Chapter 1

Introductory remarks and Scope of the thesis
Nutrients and medicines, when present in the blood stream, may diffuse freely to and into surrounding tissues, but not so from the blood into the brain. Indeed, contrary to the homeostasis of the extracellular environment in many other tissues, that within brain is strictly regulated. Thus, neurons, constituting approx. 90% of all cells within the brain, are protected from potential toxic compounds and at the same time are optimally supplied with glucose, oxygen and other nutrients. Of critical importance in executing and maintaining this dual role of careful regulation of in- and outbound transport at the border of the two interfaces (i.e., blood and brain), is the blood-brain barrier (BBB) – an anatomical organization of tightly sealed endothelial cells, basal lamina and astrocytic end-feet (Fig. 1). Molecules, depending on their physical properties, may enter endothelial BBB cells by direct diffusion through the luminal plasma membrane, via membrane-embedded transporters or by means of other internalization processes, linked to transcellular transport. Yet, already at the cell surface of the endothelial cells the first line of protection is active, in particular efflux pumps such as those belonging to the family of multidrug resistant proteins, that might recognize exogenous molecules as harmful, resulting in their expulsion back into the circulation. However, even molecules escaping this first line of defense, require an appropriate transcellular transport route that avoids their confrontation with proteolytic and other degradative enzymes, before reaching the abluminal membrane, which has to be crossed to finally enter the brain.

However, the BBB not only protects the brain from an undesirable invasion of exogenous compounds and biological entities, but at the same time precludes delivery of therapeutics in case of efforts to cure brain-related diseases, like Alzheimer, Parkinson and brain tumors. With very few exceptions, current pharmaceuticals applied in the treatment of brain diseases acquire access into the tissue by passive diffusion across the BBB. This requires the compounds to meet the criteria of being lipid-soluble, small molecules (< 500 Da) with a restricted number of hydrogen-bond forming moieties. Indeed, most of the currently applied central nervous system (CNS) treatment modalities are either well known psychoactive natural products or synthetic compounds with accidentally discovered activities (barbiturates, benzodiazepines, etc.). Once pharmacological activity is attributed to a certain moiety of the drug molecule, its physicochemical and pharmacokinetic properties might be adjusted by chemical modifications to further improve brain penetration. For example, acetylation of morphine to heroin produces a dramatic increase in the pharmacological response, which is related to the enhanced lipophilicity of heroin over morphine, and as a result, its facilitated diffusion. In passing, for such drugs to become effective and economically of interest, bioavailability is another obvious criterion to be met. In case of morphine, its relatively low bioavailability is compensated by its high potency, making it one of the most robust analgesics. The successful marketing of many other psychoactive drugs, such as the antidepressants fluoxetine and sertraline hydrochloride, is based on the very same criteria.

Nearly 1,000 known genetic and neurodegenerative diseases affect the brain. To
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Figure 1: Anatomical and functional organization of the Blood-brain barrier. (a) Orchestrated by neurons, different cell types act in harmony to assure optimal conditions for normal brain functioning and formulate the modern concept of the BBB – the neurovascular unit. Endothelial cells of brain capillaries are neatly zipped with tight and adherence junctions. The basal lamina additionally supports the structure. Pericytes, cells with smooth muscle-like properties, regulate dynamically the blood flow. Astrocytic end-feet mediate communication with deeper brain structures. (b) Glucose – the primary energy substrate – and building blocks for the brain own needs, are delivered with specialized transporters. Foreign molecules or endogenous threats are exported back into the capillary lumen using efflux pumps (P-glycoprotein, Multidrug Resistance-associated Proteins) or internally digested (MAO – monoamine oxidase, γ-GT – γ-glutamyl transpeptidase). Nutrients (LDL, iron), hormones (insulin, leptin), signaling molecules (interleukins) access the brain via receptor-mediated transport across the blood-brain barrier. (adapted from Wikipedia)

potentially cure such diseases advanced therapies are commonly based on targeting the molecular cause of the disease, rather than merely treating symptoms. Thus, whereas small, hydrophobic drugs often suffice for (symptomatic) treatment of psychological disorders, a more advanced approach may rely on neuron(s) specific correction of protein expression or functional regulation.
Figure 2: **Receptor-mediated transport of drug-loaded nanoparticles across the Blood-brain barrier.** Nanoparticles, decorated with surface ligands, bind specifically to endothelial receptors, resulting in their uptake (1). Intracellularly, the nanoparticles are transported in vesicles, that move in apical (luminal) to basal (abluminal) direction (2), escaping degradation in lysosomes. When the opposing membrane is reached, the vesicle opens towards the brain tissue and releases the nanoparticles (3).

Hence, emerging treatment modalities that involve the use of proteins, antibodies, or (small interference) RNAs necessitate the design of ‘proper’ formulations for reaching the desired target site. Translated into ready-to-use pharmaceuticals, the effect-producing compound should be well protected (i.e., its structural and functional integrity should be maintained) on its way from the site of administration to the site of action. For example, in case of oral administration the compound should be resistant to digestive enzymes. Following intravenous administration, it should be immunologically inert and not be modulated by interactions with serum proteins. Furthermore, the compound should be insensitive to a first-pass effect (i.e., should not be metabolized in the liver), and capable of crossing biological barriers to assure optimal bioavailability, which is also attained by facilitating a preferential accumulation in the desired tissue. The latter can be achieved by appropriate targeting, so as to minimize adverse effects due to unfavorable tissue distribution.

All these issues and challenges are addressed within the research field known as Nanomedicine. With a focus on drug delivery into the brain, the concept of nanoparticle-mediated drug transport is schematically depicted in Fig. 2.

In this simplified scheme, a nanoparticle, which could be a lipid or polymer-
based carrier, encloses within its aqueous volume a drug solution or, alternatively, drugs of a more hydrophobic nature could be part of the lipidic or polymeric boundary phase. To ensure specific targeting of the carrier towards the endothelial cells that constitute the BBB, the surface of the nanoparticle is decorated with ligands with specificity towards distinct BBB receptors, specifically receptors that after internalization engage in transcellular transport, known as transcytosis. Thus when the administered nanoparticles eventually reach the brain capillaries, they should bind to the endothelial cells, followed by receptor-mediated endocytosis, thereby ensuring subsequent particle processing along the pathway of transcytosis, causing the release of the nanocarrier at the opposing membrane surface that faces the brain tissue. The work presented in this thesis aimed at identifying novel transcytotic receptors and their corresponding ligands at the BBB to enable the development of improved nanocarriers for specific delivery of drugs into the brain.

The current trends in the development of transport strategies for advanced drug delivery into the brain are summarized in Chapter 2. The use of invasive techniques to produce transient openings in the BBB for therapeutic applications is losing popularity. Instead, efforts are focused on designing ‘clever’ nanofomulations, that are able to target the brain capillaries, addressing apical receptors, and inducing transcellular transport.

In Chapter 3, novel receptors with a potential interest for receptor-mediated transcellular transport of nanocarriers are proposed. In search for these novel entities, efforts were made to isolate glycosylphosphatidylinositol anchored proteins (GPI-APs) from human brain endothelial cells that were subsequently identified by mass spectrometry. The brain tissue specificity of one of the identified GPI-APs, i.e. prion protein, led us to investigate its transcytotic potential at the BBB.

In Chapter 4, the extent of transcytosis and the nature of the intracellular compartments involved in the transport of nanoparticles across an in vitro BBB cell model were studied. Nanoparticles, non-targeted and charge- (PEI) or ligand-modified (prion), were investigated. The possible involvement of caveolae, i.e., membrane domains specifically enriched in cholesterol and GM1, in transcytosis and the potential of the prion protein to trigger transcytosis of nanoparticles, prompted us to search for GM1-binding and prion-binding ligands by means of a phage display screening procedure. In this manner, several GM1- and prion-binding peptides were identified, synthesized and subsequently coupled to nanoparticles composed of polymers (polymersomes). The interaction and transcellular transport of the targeted polymersomes were investigated in both an in vitro BBB cell model and in vivo (Chapter 5).

A detailed understanding of the transcellular transport mechanism(s), i.e., the driving force in apical to basal cargo movement, is essential for the rational design of nanoparticles for transcellular drug delivery. In Chapter 6, the transcytosis of GM1-targeted polymersomes, displaying high affinity to BBB endothelial cells, was studied in detail. The data reveal a mechanism of intracellular processing
and basolateral expulsion of nanoparticles that parallels the pathway of multivesicular bodies (MVBs) formation.

In Chapter 7, the natural migratory properties of neural stem cells (NSCs) across the BBB were characterized in vitro with the aim of devising a natural biological carrier for drug delivery into the brain. To preserve the specificity of the physiologically relevant interaction between NSCs and the BBB, membrane vesicles were prepared from NSC plasma membranes. Their capacity to traverse the in vitro and in vivo BBB and hence to serve as potential drug carriers, was studied. Finally, the overall results and future perspectives are discussed in Chapter 8.