

Invited review

Targeting blood-brain-barrier transcytosis – perspectives for drug delivery



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ABSTRACT

Efficient transcytosis across the blood-brain-barrier (BBB) is an important strategy for accessing drug targets within the central nervous system (CNS). Despite extensive research the number of studies reporting successful delivery of macromolecules or macromolecular complexes to the CNS has remained very low. In order to expand current research it is important to know which receptors are selective and abundant on the BBB so that novel CNS-targeting antibodies or other ligands could be developed, targeting those receptors for transcytosis. To do that, we have set up a proteomics- and transcriptomics-based workflow within the COMPACT project (Collaboration on the Optimization of Macromolecular Pharmaceutical Access to Cellular Targets) of the Innovative Medicines Initiative (IMI) of the EU. Here we summarise our overall strategy in endothelial transcytosis research, describe in detail the related challenges, and discuss future perspectives of these studies.

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Reaching macromolecular drug targets in the brain has remained a great challenge in biomedical research and clinical sciences despite rigorous efforts for more than two decades. This is because the blood-brain-barrier (BBB) represents one of the most tightly regulated and complex biological barriers in mammals with a function to protect the brain from damage of unwanted blood-borne molecules. This is essential for maintaining correct neurological functions. However, in case of neurological disease, the delivery of therapeutic bioactive macromolecular compounds to

affected areas of the central nervous system (CNS) is highly desired but notoriously difficult to achieve, especially when the general protective barrier function of the BBB is to be maintained. This explains the failure of clinical trials of numerous biologicals targeted to the CNS (Pardridge, 2015a).

To overcome this problem, many attempts have been made to find specific targeting ligands, endogenous proteins or antibodies against certain receptors (e.g. transferrin receptor (TFRC) and insulin receptor (INSR)) that could be used to induce receptor-mediated transcytosis of therapeutic biologicals across the BBB. For example, TFRC antibody chimeras have been successfully used in animal models of Parkinson's disease, stroke, Alzheimer's disease and lysosomal storage disease for enhancing brain accumulation of therapeutic proteins (Watts and Dennis, 2013; Pardridge, 2015b;

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Lajoie and Shusta, 2015; Niewoehner et al., 2014). INSR targeted antibody and therapeutic protein fusions have shown great potential in non-human primates for treating mucopolysaccharidosis type I (MPS-I) and Alzheimer's disease, and also in brain delivery of GDNF, TNFR, erythropoietin and paraoxonase-1 (Lajoie and Shusta, 2015). To deliver drugs across the BBB independently of specific targeting ligands, the barrier can be temporarily opened in some conditions by using microbubbles and focussed ultrasound. Initial safety and efficacy data, incl. the treatment of a brain tumour patient, are promising and indicate that concerns about risks related to the loss of BBB integrity could be managed (Piper et al., 2016; Lipsman et al., 2014; Sunnybrook). However, the advantages and disadvantages compared to the receptor-mediated BBB transcytosis need further thorough evaluation.

Receptor-mediated transcytosis across the BBB is an attractive strategy for reaching targets within the CNS for various reasons. For example, it could enable the transport of macromolecules or macromolecular complexes that would be unfeasible in carrier-mediated transport systems (i.e. channels and carriers) (Pardridge, 2012). Furthermore, alternative CNS entry routes such as via injection into the cerebrospinal fluid (CSF) are generally regarded as unfavourable because it requires hospitalization of the patient, because of the risk of infection, and due to the rapid drug export from the CSF back to blood circulation (Pardridge, 2012).

The architecture of brain microcapillary network is favourable to receptor-mediated transcytosis too. The distance between adjacent vessels in the brain is in the range of 40–60 μm (Pardridge, 2015a; Nicholson, 2001; Duvernoy et al., 1983). Taking account the size of neurons, the cell body of a neuron is no further than 10–20 μm from the closest capillary on average (Wong et al., 2013; Schlageter et al., 1999). This means that the diffusion distance of the traversed macromolecular compounds is short enough to diffuse to neurons in the time scale from seconds to tens of minutes (but could be longer in a crowded environment), depending on the molecular weight of the compound (Atkins and de Paula, 2006). However, achieving transcytosis across the BBB requires thorough understanding of brain microendothelial cells surface proteins.

One of the crucial questions is how to find receptors which are (a) selective to the brain microvasculature, (b) expressed at sufficiently high levels and (c) could be used for transcytosis. Furthermore, once these receptors have been identified, it is an additional challenge to find ligands/antibodies capable of taking advantage of transcytosis of these receptors for drug delivery. These questions address the issue how to reach the interstitial space of the brain tissue. However another layer of complexity is added when the drug target is in the intracellular environment of neurons, requiring cell uptake and delivery to required cell compartment.

Within the COMPACT project (Collaboration on the Optimization of Macromolecular Pharmaceutical Access to Cellular Targets) of the Innovative Medicines Initiative (IMI) of the EU (<http://www.imi.europa.eu/content/compact>), we have developed and set up a workflow to identify brain microvascular cell surface specific receptors (Fig. 1) with an aim to use them for drug delivery across the BBB. The workflow is based on analysing the cell surface proteome and whole cell transcriptome.

The need to thoroughly characterise individual molecular constituents of the BBB for finding novel BBB-specific receptors for drug delivery has been recognized by many. Recent advances in proteomics and transcriptomics have enabled detailed comparisons of human and small animal model BBB properties, which in turn helps to select human relevant brain-specific receptors for drug delivery with a possibility to study them in animal models (Ohtsuki et al., 2014; Enerson and Drewes, 2006; Zhang et al., 2014). Even though the gene expression profile of BBB endothelial cells is heterogeneous (Macdonald et al., 2010), by analysing brain

microvascular endothelial cell transcriptome, it is possible to find leads for brain-specific transporters and other proteins with drug delivery potential (Daneman et al., 2010). Recent data indicate that by combining transcriptomic analysis and proteomic profiling of brain endothelial cells in mice, new target receptors can be discovered that mediate therapeutic antibody transcytosis to brain more efficiently than TFRC and INSR binding antibodies in mice (Zuchero et al., 2016). For exploiting unknown receptors for BBB transcytosis, assays such as phage display panning has been used (Jones et al., 2014; Muruganandam et al., 2002; Smith et al., 2012).

In order to identify brain microvasculature selective receptors it is essential to profile microvascular cells also from other tissues for comparison. Because many nanoparticle drug delivery formulations accumulate in liver and lung, the first choice is to include microvascular cells from these organs to the comparison (Fig. 1). However, it must kept in mind that drug formulations can accumulate in those vessels not only in receptor-specific manner; nonspecific physical capture can be important too. There are two principal strategies for profiling the cells, comparing either (a) cell transcriptomes, e.g. by next generation sequencing (NGS) (Fig. 1, A), or (b) cell surface proteomes by LC-MS/MS (Fig. 1, B). Both strategies have clear advantages and disadvantages. NGS is sensitive enough for allowing the analysis of RNA from freshly isolated primary microendothelial cells without requiring prior cell culture, a process that potentially impacts the gene/protein expression profile of isolated primary cells. Freshly isolated mRNA samples are even available from commercial vendors. However, NGS based mRNA expression profiles may not fully match in a qualitative and quantitative manner the expressed cell proteome. Furthermore, it is also unclear whether membrane proteins, even when effectively translated, are sorted to the plasma membrane for being exposed to potential targeting ligands or targeting antibodies.

These drawbacks can partially be addressed by performing a cell surface proteomics experiment. Cell surface proteins can be labelled, for example with biotin by using appropriate kits, and pulled down using magnetic beads. By analysing protein composition of these samples it is possible to claim with high confidence which receptors are presented on cell surface and therefore accessible to targeting ligands and antibodies. However, in order to obtain enough material for proteomics it may be necessary to subculture the isolated primary microendothelial cells first, before extracting the proteins. It is well documented that this process can considerably change the gene/protein expression profile of cells, for example the loss of certain tight junction proteins (Cecchelli et al., 2007; Abbott et al., 2006; Nakagawa et al., 2009). Furthermore, proteomic profiling does not aid in the discovery of non-protein receptors such as gangliosides and/or glycans. All these aspects must be taken into account when interpreting the cell surface proteomics data.

In order to minimise risks associated with the both strategy it is important to conduct mRNA sequencing and proteomics experiments in parallel. Understanding similarities and differences of identified receptors is highly informative and can help selecting as robust targets as possible for the drug delivery systems (DDSs) being developed. However, these technical challenges are not the only challenges that need to be met, as summarised below (Fig. 2).

Long term goals of most investigations related to drug delivery systems are associated with their applicability to treat human diseases (Fig. 2, A). This means that already in the first instance it is important to include human primary tissue samples for the transcriptomic and proteomic analyses. However doing that is related to several practical questions, such as the availability of mRNA and cells samples from commercial providers. Even if readily available, there might be great differences in terms of the age, sex and health/disease status of donors of the samples, possible contamination

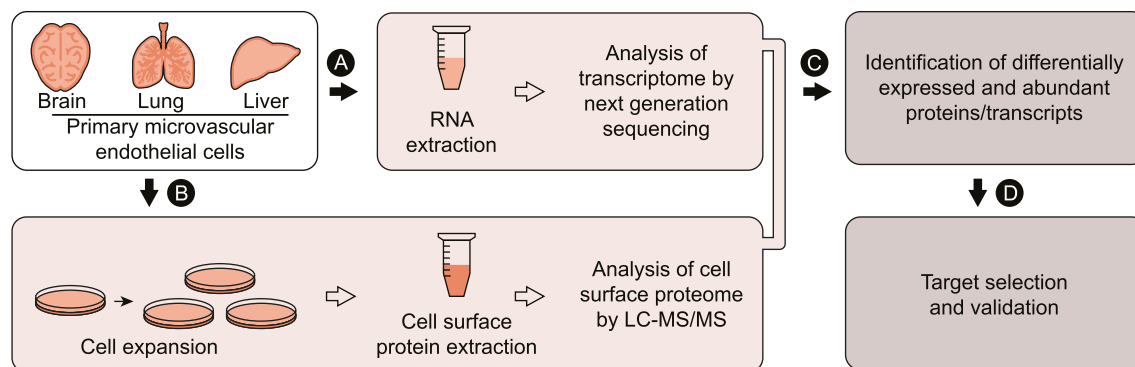


Fig. 1. Overview of the workflow for finding novel brain microvascular endothelial cell specific receptors for the delivery of biologicals across the BBB. Primary microvascular endothelial cells from brain, lung and liver are obtained from a commercial vendor. (A) RNA is extracted from these cells and subjected to transcriptome analysis using next generation sequencing. (B) Cells are expanded and cell surface proteins are isolated and analysed by LC-MS/MS in parallel. (C) The analysis will reveal which cell surface proteins/receptors and which transcripts are abundantly and more specifically expressed in brain microvasculature as compared to the endothelial cells in liver and lung. (D) This helps identifying novel potential targets that could be exploited for drug delivery across the BBB via transcytosis.

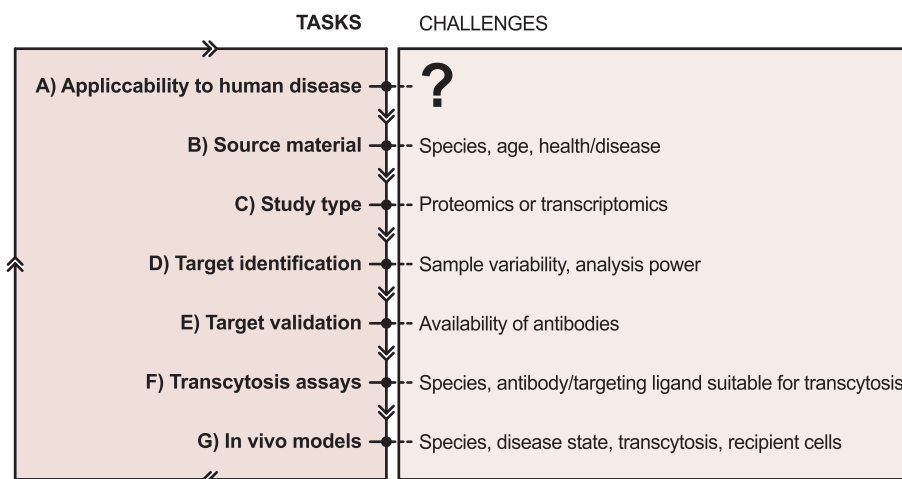


Fig. 2. Challenges of the BBB endothelial transcytosis research. The key goal of (A) developing CNS therapies for human disease dictates the downstream workflow. For discovering novel BBB selective receptors, (B) selection of suitable source cells/RNA must be obtained for (C) the analysis of proteome/transcriptome of selected tissue samples. (D) Target identification and (E) validation are associated with several challenges too, often related to the commercial availability of required materials. Alignment of accessible (F) transcytosis assays and (G) *in vivo* models pose both technical challenges and the critical question of translatability of the results to human disease (A). See text for details.

from other cell types, and the quality of the tissue itself used for isolation, such as post-explantation (post-mortem) interval etc. (Fig. 2, B). This, in turn, will obviously increase the variability of the data set and can considerably decrease the power of the subsequent data analysis. Sample variability issues can be partially controlled by using animal tissues for primary cell isolation instead of using human primary cell samples provided by contract research organisation (CROs). However, this can lead investigators too far from the initial goal of developing a DDS for treating human diseases. In our experience it can be more informative to collect as detailed information as possible about the used human primary cell/mRNA samples and to perform principal component analysis (PCA) on both, the proteomics and NGS data sets (Fig. 2, C) to understand the factors of sample variance. PCA is a mathematical technique that can be used to transform complex data set variables into a set of new independent abstract variables (principal components) in order to visualise patterns within the data (Jolliffe, 2002). Because PCA is a powerful tool for describing data set variability and for ensuring that the gene expression profile of replicate samples cluster together, it is an important step for estimating the robustness of the data set.

Once PCA has indicated that enough sample variability

originates from the tissues that the microendothelial cells were derived from it is possible to move on with target identification (Fig. 2, D). Care must be taken also at this step because both in proteomic and transcriptomic experiments large numbers of proteins/genes are identified which can lead to the detection of number of potentially false positive candidates, even when carefully corrected for multiple comparisons. This again is related to sample variability. In our experience it may be more robust to rely on false discovery rate (FDR) analysis to identify differentially expressed proteins/genes rather than strict p-value and fold change cut-offs.

Once differentially expressed candidate receptors have been identified it is essential to validate these targets in various models (Fig. 2, E). For example, while validation in primary human cell/mRNA samples can confirm correct target identification in the starting material, it doesn't indicate whether these receptors are similarly expressed in the used *in vitro* BBB transcytosis models and in model organisms. Because of their exploratory nature, proteomic and transcriptomic analyses can lead to the identification of previously poorly studied BBB selective receptor candidates. Receptors with poorly characterised functions may be highly interesting target candidates nevertheless. When this occurs, the lack of

commercially available suitable antibodies can be a serious challenge, potentially requiring generation of custom made antibodies which can often be a lengthy and costly process. Even when antibodies are available for validating BBB-selective membrane proteins, despite binding the antigen, they still may not be suitable for functional experiments, e.g. for analysing transcytosis using *in vitro* BBB models or for *in vivo* drug delivery. For example, this can be related to the epitope recognized by the antibody, its (lack of) cross-species reactivity, as well as the antibody avidity/affinity which all may impact functional studies. Experiments with anti-TFRC antibodies revealed that avidity (mono- or bivalent binding) and affinity is important for antibody delivery into the CNS. Even more, the lack of brain exposure does not disprove a selected receptor as a suitable target, if strong binding of the tool antibody prevents its dissociation at the brain side of BBB (Watts and Dennis, 2013; Bien-Ly et al., 2014; Yu and Watts, 2013). In addition, a “silent” binding of the DDS to its target receptor may be preferred, since interference with endogenous ligand binding may block its natural function and bears a toxicity risk for later therapeutic use (Fig. 2, F).

Next, when moving to *in vivo* model systems, another layer of complexity emerges (Fig. 2, G). In addition to above-mentioned challenges related to choice of species, disease state and transcytosis capacity, it is important to consider the exact target of the DDS. For example, when drug target is located in the in interstitial space of the brain, e.g. extracellular amyloid beta deposits, mere transcytosis of the DDS might be sufficient for observing a therapeutic effect. However, for intracellular targeting of neurons or other brain residing cell types, the transcytosed DDS needs to cross in addition the plasma membrane of the target cell. An important question still remains, whether the BBB-targeted DDS with transcytosis capacity is also suitable for subsequent cellular internalization, efficient endosomal escape and the delivery of the therapeutic payload to the desired cellular compartment. Answer to those questions must be determined experimentally, e.g. by analysing whether the transcytosed drug concentration in brain parenchyma is sufficient for the anticipated biological activity and whether the observed activity is adequate for therapeutic effect.

Within the COMPACT consortium we are actively tackling several of the above-described challenges. The multifaceted nature of the overall aim to discover novel BBB selective receptors with transcytosis capacity requires careful planning in every step. Several potential candidate receptors have already been identified whereas target validation and applicability testing is currently ongoing. Despite task complexity we have demonstrated that by capitalizing on individual expertise of different partners in the consortium, great progress can be made in the BBB endothelial transcytosis research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2016.08.025>.

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