VEHICULATION OF SUBSTANCES ACROSS THE BLOOD-BRAIN BARRIER MEDIATED BY MOLECULAR TROJAN HORSES

MAIN SCOPE: PHYSIOLOGY AND PHYSIOPATHOLOGY

SECONDARY SCOPES: 1. PHARMACOLOGY AND THERAPEUTICS

2. CELLULAR BIOLOGY

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ABSTRACT

The blood-brain barrier (BBB) is a specialized system that comprises brain microvasculature endothelial cells, basement membrane and various types of cells, including astrocytes, pericytes and neurons located close to the endothelium. The BBB is a key element for the central nervous system (CNS), as it shields the brain from toxic substances in the blood, supplies brain tissues with nutrients and filters harmful compounds from the brain back to the bloodstream. Understanding of its complex structure is essential to make a step further in the discovery of new effective brain treatments. Actually, gene therapy is bringing about new horizons in the treatment of brain disorders, however, it has to solve the transport across the BBB of its large therapeutic molecules. Compared to other gene therapy techniques, such as viral gene delivery or cationic liposome delivery, the Trojan horse liposome technique seems to have some advantages. Trojan horse liposomes, together with avidin-biotin and fusion protein technologies, are three different techniques that researchers are studying to carry drugs into the brain via a receptor-mediated transcytosis. During the last few years a lot of studies in animal models *in vitro* and *in vivo* have emerged, but research has not yet reached clinical trials.

RESUM

La barrera hematoencefàlica és un sistema especialitzat que està format per cèl·lules endotelials, membrana basal i diversos tipus de cèl·lules com astròcits, pericits i neurones localitzats a prop de l'endoteli. La barrera hematoencefàlica és un element clau per al sistema nerviós central, ja que protegeix el cervell de substàncies toxiques provinents de la sang, proveeix el teixit cerebral de nutrients i filtra compostos nocius del cervell cap a la circulació sanguínia. La seva estructura és complexa, però el seu coneixement és essencial per al descobriment de nous tractaments cerebrals més efectius. Actualment, la teràpia gènica està obrint nous horitzons pel que fa al tractament de malalties del sistema nerviós, tot i així, encara ha de resoldre el problema de com fer que les seves molècules terapèutiques grans travessin la barrera hematoencefàlica. La tècnica que utiliza liposomes com a cavalls de Troya moleculars sembla presentar alguns avantatges respecte d'altres tècniques, com el transport a través de virus o de liposomes catiònics. Aquesta

tecnologia, junt amb les de l'avidina-biotina i de la proteïna de fusió, són tres tècniques differents que els investigadors estan estudiant per al transport de fàrmacs a través de la barrera via transcitosi mitjançada per receptors. Durant els últims anys s'han fet molts estudis en models animals *in vitro* i *in vivo*, però encara no s'ha aconseguit arribar als assaigs clínics.

INTEGRATION OF THREE DIFFERENT SCOPES

This assignment is framed in the Physiology and Physiopathology scope, and also in the Pharmacology and Therapeutics and Cellular Biology scopes.

- **Physiology and Physiopathology**: this work describes the physiological functioning of the BBB which is essential for the homeostasis of the central nervous system. Given that the BBB impedes the pass to the brain of therapeutic substances or substances used for diagnosis to the brain, different transport mechanisms and strategies directed to cross the BBB are studied.

- **Pharmacology and Therapeutics**: this work studies different drug delivery techniques that can be used to cross the BBB. Besides, it gives special attention to gene therapy and the Trojan horse liposome technique.

- **Cellular biology**: this work describes different types of transport across the BBB and focuses on receptor-mediated transcytosis. This implies processes of cellular internalization. Besides, the detailed description of the structure of the BBB is based on physiology as well as cellular biology.

1. INTRODUCTION

1.1 THE BLOOD-BRAIN BARRIER

The blood-brain barrier (BBB) is a complex structure essential to maintain the central nervous system (CNS) homeostasis. This barrier is necessary to prevent certain hazardous substances from entering to the central nervous system and to facilitate the entrance of nutrients and chemical signals.

The BBB is formed by the microvasculature of the brain and its particular properties are given by the expression of BBB-specific genes (1). There are different cells that comprise the brain microvasculature, and all contribute to its regulation including permeability. Within the microvasculature there are endothelial cells and pericytes which share a common capillary basement membrane. There is approximately one pericyte for every two to four endothelial cells. Furthermore, more than 99% of the brain surface or abluminal surface of the capillaries is covered by astrocytic foot processes. The capillaries are also innervated by nerve endings of either intra- or extra-cerebral origin. The distance between the astrocyte foot process and the capillary endothelial cell and the pericyte is only 20 nm. Therefore, the interrelationships between them are as intimate as any cell-cell interactions in biology. The space filled by the basement membrane and situated between the endothelium/pericyte and the astrocyte foot process forms the interface between blood and brain (2).

Brain capillaries are much less permeable than capillaries in most other tissues so that even small molecules cannot pass the capillary walls. This is caused mainly from very extensive tight junctions between the endothelial cells. Compared to capillary walls of other organs, which present less tight junctions and so let water flow, brain capillaries prevent water-soluble substances from passing through them (3).

However, certain areas of the brain, most of which are situated close to the ventricle and are therefore called circumventricular organs (CVO), have endothelial cells that do not form tight junctions. These areas together comprise less than 1% of the brain, and the endothelium is

fenestrated with circular pores which allow free exchange of molecules between the blood and the adjacent neurons. The epithelial cells, which delimit the circumventricular organs, however, impede diffusion into the rest of the brain and the cerebrospinal fluid (CSF). Therefore, substances that have entered these areas do not have unrestricted access to the rest of the brain (2).

Other particular characteristics of the BBB are the lack of lymphatic drainage and the absence of major histocompatibility complex (MHC) antigens. The BBB has strict limit for the passage of immune cells, especially lymphocytes, and its immunity is constituted by the association between BBB endothelial cells, perivascular macrophages and mast cells. Additionally, the BBB immunity is reinforced by local microglia cells (4).

1.2 PERMEABILITY AND TRANSPORT ACROSS THE BBB

The brain is protected against peripheral neurotransmitters, cytotoxins or microorganisms because passive diffusion is only possible for small molecules (less than 400-500 Da) which are highly lipid soluble. This property, which is a protection, is a disadvantage for hydrophilic molecules and hence hydrophilic drugs (1). As an example, certain drugs such as barbiturates are highly lipid-soluble and act rapidly, and other drugs, such as penicillin, have low lipid solubility and pass the BBB with difficulty. The need for such a barrier is due to the delicate balance of neuronal excitability that must be kept out of disturbing agents (3).

But not all molecules which are necessary for the CNS can passively diffuse through the BBB, and thus the only way to gain access to the brain is through specific transporters located on both luminal and abluminal membranes of the BBB. Glucose, amino acids or insulin are examples of molecules that use this type of transport. Glucose is an example of a water-soluble substance that reaches high concentrations in the brain. This is essential as the neurons depend, almost only, on glucose as a source of energy. The glucose transporter GLUT 1 is specific to brain capillaries (3).

Macromolecules, such as some growth factors and cytokines, are to a limited extend carried from blood plasma to the brain, by receptor-mediated transport. Insulin or transferrin are also transferred across the barrier via this type of transport (3).

The BBB is able to actively pump ions that are present in different concentration in the brain extracellular fluid and in the blood plasma such as Na^+, K^+ or Ca^{2+}. Organic acids are as well actively pumped out of the brain by specific transporters (3).

Transport of small and large molecules through the BBB is restricted and, moreover, the BBB is equipped with efflux transporters that actively expel substances out of the brain. One of the most characterized efflux transport is P-glycoprotein, and it is responsible for the transports of many drugs out of the brain (3).

Among the different types of transport present in the BBB, the receptor-mediated transcytosis has been chosen to illustrate how new treatment strategies use endogenous mechanisms to overcome the difficulty to access into the brain. Various techniques can be used to deliver drugs through receptor-mediated transcytosis, however this work will focus on one of these techniques to have a more detailed understanding of its engineering and how gene therapy can beneficiate of its advantages compared to other existing gene therapies.

2. OBJECTIVES

This work pretends to review the actual research about BBB structure and drug delivery across the barrier. The specific objectives are the following:

-To describe the structure of the BBB and how it confers its particular characteristics.

-To explain ways of transport across the BBB and go through the various types of transport mechanisms and the different types of transporters.

-To explain in a detailed manner the receptor-mediated transcytosis, its main receptors and include a detailed description of the molecular Trojan horses.

-To describe different drug delivery techniques that use receptor-mediated transcytosis.

-To give a brief account of the importance of gene therapy in the future of effective brain treatments and the different strategies that have been used to date.

-To describe thoroughly the Trojan horse liposome gene delivery technique, referring to its actual engineering.

-To give general aspects of the research and future perspectives of the Trojan horse liposomes as well as those of other techniques.

3. MATERIAL AND METHODS

This assignment is a bibliographic research. The sources belong to cientific articles collected in Pubmed and books specialized in the central nervous system and drug delivery to the brain.

The first search in Pubmed was based in the key words “blood-brain barrier” AND “antibody” AND “trojan horse”. From the results obtained, a recent review was chosen. Some of the bibliography of this review was used to obtain more information. Besides, other searches were done with key words such as “Trojan horse liposomes” or “gene therapy” or “receptor-mediated transcytosis”. Among the articles used, some are reviews and others research papers.

A lot of articles date from 2007 onwards to have recent and actualized information, but some anterior interesting bibliography has also been used.

To complement the search, various useful books were found using the library catalog. The first search was with the key word “blood-brain barrier” and the following searches used, as key words, “central nervous system” and “Trojan horse”. Some books were available online and others were found in the libraries of the Faculty of Pharmacy and the Faculty of Medicine.

4. RESULTS

4.1 STRUCTURE OF THE BLOOD-BRAIN BARRIER

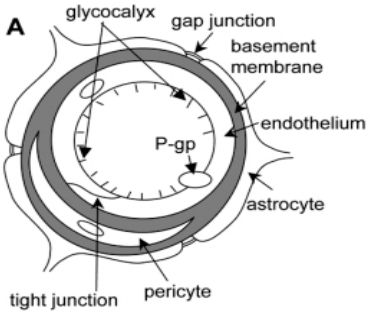

Figure 1. Schematic outline of the capillary of the BBB in a transverse section showing its different components. (5)

The BBB is comprised of endothelial cells of brain microvasculature and also other types of cells as shown in figure 1. These cells will be explained in the present chapter.

4.1.1 Endothelial cells

Brain capillaries are continuous and their walls are composed of one or more endothelial cells. The main features that distinguish endothelial cells from those that do not belong to brain vessels form the structural basis of the BBB. These include the presence of tight junctions between cerebral endothelial cells, reduced endothelial plasmalemmal vesicles or caveolae, albondin, glycocalyx and increased numbers of mitochondria (2).

Within the endothelial cells of brain capillaries there are enzymes which inactivate some substrates. These enzymes are located not only within the endothelial cells, but also in brain tissue generally (6). Enzymatic activity is carried by enzymes such as alkaline phosphatase (ALPase), acid phosphatase (ACPase), 5'-nucleotidase (5'-N), adenosine triphosphatase-Na^+, k^+-ATPase, nucleoside diphosphatase (NDPase) (5).

4.1.1.1 Junctional complex

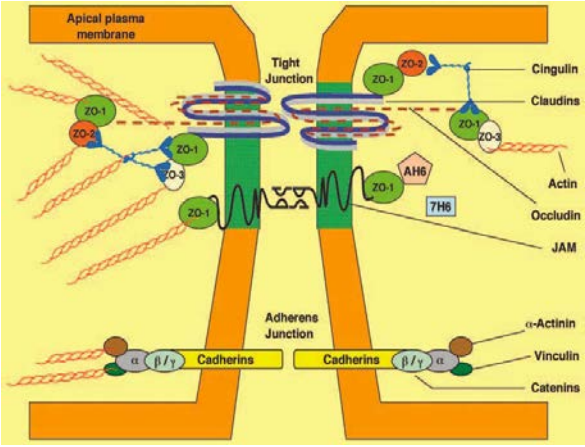

Figura 2. Major proteins associated with the junctional complex at the BBB. (2)

The most differential characteristic of these endothelial cells is tight junctions. These junctions are characterized by fusion of the outer leaflets of adjacent plasma membranes at intervals producing a pentalaminar appearance and forming tight or occluding junctions that prevent paracellular diffusion. These tight junctions form the most apical element of the junctional complex, which includes both tight and adherens junctions. Tight junctions extend circumferentially around cerebral endothelial cells; hence, their name zonula occludens (2,5). Figure 2 shows the junctional complex and the major proteins associated to tight and adherens junctions.

The physiologic correlate of tightness in epithelial membranes is electrical resistance. It is estimated to be approximately 4000-8000 Ω/cm^2 compared to leaky epithelia which generally exhibits electrical resistances between 100-200 Ω/cm^2 (2).

Tight junctions are composed of an intricate combination of trans-membrane and cytoplasmic proteins linked to an actin-based cytoskeleton that allows these junctions to form a seal while remaining capable of rapid modulation and regulation (2). Three integral proteins –claudin 1 and 2, occluding and junction adhesion molecule (JAM)- form the tight junction (2). Claudins form dimmers and bind homotypically to claudins on adjacent endothelial cells to form the primary seal of the tight junction. In the other hand, occludin is a regulatory protein, whose presence at the BBB is correlated with increased electrical resistance across the barrier and decreased paracellular permeability. Besides, occludin is not present in non-neural vessels thus differentiating the tight junctions of cerebral and non-neuronal vessels (2). The third type of integral proteins that are localized at the tight junctions are junctional adhesion molecules (JAM) which are members of the immunoglobulin superfamily, and can function in association with platelet endothelial cellular adhesion molecule 1 (PECAM) to regulate leukocyte migration. Overexpression of JAM in cells that do not normally form tight junctions increases their resistance to the diffusion of soluble tracers, suggesting that JAM contributes to permeability control (2).

Tight junctions are also made up of several accessory proteins that are necessary for structural support such as ZO-1 to 3, AF-6, 7H6 and cingulin. The zonula occludens (ZO) proteins 1-3 belong to a family of proteins involved in the coupling of transmembrane proteins to the cytoskeleton. The ALL-1 fusion partner from chromosome 6 (AF-6) is associated with ZO-1 and serves as a scaffolding component of tight junctional complexes by participating in regulation of cell-cell contacts via interaction with ZO-1. 7H6 antigen is a phosphoprotein found at tight junctions that is impermeable to ions and macromolecules. Lastly, the double-stranded myosin-like protein cingulin is localized at the tight junction and found in endothelial cells as well and it appears to serve as a scaffolding protein that links tight junction accessory proteins to the cytoskeleton (2).

The primary cytoskeleton protein, actin, has known binding sites on all ZO proteins and on claudin and occludin. Tight junctions are localized at cholesterol-enriched regions along the plasma membrane associated with caveolin-1. Caveolin-1 interacts with and regulates the activity of several signal transduction pathways and downstream targets. Furthermore, several cytoplasmic signaling molecules are concentrated at tight junction complexes and are involved in signaling cascades that control assembly and disassembly of tight junctions (2).

Adherens junctions are located near the basolateral side of endothelial cells. Adherens junction proteins include different types of catherins, which are single-pass transmembrane glycoproteins that interact homotypically in the presence of Ca^{2+}. These catherins are not specific for cerebral endothelial junctions being present in endothelium of non-neural blood vessels as well. Cadherins are linked intracellularly to a group of proteins termed catenins. Catenins are part of the system by which adherens and tight junctions communicate (2).

4.1.1.2 Plasmalemmal vesicles or caveolae

Within endothelial cells there are endothelial plasmalemmal vesicles or caveolae. These are non-coated structures also referred to as pinocytotic vesicles. Free cytoplasmic caveolae are spherical structures. The decreased number of vesicles in cerebral endothelium implies limited transcellular traffic of molecules. Endothelial caveolae are either endocytic or transcytotic. The permeant molecules can either be internalized within endothelial cells by endocytosis or may be translocated across the cell to the interstitial fluid, a process termed transcytosis. Both

endocytosis and transcytosis may be receptor-mediated or fluid phase and require ATP. Receptor-mediated transcytosis of caveoli are involved in transport of low density lipoprotein (LDL), β-very low density lipoprotein (VLDL), transferrin, insulin, albumin, ceruloplasmin, and transcobalamin across the endothelium (2).

4.1.1.3 Albondin

Albondin is a 60-kDa albumin-binding sialoglycoprotein that is expressed selectively by vascular endothelium and is present on the luminal surface of continuous endothelium. It binds albumin apparently not only to initiate its transcytosis via caveolae but also to increase capillary permselectivity. Low expression or lack of expression of albondin in brain-derived microvascular endothelial cells accounts for restricted albumin passage into brain (2).

4.1.1.4 Mitochondria

Most of the mitochondria in cerebral endothelium are located in the vicinity of the nucleus, but occasional mitochondria occur throughout the cytoplasm and these tend to be parallel to the cell surface. Increased mitochondria in cerebral endothelium may provide the metabolic work capacity for maintaining the ionic gradient across the BBB (2).

4.1.1.5 Glycocalyx

The glycocalyx is a negatively charged, surface coat of proteoglycans and adsorbed plasma proteins lining the luminal surface of the endothelium. Therefore it works at the first line of the BBB. It is thought that it contributes to the vasculoprotective effects of the vessel wall and that is involved in maintaining vascular permeability. Glycocalyx harbours a wide array of enzymes that might contribute to its vasculoprotective effect. The glycocalyx damage shifts the balance towards a pro-oxidant state. These observations are of particular interest because altered vascular permeability attenuated the earliest characteristics of atherogenesis. Moreover, it is known that

endothelial glycocalyx is disturbed in various types of vascular diseases and that inflammation induces glycocalyx shedding (5).

4.1.2 Pericytes

In the CNS, pericytes have an oval to oblong cell body arranged to the vessel long axis. The cell body of the pericyte consists of a prominent nucleus with limited perinuclear cytoplasm from which extend cytoplasmic processes that also run parallel to the long axis of the blood vessel; secondary processes arise along the length of the primary process and partially encircle the vascular wall. Pericytes may be “granular” or “agranular” depending on whether cytoplasmic lysosomes are abundant or sparse respectively. The cerebral pericytes are exclusively granular and are rich in cytoplasmic plasmalemmal vesicles. Although pericytes are separated from endothelium by the basement membrane, there are gap-junctions between them. Cytoplasmic processes of the pericyte indent the endothelial cell and vice versa, forming the so-called “peg-and-socket” contacts. Three major functional roles have been ascribed to pericytes associated with CNS microvasculature. They include contractility, regulation of endothelial cell activity and a role in inflammation (2).

4.1.3 Basement membrane

The basement membrane is a specialized, extracellular matrix, which separates endothelial cells and pericytes from the surrounding extracellular space. In adults this membrane is 30-40 nm thick and is synthesized by both astrocytes and endothelial cells which are connected with the basement membrane via fine filaments. The basement membrane has an inner electron-dense layer called the lamina densa; and less electron-dense layers called the laminae rarae. The basement membrane is composed of laminin, collagen IV, proteoglycans, notably heparan sulphate, fibronectins, nidogen and entactin. The chemical composition of these individual basement membrane components differ among various organs. The subendothelial basal lamina is no impediment to the extracellular flow of tracers such as horseradish peroxidase. The basal lamina of capillaries forms a negatively charged screen or filter controlling the movement of

charged solutes between blood and the brain interstitial fluid. Large, charged molecules such as ferritin do not cross the basal lamina. The subendothelial basal lamina also serves as a repository for growth factors such as basic fibroblast growth factor and heparin binding proteases and protease inhibitors. Therefore regulated release of growth factors and proteases from the basal lamina reservoir could play a role in angiogenesis and the invasion of the interstitium by tumor cells (2).

4.1.4 Astrocytes

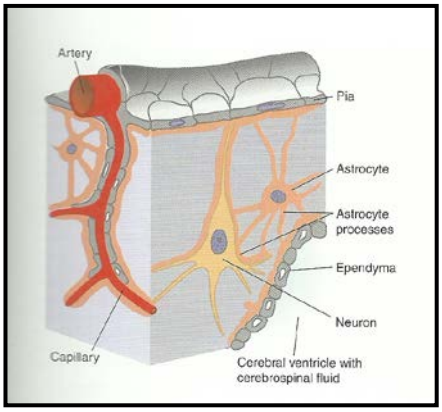

Figura 3. Relationship between astroglia and neurons, blood vessels and the cerebrospinal fluid (CSF). (3)

Astrocytes are one of the three types of glial cells. Glial cells do not take part in the fast and precise information processing in the brain, nevertheless are of crucial importance to proper functioning of neurons. In fact, the number of glial cells is much higher than the number of neurons. The name glia derives from the older notion that glial cells served as a kind of glue, keeping the neurons together. Astrocytes have numerous processes that contact capillaries and the lining of the cerebral ventricles. They serve important homeostatic functions by controlling the concentrations of ions and the osmotic pressure of the extracellular fluid (water balance), thereby helping to keep the neuronal environment optimal. Astrocytes also take part in repair processes and have structural features that make them well suited to control the extracellular environment of the neurons (3).

First of all, they have numerous short or long processes that extend in all directions, as shown in figure 3. Thus, astrocytes have a very large surface area that enables efficient exchange of ions and molecules with the extracellular fluid (ECF). Furthermore, some processes contact the surface of capillaries with expanded end-feet and cover 99% of the brain surface of the capillary basement membrane. Some other processes form a continuous, thin sheet (membrana limitans or glia limitans) where nervous tissue borders the cerebrospinal fluid (CSF), that is, in the cavities inside the CNS and against the connective tissue membranes on its exterior. And finally some processes

contact neuronal surfaces as well; in this manner, parts not contacted by neurons are covered by glia (3).

Astrocytes are coupled by numerous gap junctions (nexus), allowing free passage of ions and other small particles among them. Thus, apart from allowing electric currents to spread, astrocytes form continuous, large fluid volumes for distribution of substances removed from the ECF (3).

Although glial cells do not send precise signals over long distances, they can produce brief electric impulses (currents) by opening of membrane channels for Ca^{2+}. Such an opening can be evoked by binding of neurotransmitters to receptors in the glial cell membrane. Thus, neuronal activity can directly influence the astrocytes, whereas the latter affects neuronal activity. Owing to the electric coupling (nexus) of the astrocytes, the "calcium signal" can presumably spread rapidly in networks of astroglial cells, and consequently influence many neurons almost simultaneously, which, among other roles, can help synchronize the activity of neurons in a group. When activated, the astrocytes increase local blood flow (3).

The intimate contact with neurons, capillaries, and the CSF places astroglial cells in a unique position to control the environment of the neurons, that is, the extracellular (interstitial) fluid of the brain. Such control is vitally important for three main reasons. First, neurons are exquisitely sensitive to changes in extracellular concentrations of ions and neurotransmitters. Second, the osmotic pressure (the water concentration) must be tightly controlled because the brain cannot expand in the skull. Third, adding even minute amounts of a substance may produce a substantial increase in its extracellular space in the brain. Besides, the tortuous shape of the extracellular space hampers free diffusion of particles (3).

With regards to extracellular ions, the control of K^+ is particularly important. Neuronal excitability is strongly influenced by small changes in the amount of K^+. Astrocytes help remove K^+ to prevent high toxic concentrations of this ion. Furthermore, astrocytes contribute to extracellular pH control by removing CO_2 (3). Also, extracellular neurotransmitter concentrations must be tightly controlled, because proper synaptic functioning requires that their extracellular concentrations be very low, except during the brief moments of synaptic release. Astrocytes help to remove them from the synapses (3).

Astrocytes are also involved in the control of the extracellular osmotic pressure, that is, in controlling the water balance of the brain. Of particular interest in this respect are channels for transport of water –aquaporins- that are present in the membranes of astrocytes. Aquaporins in the brain are most abundant on the glial processes that are in close contact with capillaries and the CSF. Exchange by astroglial cells of small neutral molecules, such as the amino acid taurine, may be another mechanism to control extracellular osmolarity. Finally, the layer of astrocytic processes surrounding brain capillaries helps to prevent many potentially harmful substances from entering the brain (3).

4.2 TRANSPORT ACROSS THE BBB

Transport of substances in either direction of brain to blood, or blood to brain, requires movement across the capillary endothelial plasma membranes. The luminal and abluminal membranes of the capillary endothelium are separated by 100-300 nm of endothelial cytoplasm. Therefore, solute transfer across the capillary endothelial barrier is a process of transport through two membranes in series. However, in order for a molecule to move from blood to the brain interstitial space beyond the astrocyte foot process, the molecule must also escape the immediate perivascular space bordered by the plasma membranes of the capillary endothelial cell, pericyte, and astrocytic foot processes. Many "enzymatic BBB" mechanisms may operate within this space. The actual transport of nutrients or drugs across the BBB may be the result of a complex interplay between active efflux systems located on the endothelial plasma membrane, active transporters within the astrocytic foot process, and ectoenzymes present on the pericyte plasma membrane (2), as shown in figure 4.

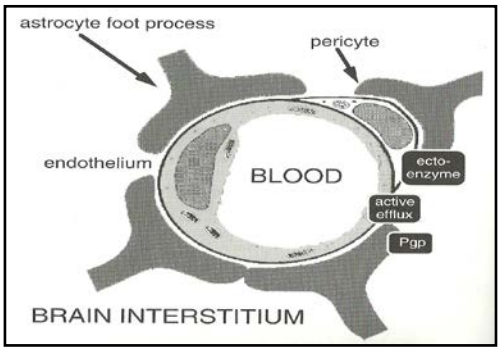

Figura 4. Intimate relationship of active efflux systems within the brain capillary endothelial membrane, ectoenzymes in the pericyte, and p-glycoprotein (Pgp) in the plasma membrane of astrocyte foot processes (7)

The bottleneck in the development of new drugs for the brain is the permeability of BBB. Unfortunately, only small molecules which are both lipid soluble and have a molecular weight <400 Da (8), and are not bound by plasma proteins or are not a substrate of an efflux transport,

can cross the BBB by passive diffusion in pharmacologically significant amounts (9). In this case, substances dissolve in the cell membrane and cross the barrier, e.g. alcohol, nicotine, and caffeine. Actually less than 5% of all drugs are effective in the brain (8). In the absence of the lipid-mediated pathway, circulating molecules may gain access to brain only via transport on certain endogenous transport systems within the brain capillary endothelium (9). Products of biotechnology such as recombinant growth factors and enzymes, monoclonal antibodies (MAb), antisense RNA drugs, short interfering RNA (siRNA), or gene therapy are large molecules which need a type of transport to get through the BBB (8).

4.2.1 Transport mechanisms at the BBB

There are various transport mechanisms at the BBB, as shown in figure 5. Drugs may use these to cross the barrier. As explained above, the paracellular aqueous and the transcellular lipophilic pathways are difficult as a result of the tight junctions and the size of lipid-soluble molecules, respectively (6).

The rest of the molecules need other types of transport across the barrier. Proteins such as glucose, amino acids or purines are transported via a carrier-mediated transport through the BBB (6). Carrier-mediated transport involves the binding of a solute to a protein transporter on one side of the membrane that triggers a conformational change in the protein, resulting in the transport of the substance to the other side of the membrane, from high to low concentration. If compounds need to be moved against a concentration gradient, ATP may provide the energy to facilitate the process (4).

In addition, various nonselective transporters are responsible for the active transport of endogenous and xenobiotic compounds. Pgp is one of the best characterized transporters, but other transporters, such as multidrug resistance proteins (MRP) or other cation or anion transporters, are present as well. These nonselective transporters are mainly responsible for the limited uptake of several drugs in the brain. Carrier-mediated transport and active efflux transport will be explained in the next chapter (6). Furthermore, specific receptor-mediated transcytosis can transport molecules such as insulin or transferrin (6), and this process will be described in more detail in chapter 4.3.

On the other hand, adsorptive transcytosis is a vesicular transport triggered by an electrostatic interaction between a positive charged substance, usually the charged moiety of a peptide, and a negatively charged plasma membrane surface (4) that can carry lectins or cationic proteins across the BBB (6). In the case of some lectins (glycoproteins), the transport across the BBB is mediated by wheat germ agglutinin (WGA). Lectin-bound WGA causes depletion of membrane proteins, which in turn leads to lipid transitions and results in a significant alteration in membrane fluidity and permeability. These membrane modifications make possible its transcytosis. On the other hand, cationic proteins have a net positive charge that binds the anionic sites on the brain capillary endothelium, and this binding triggers the absorptive-mediated endocytosis and transcytosis. Even though, there are proteins that are naturally cationic, like protamine and histone; some proteins can be cationizated to enhance their cellular uptake by adsorptive-mediated transcytosis, like the cationized human serum albumin (HSA) or cationized monoclonal antibodies (MAbs) (7).

Last, cell-mediated transcytosis is a more recently identified route of drug transport across the BBB, although it is a well established mechanism for some pathogens such as HIV entry into the brain. This transport relies on immune cells such as monocytes or macrophages to cross the intact BBB. Unlike other transports, cell-mediated transcytosis is unique in that it can be used virtually for any type of molecules or materials (4).

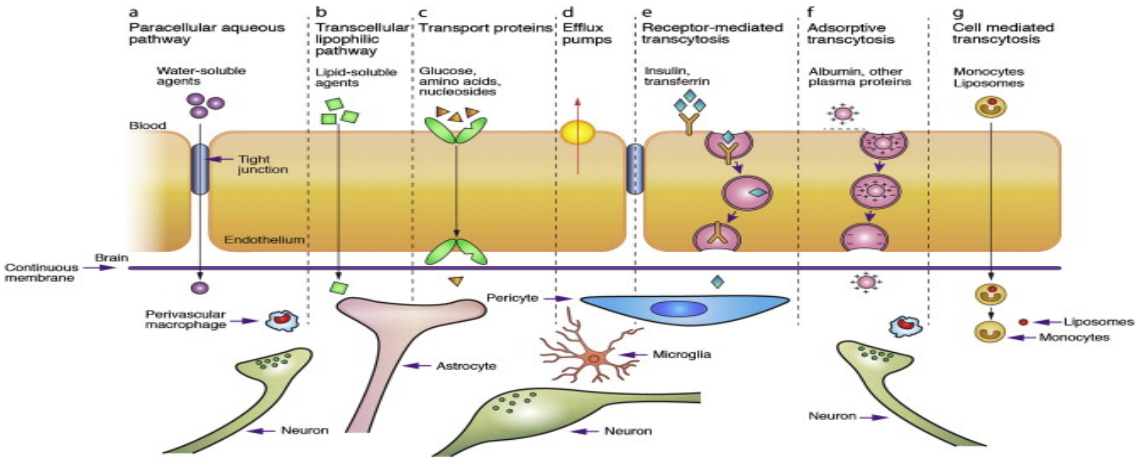

Figure 5. Various transport mechanisms of drugs through the BBB (4)

4.3 TYPES OF TRANSPORTERS AT THE BBB AND DRUG STRATEGIES

There are three broad classes of transporters within the BBB.

4.3.1 Carrier-mediated transporters (CMT) for small molecules

The CMT include the GLUT1 glucose transporter, the LAT1 large neural amino acid transporter, the CAT1 cationic amino acid transporter, the MCT1 monocarboxylic acid transporter, and many other transporters that mediate either the influx of nutrients, hormones, or vitamins from blood into brain, or the bi-directional movement of these molecules between the blood and brain compartments. The CMT system may be expressed at both luminal and abluminal membrane. In the latter situation, another CMT system must function at the abluminal membrane, so as to mediate the transport of the solute across both membranes (10).

4.3.2 Active efflux transporters (AET) for small molecules

The AET include P-glycoprotein and other members of the ATP-binding cassette (ABC) gene family. However, there are several MRP transporters, which also belong to ABC gene family. The energy-dependent ABC transporters at the BBB work in concert with an energy-independent transporter, generally a member of the Solute Carrier (SLC) gene family, to mediate the active efflux of metabolites and drugs from brain to blood. ABC transporter is expressed at one of the two endothelial membranes, while an SLC transporter is expressed at the opposite membrane (10). Figure 6 shows an example of an active efflux transport.

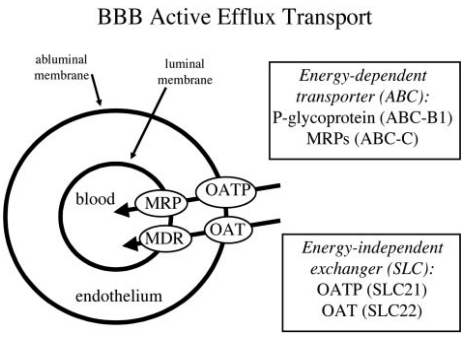

Figure 6. Example of an active efflux transport, it shows the members of the ABC gene family at the luminal endothelial membrane, and the members of the SLC gene family at the abluminal endothelial membrane, although it could be the opposite (9).

4.3.3 Receptor-mediated transporters (RMT) for large molecules

The RMT include receptors such as the IR, transferrin receptor (TfR) (10), leptin receptor (OBR) (2), Fc receptor (FcR), type 1 scavenger receptor (SR) and insulin-like growth factor receptors 1 and 2 (10).

This pathway involves several sequential steps. First, there is binding of the circulating peptide or peptidomimetic MAb to an specific receptor on the luminal membrane and this is followed by endocytosis of the receptor-ligand complex. Following entry into the preendosomal compartment immediately distal to the plasma membrane, the peptide is triaged into one of the following RMT systems (7) shown in figure 7.

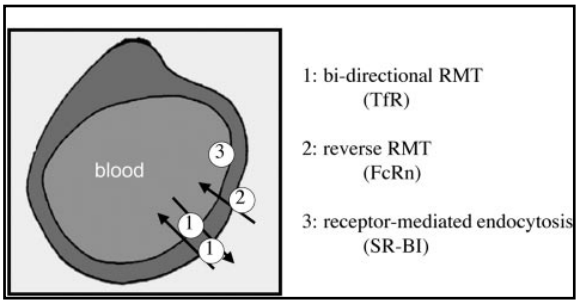

Figure 7. Types of RMT systems. (9)

The TfR is an example of a bidirectional RMT system that causes both the receptor-mediated transcytosis of holo-transferrin in the blood to brain direction, and the reverse transcytosis of apo-transferrin in the brain to blood direction. The neonatal Fc receptor (FcRn) is an example of a reverse RMT system that functions only to mediate the reverse transcytosis of IgG in the brain to blood direction, but not in the blood to brain direction. The type 1 scavenger receptor (SR-VI) is an example of a receptor-mediated endocytosis system that mediates the uptake of modified low-density lipoprotein (LDL) from the blood compartment into the intraendothelial compartments, and this endocytosis is not followed by exocytosis into brain interstitial fluid (9).

4.3.4 Drug strategies using endogenous BBB transport

In drug industry, scientist may alter the structure of a lead molecule to increase CMT affinity. For example, L-Dopa is a form of dopamine, and gabapentin is a form of gaba, and both drugs are effective drugs because their affinity to CMT in the BBB has been enhanced thanks to structure-activity relationships (SAR). Cloning and expression of a BBB AET by a drug developer it can be used to isolate “co-drugs” by High Throughput Screening (HTS). A co-drug inhibits a BBB AET

system, and thereby increases brain permeation of a pharmacologically active molecule that has limited brain penetration, owing to its export form brain via the BBB AET systems (9).

The delivery of large molecule drugs to the brain via the BBB RMT systems requires the use of molecules that can ferry them across. Receptor-specific ligands or peptidomimetic monoclonal antibodies (MAb) can cross the BBB on an RMT system. Such molecules may act as a molecular Trojan horse, and ferry across the BBB a large molecule, such as a recombinant protein, a therapeutic MAb, an antisense agent, a non-viral plasmid DNA therapeutic, or an RNA interference (RNAi) drug (9).

4.3.5 Receptor-mediated transcytosis

It is also known as the molecular Trojan horse approach (1). A BBB molecular Trojan horse, as said above, is an endogenous peptide or peptidomimetic monoclonal antibody (MAb) that crosses the BBB via RMT on one of several endogenous BBB receptors. Transcytosis, means to be endocytosed and exocytosed, thus crossing the BBB. But not all RMT, as described in the anterior chapter, enable transcytosis. The most useful transporters are TfR and IR (11).

For this reason, receptor-mediated transcytosis follows a three-step mechanism. First, the receptor-ligand complex is endocytosed at the luminal (blood) side. Second, the complex moves through the endothelia cytoplasm and third, there is the exocytosis at the abluminal (brain) side (4).

4.3.5.1 Insulin receptor

Insulin is a neuromodulator substance in the central nervous system (7). Insulin is not made in the brain and brain insulin arises from blood via transport across the BBB on the endothelial IR (10). There are also IRs widely distributed throughout the brain and insulin

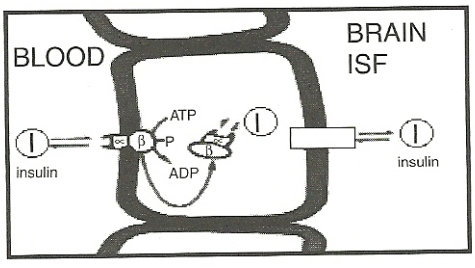

Figure 8. Model for insulin receptor-mediated transcytosis through the BBB. ISF =interstitial fluid (7).

concentrations are readily measurable in the brain (7). Figure 8 shows how insulin can cross the BBB via receptor-mediated transcytosis.

The use of insulin as a Trojan horse could lead to hypoglycemia. Therefore, a peptidomimetic MAb against the human insulin receptor (HIR), HIRMAb, has been used (11).

4.3.5.2 Transferrin receptor

Brain iron originates from transferrin (Tf) in blood, which is transported into brain on the BBB TfR (10). There is evidence that the TfR is expressed at both luminal and abluminal membranes of the brain capillary endothelium (7). The RMT of Tf through the BBB follows a bidirectional system. The holo-transferrin (iron-bound Tf) is transcytosed from blood to brain and then endocytosed into brain cells, once within brain cells, Tf-Fe complex dissociates wherein brain cell ferritin can absorb the iron. Finally the apo-transferrin (iron-free Tf) is then transcytosed back to blood (7).

The use of Tf as a Trojan horse is problematic, because the BBB TfR binding site for Tf is completely saturated by the endogenous Tf in plasma. Therefore, various species-specific MAb have been engineered (11). This antibodies bind to an epitope of the TfR that is different to the transferrin binding site (4).

4.3.5.3 Properties of BBB molecular Trojan horses

The characteristics of BBB molecular Trojan horses must enable the targeting of drugs to brain in pharmacologically active amounts. First, the Trojan horse must target a BBB receptor that is a trancytosis, not an endocytosis, system. IR and the TfR are the most used ones. Second, the Trojan horse should bind the BBB receptor with high affinity. Third, the high affinity binding of the molecular Trojan horse to the receptor must be retained following fusion or conjugation of the drug to the Trojan horse. Fourth, high activity of the drug must be retained following fusion or conjugation of the drug to the Trojan horse. Fifth, the brain uptake of the Trojan horse-drug molecule must be high. Sixth, *in vivo* CNS pharmacologic effects should be demonstrable following intravenous administration. The best assay of Trojan horse efficacy is an *in vivo* model, wherein *in*

vivo pharmacologic effects in brain are demonstrated following the intravenous administration (11).

The most potent molecular Trojan horses are MAbs against mouse or rat TfR and the human HIR. Other ligands have been tested, but they demonstrate limitations in terms of specificity and/or global distribution of the transgene (12). For drug delivery in humans, genetically engineered forms of the HIRMAb have been produced, including a chimeric HIRMAb and a humanized HIRMAb (11).

Table 1. Species-specific peptidomimetic monoclonal antibodies for receptor-mediated transport of drugs across the BBB. (8)

Species to be targeted	Peptidomimetic monoclonal antibodies
Mouse	8D3 rat mAb to mouse transferrin receptor (TfR)
Rat	OX26 murine mAb to rat TfR
Rhesus monkey	83-14 murine mAb to human insulin receptor
Human	Genetically engineered chimeric mAb to human insulin receptor (HIR)

4.4 DELIVERY OF DRUGS TO THE BRAIN THROUGH RMT

There are three major technologies for delivery of drugs to the brain through RMT, and these are shown in figure 9 (8):

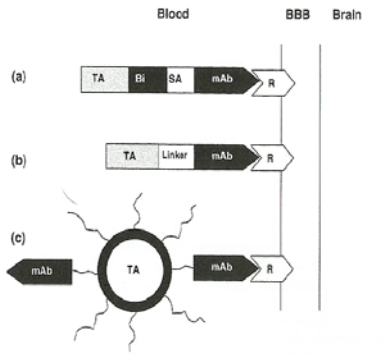

Figure 9. Technologies for delivery of drugs through RMT. (a) Avidin (streptavidin)-biotin technology. (b) Fusion protein technology. (c) Trojan horse liposomes (THLs). SA, streptavidin; Bi, biotin; R, receptor. (8)

4.4.1 Avidin (streptavidin)-biotin technology

Oligopeptides, antisense RNA, or siRNa can be delivered with avidin (streptavidin)-biotin technology (8).

In this approach, a conjugate of the transport vector and avidin or neutral forms of avidin, such as neutral light avidin (NLA) or streptavidin (SA), are prepared in parallel with monobiotinylation of the drug. Owing to multivalancy of avidin or SA binding of biotin, a drug that had higher degrees of biotinylation than the monobiotinylated form would form high molecular weight aggregates upon binding to the vector/avidin or vector/SA conjugate, and would be removed rapidly from blood (7). This is a "2 vial" approach, where the biotinylated drug is prepared in one vial, and the MAb/SA is prepared in the second vial. The two vials are mixed just prior to administration, resulting in rapid formation of the drug-biotin/SA-MAb conjugate (13).

Avidin is a protein which is glycosylated and has a highly cationic charge. Although avidin is a protein found only in birds and is not produced in humans, studies have shown that avidin or SA do not cause immunogenic consequences. Avidin has a rapid plasma clearance. Given the fact of its rapid removal, it would be anticipated that a conjugate of a BBB-targeting vector and avidin would also be rapidly removed from plasma. In order to optimize the plasma pharmacokinetics, subsequent studies employed conjugates of the peptidomimetic MAb and neutral forms of avidin such as NLA or SA, and showed identical rates to that observed with transferrin (13).

Furthermore, the genetic engineering of fusion genes of avidin or SA and BBB drug-targeting vectors also decreases the plasma clearance (13).

4.4.2 Fusion protein technology

Recombinant growth factors and monoclonal antibodies can be delivered with fusion protein technology (14). Fusion or chimeric proteins are created through the joining of two or more genes which originally coded for separate proteins. Translation of this fusion gene results in a single polypeptide with functional properties derived from each of the original proteins (14).

Recombinant protein neurotherapeutics can be delivered across the BBB following the genetic engineering, expression and purification of recombinant fusion proteins. The neurotrophin-antibody fusion protein is an example. Neurotrophin is a therapeutic protein that cannot be transported. In this approach, neurotrophin is fused to the carboxyl or amino terminus of either the heavy or the light chains of the genetically engineered human insulin receptor monoclonal antibody (HIRMAb). A fusion protein of the chimeric HIRMAb and a neuroprotective neurotrophin is genetically engineered, expressed and shown to retain the bifunctional properties of the fusion protein. The fusion protein can bind both the HIR with high affinity to enable transport across the BBB and then the neurotrophin receptor on brain cells, to induce neuroprotection (15).

MAb-based therapeutics may also be delivered across the BBB with fusion protein technology. An example, as seen in figure 10, is the genetic engineering, expression, and validation of a fusion protein of the chimeric HIRMAb and a single chain Fv (ScFv) antibody to the Aβ amyloid peptide of Alzheimer's disease (AD). Anti-Aβ antibodies are potential therapeutics of AD, as these agents disaggregate the amyloid plaque of AD following the intra-cerebral injection of the anti-Aβ antibody. The aim of either active or passive immunization therapy of AD is to use MAb-based therapeutics to deplete the brain of Aβ amyloid plaque. However, in the case of either active or passive immune therapy of AD, it is necessary for the anti-Aβ MAb to cross the BBB in both the blood to brain and brain to blood directions. There is no IgG transporter at the BBB to mediate the blood to brain transport of these large molecules. Therefore, IgG therapeutics do not cross the BBB in the blood to brain direction, since these molecules lack affinity for any BBB receptor/transporter. However, MAb-therapeutics can be made to cross the BBB via RMT following the re-engineering of these molecules as fusion proteins with a BBB molecular Trojan horse. The structure of a genetically engineered fusion antibody of the HIRMAb and the anti-Aβ ScFv is shown in figure 10. This fusion antibody is a tri-functional molecule that is comprised of three domains. The first domain is the HIRMAb at the head of the molecule, which mediates the RMT of the fusion antibody from blood to brain across the BBB via the endogenous BBB HIR. The second domain is the anti-Aβ ScFv at the tail of the

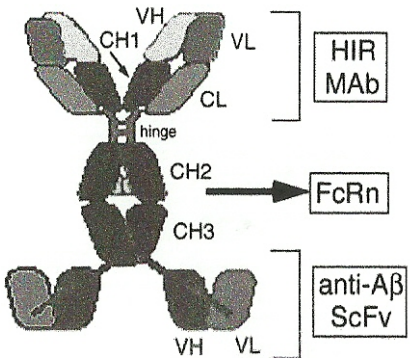

Figure 10. Structure of a genetically engineered fusion protein of the chimeric HIRMAb and a single chain Fv (ScFv) antibody to the Aβ amyloid peptide (15).

fusion antibody, which allows binding to and disaggregation of Aβ amyloid plaque within the brain behind the BBB. The third domain is the CH2-CH3 interface in the midsection of the molecule, which is the binding site for the BBB FcRn. The BBB FcRn mediates the efflux of the fusion antibody in the brain to blood direction via reverse transcytosis across the BBB *in vivo*. All three functionalities of the fusion antibody are retained following genetic engineering and expression of this molecule. The intra-cerebral injection of the fusion antibody into double transgenic AD mouse brain results in a 40% clearance of Aβ amyloid plaque within 48h (15).

4.4.3 Trojan horse liposomes (THLs technology)

Non-viral plasmid DNAs or genes encoding shRNAs are examples of large molecules that may be shuttled to the brain with THLs. This technique will be explained in detail in the following chapters.

4.5 GENE THERAPY OF THE CENTRAL NERVOUS SYSTEM

Nowadays just few brain disorders can be treated effectively, and these include affective disorders, chronic pain, epilepsy and migraine headache. Parkinson's disease (PD) patients are given L-DOPA for dopamine replacement therapy. L-DOPA is an example of a BBB drug targeting strategy. However, there is no treatment that stops the neurodegeneration of PD. The same happens with other neurodegenerative diseases such as AD, Huntington's disease, and amytrophic lateral sclerosis. In the case of multiple sclerosis, the actual therapy does not permanently stop the progression. The human immunodeficiency virus infects the brain early in the course of acquired immune deficiency syndrome and the highly active antiretroviral therapy (HAART) which is effective in the periphery, does not access completely into the brain due to the efflux transport of some HAART drugs. Other serious brain disorders such as brain cancer or stroke lack effective drug therapy (9).

Many of these brain diseases are due to genetic mutations. Some examples include Huntington's disease, familial AD or amyotrophic lateral sclerosis. For this reason, gene therapy is an approach that seems to fulfill the need of an effective treatment. However, the development of genes as drugs brings into focus the need to discover new techniques to target genes across the BBB. To

develop new strategies, a thorough understanding of the BBB structure and functions is primordial (7).

The most widely applied method of brain gene delivery is craniotomy, with the intracerebral introduction of therapeutic genes formulated in either viruses or cationic liposomes. In parallel with the craniotomy-based methods of drugs or gene delivery to the brain, there is a line of investigation aimed at disrupting the BBB. Both, disrupting the BBB and craniotomy, are invasive procedures and may induce chronic neuropathologic effects in brain (7).

Another approach is the noninvasive administration of gene medicines to the brain via BBB endogenous transport systems. This approach has two advantages (7). First, a global distribution of the transgene throughout the brain (16). In contrast, when gene medicines are delivered by craniotomy approaches, the treatment volume is reduced due to the limitations of diffusion within the brain. The second advantage is that the gene formulation can be given by an intravenous or subcutaneous route of administration that is no more invasive than that used by insulin-dependent patients. The formulations of gene medicines that are used in present practice are based on viruses, cationic liposomes, or naked DNA/polylysine conjugates (7). Viruses have been used as brain DNA delivery systems with disappointing results associated with preexisting immunity, immunological response induced by viral coat proteins, and inflammation that led to demyelination. Therefore, nonviral approaches, such as cationic liposomes, appeared (16). In this approach, cationic lipids form complexes with DNA (7). Cationic liposomes are widely used for transfection of DNA *in vitro*. However, cationic lipid-DNA complexes *in vivo* are unstable or form large molecular weight aggregates that deposit in the pulmonary vascular bed, which decreases its bioavailability (16). On the other hand, naked DNA/polylysine conjugates are a result of the conjugation between a receptor ligand and a polylysine, using approaches that include either chemical linkages or avidin-biotin technology. The polycationic polylysine then binds to the polyanionic DNA and goes through a receptor-mediated transport. However, although it proves to be effective in cell cultures, it is apparently less effective *in vivo* (7).

An alternative is the THL technology that will be explained in the next chapter.

4.6 TROJAN HORSE LIPOSOMES

THLs are pegylated liposomes containing a supercoiled plasmid DNA molecule or genes encoding shRNAs in the interior of the liposome (16). Therefore, it is an appropriate technique for gene therapy.

4.6.1 Structure of a Trojan horse liposome (THL)

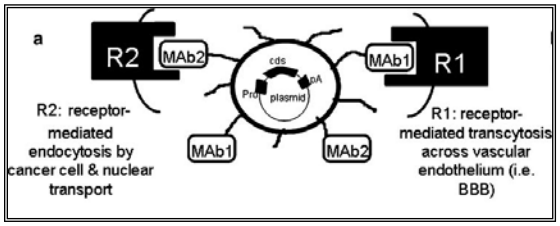

Figure 11. Engineering of a Trojan horse liposome. A supercoiled plasmid DNA is encapsulated in the interior of the THL. The plasmid encodes for a coding sequence (cds), the expression of which is under the influence of a promoter (pro), that is, SV40, and a polyadenylation sequence (pA). The surface of the liposome contains a lot of strands of polyethylene glycol (PEG) to stabilize the complex in blood. About 1-2% of these strands are conjugated with MAbs, which trigger transport (16).

Figure 11 shows the structure of a THL encapsulating a plasmid DNA. A supercoiled plasmid DNA is encapsulated in the interior of the THL. The plasmid encodes for a coding sequence, the expression of which is under influence of a promoter, and a polyadenylation sequence. The promoter eliminates ectopic transgene expression and enables the expression in targeted regions of the CNS (16). The encapsulation of DNA and sizing of the THL are completed by forced extrusion through a series of polycarbonate filters of reduced pore size to form liposomes of 80-100 nm diameter. The DNA in excess, i.e. either free DNA or DNA bound to the exterior of the liposome, is removed from the preparation by digestion with a mixture of DNA endonuclease I and exonuclease II to avoid interference with the conjugation to the target MAb (17).

In order to achieve maximum targeting, liposomes should remain in the systemic circulation for a relatively long time, although several formulations of liposomes used in the past were rapidly removed from the circulation by the reticuloendothelial system (RES). Furthermore, it is known that coating liposomes with antibodies leads to enhanced uptake of the immunoliposomes by the RES. Liposomes containing amphipathic polyethylene glycol (PEG) derivatives are not readily taken

by the macrophages in the RES. PEG is useful because of its easy preparation, low cost, controllability of molecular weight and linkability to lipids or proteins including antibodies. For this reason, the surface of the liposome contains thousands of strands of polyethylene glycol (PEG) which stabilize the complex in blood. Approximately 1-2% of the PEG strands are conjugated with a MAb, which triggers transport of the THL across barriers *in vivo*. MAbs are almost always species specific, thus THLs are engineered with one or different type of MAb to target both the BBB and brain cells, depending on the origin of the receptor (animal or human) or in case the receptors in both barriers are different. The targeted receptor must be present in both BBB and brain cell membranes to transport drugs into the brain cells. IR and TfR are highly expressed in both barriers, and so MAbs against these two receptors enable the sequential receptor-mediated transcytosis of the THL across the BBB followed by the receptor-mediated endocytosis of the THL into the brain cells (12).

THLs are engineered with a mixture of naturally occurring lipids that has been optimized for the encapsulation of the plasmid DNA (12).

Liposomes are comprised of 93% of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospocholine (POPC), 3% of didodecyldimethylammonium bromide (DDAB), 3% of distearoylphosphatidylethanolamine (DSPE)-PEG2000, and 1% of DSPE-PEG2000-maleimide. The maleimide functional group allows for covalent conjugation of a thiolated MAb via a stable thioether linkage (12).

Plasmid DNA-based gene therapy with THL technology involves episomal gene expression and must be given on a chronic basis, which arises concerns about potential toxic side effects from repeated dosing. However, toxicity studies have shown that there is no toxicity as a result of chronic dosing (12).

Formulation optimization can enhance the efficacy of this technique as for example the recent use of ethanol-mediated DNA condensation to increase the efficiency of DNA encapsulation (18). Avidin-biotin technology may also facilitate conjugation of ligands to THLs (19).

Another application suitable for the THL technology is RNA interference (RNAi), which represents one of the most potent mechanisms of gene downregulation. RNAi has been extensively demonstrated in cell culture by lipofection with RNA duplexes. However, the delivery of short RNA fragments into cells *in vivo* in mammals is problematic owing to the rapid degradation of the RNA.

Short hairpin RNA (shRNA) mimics the structure of the RNAi duplex, and shRNA can be produced in cells following the delivery of expression plasmids encoding the shRNA. This shRNA is then processed in the cell by the enzyme dicer to form an RNA duplex with a 3'-overhang and this short RNA duplex mediates RNAi or post-transcriptional gene silencing. RNAi activity has been shown in cell culture by transfecting cells with plasmids producing shRNAs, using gene delivery systems comprised of either cationic polyplexes or retroviral vectors. However, as it has been said above, cationic DNA polyplexes (i.e. lipofection) or retroviral vectors do not cross the BBB (17).

RNAi-based gene therapy offers promise for the treatment of cancer and other brain disorders like AD. Recent studies demonstrated its efficacy directed at the human epidermal growth factor (EGFR) in an experimental human brain tumor model in mice (17).

In this case THLs may be engineered with shRNA expression vectors driven by the U6 promoter and encoding a T5 terminator sequence for RNA polymerase III after the 3'-end of the shRNA (17).

4.6.2 Important aspects of THL design

There are three important aspects in the design of the THL that may substantially affect the levels and specificity of the expression of the exogenous gene to be delivered (17):

1) Targeting ligand: the targeting ligand represents an important factor in determining the levels of expression of the exogene to be delivered with the THL. Studies then show that IR represents the preferred pathway for delivery of transgenes to cells with this technology.

2) Introduction of regulatory sequences in the plasmid DNA: cis-regulatory sequences are important regulatory elements that modulate the expression of transcripts. These sequences are short (~200bp) and do not affect the final nucleotide load to THLs.

3) Use of tissue specific promoters in the plasmid DNA: these promoters prevent ectopic expression and allow brain cell-specific expression of the transgene of interest *in vivo* in animal studies.

4.6.3 Technique validation

The THL plasmid DNA gene transfer technology has been validated in multiple animal models in mice, rats, and Rhesus monkeys, and so it is possible to deliver transgenes to brain. The ectopic expression of the transgene can be eliminated by the combined use of THLs and plasmid DNA engineered with tissue-specific gene promoters. Transgene expression is shown to be reversible because the plasmid DNA is not integrated into the host genome and this property is advantageous, since the integration of viral genomes into the host DNA can lead to mutagenesis. An increase in the duration of plasmid DNA expression is possible with the incorporation of chromosomal elements to the plasmid DNA. THLs can also be administered chronically without toxicity or immune reactions (16).

4.7 RESEARCH AND FUTURE PERSPECTIVES

In vivo applications of THLs were initially investigated with luciferase and lacZ reporter genes. THLs were constructed with the expression plasmid of the luciferase reporter gene, and engineered with either the TfRMAb for rodents or the HIRMAb for Rhesus monkeys (16). Tissue-specific gene expression with the combined use of THLs and the opsin promoter was also demonstrated *in vivo* in the Rhesus monkey (20). *In vivo* efficacy of THLs has been investigated in a model of mucopolysaccharidosis (MPS), a lysosomal storage disorder that affects the CNS. The non-viral plasmid DNA encoded a lysosomal enzyme, β-glucuronidase (GUSB). After intravenous Trojan horse liposome administration there was an increase of brain GUSB enzyme activity (21). The therapeutic efficacy of THLs has been also demonstrated *in vivo* in a model of PD (22). PD is associated with a loss of dopaminergic neurons in the substantia nigra and the rate limiting enzyme in the synthesis of dopamine is tyrosine hydroxylase (TH), thus, a potential treatment for PD is TH gene replacement therapy. In that study, pegylated immunoliposomes (PIL) nonviral gene transfer technology enabled normalization of striatal tyrosine hydroxylase activity. On the other hand, plasmid DNA that produces short hairpin RNA for the purposes of silencing genes via pegylated immune liposome (PIL) gene targeting technology in brain cancer was first studied with the luciferase gene as the target and the result showed an effective gene therapy delivery. Following these results, the ability of knocking down the EGFR which is expressed in 90% of

primary brain cancers, was evaluated. This resulted in an increase in survival time in adult mice (23).

A lot of research has been done using the avidin-biotin approach. Table 2 summarizes *in vivo* CNS pharmacologic effects in brain following intravenous administration of large molecule drugs using this technique (11).

For drug delivery in humans, genetically engineered forms of the HIRMAb have been produced, including a chimeric HIRMAb and a humanized HIRMAb. Innovation in brain drug development is now focused on the engineering of bifunctional fusion proteins of a BBB Trojan horse and the therapeutic protein. To date, HIRMAb fusion proteins have been engineered for multiple types of biopharmaceuticals as shown in Table 3 (24). Table 4 shows recent *in vivo* pharmacological effects in the brain following the administration of Trojan horse fusion proteins (24).

The future step is to translate these technologies to humans with the use of human-specific antibodies that are genetically engineered to reduce immunogenicity such as the chimeric and humanized HIRMAb (16). Finally, it is worth adding a comment about a recent *in vivo* study which opens a debate in whether monoclonal antibodies targeting the TfR undergo receptor-mediated transcytosis into the brain. Researchers argued that many studies assume this transcytosis with the use of indirect outcome measures such as protein expression or enzymatic activity. Studies that examined direct evidence by using radiolabelling and immunohistochemical approaches concluded that there was transport into the endothelial cells but not exocytosis into the brain (25). Nevertheless, studies performed about delivery of drugs through Trojan horse liposomes and other strategies directed to avoid BBB are of relevant importance for the treatment of CNS disorders.

Table 2 ***In vivo*** **CNS pharmacologic effects in brain following intravenous administration of large molecule drugs using the avidin-biotin technique.**

Drug	*In vivo* CNs pharmacological effect	Reference
VIP	Increase in cerebral blood flow	(26)
BDNF	Complete neuroprotection of hippocampal CA1 neurons in translent forebrain ischemia	(27)
BDNF	65-70% reduction in stroke volume in permanent or reversible middle cerebral artery occlusion	(28, 29)
FGF-2	80% reduction in stroke volume in permanent middle cerebral artery occlusion	(30)
$A\beta^{1-40}$	Imaging brain amyloid *in vivo* with peptide radiopharmaceutical	(31)
EGF	Early detection of brain cancer *in vivo* with peptide radiopharmaceutical	(32)
PNA	Imaging gene expression *in vivo* with antisense radiopharmaceutical	(33)

VIP, vasoactive intestinal peptide; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; PNA, peptide nucleic acid.

Table 3 **IgG Fusion proteins engineered for targeted brain delivery**

Category	Protein therapeutic	Reference
Neurotrophins	BDNF	(34)
	GDNF	(35)
	Erythropoietin (EPO)	(36)
Enzyme	IDUA	(37)
	IDS	(38)
	GUSB	(39)
	Paraoxonase (PON)-1	(40)
Decoy receptor	Tumor necrosis factor receptor (TNFR) type II	(41)
Monoclonal antibody	Antiamyloid antibody (AAA)	(42)
Other	Avidin	(43)

BDNF, brain-derived neurotrophic factor; GDNF, glial-derived neurotrophic factor; IDUA, α-L-Iduronidase; IDS, Iduronate-2-sulfatase

Table 4 ***In vivo*** **pharmacological effects in the brain following the administration of Trojan horse fusion proteins**

Species	Route	Drug	Disease	Reference
Rat	Intracranial	EPO	Stroke	(44)
Mouse	Intravenous	EPO	Stroke	(45)
	Intravenous	EPO	Parkinson's disease	(46)
	Intravenous	GDNF	Parkinson's disease	(47)
	Intravenous	AAA	Alzheimer's disease	(48)
	Intravenous	IDUA	Hurler's syndrome	(49)

EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; AAA, antiamyloid antibody; IDUA, α-L-Iduronidase

5. CONCLUSIONS

- The BBB is formed by endothelial cells, pericytes, astrocytes and a basement membrane and these components are responsible for its particular properties.
- Endothelial cells from brain capillaries have specific features that help to maintain a restrictive permeability.
- Endothelial cells and pericytes share a common basement membrane which controls movement of charge solutes and serves as a repository of growth factors.
- Astrocytes are comprised of various foot processes that can contact different structures and this property makes them capable of maintaining the optimum neuronal environment.
- The BBB has a restricted transport. The paracellular pathway is limited by tight junctions and only small lipid soluble substances can pass by passive diffusion. The rest of molecules, even if they are essential, need an specific transporter.
- The presence of efflux transporters in the BBB makes the transport of its substrates even more difficult. This protective property is a disadvantage for most drugs.
- Other types of transports across the BBB are the carrier-mediated transport, adsorptive-transcytosis, receptor-mediated transcytosis and cell-mediated transcytosis.
- Carrier-mediated transporters serve mainly to transport nutrients into the central nervous system and RMT include the insulin or TfRs which enable transcytosis of its respective substrates into the brain, transcytosis from the brain into blood and endocytosis into endothelial cells.
- Some drug strategies may be used to enhance transport across the BBB such as modifying the structure of a drug to act as a substrate of a carrier-mediated transporter, inhibiting active efflux system or using receptor-specific ligands or MAbs, as molecular Trojan horses, to ferry drugs into the brain via receptor-mediated transcytosis.

- A useful molecular Trojan horse needs to achieve certain necessary properties and for this reason the most used ones are MAbs.

- Avidin(streptavidin)-biotin, fusion protein and Trojan horse liposomes are three different technologies used to deliver drugs through receptor-mediated transcytosis. As gene therapy becomes a new approach to treat many brain diseases, the Trojan horse liposome technique seems to have advantages to other formulations used in present practice.

- A Trojan horse liposome is comprised of three principal elements. First, a liposome which can encapsulate both plasmid DNA or shRNA. Second, PEG which stabilize the complex in blood and third, a MAb which enables the binding to an specific receptor. Besides, the construction of a THL has been optimized to enhance its efficacy and the technique has been validated in multiple animal models.

- *In vivo* efficacy of THL has been demonstrated in many animal studies and in various disease models and the same happens with the avidin(streptavidin)-biotin technology.

- Innovation in drug development is focused on the fusion protein technique. To date, HIRMAb fusion proteins have been engineered for multiple classes of biopharmaceuticals.

- Most of the research that has been done seems to indicate that these techniques can be useful to treat CNS disorders.

- A future step would be reaching clinical trials using chimeric o humanized MAbs, which could be useful in crossing the human BBB.

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