparticles) may be formed from poly(butylcyanoacrylate) with an average diameter of 230 nm. Nanoparticles have been used to deliver the peptide dalargin to the CNS (Fig. 6) (Engelman & Steitz 1981; Emr & Silhavey 1983; Kreuter et al 1995). Dalargin is a hexapeptide analogue of encephalin which is stable in plasma but has little central analgesic action when injected intravenously. If dalargin is absorbed onto the surface of nanoparticles and these particles are then coated with the detergent polysorbate-80 and the complex injected into mice, a pronounced analgesic effect is obtained reaching a maximum in 45 min (Fig. 7). Little effect is produced with dalargin, nanoparticles or polysorbate-80 alone. The effect is also dose-dependent with a greater absorption of dalargin onto the particles and is reversible by naloxone (Engelman & Steitz 1981; Emr & Silhavey 1983; Kreuter et al 1995). Presumably the detergent enables the particles to penetrate the BBB and the dalargin is released from the nanoparticles in effective amounts within the brain. In contrast, attempts to use liposomes to deliver drugs to the brain have been universally unsucessful (Pardridge 1991).

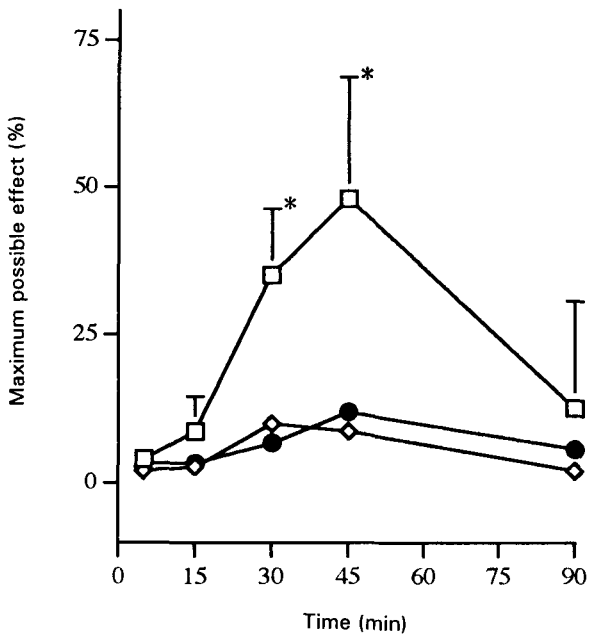


FIG. 7. Analgesia in mice produced by dalargin-loaded nanoparticles. Analgesia is expressed as the percent of the maximally possible effect after intravenous injection of the indicated dose. (n = 6, mean ± s.d.)

Exploiting existing transporters

As mentioned earlier the presence of a blood-brain barrier to polar molecules dictates that a number of transporters must be present in the cerebral endothelium in order to supply the brain with an adequate supply of nutrients. The kinetic properties of some of these transporters are shown in Table 3.

The glucose carrier GLUT-1 has a very large capacity to transport β-D-glucose across the BBB. The intestinal Na^+/D-glucose transporter will also transport β-glycosides but not as effectively as the preferred substrate glucose. Given that GLUT-1 and the intestinal transporter have a similar

Table 3. Kinetic values for some transport systems present at the BBB.

Representative substrate	Transporter	K_m (μM)	V_{max} (nmol min^{-1} g^{-1})	K_d (μL g^{-1} min^{-1})
Glucose	Hexose: Glut-1	11000 ± 1400	1420 ± 140	
Lactic acid	Monocarboxylic acid	1800 ± 600	91 ± 35	
Phenylalanine (apparent)	System-L1	30·0 ± 1·0	14·0 ± 4·0	44·0 ± 14·0
Phenylalanine (real)	System-L1	11·0 ± 2·0	25·0 ± 6·0	8·0 ± 1·0
Arginine (apparent)	Basic amino acid	40·0 ± 24	5·0 ± 3·0	
Leucine encephalin	Peptide specific	39·0 ± 3·2	160·0 ± 22·0*	0·062 ± 0·086
Arginine vasopressin	Peptide specific	2·08 ± 0·32	5·49 ± 0·74*	0·021 ± 0·044
Adenosine	Nucleoside	25·0 ± 3·0	0·75 ± 0·08	
Adenine	Purine base	11·0 ± 3	0·5 ± 0·09	

*pmol min^{-1} g^{-1}.

The K_m value is the concentration of substrate at which the transporter is half-saturated and indicates the affinity of the substrate for the system. The V_{max} is the maximal transporting capacity of the system. The Glut-1 transporter has a high affinity and a high capacity for glucose. As explained in the text many amino acids share a common transport system and will have different affinities (K_m) for the systems; because of this they will compete with each other for transport. Thus an amino acid will have a real K_m and V_{max} where their kinetic values are determined in the absence of competing amino acids and apparent values when competing amino acids are present. The K_d is a measure of the passive diffusional component to the movement of a solute and is significant in the case of the amino acids but is much smaller in the case of the more polar peptides. Means ± s.e.m. Compiled from various sources.

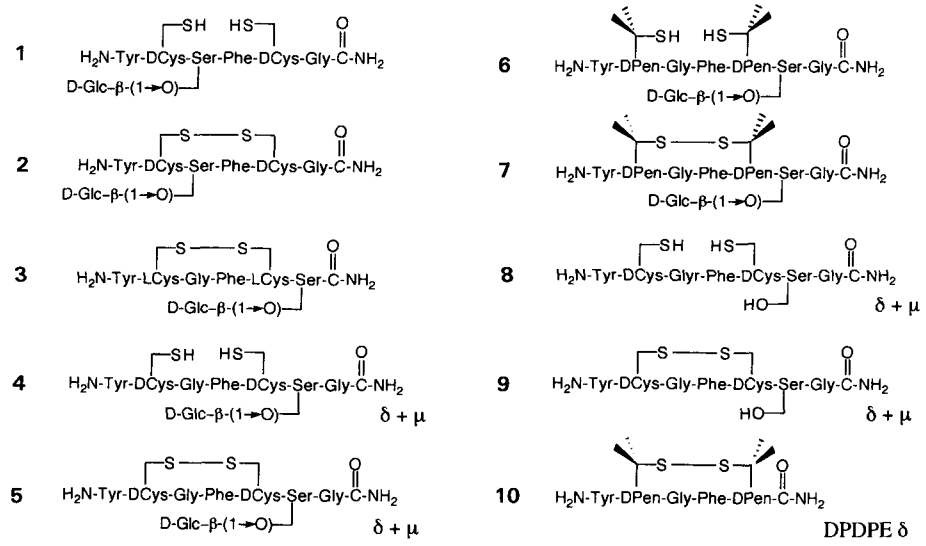


FIG. 8. Some analogues of DPDPE (D-pen^{2,5} encephalin). DPDPE is a δ-receptor agonist but is ineffective intraperitoneally. Analogues **4** and **5** and **8** and **9** are δ- and μ-receptor agonists. However only analogues **4** and **5** produce analgesia determined by the tail flick test after intraperitoneal administration. From Polt et al (1994).

structure it might be possible that peptide-β-D-glycoside conjugates may be acceptable to the brain glucose transporter (Polt 1994). Some glycopeptides administered intraperitoneally as L-serinyl-β-D-glycoside analogues of Met^5 encephalin (Fig. 8) have been shown to be transported across the BBB and bind to μ- and δ-opioid receptors in the brain. The glycopeptide encephalin analogues **4** and **5** produce a marked and long-lasting analgesia after intraperitoneal administration as determined by tail-flick and hot-plate assays in mice (Fig. 9). This analgesia is reversible with naloxone (Polt 1994). It is suggested that GLUT-1 is responsible for transporting the glycopeptide into the CNS. Interestingly, these glycopeptides have a reduced lipohilicity compared with the native encephalins but the conjugate has reactivity with both the glucose transporter and with opioid receptors within the brain (Polt 1994). The brain transport appears to be specific for β-*O*-linked glycosides as α-linked and N-linked and O-acyl linked glucose conjugates (Fig. 8) do not appear to cross the BBB and have no analgesic effects. Also only glycosides linked via the serine residue showed any CNS activity. In this connection it is interesting to note that morphine-6-glucuronide formed naturally in the liver is 10-50 times more potent in producing analgesia than morphine itself and that morphine may act as a prodrug with the 6-glucuronide producing a significant part of the analgesic effect. There is thus a good possibility that glycosylation might be applicable to the delivery of a range of drugs across the BBB.

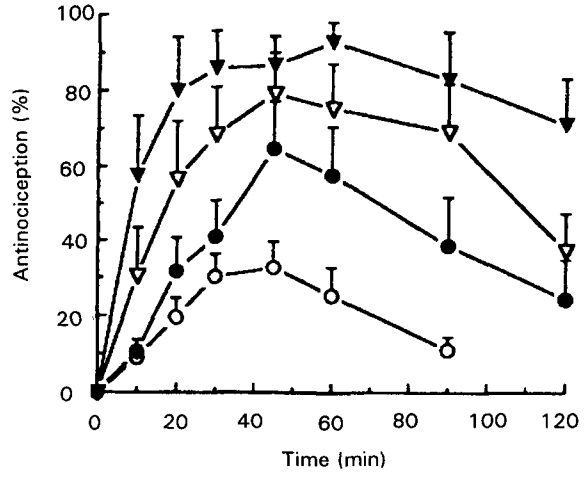


FIG. 9. Analgesia produced by DPDPE glycopeptide analogue **5**. Intraperitoneal administration of this peptide produces dose-related and long-lasting antinociception in a 55°C tail-flick test. From Polt et al (1994).

System-L transporting neutral amino acids into the CNS is another obvious target for drug analogues or complexes which might have a reactivity with this system. To date, the most successful exploitation of system-L is the delivery of L-dopa to the brain for the treatment of Parkinsonism. Dopamine given orally is ineffective as it is not a substrate for system-L and thus does not enter the brain readily. It is also subject to rapid metabolism at the level of the BBB by MAO and COMT. Fortunately L-dopa is a substrate for system-L and the enzyme L-amino acid decarboxylase (AAD or dopa decarboyxlase) within the BBB will convert L-dopa to dopamine during passage through the barrier. The dopamine can then be taken up by cells in the substantia nigra to replace their missing neurotransmitter. Although some further dopamine must be lost as the result of MAO-B and COMT activity in the CNS sufficient appears to reach the nerve cells.

The antineoplasic alkylating agent melphalan also has a reactivity with system-L (Grieg 1992). It is chemically a nitrogen mustard derivative of phenylalanine (Fig. 10). However its reactivity with system-L is only some 20% of that of phenylalanine and endogenous amino acids will compete for transport. The steric requirements for system-L are that the substrate must possess an α-amino group and

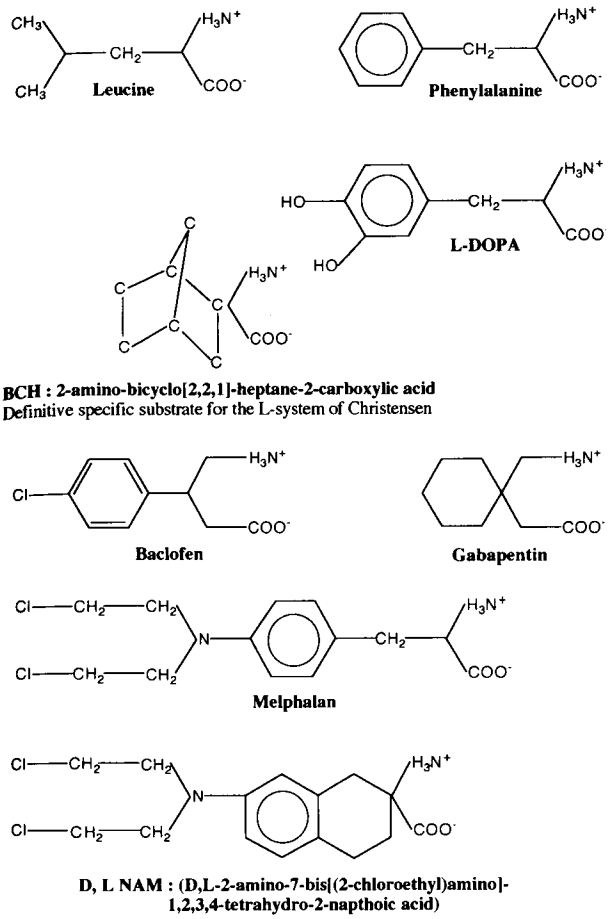


FIG. 10. Substrates known to be transported by system-L at the blood-brain barrier. All of the structures have in common an amino and a carboxylic acid group attached to a single carbon atom, with the exception of baclofen and gabapentin. In the case of these two drugs it is thought that the amino and carboxylic acid groups have sufficient flexibility to make the molecules sterically acceptable to the transport mechanism. BCH is the defining substrate for system-L and only reacts with that amino acid transporter.

a carboxylic acid group attached to the same carbon atom plus a hydrophobic side group. However there are some known exceptions (Fig. 10). The size of the hydrophobic side group does not appear to be a hindrance to transport and the amino acids phenylalanine and tyrosine have a high affinity for the transporter. The importance of the relative positions of the α-amino and carboxylic acids groups is emphasized by the fact that when the small peptide glycyl-leucine is formed reactivity with system-L is lost. Other substrates with significant affinity for system-L have been designed (Grieg 1992). The nitrogen mustard DL-2-amino-7-*bis*((2-chloroethyl)amino)-1,2,3,4-tetrahydro-2-naphthoic acid (DL-NAM) is a good example. It possesses an additional naphthoic side chain compared with melphalan (Fig. 10), which renders it more hydrophobic and confers on the molecule 20 times the affinity for system-L than phenylalanine and 100 times the affinity of Melphalan and L-dopa for the transporter. Additional advantages of DL-NAM are that it has 1.5 times the alkylating activity of melphalan and it is only 20% bound to plasma proteins compared with 80% for mephalan giving it a high therapeutic index (Grieg 1992). Its high affinity for system-L means that it competes effectively with endogenous amino acids to achieve an efficient brain uptake even though the V_{max} is lower.

The antiepileptic drug gabapentin (neurontin) also enters the CNS via system-L even though its does not fully satisfy the strict steric requirements suggested above. Its uptake into the CNS is inhibited by BCH the model substrate for system-L (Fig. 10). Gabapentin is not a substrate for the glutamate transporter and is thus unusual in that it is a γ-amino acid that is acceptable to system-L. It is suggested that the amino and carboxylic acid groups are sufficiently flexible to render it sterically acceptable to system-L. The binding site for gabapentin in nervous tissue is distinct from system-L as leucine is more potent in displacing it than is BCH. It acts as an inhibitor of branched-chain amino transferase and both it and leucine act to stimulate glutamate dehydrogenase in the CNS.

A similar situation pertains for the muscle relaxant baclofen (4-amino-3-(*p*-chlorophenyl)-butyric acid (Fig. 10) (Van Bree et al 1988). Baclofen acts at the spinal and supraspinal levels and is transported into the CNS by system-L.

A number of specific uptake mechanisms for peptides exist at the BBB (Begley 1994). Those for leucine encephalin and arginine vasopressin have been kinetically described as high affinity low capacity systems (Zlokovic et al 1989, 1990) as shown in Table 3. The uptake mechanism for leucine encephalin is remarkably specific showing little affinity for fragments of the molecule with almost the entire pentapeptide being sterically required. Fig. 11 shows the results of inhibition studies carried out in an in-situ brain perfusion technique with the addition of a number of inhibitors and peptide fragments. Under the conditions of perfusion the

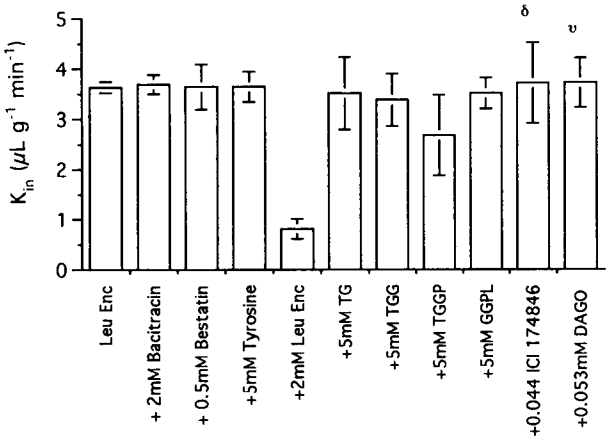


FIG. 11. Specificity of leucine encephalin uptake at the blood-brain barrier. Inhibition studies to determine the specificity of the uptake mechanism for leucine encephalin at the blood-brain barrier of the guinea-pig hippocampus determined by an in-situ vascular brain perfusion method. Vertical axis unidirectional cerebrovascular constant K_{in} μL g^{-1} min^{-1} ± s.e.m. Horizontal axis K_{in} for leucine encephalin tracer alone (control); plus 2 mM bacitracin, 0.5 mM bestatin, 5 mM L-tyrosine, 2 mM leucine encephalin, 5 mM tyrosyl-glycine, 5 mM tyrosyl-glycyl-glycine, 5 mM tyrosyl-glycyl-glycyl-phenylalanine, 0.044 mM ICI 174846 (a δ opioid ligand), 0.053 mM DAGO (a μ opioid ligand.). Only the addition of 5 mM leucine encephalin produces a significant inhibition of the K_{in} value ($P < 0.01$) and 5 mM tyrosyl-glycyl-glycyl-phenylalanine ($P < 0.05$). Data from Zlokovic et al (1987, 1989). Means ± s.e.m.

(^{3}HTyr)-encephalin molecule is stable as the addition of 2.0 mM bacitracin or 0·2 mM bestatin to the perfusate has no effect on the cerebrovascular permeability constant, K_{in}. Significant reduction of the K_{in} value, indicating competitive inhibition, was only produced by intact leucine encephalin and des-leucine encephalin. DAGO and ICI 174846, ligands for the μ- and δ-opioid receptors, respectively, had no effect in the K_{in} indicating that the carrier is distinct from the receptor producing the central nervous effects of these opioid peptides (Zlokovic et al 1989).

Transporters for macromolecules also exist at the BBB which might be utilised as vectors to transport material into the CNS. Strategies utilizing the transferrin receptor in this way have been attempted (Friden et al 1991, 1993; Pardridge et al 1991). A monoclonal antibody, to the transferrin receptor OX26 has been utilised as this avoids competition between any administered transferrin and endogenous transferrin in the experimental animal. A variety of molecules can then be attached to the monoclonal antibody. In experimental animals this approach has been used to deliver horseradish peroxidase, nerve growth factor (NGF), methotrexate and vasoactive intestinal polypeptide (VIP), across the blood-brain barrier. In some respects these studies remain controversial as there is evidence to suggest that in the intra-endothelial transport of iron by transferrin at the BBB, the iron is decoupled from the transferrin within the cells and the transferrin molecule itself may not necessarily be transcytosed. (Taylor & Morgan 1990; Ueda et al 1993). However, the NGF-OX26-transferrin complex may act as a vector to allow large proteins such an NGF to enter a protected compartment within the endothelial cells and the whole complex may not necessarily have to be exocytosed at the abluminal membrane.

Efflux mechanisms

As mentioned earlier the efflux pump P-glycoprotein is expressed constitutively at the luminal surface of the cerebral endothelial cells forming the BBB. A number of lipid soluble cytotoxic agents, such as vincristine and vinblastine and other substrates for P-glycoprotein, do not exhibit the brain uptakes that would be predicted by their lipid solubility (Begley 1992) and are outliers on the plot shown in Fig. 2. One strategy for enhancing the uptake of substrates for P-glycoprotein is to employ non-competitive or competitive inhibitors of the efflux pump. These agents will reverse multidrug resistance in peripheral tissues that have acquired it and thus have potentially a wide application in cancer chemotherapy. Table 4 shows the effect of a number of substrates and inhibitors of Pgp activity on the accumulation of [^{3}H]colchicine in-vitro into a cultured monolayer of an immortalized clone of rat brain endothelial cells (Begley et al 1994). Similar results have also been obtained in-vivo by administering therapeutic doses of vincristine to guinea-pigs (Begley & Evans 1992). In this study co-administration of vincristine and [^{3}H]colchicine increases BBB uptake of colchicine compared with control animals receiving tracer colchicine alone, suggesting an inhibition of Pgp activity in the cerebral endothelium.

A spectrum of efflux pumps exists within the CNS and contributes to the homeostasis of the brain extracellular fluid. These pumps may be located in the cerebral endothelium and also at the choroid plexus. For example penicillin, *p*-aminohippuric acid and AZT (azidothymidine), are pumped out of cerebrospinal fluid (CSF) at the choroid plexus by an organic acid pump which is probenecid-sensitive (Spector & Lorenzo 1974). Probenecid can therefore be used to increase the CSF levels of these drugs. Amino acids are cleared rapidly from CSF by saturable transport mechanisms (Davson et al 1982) as are a number of peptides (Begley & Chain 1988, 1992; Banks & Kastin 1992). Amino acids with neurotransmitter function have very low free concentrations in CSF. Thus the design of peptide- or amino acid-based drugs with a reduced affinity for these efflux mechanisms or the inhibition of these efflux mechanisms will lead to an increase in CNS levels. There are also free peptidases in CSF (Begley & Chain 1988, 1992; Begley 1994) which can hydrolyse peptides, for example leucine encephalin and angiotensin II. The substitution of D-isomers into the peptide confers resistance to the action of these enzymes in the same manner as with plasma peptidases.

Opening and Permeabilizing the BBB

A number of techniques exist which enable the permeability of the BBB to be modified. Most of these are non-selective and open the barrier to a range of solutes of varying molecular weight.

Table 4. Inhibition of Pgp activity in RBE4 cells.

		[^{3}H] Colchicine uptake (mL (μg protein)$^{-1}$)		
Inhibitor	Concn (μM)	Control	Experimental	% control
Vincristine	50	13·6411·19	53·3918·62	391**
AZT	50	14·1610·82	33·9313·24	232**
Chlorpromazine	50	12·9110·93	25·7511·88	200**
Verapamil	100	12·4511·19	17·4618·62	140*

Cells were either pre-treated with inhibitor in HBSS for 30 min (experimental), or left for 30 min in HBSS alone (control). The incubation medium was then changed for one containing [^{3}H]colchicine (18 nM, 1.38 mCi) plus inhibitor (experimental) or the same chemical and radioactive concentrations of [^{3}H]colchicine alone (control) and incubated for a further 30 min. An increase of [^{3}H]colchicine accumulation into the cells is interpreted as an inhibition of the efflux activity of Pgp. Mean ± s.e.m. Experimental values were significantly different from control values by unpaired Student's *t*-test. From Begley et al (1994).

The most widely used technique is that of osmotic opening where a short infusion of a hyperosmotic solution, usually mannitol, is introduced into a carotid artery. Large molecules can be given access to the CNS by this technique and its therefore thought that the osmotic agent is affecting the integrity of the tight junctions between the cerebral endothelial cells. One explanation that has been offered is that the osmotic agent is shrinking the endothelial cells and physically opening paracellular pathways. Osmotic blood-brain barrier disruption coupled with the infusion of chemotherapeutic drugs has been used to treat a number of central nervous tumours with marked improvements in survival time and little apparent ill effect from the BBB disruption (Gumerlock & Neuwalt 1992). Osmotic BBB disruption can be used to deliver therapeutic substances that would otherwise be excluded from the CNS because of their size, including the possibility of introducing genetically-engineered retroviruses in order to replace defective genes.

The peptide bradykinin and some of its analogues increase BBB permeability by apparently permeabilizing the tight junctions via B2 receptors (Unterberg et al 1984); this general phenomenon is referred to as receptor-mediated permeabilization (RMP). Similar permeabilization can be achieved with leukotrienes, histamine and 5-hydroxytryptamine. In in-vitro cultures of monolayers of bovine cerebral endothelial cells leucine encephalin, α-adrenergics, arachidonic acid, bradykinin, aluminium, phorbolmyristate esters, and α-thrombin all increase permeability of the monolayer, whilst angiotensin II, saralasin (an angiotensin analogue), β-adrenergics, lowered temperature, and 2-deoxyglucose all reduced the permeability of the monolayer (Grieg 1992).

Some of these factors used to permeabilize the barrier are more selective than others. As previously mentioned, the BBB exists for the specific purpose of protecting the brain from potential toxins and providing a separate and stable environment within which the neurones can perform their integrative functions. Thus any non-selective opening of the barrier will transitorily disturb the brain fluid environment and cause possible long term damage.

Conclusions

Clearly then, there is considerable scope for optimizing drug delivery to the brain in order to treat a whole range of central nervous diseases from neoplasm to degenerative disorders and psychiatric disturbance.

There still remains a great need for a systematic study of molecular structure and properties of drugs in relation to their BBB penetration. For substances that enter passively, the aim is to extend the plasma half life, increase the penetration of the blood brain barrier, retain and enhance central activity and reduce reactivity with central nervous mechanisms degrading or removing the drug from the CNS. In practice it is unlikely that all of these ideals will be satisfied for a single drug, but optimizing some of them will probably produce gains in most cases.

Specifically designing drugs which mimic the substrates for uptake mechanisms located in the BBB, appears to hold great promise, particularly if a range of chemical groups can be designed which can be attached to a variety of compounds rendering them substrates with a high affinity for particular carriers. The two transporters GLUT-1 and system-L have the highest capacity and perhaps offer the best chance for delivering substances to the brain in quantity. However it may not be necessary to deliver large quantities of some substances to the brain which possess very potent central activities and which may influence, for example, behavioural activity. In these cases lower-capacity transporters or an enhanced passive diffusion might suffice.

Opening and permeabilizing the barrier are generally non-selective and are useful in allowing an acute entry of substances into the brain where a once-only opening is required in order to introduce a genetic vector or potent drug. Non-selectively lifting the protective BBB repeatedly may carry some risks but these risks might be clinically insignificant in relation to those of the disease being treated.

The development of in-vitro models of the BBB by growing tight layers of cerebral endothelial cells in culture will play a substantial role in screening potential drugs in relation to their physical properties and BBB penetration. It will probably be impossible to recreate all of the characteristics of the BBB in-vitro, and in-vivo testing will always be required to evaluate the intact barrier and the central effectiveness of a test compound.

Conversely an understanding of the factors involved in drug penetration of the BBB is also essential in optimizing the design of drugs for peripheral use where their passage through the BBB is undesirable. There must be many lead compounds whose development has been abandoned as the result of disastrous unwanted central actions.

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