Laboratory of Neurobiology

The Blood-Brain Barrier *in vitro* Using Primary Culture: Implications for Studies of Therapeutic Gene Expression and Iron Transport

PhD Thesis by
Annette Burkhart

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Preface

This thesis: "The Blood-Brain Barrier *In Vitro* Using Primary Culture: Implications for Studies of Therapeutic Gene Expression and Iron Transport" has been submitted to the Faculty of Medicine, Aalborg University, Denmark. The experimental work in this thesis has been carried out in the Laboratory of Neurobiology, The Biomedicine Group, Department of Health Science and Technology, Aalborg University, Denmark from October 2011 to October 2014.

In December 2011 I spend three week in the laboratory of István Krizbai at the Institute of Biophysics, Biological Research Centre of the Hungarian Academy of Science in Szeged, Hungary, where I observed their work on establishing an *in vitro* Blood-Brain Barrier model based on primary cells isolated from rat brains, with the purpose of establishing the model in our own laboratory afterwards.

During my PhD study I have been supervising students in the two disciplines Medicine with Industrial Specialisation and Medicine, and I have taken PhD courses corresponding to 31 ECTS points. Together these activities correspond to a full year of my PhD study.

The thesis is based on three original experimental studies; one has been submitted, and the remaining two are in preparation for submission. The thesis is composed of a general introduction encompassing the topics being explored in the three manuscripts, the objectives of the thesis, the results presented as three manuscripts in article form, a general discussion and finally conclusions and perspectives. The thesis also contains two published review articles.

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I would, additionally, like to express my deepest gratitude to my co-supervisor Assistant Professor Louiza Bohn Thomsen. Her door was always open and she was an endless source of knowledge, advice and solutions to my problems during last three years. Torben and Louiza are both thanked for all their help during the writing of this thesis.

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Last but not least, I would like to thank all of my family and friends for their support and encouragement throughout the last three years. A special thanks to Andreas for always supporting me and for his endless patience, when I started to share results, frustrations and happy experiences from my work. I know it only made sense to me, but he was always sweet to listen and try to help anyway, especially during the writing of this thesis.

List of abbreviations

ABCB1	ATP-binding cassette, subfamily B, member 1
ABCG2	ATP-binding cassette, subfamily G, member 2
α-SMA	Alpha Smooth Muscle Actin
BBB	Blood-Brain Barrier
BCECs	Brain Capillary Endothelial Cells
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BRCP	Breast Cancer Resistance Protein
cDNA	Complement Deoxyribonucleic Acid
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Da	Dalton
DCT1	Divalent Cation Transporter 1
DMT1	Divalent Metal Transporter 1
Dcytb	Duodenal Cytochrome b
EPO	Erythropoietin
GDNF	Glial Derived Neurotrophic Factor
GH1	Growth Hormone 1
GPI	Glycosylphosphatidylinositol
hBMEC	Human Brain Microvascular Endothelial Cell line
hCMEC/D3	Human Cerebral Microvessel Endothelial Cell line
JAK2	Janus Kinase 2
JAMs	Junctional Adhesion Molecules
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
MTP-1	Metal Transporter Protein-1
NLS	Nuclear Localisation Signal
NRAMP	Natural Resistance-Associated Macrophage Protein
NVU	Neurovascular Unit
PECAM	Platelet Endothelial Cell Adhesion Molecule
PDGFRβ	Platelet Derived Growth Factor Receptor Beta
PgP	P-glycoprotein
RNA	Ribonucleic Acid
RBE4s	Rat Brain Endothelial Cell line 4
RBECs	Rat Brain Endothelial Cells
SLC11A2	Solute Carrier Family 11, member 2
Steap	Six-Transmembrane Epithelial Antigen of Prostate
SV40	Simian Virus 40
TEER	Trans Endothelial Electrical Resistance
TGF-β	Transforming Growth Factor Beta
VE-cadherin	Vascular Endothelial cadherin
ZO	Zonula Occludens
Å	Ångström

Abstract

The brain is protected from the entry of unwanted substances circulating in the blood by means of the blood-brain barrier (BBB) formed by the brain microvasculature. This BBB is composed of non-fenestrated brain capillary endothelial cells (BCECs) with intermingling tight junctions preventing substances from entering the central nervous system (CNS) via a transcellular route. The BCECs are invested on their abluminal side by astrocytes and pericytes that maintain and regulate many properties of the BBB. The BCECs express a variety of transporters that facilitate the transport of essential molecules into the CNS.

The presence of the BBB is a huge obstacle for the treatment of CNS diseases, as many potentially CNS active drugs are unable to reach their site of action within the brain. To further explore this obstacle preventing molecules from entering the brain, *in vitro* BBB models are being developed to enable an experimental condition where the BBB permeability of a given drug can be tested early in its development. *In vitro* BBB models consist of BCECs cultured on semipermeable culture inserts either alone, or together with astrocytes and/or pericytes, and many current *in vitro* BBB models exhibit important characteristics of the BBB as they occur *in vivo.* Several strategies have been explored to enable otherwise impermeable molecules to passage through the BCECs. One strategy involves gene therapy to the BCECs, with the principle being to genetically modify BCECs to produce and secrete therapeutic proteins into the brain.

The first part of this PhD thesis involves the establishment and characterization of three different *in vitro* BBB models based on primary cells isolated from the rat brain. The BCECs were cultured alone in a mono-culture, in co-culture with astrocytes and in triple culture together with astrocytes and pericytes. The co-culture and triple culture models were found to be superior to the mono culture with respect to many important BBB characteristics, like high expression of tight junction proteins and BBB specific transporters, low permeability and high transendothelial electrical resistance (TEER). The co-culture model was found to be the best suited model for the remaining part of this PhD thesis due to its consistent expression of many of these properties.

The second part of the thesis deals with gene therapy at the BBB. Cells at the BBB are supposed to be difficult to transfect using non-viral carriers due to the lack of cell-division *in vivo*. The transfection efficiency of BCECs cultured in polarised conditions with confined BBB properties was, therefore, compared to that of non-polarised, highly dividing BCECs. The transfection efficiency was found to be independent of cell division. Additionally, transfection did not influence the barrier properties of the BCECs.

The strategy of genetically modifying BCECs into protein factories for delivery of therapeutic molecules to the brain was, additionally, investigated using primary isolated BCECs. The BCECs were successfully transfected and the therapeutic proteins were observed both on gene and protein level. It was, however, not

possible to detect secretion of the recombinant proteins into the surroundings after transfection. Therefore, additional investigations are needed to verify the possibility of using BCECs as cellular factories for secretion of therapeutic proteins into the brain.

Many studies on drug delivery to the brain target the transferrin receptor, which is expressed on the BCECs to enhance the drug delivery to the brain. The transferrin receptor is normally involved in the uptake and transport of iron into the brain. However, the exact mechanisms by which iron is delivered to the brain remain highly debated. The transferrin receptor is thought to either undergo transcytosis into the brain to directly deliver iron to the brain parenchyma, or it may undergo endocytosis within the BCEC by a mechanism where iron is detached from transferrin and its receptor within the endosome and subsequently become transported into the brain as non-transferrin bound iron mediated by divalent metal transporter 1 and ferroportin. The latter transport mechanism would imply that transferrin is unable to deliver its cargo within the brain, as it probably does not fuse with the abluminal membrane of the BCEC. Therefore, in the third part of the thesis, the expression of iron-related proteins was investigated at the BBB in order to elucidate the pathway of the transferrin receptor within the BCECs. We were able to show expression of all proteins necessary for the endocytosis pathway, which were additionally confirmed in brain capillaries isolated from rats. In addition to astrocytes, pericytes were found to be a source of ferrooxidase activity within the brain, and these cells might, therefore, be implicated in controlling the transport of iron into the brain parenchyma. The exact pathway of the transferrin receptor was not fully elucidated, as both pathways seem possible.

Dansk resume / Resume in Danish

Hjernen beskyttes mod uønskede stoffer, som cirkulerer i blodet, pga. tilstedeværelsen af blod-hjerne barrieren (eng: Blood-brain barrier (BBB)), som findes i hjernens kapillærer netværk. BBB udgøres af hjernekapillærer, der er opbygget af endothel celler (eng: brain capillary endothelial cells (BCECs)). BCECs er endvidere tæt omgivet af astrocytter og pericytter, som regulerer og understøtter BCECs barrierefunktioner. BCECs er tæt forbundne via særlige kontaktproteiner kaldet "tight junctions". Dette medfører, at stoffer ikke kan trænge ind imellem cellerne ved paracellulær transport, men i stedet tvinges igennem cellen, hvilket giver BCECs stor kontrol over hvilke stoffer, der får lov til at passere ind i hjernen. BCECs indeholder flere transportproteiner, som sikrer transport af vigtige næringsstoffer ind i hjernen. Tilstedeværelsen af BBB er samtidigt en stor farmakologisk udfordring, eftersom mange af de lægemidler, der udvikles til behandling af sygdomme i hjernen ikke kan trænge igennem BBB og dermed kan nå frem til de nerveceller, hvor deres virkning var tiltænkt. Som svar på dette problem er der udviklet *in vitro* BBB modeller, som gør det muligt at teste et lægemiddels gennemtrængelighed tidligt i udviklingsforløbet. *In vitro* BBB modeller består af BCECs, som dyrkes på et semi-permeabelt filter enten alene eller sammen med astrocytter og pericytter. Nutidens *in vitro* BBB modeller udviser mange af de vigtige egenskaber, der ses hos BBB *in vivo*. Forskere har udtænkt flere forskellige strategier for, hvordan lægemidler kan transporteres ind i hjernen. En af disse strategier er genterapi ved BBB, hvormed man genetisk modificerer BCECs til at producere og udskille et terapeutisk protein, som ellers ikke kunne opnå transport gennem BBB. Det terapeutiske protein, som f.eks. growth hormone 1 (GH1), erythropoietin (EPO) og brain derived neurotrophic factor (BDNF), bliver dermed frigivet fra BCECs ved sekretion for efterfølgende at blive optaget i nervecellerne.

Den første del af denne Ph.d. afhandling omhandler opsætning og karakterisering af tre *in vitro* BBB modeller baseret på primære celler isoleret fra rotte hjernen. BCECs blev enten dyrket alene (mono-kultur), sammen med astrocytter (co-kultur) eller sammen med både astrocytter og pericytter (triple-kultur). Co- og triple kulturerne viste sig at være overlegne i forhold til mono kulturen. De udviste mange vigtige BBB egenskaber, som f.eks. høj udtryk af tight junctions og transport proteiner, lav permeabilitet og høj transendothel elektrisk resistans (TEER). Co-kulturen blev vurderet til at være den bedst egnede model til de øvrige studier.

Anden del af denne Ph.d. afhandling omhandler genterapi af BBB. Tidligere studier har vist at det er svært at transfektere BCECs ved hjælp af ikke virale gentransportere, eftersom disse celler ikke er aktivt delende *in vivo*. Transfektionseffektiviteten af polariserede BCECs med BBB egenskaber var mindst lige så høj som i ikke polariserede aktivt delende BCECs. Transfektionseffektiviteten viste sig derfor at være uafhængig af

cellens delingsstadie. Det var endvidere muligt at transfektere de polariserede BCECs uden at ødelægge deres barrier egenskaber.

Strategien med genetisk at omdanne BCECs til små proteinfabrikker, som kan levere terapeutiske molekyler til hjernen blev endvidere undersøgt i de primære BCECs. BCECs blev succesfuldt transfekteret og tilstedeværelsen af de rekombinante proteiner, GH1, EPO og GH1 blev observeret på både gen- og protein niveau. Det var dog ikke muligt at påvise, hvorvidt BCECs også udskiller disse proteiner til deres omgivelser. Flere studier er derfor påkrævet, inden det med sikkerhed kan afgøres om BCECs kan bruges som protein fabrikker med henblik på lægemiddel transport ind i hjernen.

Forskere har i mange år forsøgt at levere lægemidler til hjernen ved hjælp af transferrin receptoren, som findes på BCECs. Dette gøres for at øge transporten af lægemidlet til hjernen i forhold til kroppens andre organer, da det kun er på kapillærerne i hjernen, denne transporter er udtrykt. Transferrin receptoren er afgørende for jerns transport ind i hjernen. Den tredje og sidste del af denne ph.d. afhandling omhandler derfor transport af jern over BBB. Det er meget omdiskuteret, hvordan jern bliver transporteret ind i hjernen og der findes i hvert fald to teorier. I den første undergår transferrin receptoren ”transcytose” igennem BCECs, hvormed receptoren afleverer jernet på hjernesiden af BCECs. I den anden teori undergår transferrin receptoren ”endocytose”, hvormed transferrin receptoren og jernet blot bliver optaget i cellen i små endocytotiske vesikler. Inde i disse vesikler frigives jernet fra transferrin receptoren og transporteres efterfølgende ind i hjernen ved hjælp af en divalent metal transporter (DMT1) og ferroportin. Endocytose-teorien indikerer derfor at transferrin receptoren ikke transporteres hele vejen igennem BCECs, og derfor ikke kan levere lægemidler til hjernen.

Tilstedeværelsen af de proteiner som er involveret i jerns transport til hjernen blev undersøgt i primære BCECs dyrket med definerede BBB egenskaber, og det var muligt at påvise tilstedeværelsen af alle de proteiner, som er nødvendige for endocytose. Ydermere fandt vi ferrooxidase aktivitet i både pericytter og astrocytter. Disse celler kan derfor være med til at kontrollere mængden af jern, der transporteres ind i hjernen. Ifølge vores studie ser det derfor ud til at begge teorier er en mulighed.

List of manuscripts

Study I: **Non-viral Gene Transfection of Brain Capillary Endothelial Cells Grown in Primary Culture with Confined Blood-Brain Barrier Properties**
Annette Burkhart, Louiza Bohn Thomsen, Maj Schneider Thomsen, Jacek Lichota, Csilla Fazakas, István Krizbai, Torben Moos.
Submitted, 2014.

Study II: **Transfection of Brain Capillary Endothelial Cells for Protein Synthesis and Secretion: Evaluation on the Strategy for Drug Delivery to the Brain**
Annette Burkhart, Louiza Bohn Thomsen, Torben Moos.
Manuscript in preparation

Study III: **Iron Transport Mechanisms in Cultured Rat Brain Capillary Endothelial Cells with Confined Blood-Brain Barrier Properties**
Annette Burkhart, Tina Skjørringe, Louiza Bohn Thomsen, Torben Moos
Manuscript in preparation

Reviews:

Accessing Targeted Nanoparticles to the Brain: The Vascular Route.
Annette Burkhart, Minaz Azizi, Maj Schneider Thomsen, Louiza Bohn Thomsen, Torben Moos.
Current Medical Chemistry, 2014 Jul 15 [Epub ahead of print]

Nanoparticle-derived Non-viral Genetic Transfection at the Blood-Brain Barrier to Enable Neuronal Growth Factor Delivery by Secretion from Brain Endothelium
Louiza Bohn Thomsen, Annette Burkhart Larsen, Jacek Lichota, Torben Moos.
Current Medical Chemistry.2011;18(22):3330-4.

Introduction

Central nervous system (CNS) diseases are becoming more prevalent. However, the treatment of the CNS diseases is complicated due to the inability of many drugs to access the brain because of the presence of the blood-brain barrier (BBB). The drugs are, therefore, not able to reach their site of action within the brain. Many neuropharmacological drug candidates with great potential are being developed. However, due to their poor BBB permeability, it is estimated that at least 98 % of all drug candidates developed for treatment of CNS diseases never make it into the clinic [154].

Two major vascular barriers regulate and limit the exchange of molecules between the circulation and the CNS, namely the blood-cerebrospinal fluid (CSF) barrier and the BBB. The blood-CSF barrier is formed by the choroid plexus epithelium located at the interface between the blood and the CSF. The BBB is located at the brain microvasculature and is mainly formed by the capillary endothelium [5, 6, 111]. The area of the BBB is about 1000 times larger than that of the blood-CSF barrier. Neurons are rarely more than 8-20 µm from a brain capillary, but may be millimetres from the ventricular and subarachnoid CSF compartments [6]. Therefore, the BBB is the most relevant barrier with regards to drug delivery to the brain and hence the main focus of this PhD thesis.

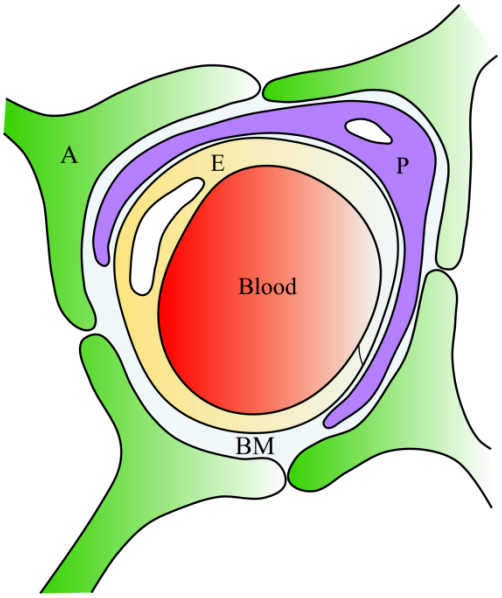

Figure 1. *The blood-brain barrier (BBB). Brain capillary endothelial cells (BCECs) (E) constituting the brain capillaries separate the blood from the brain parenchyma. The brain capillary endothelial cells are tightly connected by tight junction proteins. Pericytes (P), which is embedded in the basement membrane (BM), are distributed along the brain capillaries surrounding the BCECs. Astrocytic endfeet (A) are in close contact with the BCECs at their abluminal side. Together with the basement membrane, the BCECs, pericytes and astrocytes make up the neurovascular unit (NVU).*

The blood-brain barrier (BBB)

The BBB is formed by the brain capillary endothelial cells (BCECs). These cells line the cerebral microvasculature. Compared to endothelial cells of other organs, BCECs have a set of unique features, including a robust expression of tight junction proteins, lack of fenestrations and a low number of cytoplasmic vesicles, resulting in low vesicular trafficking across the BCECs compared to capillary endothelial cells in many other organs of the body [5, 6]. Pericytes and astrocytes form intimate contact with the BCECs and both are important for the formation and maintenance of the BBB phenotype. Together with the BCECs, pericytes and astrocytes are referred to as the neurovascular unit (NVU) [2, 14, 44]. Pericytes are embedded in the basement membrane, which they share with the BCECs. Pericytes are distributed discontinuously along the length of the capillaries, and there is about one pericyte for every two to four endothelial cells [155]. Astrocytic endfeet form a complex network surrounding more than 99 % of the capillaries (Fig. 1). Astrocytes secrete important factors like transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), leukaemia inhibitory factor and glia cell-derived neurotrophic factor (GDNF) that regulate the BBB phenotype [2, 84, 188, 195, 205]. Cultured astrocytes implanted into areas with normally leaky vessels have been found to increase the tightness of the endothelium [86], emphasising the important role of astrocytes in establishing and maintaining the BBB phenotype.

BCECs are thin and non-fenestrated cells that are closely joined together by adherens- and tight junction proteins [5, 111]. Adherens junction's proteins are primarily responsible for holding the cells together and consequently giving the BCECs structural support. Adherens junctions are necessary for the formation of tight junctions, since the lack or disruption of adherens junction leads to disruption of the tight junctions and thereby a disruption of the barrier properties [183]. Tight junction proteins are mainly responsible for the polarisation of BCECs, which results in the clear definition of a luminal and abluminal membrane. The tight junction proteins force most molecules to take a transcellular route across the BBB, rather than a paracellular route [205]. Adherens junction proteins include vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule (PECAM). These proteins span the intracellular cleft and are linked to the cell cytoplasm by scaffolding proteins. The tight junctional complex is primarily composed of proteins like occludin and claudin, with claudin 3 and 5 being the most important barrier forming proteins. The membrane spanning region of two tight junction proteins from two neighbouring cells interacts and binds each other across the intercellular cleft. They are linked to a number of cytoplasmic scaffolding proteins, called zonula occludens (ZO1-3), that are further linked to the cells actin skeletal system [5, 20, 206]. The presence of the tight junction proteins results in a high *in vivo* transendothelial electrical resistance (TEER) above 1000 Ω*cm^2 in rats [35, 51]. Therefore, tight junctions are important in regulating the paracellular transport of polar substances into the brain. Finally, junctional adhesion molecules (JAMs) are also expressed at the

intercellular junction between two adjacent BCECs. These are believed to be important cell adhesion molecules for leukocytes (Fig. 2) [50, 206].

The restricted paracellular transport across the BBB creates a demand for specific transport mechanisms that can supply the brain with required nutrients. A functional BBB express a number of active transport mechanisms that ensure the transport of essential nutrients into the CNS. These include passive diffusion of small lipophilic molecules, carrier mediated transport, receptor mediated transport and absorptive mediated transport [5]. Small molecules like O_2, CO_2 and ethanol passively diffuse across the BBB due to their lipophilic nature, while glucose and amino acids are carried via specific solute transporters expressed on both the luminal and abluminal side of the BCECs. Macromolecules, like insulin and transferrin enter the brain via binding to their respective receptors expressed on the BCECs, which triggers an endocytotic event and subsequently transport across the BBB. Positively charged macromolecules like albumin can also be absorbed by the BCECs via a non-specific transcytotic mechanism (Fig. 2) [5, 20].

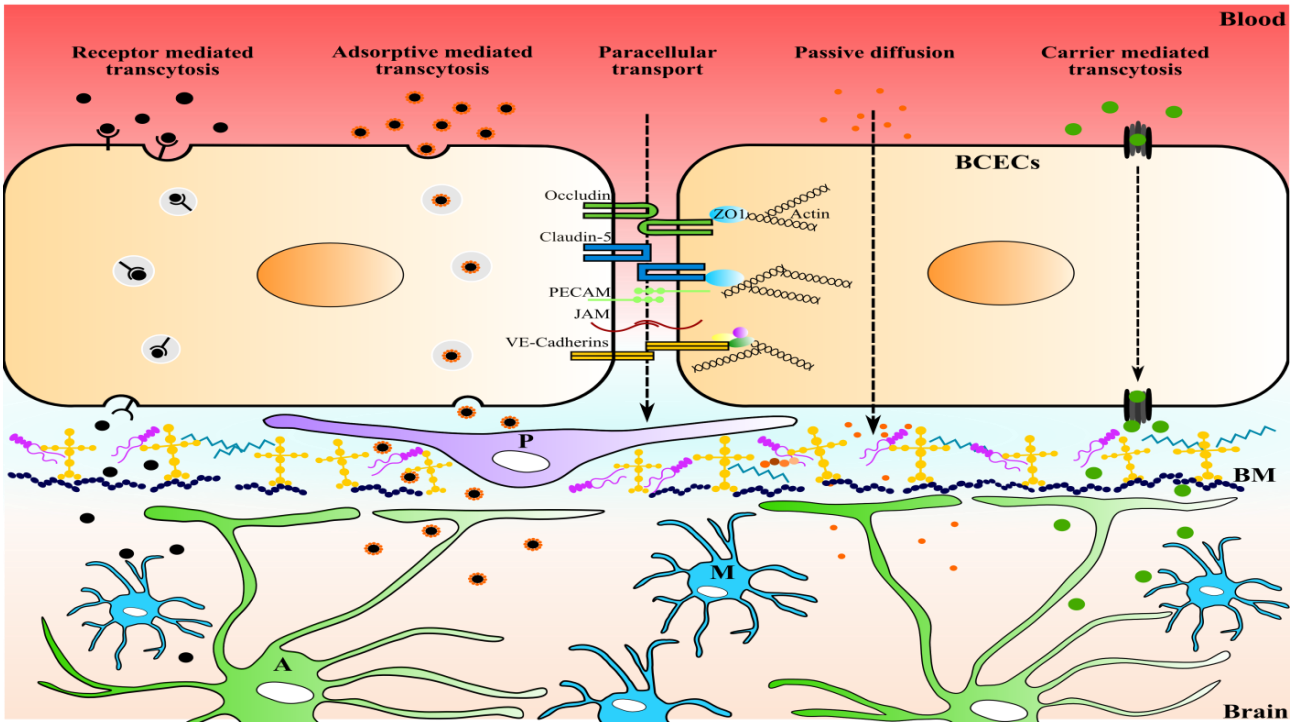

Figure 2: *Paracellular and transcellular routes across the blood-brain barrier (BBB). Two adjacent brain capillary endothelial cells (BCECs) are tightly connected by the membrane spanning tight junction proteins, claudin-5 and occludin and the adherens junction proteins, platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial cadherin (VE-cadherin). These proteins force most molecules to take a transcellular route into the brain. Junctional adhesion molecules (JAMs) are believed to be important for cell adhesion of leukocytes. There are four different transcellular transport route across the BCECs. Small lipophilic molecules can access the brain via passive diffusion across the BCECs, while molecules like glucose, amino acids and nucleotides are carried across the BCECs via specific solute carriers. Macromolecules like transferrin and insulin enter the brain via receptor mediated transcytosis, while positively charged molecules like albumin enter the brain via absorptive mediated transcytosis [34].*

BCECs are also characterised by a robust expression of ATP-dependent efflux pumps that are capable of transporting a diverse range of substances, which have entered the brain, back into the blood. The most significant efflux transporters are the breast cancer resistance protein (BCRP, also referred to as ATP-binding cassette, subfamily G, member 2 (ABCG2)) and the P-glycoprotein (PgP, also referred to as ATP-binding cassette, subfamily B, member 1 (ABCB1)). Since these transporters are ATP-dependent, their transport can easily occur against a concentration gradient, as mitochondria are abundant in the BCECs. The efflux transporters are a major obstacle for the delivery of drugs to the CNS, since they show a broad substrate specify and considerable overlap in function [5, 19, 185].

In vitro BBB models

The development of *in vitro* BBB models has resulted in an extensive knowledge on the physiology and pathology of the BBB. Experimental conditions are often more controllable *in vitro* than *in vivo*, which emphasises the need for these models. Additionally, the low permeability of CNS acting drugs has also created a huge demand for *in vitro* BBB models that would be able to predict drug permeability *in vitro* prior to animal studies. Although the *in vitro* BBB models are unable to mimic the full complexity of the *in* vivo BBB, *in vitro* models have a lot of advantages to the use of laboratory animals [177, 205].

The field of *in vitro* BBB models was pioneered by the first successful isolation of brain capillaries from rat brains by Ferenc Joó and colleagues in 1973 [92]. Since then the field of *in vitro* BBB models has dramatically expanded, and today a variety of BCECs have been isolated from rats [3, 143], mice [209], bovine [49, 68, 176], porcine [81, 147, 159] and human brain microvessels [93, 101, 187, 204]. For the study of the BBB both primary isolated BCECs and immortalised BCECs have been used. Immortalised BCECs often originate from primary isolated BCECs, either by spontaneously differentiation or by immortalisation by introduction of e.g. the simian virus 40 antigen [205]. The most widely used immortalised cells are the rat brain endothelial cell line (RBE4s) [175] and the human cerebral microvessel endothelial cell line (hCMEC/D3) [204]. Immortalised BCECs tend to loose many of their BBB properties when cultured *in vitro*, which results in less tight *in vitro* BBB models compared to primary isolated BCECs [173]. The diminished need for laboratory animals is by far the greatest advantage of using the immortalised BCECs instead of primary isolated BCECs. The greatest advantage of the rodent models is the availability of experimental animals and the easy transition from *in vitro* to *in vivo* experiments. The rodents are, however, rather small, which results in a low amount of cells per brain, compared to the number of cells extracted from one bovine or porcine brain [51, 173, 205]. Human *in vitro* BBB models are obviously of high value for the scientific and industrial community. However, the access to the material is limited and the available human BCECs are often isolated from surgical material, which cannot be classified as healthy tissue [51, 205].

Despite the restricted access to human tissue, human BCECs have been isolated for more than two decades. Unfortunately, the yield is very low and the cells seem to be less robust than cells of porcine or bovine origin [51]. Recently, human *in vitro* BBB models based on pluripotent stem cells have been developed and they seem to be a promising tool in the study of the human BBB *in vitro* [40, 113].

In general, the biggest challenge with the *in vitro* BBB models has been to maintain the *in vivo* characteristics of the isolated BCECs when cultured *in vitro*. By mimicking the microenvironment of the *in vivo* situation as closely as possible and by keeping the passage number of the cells to a minimum, it is possible to restore many of the important *in vivo* characteristics [205]. Culturing BCECs together with astrocytes [3, 49, 68, 80, 159] and/or pericytes [78, 143, 144] has been shown to up regulate tight junction's proteins. The addition of soluble factors, like cAMP and hydrocortisone has also been shown to assist in the re-establishment of the *in vivo* characteristics, such as increased tight junction expression [78, 167, 176, 205]. Another major obstacle in designing an *in vitro* BBB is the difficulty of obtaining pure cultures of primary BCECs. However, by adding puromycin to the growth media, and thereby exploiting the high expression of efflux pumps, it is possible to obtain almost pure cultures of BCECs [37, 167]. Furthermore, the use of plasma derived serum, instead of foetal calf serum has been shown to inhibit the growth of fibroblast and smooth muscle cells, which are the most frequent types of contaminating cells, when isolating BCECs [37, 191]. This is due to the absence of platelet derived growth factor in plasma derived serum, a factor which stimulates the growth of the contaminating cells [4, 191].

Different types of *in vitro* models have been developed. The most widely used model is the static *in vitro* BBB model, which is based on the insertion of a semipermeable membrane culture insert into a culture plate. The simplest model is the monoculture of BCECs, where BCECs are grown on the upper side of the culture insert. The objective in all *in vitro* BBB models is to obtain the same polarised conditions as those found *in vivo*. By culturing the BCECs on the upper side of the culture insert, the BCECs will form a monolayer to which the following can be defined: a luminal membrane facing the capillary lumen, a basement membrane facing the abluminal brain side and a lateral membrane facing the neighbouring cells. The cell-cell contacts between two adjacent BCECs will be tightly connected by tight junction and adherens junction proteins. The polarised characteristics can be increased by culturing the BCECs together with astrocytes or pericytes either on the back of the culture insert (contact co-culture) or on the bottom of the well (co-culture), with both sides corresponding to the brain side. The most anatomical correct model is the triple culture model, where pericytes are cultured on the back of the culture insert containing BCECs, and astrocytes cultured on the bottom of the well (Fig. 3) [143, 144].

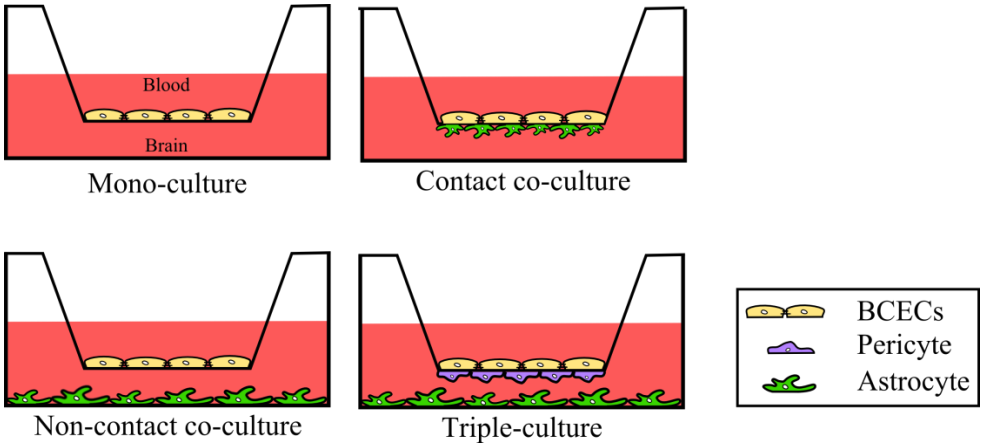

Figure 3. *In vitro blood-brain barrier (BBB) models. In vitro BBB models are based on a semipermeable culture insert that is inserted into a culture well. Brain capillary endothelial cells (BCECs) cultured on the upper side of a culture insert form a polarised monolayer to which a luminal blood side and an abluminal brain side can be defined. The simplest model is a monoculture of BCECs. BCECs can also be cultured in co- culture with astrocytes, with the astrocytes residing on the abluminal brain side either in a contact or a non-contact co-culture model. The anatomically correct model is the triple culture of BCEC, pericytes and astrocytes, where pericytes are seeded on the bottom of the culture insert and astrocytes at the bottom of the well.*

It is possible to measure the paracellular permeability of the static *in vitro* BBB model by inserting electrodes into each chamber. The two electrodes, separated by the endothelial cell layer, will thereby measure TEER across the BCECs monolayer [51, 205]. Besides the TEER measurements, the paracellular permeability of selected tracers with known molecular weight is also used to validate the integrity of the *in vitro* BBB model. The tracers are labelled with a fluorescence dye or an isotope. The most widely used fluorescence tracers include sodium fluorescein (molecular weight (MW) 376 Dalton (Da)) or dextran of various sizes (MW 1-150 kDa). Among the isotope labelled tracers, sucrose (MW 342 Da), inulin (MW 5 kDa) and mannitol (MW 182 Da) are the most widely used [51, 205]. Finally, albumin (MW 67 kDa) is used as a transendothelial marker [51, 170]. TEER measurements using the static model is, generally, much lower than those reported *in vivo* (>1000 $\Omega*cm^2$ for rats), while models based on bovine or porcine BCECs are closer to the *in vivo* measurements with reported TEER values around 500-1800 $\Omega*cm^2$ [81, 159]. However, Gaillard and colleagues analysed the relationship between TEER and paracellular permeability of sodium fluorescein using primary isolated rat BCECs, and found that at TEER values above 131 $\Omega*cm^2$, the permeability of sodium fluorescein was independent of TEER [66]. Therefore, it has been widely accepted that an *in vitro* model based on cells isolated from rats, are considered decent an *in vitro* model of the BBB, when it reaches TEER values above 131 $\Omega*cm^2$ [51, 173].

The scientific applications of *in vitro* BBB models are extensive. *In vitro* BBB models can be used to study the BBB in relation to BBB penetration of drugs or other molecules of interest, understanding BBB specific

transport mechanisms and the possible modulation of these, the BBB role in CNS pathologies, and the possibility of genetically engineering BCEC for gene therapy [173]. *In vitro* BBB models are to date a valuable tool to study the BBB and are responsible for much of the knowledge we, currently, have on BBB physiology, pharmacology and pathology.

Drug delivery to the brain

The most critical role of the BBB is to protect the brain from toxic substances circulation in the blood, and to create a uniform extracellular environment to ensure that neurons and glia cells thrive. The major side effect is that the BBB complicates the delivery of diagnostic and therapeutic agents into the brain [154, 163]. In general, five factors affect the BBB permeability of a drug and based on the Lipinski's rule of five [112]. Hence, in order for a molecule to pass through the BBB it should have a MW below 400-500 Da, but as soon as a molecule reaches a MW higher than 200 Da, its BBB permeability decreases 100 fold [125]. The molecule must be lipophilic in order to passively diffuse across the hydrophobic phospholipid bilayer of the cell membrane and the molecule should not have a polar surface area exceeding 60-90 Ångström (Å). Moreover, the addition of hydrogen bonds to a molecule is negatively affecting the BBB permeability of the molecule. Finally, a molecule with a significant electrostatic charge will not be able to passively diffuse into the brain [125, 154, 163].

Invasive approaches

One of the less invasive approaches used to sneak therapeutic drug pass the BBB is temporary physiochemical opening of the tight junctions. Exposing the BCECs to an osmotic shock causes the endothelial cells to shrink, thereby stretching the tight junctions, resulting in an increased paracellular drug delivery route to the brain [72, 163]. For this purpose high concentration of mannitol is often injected through the carotid artery [153]. Mannitol is often used together with anticancer drugs for the treatment of brain tumours to improve the brain uptake of the drug [54]. The disruption must, however, be temporary; otherwise it will result in an unwanted high influx of serum proteins, altered glucose uptake, activation of the heat shock response, and as a consequence increased neuronal cell death [154, 163]. Vasoactive amines, such as bradykinin analogues, have also been reported to open the BBB [163]. However, structural brain damage in areas of BBB opening and seizures has been reported with this strategy [179].

A highly invasive approach is neurosurgery, in which the BBB is bypassed by placing a catheter directly into the CSF or parenchymal space, whereby high drug concentrations can be delivered to the brain. This ensures high drug concentrations in the brain near the site of administration and low concentrations outside the CNS. The pharmacological effect of this strategy may, however, be limited due to poor diffusion in the brain

tissue. This type of drug delivery strategy carries a large risk of infection due to the need for repeated craniotomy and also because increased interstitial fluid production carriers a risk for increased intracranial pressure [163, 192].

Non-invasive approaches

With Lipinski's rule of five in mind, several attempts have been made to chemically modify poor BBB permeable CNS drugs. One of the most common chemical modifications involves lipidization, in which the drug is modified by converting polar groups into non-polar groups, thereby increasing the permeability of the drug. There are, however, disadvantages to this approach. The modifications often results in the drug losing its CNS activity, or that the drug becomes a substrate for the efflux pumps. Additionally, by increasing the drugs lipophilicity, the drugs also become more permeable to all other biological membranes in the body, which dramatically increases the plasma clearance. Plasma protein binding is also enhanced, resulting in decreased BBB permeability. Lipidization might increase the molecular weight of the drug, resulting in a size above 400 Da, which will have the opposite effect on brain penetration [125, 192]. Prodrugs are another interesting approach. By amidation or esterification of amino, carboxyl and hydroxyl groups, the drug will increase its lipid solubility and thereby its uptake into the brain. The groups will subsequently be hydrolysed and the active drug will be able to reach its active site within the brain parenchyma [163].

The carrier-mediated transport systems are as previously mentioned responsible for the transport of nutrients, vitamins and hormones into the CNS. Carrier-mediated transport is well suited for the transport of small substances with a molecular weight below 600 Da. Therefore, efforts have been made to modify drugs or prodrugs such that they become substrates for the transporters of this system. This approach is mainly based on two strategies; the modification of a drug to have a pseudo-nutrient prodrug structure, which can be recognised by the carrier mediated transport system, or to conjugate a drug into a nutrient substrate of the carrier mediated transport system [125]. Among the different transporters within this system, the carriers for neutral amino acids and glucose have been found to have the highest transport capacity [125, 197]. The neutral amino acid carrier has for example been used to deliver the metabolic precursor for dopamine, L-Dopa to Parkinson's patients. This prodrug is converted at the site of action to dopamine by decarboxylation enzymes present in the brain. Also the glucose transporter has been widely targeted for drug delivery into the brain. This transporter carries hexose carbohydrates, e.g. D-glucose, across the BBB. Therefore, conjugating the drug with D-glucose has revealed promising results. A major drawback of using prodrugs is the possibility of producing toxic metabolites [125, 163].

The most inventive approach to deliver a drug to the brain is to design vectors or nanocarriers that could ferry the drug across the BBB and release the drug at its site of action, without altering the drugs physiochemical properties. The macromolecule transport routes, receptor mediated transcytosis and

absorptive mediated transcytosis provides the basis for this strategy. An example of a widely used carrier is liposomes, which are able to encapsulate both lipophilic and hydrophilic substances. Liposomes can be conjugated with targeting ligands that enhance its brain uptake. A well-studied ligand is the OX26 antibody that targets the rat transferrin receptor, which is abundantly expressed by the BCECs [34, 91].

Bypassing the BBB is not the only challenge in CNS drug delivery. Once inside the brain, efflux transporters are able to clear the drug from the brain and return it to the circulation. BCECs express a large variety of efflux transporters, which recognise a wide diversity of molecules and they even show some degree of overlap [19]. A strategy to maintain high brain concentration of a drug, which is a substrate for the efflux transporters, is to use a substrate which inhibits one or several efflux transporters, e.g. the immunosuppressant cyclosporine A has been used to inhibit the efflux transporter PgP and thereby increase the brain delivery of escitalopram, a selective serotonin reuptake inhibitor, used to treat patients suffering from depression [125, 148].

Gene therapy

Gene therapy may be another promising approach to overcome the restraints of the BBB to treat human diseases affecting the CNS. This strategy involves delivery of genetic material into cells in order to silence or enhance the production of a protein [164]. Defective genes can be silenced by delivery of small interfering ribonucleic acid (RNA) sequences into the target cells. The RNA will then interfere with the translation of the messenger RNA (mRNA) encoding the defective protein and promote the degradation of the mRNA [106]. Proteins can be enhanced by the delivery of complementary deoxyribonucleic acid (cDNA) into the target cells. The target cell will then subsequently transcribe the cDNA and translate it into the protein of interest [164, 194]. Systemic delivery of genetic material will result in rapid breakdown by nucleases and clearance by phagocytic cells. Therefore, the genetic material could be protected by a carrier, which will be able to ferry the genetic material to the cells inferior [164].

Generally speaking, there are two types of gene carriers; viral and non-viral. Viral vectors are simple biological agents known for their ability to carry and protect genetic material throughout the body. They express proteins on their surfaces that determine which cell they will enter, and they have the ability to integrate the genetic material into the host genome. Viral gene carriers are recombinant viruses that have been genetically modified to eliminate their pathogenicity, while they retain their high transfection efficiency. The most widely used viral gene carriers include adeno-associated virus, adenovirus vectors, herpes and retroviruses [107]. However, there are some considerable risks associated with the use of viral gene carriers despite the removal of the pathogenic components. Integration of the genetic material into the host genome carries a risk for insertional mutagenesis, which can activate oncogenes or silence tumour suppressor genes, leading to cancer development. Additionally, the surface antigens on the viral surface

might be recognised by the immune system, leading to an immune reaction and subsequent degradation of the genetic material [107].

Non-viral vectors are easy to synthesise, exhibit lower immunogenicity and are thereby safer to use than the viral vectors. They do, however, have considerably lower transfection efficiency compared to the viral vectors. This is because of the cellular, metabolic and physico-chemical obstacles non-viral vectors face in order to deliver the plasmid DNA to the nucleus [103, 104]. Non-viral gene delivery can be divided into two categories; physical non-viral delivery involving the direct delivery of the DNA to the cytosol, whereas chemical non-viral vectors needs to undergo cellular uptake followed by subsequent endosomal escape [164].

Chemical non-viral delivery systems are often comprised of an expression vector into which synthetic DNA is inserted. This is often in complex with a cationic polymer (polyplex), a lipid (lipoplex) or a mixture of these, referred to as a lipopolyplex [104]. The delivery of genetic material into a cell includes several critical phases, with the first being cellular uptake (Fig. 4). Most non-viral vectors are designed to have excess positive charges that can condensate the negatively charged DNA and, additionally, interact with the negatively charged cell surface [104, 164]. Targeting a receptor on the cell surface might also increase the cellular uptake [71]. The DNA complex will consequently be internalised by the cell via an endocytotic pathway. The exact mechanism of internalisation might be determined by the size of the DNA complex. Complexes above 500 nm enter the cell cytoplasm in clathrin coated pits, while smaller complexes less than 200 nm enters in non clathrin coated pits [103]. Once inside the endosomes the DNA complexes will need to undergo endosomal escape, in order to avoid fusion with the main degradative compartment of the cell, namely the lysosomes. Liposomal formulations are believed to interact with the endosomal lipid membrane and thereby facilitate its release. Polymers are believed to escape from the endosome by means of the proton sponge mechanism. Polymers have an extensive buffering capacity and are, therefore, able to buffer the endosomal pH by increasing the proton and chloride content within the endosome, resulting in an osmotic swelling and eventually disruption of the endosome. The DNA complexes are, thereby, released into the cytosol [103, 104, 164]. Regardless of these endosomal escape mechanisms, a large portion of the internalised DNA complexes will be degraded in the lysosomes and only a small portion will reach the cytosol [104]. Once inside the cytoplasm the DNA must be rapidly delivered to the nucleus for transcription, since the cytosol contains cytosolic nucleases that can degrade the DNA within 50-90 min [164]. The nuclear envelope is the ultimate obstacle for non-viral gene carriers, as these do not contain mechanisms for nuclear internalisation, like the viral vectors. The DNA complex can enter the nucleus using three different routes. The first is during cell division in which the nuclear membrane is temporarily disassembled. The second is by means of passive diffusion into the nucleus through nuclear pores; however, this is only possible for molecules smaller than 40 kDa, which is not the case for many DNA complexes. Finally, molecules larger

than 60 kDa can via an energy dependent transport mechanism enter the nuclear pores if they contain a nuclear localisation sequence (NLS) [103, 104, 164]. Higher transfection efficiencies have been reported in diving cells, suggesting that DNA enters the nucleus during disassembly of the nuclear envelope [28, 139]. However, accumulating evidence in non-dividing cell does also suggest that DNA is capable of entering the nucleus by active transport though the nuclear pores [28, 59, 196].

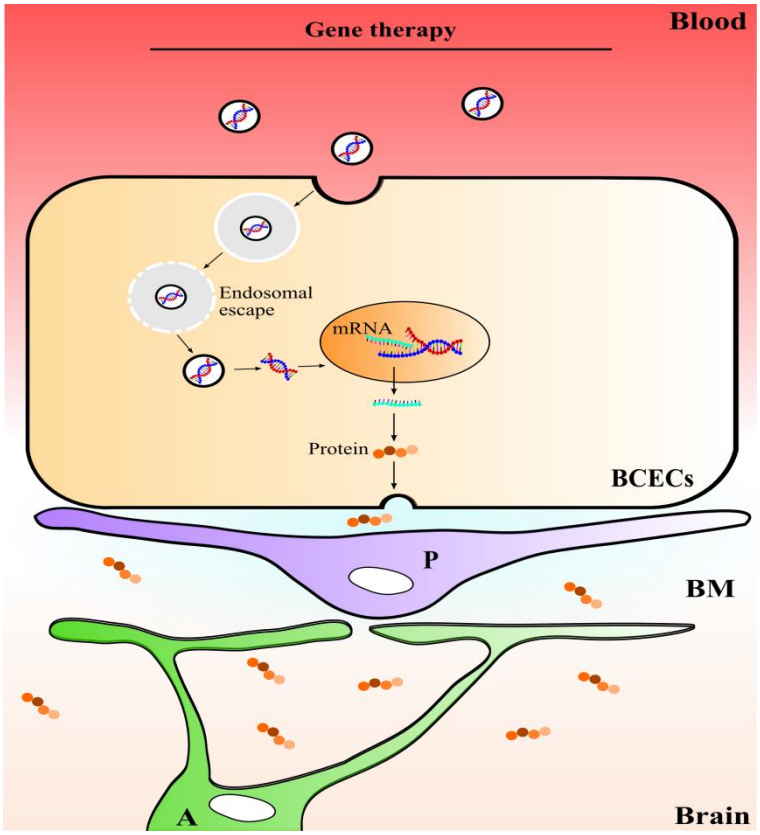

Figure 4*: Gene therapy at the blood-brain barrier (BBB). The gene carrier complex is internalised into the brain capillary endothelial cells (BCECs) via endocytosis. In order to avoid degradation, the gene carrier complex undergoes endosomal escape and reaches the cytosol. The DNA complexes travel to the nucleus, where they are transcribed into mRNA and subsequently translated to proteins. The recombinant proteins will then be secreted into the brain parenchyma, if they are naturally occurring endocrine proteins [34].*

Therapeutic polypeptides in gene therapy

Many therapeutic polypeptides have been identified to have a neuroprotective effect after traumatic or ischemic brain injury. However, their use in the treatment of acute ischemia or neurodegenerative diseases has been limited due to their poor BBB permeability. However, by means of non-viral gene therapy it would be possible to turn the BCECs into protein factories for synthesising and secreting the therapeutic proteins into the brain. This would, thereby, enable the entry of these proteins into the brain to reach the neurons inside the brain [88, 111, 194]. This therapeutic strategy could be beneficial in diseases like the lysosomal

storage diseases, depression or in neurodegenerative diseases like Parkinson's, Alzheimer's and, Huntington's disease, since these diseases could benefit from the widely increased availability of enzymes or growth factors within the CNS. In this PhD thesis, brain derived neurotrophic factor (BDNF), erythropoietin (EPO) and growth hormone 1 (GH1), all proteins of therapeutic interest, will be considered and their neuroprotective and therapeutic benefits will briefly be introduced in the next paragraph.

Brain derived neurotrophic factor

BDNF is a small dimeric protein and the most abundantly distributed member of the neurotrophin family. BDNF is particularly recognised for its role in long-term potentiation, learning and memory, but it is also generally important for the functioning and well-being of the CNS [16]. For this reason BDNF has been recognised as a key player in depression, since it seems to be a downstream target of many antidepressant drugs. Decreased levels of BDNF are often seen in depressive patients, and BDNF have been found to be upregulated by antidepressant drugs [15, 95]. BDNF alterations have also been reported in postmortem brains of Alzheimer's disease [16, 166]. A normal feature of neurological diseases is the loss of neurons in specific areas of the brain. Neurotropic factors have the ability to protect neurons from dying, induce neuronal sprouting and increase neuronal function and metabolism [9]. Site specific elevated BDNF levels have been reported as a consequence of viral gene delivery into the brain in a mouse model of Alzheimer's disease. The elevated levels of BDNF restored gene expression of some of the abnormal genes, which characterized the Alzheimer's mouse model, improved cell signalling and improved learning and memory function [140, 141].

Erythropoietin

EPO is a low molecular weight glycoprotein which primary role for many years was believed to be erythropoiesis. However, EPO is now recognised as a multifunctional growth factor with a significant role in the CNS [36, 117]. The main site of EPO production in adults is the kidney, however, EPO and its receptor have also been found to be expressed in the CNS. Astrocytes are the main sources of EPO in the CNS [119], while BCECs, neurons, microglia and oligodendrocytes express the EPO receptor [142, 214]. Brain EPO is smaller than systemic EPO, and the effects of brain EPO *in vitro* is higher in lower concentrations compared to the effects of systemic EPO [29]. EPO is particularly expressed in brain regions containing neurons vulnerable to ischemic insult, such as the hippocampus and cerebral cortex [118, 119, 142]. Its expression is significantly and rapidly increased as a consequence of hypoxia [23, 118, 119], indicating a neurotrophic and neuroprotective effect of EPO in the CNS. Several *in vitro* [108, 138, 178, 203, 211] and *in vivo* [26, 38, 52, 70, 152] studies have shown that EPO are able to protect neurons during hypoxia, indicating that EPO has beneficial effects in the treatment of stroke patients. This has also been observed in a clinical trial [58]. Additionally, EPO has been seen to exert its neuroprotective effects in an animal model of Parkinson disease, by decreasing the loss of dopaminergic neurons, and as a consequence significantly increase the locomotor

activity [70, 213]. Similar results were obtained by viral gene delivery of EPO into the brain of a rat model of Parkinson's disease [212]. Furthermore, the protective effects of EPO have been observed in animal models of Schizophrenia, Epilepsy, multiple sclerosis and amyotrophic lateral sclerosis [26, 57, 73, 99, 210].

Growth hormone 1

GH1 is a pleiotropic hormone expressed from the pituitary gland under hypothalamic control of the growth hormone releasing hormone and its receptor [18]. GH1 is an important hormone stimulating many different systems, including the CNS, both during development and adulthood [13]. During development GH1 is believed to stimulate the neurogenesis of neurons, astrocytes and endothelial cells. However, this effect of GH1 is also observed in the adult brain [8]. In vitro GH1 promotes the proliferation of neuronal stem cells and is, therefore, suggested to play a role in an injury-related recovery processes within the brain [165]. In comparison to both BDNF and EPO, GH1 is recognised as a neuroprotective growth factor [13]. Similar to BDNF, GH1 also improves memory and learning processes in both human and animals [76, 77] and in comparison to EPO, GH1 has been suggested to reduce hypoxia induced injury in an animal model of hypoxia [110]. GH1 has, additionally, been studied in patients with completed stroke and were found to be able to decrease muscle fatigue [189].

The transferrin receptor as a target for drug delivery to the brain

The transferrin receptor is an obvious target for drug delivery to the BBB, since this receptor is abundantly and exclusively expressed on the capillary endothelium of the brain [12, 87], and it has for the same reason also been extensively used as a brain specific target to enable delivery of drug complexes to the brain. However, since the exact pathway of the transferrin receptor within the BCECs is not fully elucidated, there is conflicting information on the brain penetration of the complexes using this receptor strategy. Most vector based delivery system is based on conjugation of the OX26 anti-rat transferrin receptor antibody to a drug or to nanocarriers encapsulating the drug [34].

In general, there are two strategies for transferrin receptor targeting. Targeting can be achieved using the ligand transferrin or by the use of antibodies directed against the receptor. The concentration of transferrin in plasma is, relatively high [130], meaning that the transferrin receptor is saturated by plasma circulating transferrin. Therefore, drug complexes using transferrin as the ligand, needs to compete with the naturally occurring transferrin, which limits the *in vivo* application of this exogenous transferrin as a drug carrier. Antibodies that bind to the transferrin receptor show more promise. The OX26 antibody binds an extracellular domain on the rat transferrin receptor, distinct from the transferrin binding site, and does thereby not interfere with the binding of transferrin [65].

The group of William Pardridge and others have reported transcytotic delivery of drugs or therapeutic relevant proteins, like neurotropic factors to the CNS by transferrin receptor targeting [64, 105, 156]. Others, on the other hand have reported that OX26 mainly accumulates in, or associates with, the brain endothelium instead of the parenchymal compartment [71, 132]. Therefore, there is conflicting evidence on whether the OX26 antibody is delivered to the brain by means of receptor mediated transcytosis of the transferrin receptor, or if the receptor becomes internalised into the BCECs by endocytosis, followed by retro-exocytosis to the luminal membrane. However, recent studies have suggested that the dissociation of the anti-transferrin receptor antibody is related to the affinity of the antibody to the transferrin receptor. Antibodies of lower affinity seem to be able to easier dissociate from the receptor and accumulate in the brain parenchyma, compared to high affinity antibodies [217]. Therefore, in order to fully understand the pathway by with the transferrin receptor transports iron into the brain, more information on iron transport to the CNS and the fate of the transferrin receptor after internalisation into BCECs is needed.

Iron transport into the brain

The mechanisms of iron transport into the brain have yet not been fully characterised. It is, however, generally accepted that the initial step involves binding of transferrin bound iron to the transferrin receptor. In general two different mechanisms of iron transport into the brain have been proposed. The first proposal relates to iron transport in other cells e.g. the intestinal epithelium, where iron is internalised by receptor mediated endocytosis, followed by detachment of iron from transferrin inside the endosome, translocation across the endosomal membrane and finally transport of iron across the abluminal membrane. The second proposal suggests that iron is transported across the BBB by means of receptor mediated transcytosis of the transferrin receptor [11, 130, 134]. In both proposals transferrin remains bound to the transferrin receptor, which is recycled to the luminal membrane (Fig. 5).

Iron and transferrin

In the blood iron is bound to transferrin. In aqueous solutions iron can exist in two forms; the reduced state Fe^{2+}, referred to as ferrous iron, and its oxidative state Fe^{3+}, referred to as ferric iron. The change from ferrous iron to ferric iron is the basis of many biological redox reactions. However, the reaction also gives rise to the possibility of producing potentially harmful free radicals by the reaction with oxygen [133]. The transferrin molecule is composed of a single polypeptide chain with two domains of equal size. Each domain contains an iron binding site; hence each transferrin molecule can carry two iron molecules in their ferric form. Non-iron bound transferrin is referred to as apotransferrin, while the binding of one or two iron atoms is referred to as monoferric transferrin and diferric transferrin, or simply holotransferrin [11, 130, 134]. The

binding of iron is pH dependent. At a physiological pH of 7.4 the affinity of transferrin to iron is very high, making the binding almost irreversible, but at pH levels around 6.5 the binding of transferrin to iron is lowered [136, 198]. The primary role of transferrin in plasma is to function as an iron carrier that accepts iron released from cells and transport it to other cells [130]. In order for iron to be transported into the brain it must pass the BBB. BCECs express transferrin receptors on their luminal side facing the capillary lumen [12, 87]. Due to the hydrophilic nature of the transferrin molecule it is not able to enter the BCECs by its own, making the transferrin receptor highly responsible for the transport of iron into the brain [134].

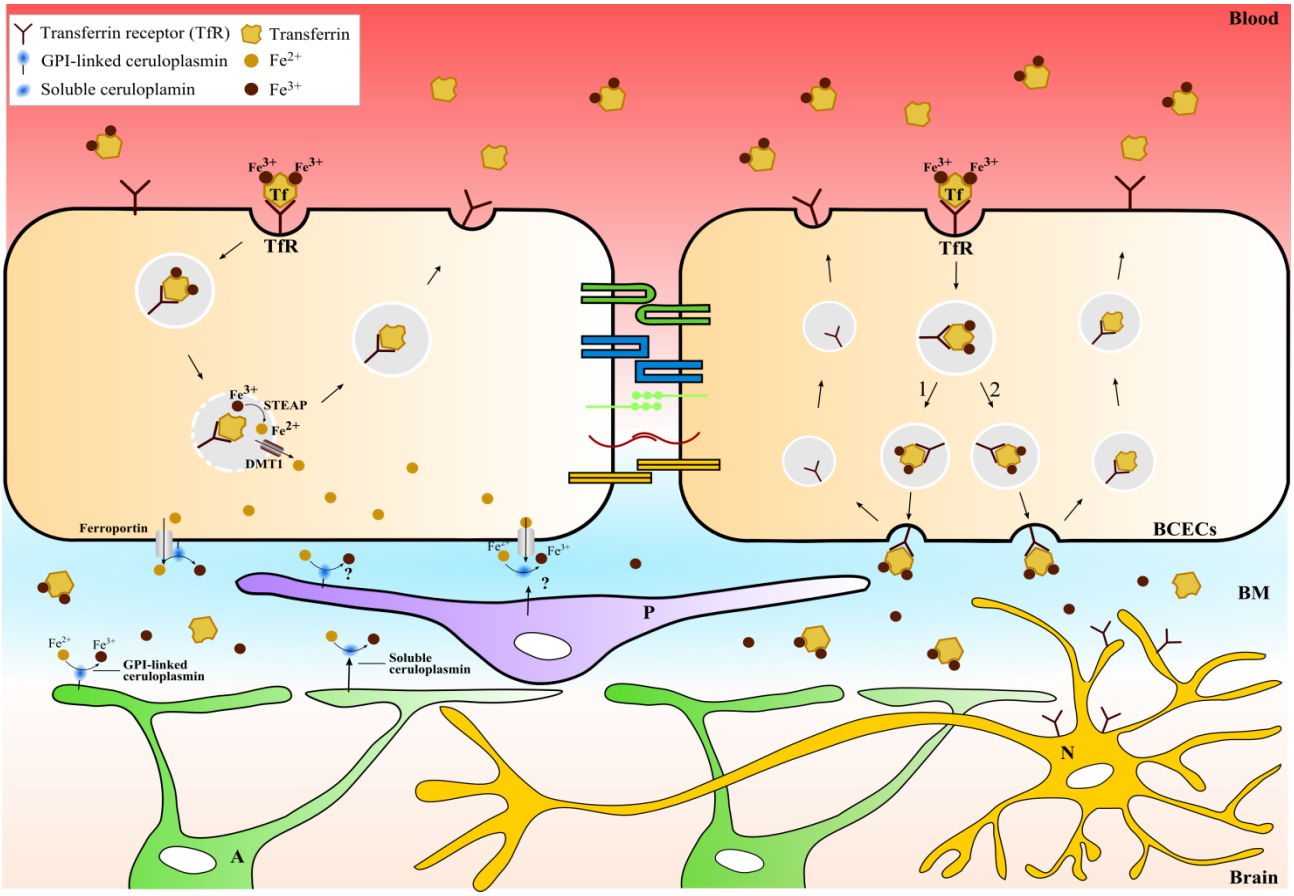

Figure 5. *Iron transport across the BCECs. Two different transport routes for iron have been suggested. The first route (left side) involves endocytosis of holotransferrin (Tf) via binding to the transferrin receptor (TfR) expressed on the luminal side of the brain capillary endothelial cells (BCECs). Within the endosome, ferric iron (Fe^{3+}) dissociates from transferrin and becomes reduced to ferrous iron (Fe^{2+}) by ferrireductases, like the six transmembrane epithelial antigen of prostate (Steap) protein. Ferrous iron is then translocated out of the endosomes to the cytosol by divalent metal transporter 1 (DMT1). Transferrin and the transferrin receptor are recycled back to the blood. Ferroportin residing in the cell membrane transports ferrous iron out of the cytosol and into the brain parenchyma. Astrocytes (A) express ceruloplasmin in both GPI anchored and soluble form, which can oxidate ferrous iron to the less toxic ferric form. We hypothesised whether this ferrooxidase activity could also be present in pericytes (P). The second route (right side) involves transcytosis of holotransferrin bound to the transferrin receptor though the BCECs. At the abluminal side either transferrin-bound iron or unbound iron is released into the brain, while the transferrin and/or transferrin receptor recycles to the luminal membrane. Neurons (N) express transferrin receptors. Within the brain parenchyma iron may also bind to other molecules released by the astrocytes, e.g. like citrate.*

Transferrin receptor

The human transferrin receptor is a transmembrane glycoprotein consisting of two identical subunits, that each can bind one transferrin molecule [133]. At physiological pH of about 7.4, the transferrin receptor binds diferric transferrin with 10 fold higher affinities than monoferric transferrin and 2000 fold higher affinity than apotransferrin. However, as the pH decreases the affinity for apotransferrin increases [11, 198]. After binding of diferric transferrin to the transferrin receptor, the complex becomes endocytosed via clathrin coated pits that results in uncoated endosomes. The pH within the endosomes decreases to 5.5 due to an endosomal ATPase [130, 133], resulting in a lower affinity of iron to transferrin [11]. This causes a release of iron from transferrin and probably a reduction of ferric iron to ferrous iron, which is subsequently transported out of the endosome into the cell cytoplasm [133]. The transferrin receptor binds apotransferrin with high affinity when the pH is lowered. Therefore, the transferrin molecule is believed to remain bound to the transferrin receptor, which is then recycled to the luminal membrane by exocytosis. At the luminal membrane the pH increases and apotransferrin is released and replaced by diferric transferrin, and the cycle is repeated [11, 133].

Iron transport into the brain has also been proposed to occur via receptor mediated transcytosis of the transferrin receptor [134]. Since no transport of transferrin though the BCECs has been observed, it was proposed that astrocytes play an important role in the release of iron from the transferrin at the abluminal membrane, before the apotransferrin-transferrin receptor complex is recycled to the luminal membrane of the BCECs [134]. Several studies have reported brain delivery of the OX26 antibodies, which targets the transferrin receptor complex [64, 105, 156], while others have reported accumulation of the antibody within the BCECs using the same strategy [71, 132]. The transcytosis of the antibodies into the brain does, however, seem to be influenced by the affinity of the antibody to the transferrin receptor [217].

The number of transferrin receptors on the surface of cells highly determines the cellular uptake of iron; however, the number of transferrin receptors is determined by the iron supply and the rate of cellular proliferation. During iron deficiency the synthesis of transferrin receptors are increased, while it is inhibited by increased iron supply. Additionally, proliferating cells have a higher number of transferrin receptors than their quiescent counterparts [133, 172].

Ferrireductases and divalent metal transporter 1 (DMT1)

The transport of ferrous iron out of the endosome has been proposed to be mediated by the divalent metal transporter 1 (DMT1), also known as the divalent cation transporter 1 (DCT1). DMT1 is a member of the natural resistance-associated macrophage protein (NRAMP) family and encoded by the solute carrier family 11, member 2 (SLC11A2) gene [11, 74]. DMT1 are a proton dependent transporter of metals that has a functional pH optimum around 5.5. It is, therefore, able to remove iron from the acidic environment within

the endosomes [11]. As its name implies DMT1 only transports ferrous iron, however, the transferrin receptor transports ferric iron [11]. This highlights the possibility that ferric iron, released from the transferrin under the acidic conditions within the endosome, is reduced to ferrous iron. Ferrireductases like the six-transmembrane epithelial antigen of prostate (Steap) family have been shown to perform this function in erythorid cells [98, 149], and might, therefore, also be responsible for this action within the endosomes of other cells [11]. Steap 3 has been well characterized as an endosomal ferrireductase [149], but recently Steap 2 and 4 have also been characterised as ferrireductases [150]. Steap 2 and 3 expressions have been found in the brain [149, 150] and Steap 2 has also been identified in a human brain microvascular endothelial cell line (hBMEC) [123]. Neurons, oligodendrocytes, astrocytes and the ependymal epithelium express DMT1 [186], while the expression of DMT1 in the brain capillaries remains debated. DMT1 expression has been reported in brain microvessel [31, 32, 186] and in hBMEC [123]. Others, on the contrary, have only been able to show DMT1 expression in neurons and not in the microvessels despite the use of three different anti-DMT1 antibodies [131, 135].

Ferroportin and hepcidin

Ferroportin 1 is a transmembrane protein residing in the cell membrane of cells that store or transports iron. Ferroportin, also referred to as metal transporter protein 1 (MTP-1), is the only known iron transporter in vertebrate that transports intracellular ferrous iron out of cells. However, very little is known about the exact mechanisms of this transport [11, 48]. In the BCECs ferroportin has been proposed to transport the ferrous iron, which DMT1 relocated from the endosomes into the cell cytoplasm, out of the BCECs either directed towards the luminal or abluminal side. However, the expression of ferroportin in the brain microvasculature has also been debated. Several groups have observed ferroportin expression in the brain microvasculature [208, 216] and in cultured BCECs [121, 122, 124], while others have not been able to show ferroportin expression [25, 31, 32].

Iron entry into plasma, iron consumption and iron storage is regulated by the interaction between the peptide hormone hepcidin and ferroportin. Hepcidin, a member of the defensin family of antimicrobial peptides, is a small 25 amino acid peptide with a high disulfide bonded structure. High levels of hepcidin results in decreased iron uptake, while low levels of hepcidin results in increased iron uptake [48]. Hepcidin regulates ferroportin in a post-translation fashion by directly interacting with an extracellular loop of ferroportin. This induces the binding and auto phosphorylation of the cytosolic non receptor tyrosine kinase, Janus kinase (JAK2), leading to the phosphorylation and internalisation of ferroportin. Ferroportin is then degraded within the lysosomes [145, 202]. Astrocytes express hepcidin and their secretion of hepcidin has been proposed to regulate the flux of iron from BCECs into the brain [121].

Ceruloplasmin and hephaestin

As described, ferroportin might transport ferrous iron out of cells; however, circulating iron is on its ferric form. Additionally, ferrous iron is highly toxic, since, it can react with hydrogen and oxygen to produce free radicals [11, 128]. Therefore, there is a need for ferrooxidase activity, and for this purpose the major plasma ferrooxidase enzyme ceruloplasmin has been recognised [11]. Additionally, the ceruloplasmin homolog hephaestin have also been shown to perform this function in the small intestine [10]. The mechanism of ceruloplasmin and hephaestin is still poorly understood, however, evidence suggest that ceruloplasmin ferrooxidase activity is required in order to release the metal from ferroportin [47]. Both hephaestin and ceruloplasmin have been found in the CNS. Ceruloplasmin is expressed both as a soluble and a glycosylphosphatidylinositol (GPI) anchored form. The GPI anchored form is the most abundant in the CNS, while the liver only expresses the soluble form [161]. Plasma circulating ceruloplasmin is not believed to cross the BBB. In the CNS, astrocytes have been shown to be a source of ceruloplasmin and hephaestin [97, 160, 201] and the expression of ceruloplasmin were especially found to be high in astrocytes surrounding the BBB [97].

Hephaestin is a transmembrane copper-dependent ferrooxidase, that shares 50 % homology with ceruloplasmin. The main difference between hephaestin and ceruloplasmin is within the C-terminus, where hephaestin contains an additional 86 amino acids that encodes a single transmembrane domain and a short cytoplasmic tail. Additionally, all the residues of ceruloplasmin involved in cobber binding are conserved in hephaestin. Hephaestin is, therefore, recognised as a transmembrane protein with a large ceruloplasmin like ectodomain [168]. Expression of both ceruloplasmin and hephaestin has been reported in BCECs [122, 216] and their expression of these two proteins is believed to be able to fulfil the role as ferrooxidases in the iron efflux pathway [122].

Objective of the thesis

The overall goal of the thesis is to establish an *in vitro* BBB model. Primary rat brain endothelial cells (RBECs), astrocytes and pericytes will be isolated from rat brains. The isolated RBECs will be cultured in mono culture, in co-culture with astrocytes and in triple culture with astrocytes and pericytes. The three models will be characterised and the best model will be used to study gene therapy and iron transport at the BBB.

The overall objective can, therefore, be divided into three separated aims, which is also represented in the thesis as three different experimental studies.

1. To establish and characterise three different *in vitro* BBB models, based on RBECs, astrocytes and pericytes isolated from rat brains. The best studied model will be used to analyse non-viral transfection efficiency of RBECs cultured with or without defined BBB properties, in order to estimate whether gene therapy at the BBB is highly dependent on the cells undergoing cell division.

2. To investigate whether non-viral gene therapy to the RBECs results in the DNA being transcribed and translated by the RBECs and whether the recombinant protein will subsequently be secreted into the cells surroundings. The transfection efficiency of the primary RBECs will be compared to that of the immortalised cell line RBE4s and cervix cancer cell line HeLa.

3. To investigate the mechanisms related to iron transport across the BBB. The *in vitro* BBB model will be used to clarify the expression and function of the proteins involved in iron transport into the brain, in order to better understand the regulation of iron transport of the BBB. The role of astrocytes and pericytes in iron regulation within the brain parenchyma will also be analysed.

Results

Study I:

Non-Viral Gene Transfection of Brain Capillary Endothelial Cells Grown in Primary Culture with Confined Blood-Brain Barrier Properties

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The manuscript is under review

Non-Viral Gene Transfection of Brain Capillary Endothelial Cells Grown in Primary Culture with Confined Blood-Brain Barrier Properties

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Running title: Gene therapy to the blood-brain barrier

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Abbreviations:

α-SMA, alpha-smooth muscle actin; ABCB1, ATP-binding cassette, sub-family B, member 1; ABCG2, ATP-binding cassette, sub-family G, member 2; BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; bFGF: basic fibroblast growth factor; BCRP, breast cancer resistance protein; BSA, Bovine serum albumin; CFDA SE, 5-and 6-carboxylfluorescein diacetate succinidyl ester; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GFAP, glial fibrillary acidic protein; Papp, apparent permeability; PBS, phosphate buffered saline; PECAM-1, platelet/endothelial cell adhesion molecule 1; PgP; p-glycoprotein; TEER, trans-endothelial electrical resistance; TGFβ, Transforming growth factor β; RO-201724, 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one; ZO1, zona occludens 1.

ABSTRACT

Non-viral gene therapy applied to brain capillary endothelial cells (BCECs) denotes a novel approach to overcome the restraints in the passage of the blood-brain barrier (BBB) to proteins in blood plasma, as transfection of the BCECs could result in secretion of therapeutic proteins further into the brain. We investigated recombinant protein synthesis by primary BCECs using *in vitro* cultures with pericytes and astrocytes, all isolated from rat brain. The cell cultures exhibited important BBB characteristics as revealed from robust expression of tight junction proteins, a lack of passive permeability and high trans-endothelial electrical resistance (TEER). The BCECs were transfected at different time points to monitor their relationship to the stages of cell division indicated in FACS analysis after 5-and 6-carboxylfluorescein diacetate succinidyl ester (CFDA SE) incorporation. The BCECs were transfectable at a culture stage exhibiting true BBB characteristics and importantly without altering the BBB integrity. The data indicate that non-viral gene therapy of BCECs is possible in culture conditions with an intact BBB.

Keywords: Astrocyte, blood-brain barrier, endothelium, gene therapy, pericytes, primary culture, transfection

INTRODUCTION

The blood-brain barrier (BBB) denotes the interface between the circulation and the central nervous system (CNS). It is formed by non-fenestrated brain capillary endothelial cells (BCECs) that control the flux of substances into the CNS. Other non-neuronal cells of the CNS, mainly astrocytes and pericytes, form the so-called neurovascular unit together with BCECs and support the function of the BBB to restrict both paracellular and transcellular transport pathways to the CNS [6, 49, 144, 205]. The restriction in paracellular transport is mediated especially via the robust expression of tight junction and adherens junction proteins between BCECs. These junctions lead to the formation of major gradients in both inorganic and organic solutes of the blood plasma, which explain the existence of a high transcellular endothelial electric resistance (TEER) across the BCECs [2]. The transcellular transport across BCECs is diminutive with respect to hydrophilic molecules [6, 49, 144, 205]. Furthermore, the expression of several efflux transporter proteins like the ATP-binding cassette transporters prevents exogenous substances lipophilic in nature to passage the BCECs [5, 19, 20].

In order to enable high-throughput screening for high permeability of putative CNS acting drugs, much effort has been devoted to design reliable *in vitro* models of the BBB. Primary BCECs have been isolated from a variety of animal sources [49, 63, 68, 79, 92, 144, 159, 184, 209], which have resulted in the establishment of immortalised cells lines. These have been widely used to study the BBB, but unfortunately they seem to loose many of the important BBB characteristics in culture [78, 109, 181]. Therefore, the biggest challenge has been to maintain the *in vivo* characteristics of BCECs with respect to high TEER value and low passive permeability when cultured *in vitro,* and primary isolated BCECs are the most promising tool to study the BBB *in vitro,* as it has been possible to maintain many important characteristics of the BBB *in vivo* [78, 109, 143, 181, 205]. A way to mimic the microenvironment is by culturing BCECs together with astrocytes and pericytes as co- or triple cell cultures. These culturing conditions significantly up regulate the expression of important tight junction proteins like zona occludens 1 (ZO1) and claudin-5, which lead to the intended high TEER value and low passive permeability [49, 68, 143, 144].

To overcome the restraints in the impermeability of the BBB, non-viral gene therapy applied to BCECs is considered a promising approach to treat CNS diseases with therapeutic polypeptides as transfection of the BCECs could result in secretion of therapeutic proteins further into the brain [111]. This strategy involves vascular delivery of genetic material encoding the protein of interest to BCECs leading to their subsequent gene expression and protein secretion into the brain interstitium [88, 194]. A carrier protects the genetic material from degradation and supposedly aids in ferrying it to the cells cytoplasm, where the genetic material gets transferred to the cell nucleus [104, 151]. In contrast to viral carriers, non-viral carriers do not have mechanisms for nuclear internalisation, which suggests that plasmid DNA of non-viral carriers mainly

enters the nucleus during cell division during which the nuclear membrane disassembles [59, 94, 103, 151]. The BCECs, however, do not undergo mitotic cell division *in vivo* unless present at an immature stage [174] and, therefore, possibly not accessible for non-viral gene transfection. Immortalised, endothelial cell lines are able to take up genetic material and process it into proteins [88, 194, 219], but so far evidence for *in vitro* transfection of primary BCECs with defined *in vivo*-like properties has never been demonstrated.

The present study aimed to optimise the settings for transfection of BCECs in different models of the BBB with defined *in vivo*-like properties using primary rat BCECs, pericytes and astrocytes. These primary cell types were isolated separately, co-cultured on Transwell membranes to reveal polarised conditions and characterised with respect to TEER values, permeability and expression of cell-specific markers. The BCECs were then transfected at different time points to monitor their relationship to the stages of cell division identified by FACS analysis after 5-and 6-carboxylfluorescein diacetate succinidyl ester (CFDA SE) incorporation. The *in vitro* model of the BBB enabled us to obtain de novo gene expression in BCECs even in culture conditions with high similarity of the *in vivo* situation.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Sigma-Aldrich (Brondby, Denmark, DK): Poly(vinylpurrolidone)-iodine complex (Cat. No. PVP1), Percoll (Cat. No. P1644), collagen type IV (Cat. No. C5533), fibronectin (Cat. No. F1141), poly-L-lysine (Cat. No. P6282), heparin (Cat. No. H3149), puromycin (Cat. No. P8833), hydrocortisone (Cat. No. H4001), CTP-cAMP (Cat. No. C3912), 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one (RO-201724) (Cat. No. B8279), competent CG5 Escherichia coli strain (Cat. No. G3169), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No. D9542) and mouse anti-alpha-smooth muscle actin (α-SMA) (Cat. No. A5228). DNase I (Cat. No. 10104159001), Collagenase/Dispase (Cat. No. 109113), insulin transferrin sodium selenite (Cat. No. 11074547001) and basic fibroblast growth factor (bFGF) (Cat. No. 1363697) were purchased from Roche (Hvidovre, Denmark, DK). The following reagents were purchased from Life Technology (Naerum, Denmark, DK): Rabbit anti-ZO1 (Cat. No. 61-7300), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cat. No. A11034) Alexa Fluor 594-conjugated goat anti-mouse IgG (Cat. No. A11032), collagenase II (Cat. No. 17101105), Dulbecco's Modified Eagle Medium consisting of nutrient Mixture F-12 (DMEM/F-12) (Cat. No. 31331), Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 21885) and fetal calf serum (Cat. No. 10270). Bovine serum albumin (BSA) (Cat. No. EQBAH62) was purchased from Europa Bioproducts (Cambridge, United Kingdom, UK). Plasma derived bovine serum (Cat. No. 60-00-810) was purchased from First Link (Wolverhampton, United Kingdom, UK). Gentamicin sulphate (Cat. No. 17-518Z) was purchased from Lonza Copenhagen (Vallensbaek Strand, Denmark, DK). Fluorescence mounting media (Cat. No S3023) and rabbit anti-glial fibrillary acidic protein (GFAP) (Cat. No. Z0334) were purchased from DAKO (Glostrup, Denmark, DK). ^{3}H D-Mannitol (Cat. No NET101250UC) and Ultima Gold™ liquid scintillation cocktail (Cat. No. 6013326) were purchased from Pelkin Elmer (Skovlunde, Denmark, DK). Turbofect^{TM} (Cat. No. R0531), phosphate buffered saline (PBS) (Cat. No SH3025802) and all reagents for qPCR were obtained from Thermo Scientific, except primers that were synthesised by TAG Copenhagen (Frederiksberg, Denmark, DK). Clonetech pHcRed1-C1 plasmid (Cat. No 632415) and Promokine Cell proliferation kit I (CFDA SE) were purchased from BioNordika Denmark A/S (Herlev, Denmark, DK). Macherey Nagel NucleoBond®Xtra Midi EF plasmid DNA purification kit (Cat. No. 740410) was purchased from AH diagnostics (Aarhus, Denmark, DK). Hanging Cell culture inserts (Cat. No. Pirp 15R48) were obtained from Merck Milipore (Hellerup, Denmark, DK).

Cell culture

Primary cultures of BCECs were prepared from two to three week old Sprague Dawley rats using slight modifications of the protocol of Nakagawa and colleagues [143]. The rats were deeply anesthetised by a subcutaneous injection of 0.5 ml / 10 g body weight of Hypnorm/Dormicum (Fentanyl/Fluanisone mixed with Midazolam and sterile water in a ratio of 1:1:1). Heads were rinsed with 70 % ethanol and 10 % poly(vinylpurrolidone)-iodine complex before decapitation. Under sterile conditions, brains were gently dissected, and the forebrain collected in ice-cold PBS. Care was taken to remove the meninges and any visible white matter, before the cerebral cortices were cut into small pieces using sterile razor blades. The tissue was then digested in collagenase II and DNase I in DMEM-F12 at 37 °C for 75 min until terminated by diluting in DMEM-F12 followed by centrifugation at 1000 G for 8 min. The pellet was resuspended in 20 % BSA in DMEM-F12 and centrifuged at 1000 G for 20 min. The microvessels present in the pellet were further digested in Collagenase/Dispase and DNase I in DMEM/F12 at 37 °C for 50 min. The digested microvessel fragments were separated on a continuous 33 % Percoll gradient. The microvessel fragments were then collected and seeded on collagen type IV and fibronectin coated 35 mm plastic dishes. Primary cultures of BCECs were maintained in DMEM/F12 supplemented with 10 % plasma derived bovine serum, bFGF, heparin, insulin, transferrin, sodium, selenite and gentamicin sulphate (10µg/ml) and cultured in an incubator with humidified 5 % CO_2 / 95 % air at 37 °C. Puromycin was added to the cell culture media in a 4 µg/ml concentration for the first two days to obtain a pure culture of BCECs, which in contrast to pericytes are able to thrive due to their high expression of efflux pumps that scavenges the intracellular toxicity generated by puromycin [167].

Primary cultures of pericytes were obtained by prolonged culture of the isolated microvessel fragments. These microvessel fragments contain both BCECs and pericytes; however, by culturing the microvessel fragments on uncoated dishes in DMEM supplemented with 10 % fetal calf serum and gentamicin sulphate for about 10 days, pericyte proliferation and survival was favoured and BCECs disappear. The pericytes were then frozen for later use. When needed they were thawed and cultured for three days before being used in the experiments.

Primary cultures of astrocytes were obtained from neonatal Sprague Dawley rat pups. The pups were rapidly decapitated, their brains dissected and pieces of the cerebral cortex mechanically dissociated through a 40 µm nylon strainer in DMEM supplemented with 10 % fetal calf serum and gentamicin sulphate. Dissociated cells were seeded in poly-L-lysine coated culture flasks for approximately two weeks until they reached confluence. Thereafter, the cells were either frozen or seeded directly into poly-L-lysine coated 12 well culture plates for about two weeks before being used for co-culture experiments with BCECs and pericytes. It was consistently the impression that the freezing step could be performed without a reduction in the cells capacity to influence their inductive effects on the barrier formation by the BCECs.

Construction of *in vitro* BBB models

Three different types of *in vitro* BBB models were constructed, i.e. monoculture of BCECs, non-contact co-cultures of BCECs and astrocytes (aka. co-culture) and triple cultures consisting of BCECs, pericytes and astrocytes (Fig. 1a). At day three after isolation, BCECs had reached about 80 % confluence and were passaged onto collagen type IV- and fibronectin-coated 12 well polyethylene terephthalate, 1.0 µm hanging cell culture inserts at a cell density of $1*10^5$ cells/cm^2. The cells were left to adhere to culture inserts overnight. To construct non-contact cultures, BCECs were seeded on the upper side of the culture inserts, before the culture inserts were placed in the 12 well culture plates containing a confluent layer of astrocytes. To construct triple cultures, pericytes were seeded on the bottom side of the coated culture inserts at a cell density of $1.5*10^4$ cells/cm^2 and left to adhere for 4-5 hours, before the BCECs were seeded on the upper side. The culture inserts were then placed in the 12 well culture plates containing the confluent layer of astrocytes grown at the bottom of the wells. To further induce BBB characteristics, the BCECs were treated with hydrocortisone, cAMP and RO-201724 in concentrations of 550 nM, 250 µM and 17.5 µM respectively [176, 205].

Immunocytochemical analysis of the isolated primary cells

Immunocytochemical analyses were performed to determine the origin of the isolated cells. The cells were washed in 0.1 M PBS, pH 7.4, fixed with absolute ethanol / acetic acid in a ratio of 95 % : 5 % for 10 min at -20 °C. The cells were then washed 3 x 5 min in 0.1 M PBS followed by blocking of non-specific binding of primary antibodies using 3 % BSA in PBS for 30 min at room temperature. BCECs were then incubated with primary antibodies raised against ZO1 (endothelial cells), α-SMA (pericytes), or GFAP (astrocytes). The primary antibodies were all used in a dilution of 1:200 in 1 % BSA / 0.1 M PBS, pH 7.4 and incubated overnight at 4 °C. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibodies were used in a dilution of 1:200 in 1 % BSA in 0.1 M PBS, pH 7.4 and incubated for 30 min at room temperature. Nuclei were stained with DAPI. The cells were mounted on glass slides with fluorescent mounting medium, and examined in a fluorescence microscope (Axiovert 2000, Carl-Zeiss, Germany). Captured images were corrected for brightness and contrast using the Adobe Photoshop C2 software.

Trans-endothelial electrical resistance (TEER) measurements

TEER values were measured to evaluate the integrity of the various *in vitro* BBB models. TEER, which in culture conditions reflects the flux of mainly sodium ions through an intact cell layer, was measured using a Millicell ERS-2 epithelial Volt-Ohm meter and STX01 Chopstick Electrodes (Millipore, Hellerup Denmark, DK). The TEER values of coated but cell free culture inserts were subtracted from the measured TEER values, and the difference was multiplied with the size of the culture insert (1.12 cm^2). Measured TEER

values are given as Ω*cm^2. Data were analysed with the GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA) using a two way ANOVA with Bonferroni post hoc test.

Passive permeability across the BCECs

The functional integrity of the BBB models was determined using radiolabeled mannitol [80]. ^{3}H-D-Mannitol (specific activity 14.2 Ci/mmol) was added to the upper chamber at a concentration of 1 µCi/ml and incubated with the cells for two hours at 37 °C on a rocking table. Donor samples (100 µl) were taken from the upper chamber at 0 and 120 min, and receiver samples (100 µl) were taken from the lower chamber at 0, 15, 30, 60 and 120 min and replaced with 100 µl fresh media. Samples were added with Ultima Gold Scintillations fluid and counted in a LKB Wallac Rackbeta Liquid Scintillation Counter, Model 1209. The permeability studies were performed on twelve culture inserts on the second day of high TEER (day 2) (c.f. Fig. 1), and the permeability data were plotted with the total number of millimoles transported against time in each well. The flux at steady state across the culture inserts was calculated as the slope of the straight line at steady state divided by the area of the culture insert. The apparent permeability (Papp) was then calculated by dividing the observed flux at steady state (J) with the initial concentration in the donor compartment (C_{donor}). The Papp values were plotted against the TEER for the individual culture inserts. Data were analysed by the GraphPad Prism 5.0 software using a one way ANOVA with Tukey's multiple comparisons post hoc test.

RT-qPCR

Gene expression analyses were performed on BCECs cultured in mono-, co- and triple culture conditions, as described above. For each RNA sample, RNA was isolated from 20 individual culture inserts and pooled into four samples each containing five culture inserts. This procedure was further repeated twice to yield cells from two different isolations, which resulted in eight RNA samples (n = 8) for all of the three different cell culture conditions. RNA was extracted from BCECs using the GeneJET RNA Purification Kit and treated with DNase I enzyme according to the manufacture's protocol. 100 ng of each DNA-free RNA sample was used as a template for RT-qPCR. cDNA synthesis was carried out with the RevertAid Premium First Strand cDNA Synthesis Kit. To assess the expression profile of endothelial cell specific proteins, quantitative RT-PCR was performed with primers specific for claudin-5, occludin, platelet/endothelial cell adhesion molecule 1 (PECAM-1), Transferrin receptor 1 (CD-71), ATP-binding cassette, sub-family G, member 2 (ABCG2), also known as breast cancer resistance protein (BCRP)) and ATP-binding cassette, sub-family B, member 1 (ABCB1, also known as p-glycoprotein (PgP)) (Table I). Pericytes are a known lightly infiltrating source of contamination in BCECs [167]. Therefore, primers specific for α-SMA were included to analyse the degree of pericytes contamination in the three different culture setups. Beta-actin was used as a housekeeping control gene for normalisation purpose. The efficiency of the primers was analysed, and only primers with

efficiencies between 95 % and 105 % were used for further analyses. 0.5 µl cDNA and 10 pmol of each primer were used for each PCR reaction together with the Maxima™ SYBR Green qPCR Master Mix. Each sample was performed in triplicates, and not reverse-transcribed RNA and water served as negative controls. Quantitative RT-PCR was performed using the Stratagene Mx 3000P^{TM} QPCR System (Agilent Technologies, Horsholm, Denmark, DK). The PCR conditions were 95 °C for 10 min, followed by 40 cycles of: 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The relative quantities of DNA in the analysed samples were calculated by the Pfaffl method [169]. Data were analysed by the GraphPad Prism 5.0 software using a one way ANOVA with Tukey's multiple comparisons post hoc test.

Table 1: *Primer sequences used for RT-qPCR analysis.*

Primer	Reference sequence	Forward primer	Reverse primer
Claudin-5	NM_031701.2	CTACAGGCTCTTGTGAGGACTTGAC	AGTAGGAACTGTTAGCGGCAGTTTG
Occludin	NM_031329.2	CTGACTATGCGGAAAGAGTCGACAG	AGAGGAATCTCCTGGGCTACTTCAG
PECAM-1	NM_031591.1	ATTCTATAAGGACGATGCGCTGGTG	GCTGTTCAGTATCACGGTGCATTTG
TfR 1	NM_022712.1	TGGATCAAGCCAGATCAGCATTCTC	TTTCTTCCTCATCTGCAGCCAGTTT
ABCG2	NM_181381.2	GAGTTAGGCCTGGACAAAGTAGCAG	AAGAGGATGGAAGGGTCAGTGATGA
ABCB1	NM_012623.2	AATCAACAGTACACAGACCGTCAGC	CCAAAGTGAAACCTGGATGTAGGCA
HcRed1-C1	pHcRed1-C1 (613-1377)	ATGTACATGGAGGGCACCGTGAA	GTCACGTGGATTCTCATGCTCTGG
β-actin	NM_031144.3	CCTCTGAACCCTAAGGCCAACCGTGAA	AGTGGTACGACCAGAGGCATACAGGG

Measurement of endothelial cell proliferation

Endothelial cell division was measured using the PromoKine cell proliferation kit I (CFDA SE), according to manufactures protocol. CFDA SE is also known as CFSE. The fluorescent tracer (495/519) passively diffuses into cells, in where it binds covalently to intracellular proteins. CFDA SE becomes fluorescent after hydrolysis by intracellular esterases, which results in long term labelling of the cell. This label is inherited through successive cell divisions, and with every cell division, each of the two daughter cells receives approximately half of the label [114]. To analyse the cell division pattern of the BCECs, the isolated microvessels were seeded directly onto collagen type IV- and fibronectin-coated 12 well polyethylene terephthalate, 1.0 µm hanging cell culture inserts. The cells were maintained in 4 µg/ml puromycin media for two days. One day after isolation (day -2) the cells were incubated with pre-warmed 1µM CFDA SE in PBS for 15 min to label the cells. Then the labelling solution was replaced with pre-warmed culture medium for 30 minutes to ensure sufficient hydrolysis of CFDA SE. Labelled cells were cultured for zero to five days (day -2 to day 3) (Fig. 4) to allow for cell division to occur. Every 24 hours the labelled cells were terminated by detaching the cells of four individual culture inserts and fixating them in 4 % paraformaldehyde. At day 0 the cells appeared to have reached confluence, when examined in the microscope. They were, therefore, co-cultured with astrocytes and BBB characteristics were induced by supplementing the media with

hydrocortisone, cAMP and RO. TEER was measured once a day. Cell divisions were tracked using the FACS Canto™ flow cytometer (BD bioscience, Albertslund, Denmark, DK). Prior to the flow cytometic analysis the instrument acquisition parameters were calibrated using FACS 7 colour beads (BD bioscience). The cells were gated using forward and side scatters to eliminate cell debri. Unlabelled BCECs were used as a negative control. The results were analysed using the FlowJo V10 software (TreeStar, Ashland, OR, USA).

***In vitro* transfection of BCECs with HcRed1-C1**

A plasmid encoding the protein HcRed1-C1 [194] was propagated into a competent CG5 E-coli strain by heat shock and purified with ion exchange chromatography using the NucleoBond®Xtra Midi EF plasmid DNA purification kit according to the manufacture's protocol. A commercially available transfection reagent Turbofect™ was used for the transfection studies. Isolated microvessels were seeded directly on collagen type IV- and fibronectin-coated 12 well polyethylene terephthalate, 1.0 µm hanging cell culture inserts. The cells were maintained in 4 µg/ml puromycin media for two days. Transfection was performed at different time points after isolation to obtain two different experimental conditions: non-confluent cells (T_{-1}), and confluent cells with defined barrier properties (TEER > 130 $\Omega*cm^2$) [66] (T_1) (Fig. 5). Cells added with transfectants at time point T_1 were grown in co-cultures conditions with astrocytes, and supplemented with hydrocortisone, cAMP and RO to further induce barrier properties, as described above. Cells grown at T_{-1} culture conditions were cultured without astrocytes, hydrocortisone, cAMP and RO.

For each culture insert, 1 µg plasmid was mixed with 100 µl culture medium (DMEM-F12) without serum and 2 µl Turbofect™. The solution was incubated at room temperature for 15-20 min for the complexes to form. The Turbofect™ solution (102 µl) was added to the luminal compartment in droplets that were dispersed throughout the wells, and the cells were then cultured for 48 hours in an incubator at 37 °C with 5 % CO_2 / 95 % air. TEER was measured once a day in order to access the effect of transfection on the integrity of the cultures. Non-transfected cells served as a control for TEER values. TEER data was analysed in the GraphPad Prism 5.0 software using a 2-way ANOVA with Bonferroni post hoc test.

After transfection the cells were fixed in 4 % paraformaldehyde for 10 min at room temperature and stained with DAPI. Some culture inserts were, additionally, stained for ZO1 using primary rabbit antibodies and Alexa Fluor 488-congujated secondary antibodies, as described above. The culture inserts were removed from their plastic supports with a razor blade and mounted on a glass slide using fluorescent mounting medium. Since the HcRed1-C1 protein encoded by the HcRed1-C1 plasmid excites its fluorescence excitation and emission maxima at 588 nm and 618 nm, respectively, it could easily be detected in the fluorescence microscope.

The transfection efficiency was analysed using qPCR with primers specific for HcRed1-C1 (Table I). Primers specific for claudin-5 were used to assess the origin of the transfected cells. Beta-actin was used as a control for normalisation. Non-transfected cells, not-reverse transcribed RNA and water served as negative controls. For each RNA sample, RNA was collected from four individual culture insert. This was repeated twice to obtain cells from two different isolations; which resulted in a sample value of six or eight (n=6-8) RNA samples for all three conditions (T_{-1}, T_1 and non-transfected cells (T_{CRTL})). Data were analysed by the GraphPad Prism 5.0 software using a 1-way ANOVA with Tukey's multiple comparisons post hoc test.

The transfection efficiency was further analysed using Flow cytometry. At the end of transfection the cells were washed in PBS, detached from the culture insert by trypsin and fixed in 4 % paraformaldehyde. Cells expressing red fluorescence protein HcRed1-C1 were counted using the MoFlo® Astrios™ Flow cytometer system (Beckman Coulter, Copenhagen, Denmark, DK). Prior to the flow cytometric analysis the instrument parameters were calibrated using SPHERO™ ultra rainbow fluorescent particles (3 μm) (Spherotech, Lake Forrest, IL, USA). Cells were gated using forward and side scatters to eliminate cell debris. HcRed1-C1 positive cells were gated based on auto fluorescence from unlabelled BCECs to ensure less than 1 % false positive events occurred. The results were analysed using FlowJo V10 software.

RESULTS

Establishment of blood-brain barrier in primary culture

Three cell types were used to construct the three different types of *in vitro* BBB models (Fig. 1a). Based on their respective expression of the cell specific markers ZO1, α-SMA and GFAP, the three isolated cell types were identified as BCECs, pericytes and astrocytes, respectively (Fig. 1b).

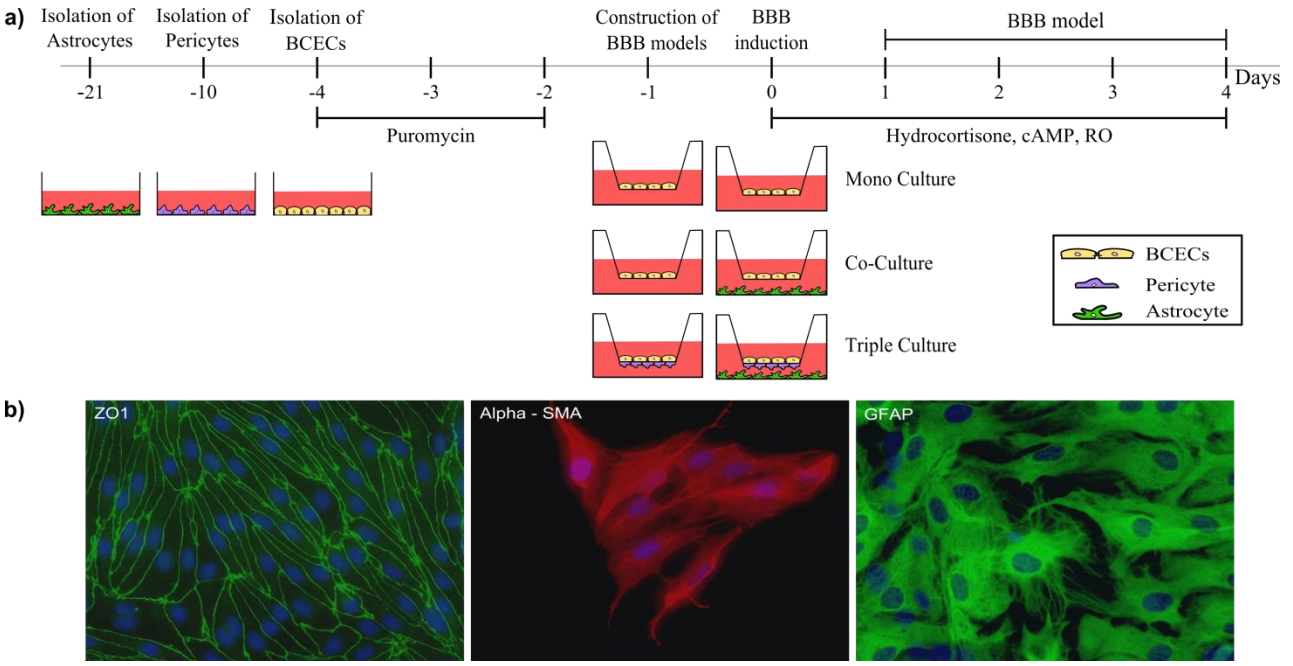

Figure 1. a) A schematic depicting the construction of the three different in vitro BBB models. *Monoculture consisting of brain capillary endothelial cells (BCECs, yellow) cultured on the upper side of the culture inserts, a non-contact co-culture of BCECs cultured on the upper side of the culture insert and astrocytes (green) cultured at the bottom of the well and a triple-culture consisting of pericytes (purple) cultured on the lower side of the culture insert, BCECs cultured on the upper side of the culture insert and astrocytes cultured at the bottom of the well. Astrocytes and pericytes were isolated in advance and cultured for approximately 21 days and 10 days, respectively. The BCECs were isolated on day -4 and cultured for three days until 80 % confluent. Puromycin was added to the media for the first two days. On day -1 the pericytes and/or endothelial cells were passaged to each side of the cultures inserts and left to adhere until the next day. On day 0 the BCECs were confluent and the culture inserts used for co- and triple culturing were moved to a 12 well culture plate containing astrocytes. The BCECs were, furthermore, stimulated with hydrocortisone, cAMP and RO.* ***b)*** *Immunocytochemical identification of the three isolated cell types using cell-specific markers. The cells were identified using antibodies against the tight junction protein ZO1, alpha smooth muscle actin (α-SMA) and glial fibrillary acidic protein (GFAP). The thorough expression of ZO1 is indicative of a substantial barrier maturity of isolated BCECs. The pericytes of the microvessel preparation is clearly identified based on their expression of α-SMA. Astrocytes of the glial cell preparation were identified by their GFAP expression. The cell nuclei in all illustrations are labelled using DAPI (blue). Magnification: x 400.*

Evaluating the integrity of the BCECs in these *in vitro* models, the TEER measurements revealed a rise in all types of cultures as a consequence to stimulation with hydrocortisone, cAMP and RO-201724. On day 2, the mono-, co- and triple culture models reached their maximal TEER values of 128 ± 46 Ω*cm^2, 299 ± 83 Ω*cm^2 and 331 ± 138 Ω*cm^2, respectively (Fig. 2a). The TEER values of co- and triple cultures were significantly higher than that of the monoculture, indicating the importance of co-culturing the BCECs with astrocytes and pericytes. The TEER values of triple culture were generally higher than that of the co-cultures albeit this difference was insignificant suggesting that the pericytes is unable to significantly raise the TEER values even further of what can be induced by astrocytes alone. Both co- and triple cultures maintained their TEER above 130 Ω*cm^2 until day 4, while monocultures failed to even reach 130 Ω*cm^2 at any experimental day examined. TEER measurements were not conducted beyond day 4, since TEER values decreased below 130 Ω*cm^2, indicating that the *in vitro* BBB model could not be considered sufficiently tight below this TEER value [66]. Additionally, on culture day 2, the passive permeability of BCECs to mannitol was analysed in culture inserts with different TEER values. It was demonstrated that higher TEER values clearly resulted in lower permeability to mannitol, indicating that TEER values above 130 Ω*cm^2 is sufficient to obtain a low permeability, which does not decrease further as the TEER increases (Fig. 2b).

The three *in vitro* models were also examined for their expression of genes signifying BCECs: The tight junction proteins claudin-5 and occludin, the adherens junction protein PECAM-1, the BBB transporter protein transferrin receptor 1 and the efflux transporter proteins ABCG2 and ABCB1. RNA was extracted from the BCECs on day 1 and used for RT-qPCR analysis. TEER was measured prior to RNA extraction and was found to be 73 Ω*cm^2 ± 39 for monoculture, 229 Ω*cm^2 ± 96 for co-culture and 243 Ω*cm^2 ± 75 for triple culture. BCECs grown in triple culture had a significantly higher expression of claudin-5 and PECAM-1 compared to the monoculture. Furthermore, ABCG2 was statistically higher expressed in the triple cultures compared to both mono- and co- cultures. This expression correlates well with the increasing TEER in the different culture models (Fig. 3). By contrast occludin, transferrin receptor 1 and ABCB1 did not show any statistic difference between the three groups.

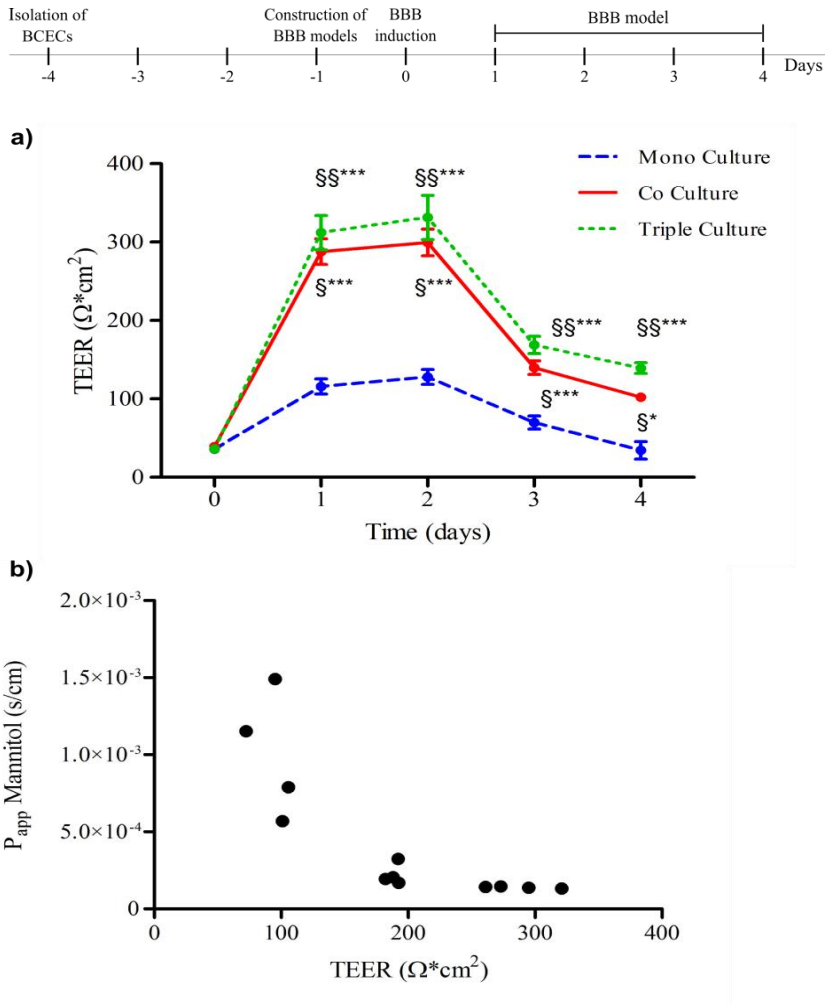

Figure 2. Maturity of the BBB in culture as revealed from measurements of trans-endothelial electrical measurements (TEER) (top) and passive permeability to mannitol (bottom). a) *Brain capillary endothelial cells (BCECs) were isolated on day -4 and cultured in media containing puromycin for two days. The different in vitro models were partly constructed on day -1, while co-culturing with astrocytes were initiated on day 0. TEER values were initially measured on day 0 just before stimulating the BCECs with hydrocortisone, cAMP and RO-201724 to increase their expression of tight junction proteins. TEER was measured daily for five days. Co-cultures of BCECs and astrocytes (red solid line), and triple cultures consisting of BCECs, astrocytes and pericytes (green dotted line) consistently displayed TEER values above 130 Ω*cm² from day 1 until day 3, which can be considered a substantial tight BBB [66]. The monoculture (blue stippled line) only containing BCECs showed a slight increase in TEER. Data are presented as means ± SEM (n = 24 culture insert of each of the three culture conditions monitored through the five days of experiments). §, significance between mono- and co-cultures, and §§, significance between mono- and triple cultures using 2-way ANOVA with Bonferroni post hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001. Significance was not observed between co- and triple cultures.* ***b)*** *The apparent permeability (Papp) of mannitol in cultured BCECs. Data are calculated based on single measurements from 12 culture inserts with TEER values ranging from 72.4Ω*cm² to 321 Ω*cm². The permeability to mannitol decreases as TEER values increase above 100 Ω*cm², which can be obtained by co-culturing the BCECs with pericytes and/or astrocytes.*

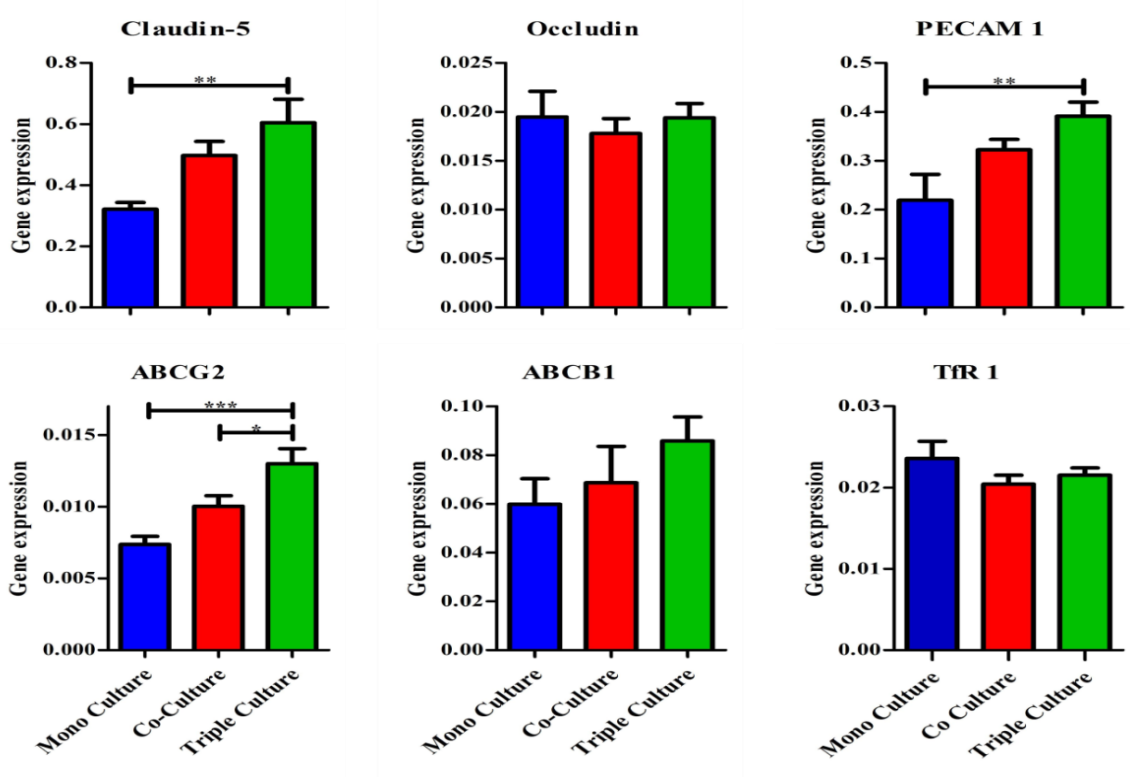

*Figure 3. Gene expression analysis of the hall mark proteins related to brain capillary endothelial cells (BCECs). RNA was obtained from BCECs grown in mono- (blue), co- (red) and triple- (green) culture conditions at day 1 (c.f. Fig 2a) and analysed for the expression of BCECs hallmark genes (claudin-5, occludin, PECAM-1, ABCG2, ABCB1 and Transferrin receptor 1 (TfR1)). The relative gene expression among the three culture conditions was statistically analysed using 1-way ANOVA with Tukey's multiple comparisons post-hoc test. Data are presented as sample means ± SEM (n = 8). *p<0.05, **p<0.01, ***p<0.001. The close contact in the triple culture setup between the pericytes and the BCECs could allow for contamination of the RNA preparation from the BCECs with pericytes. Therefore, analysis for expression of α-SMA that would reveal the presence of pericytes in the endothelial cell fraction was performed in RNA extracts from BCECs of all culture setups. The expression of α-SMA was found to be highest in monocultures and lowest in triple-cultures, but the expression level of α-SMA was very low indicating a diminutive pollution from pericytes in the fraction of BCECs (data not shown). Supporting this observation, immunocytochemical staining for α-SMA in the fraction of isolated BCECs for all three culture conditions revealed absence of cells containing α-SMA indicating that pericytes were virtually absent (not shown).*

Cell division among brain capillary endothelial cells related to the stage of blood-brain barrier maturity

Co- and triple cultures were superior to monoculture with respect to TEER values and passive permeability (Fig. 2). Since no significant difference was found in TEER between co- and triple culture, the co-culture was decided sufficient for further analyses concerning cell division and transfection studies. However, in order to establish a model in which different stages of barrier maturity could be analysed, the setup for establishing the co-culture model was slightly modified. The BCECs were seeded directly onto the culture inserts on the day of isolation (-3) (Fig. 4), and then visualised in a phase contrast microscope and found to

be confluent on day 0. From day 0 the previous described setup (Fig. 1a) was followed with BBB induction and co-culturing with astrocytes at day 0. BBB properties were present from day 1 to 3 with TEER value above 130 $\Omega*cm^2$ (Fig. 4).

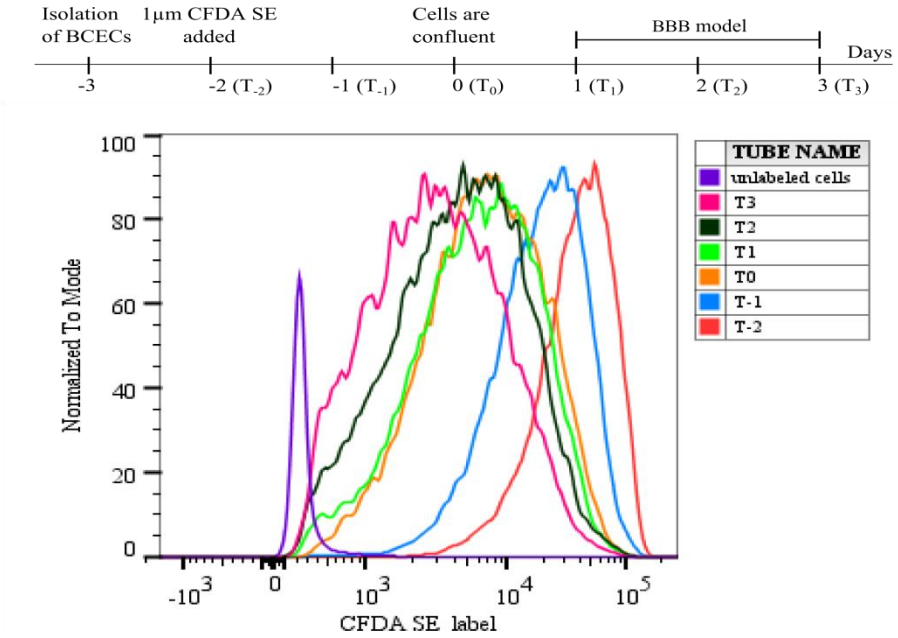

Figure 4. Analysis of the cell proliferation of brain capillary endothelial cells (BCECs) from barrier culture days -2 to 3 using a CFDA SE assay. *BCECs were isolated on day -3 and seeded directly onto cultures inserts. 1µM CFDA SE was added to the cells at day -2. After 30 min of incubation all the cells were labelled with CFDA SE and the first group were terminated (T_{-2}) (red). The CFDA SE label is inherited by the daughter cells through successive cell divisions, and with every cell division, each of the two daughter cells receives half the label [114]. Every day for five days one group (T_{-1} to T_3) (blue, orange, green, black and purple, respectively) were terminated. The BCECs were microscopically visualised to be confluent on day 0, and subsequently co-cultured with astrocytes and stimulated with hydrocortisone, cAMP and RO-201724. The cells were examined on BD FACS canto ™ and analysed with the FlowJo v10 software. The cells were gated using forward and side scatter to eliminate cell debri. Unlabelled BCECs (purple) were used as a negative control.*

To characterise the proliferative activity of the BCECs; a CFDA SE assay was used. The different stages of barrier maturity were defined according to cell confluence and it was, therefore, important to investigate whether the BCECs continued their division despite of reaching confluence as visualised in the phase microscope. The newly isolated BCECs were left to adhere to the culture insert for 24 hours by which 1 µM CFDA SE was added to the BCECs on day -2. Every day from day -2 to day 3 culture inserts were terminated and the amount of CFDA SE label in the BCECs was analysed by FACS (Fig. 4). The intensity of CFDA SE was largely reduced from day -2 until day-1 and again from day -1 to day 0, indicating that the BCECs were in a highly dividing state within this timeframe (Fig. 4). After day 0 the proliferation seemed to dramatically decrease as the BCECs retained approximately the same degree of CFDA SE label. This

observation corresponded well to the microscopic observations of BCECs reaching confluence at day 0. The curves corresponding to day 1 to day 3 began to widen, indicating a more diverse population of BCEC division judged from their different CFDA SE label. Additionally, there was a small reduction in the CFDA SE label on day 3 representing a small degree of cell division at this stage. The change in the level of CFDA SE label from day 1 to day 3 was, however, far from that seen from day -2 to day 0.

Transfection of BCECs in primary culture

The co-culture model was found to exhibit clear signs of BBB integrity for approximately two days (Fig. 2a). Therefore, a 48 hour time window was available to conduct the transfection experiments. The freshly isolated primary BCECs were seeded directly onto the culture insert and two different stages of BBB maturity were defined according to the modified setup (Fig. 4). The two stages were: Stage T_{-1}, which is defined as the immature state with the BCECs still undergoing cell division, and T_1, the mature state where the BCECs grown in co-culture with astrocytes had established true barrier properties. Only stage T_1 was co-cultured with astrocytes, as transfection at the immature state (T_{-1}) was initiated prior to the day of BBB induction (Fig. 5a). The BCECs were transfected with a plasmid encoding the red fluorescence HcRed1-C1 protein and TEER was measured daily. Non-transfected cells (T_{CTRL}) served as a control for TEER. Significantly lower TEER values were found at the immature stage (T_{-1}) compared to non- transfected cells (T_{CTRL}) in the same condition. No significant differences in TEER were found between BCECs transfected at the mature state (T_1) compared to correspondingly grown non-transfected cells. Both of these BCECs grown as T_1 had TEER values above 130 $\Omega*cm^2$ at the beginning of transfection dropping only to approximately 130 $\Omega*cm^2$ at the end of transfection (Fig. 5b).

Terminating the transfection, the BCECs were fixed and morphologically analysed for red fluorescence. HcRed1-C1 positive cells were found at both stages of barrier maturity. The HcRed1-C1 protein distributed to both cytoplasm and the nucleus of BCECs (Figs. 5c and 7a), which indicated that the HcRed1-C1 encoding plasmid was successfully delivered to the cell nucleus and expressed by the BCECs independent of barrier maturity. As described previously, pericyte contamination in the BCECs fraction could not be excluded, although the amount of pericytes was determined to be very low. Immunolabeling of transfected cells were performed after transfection and this labelling showed that the HcRed1-C1 positive cells also expressed the tight junction protein ZO1, which identifies them as BCECs (Fig. 5c). The cells depicted in figure 5c were transfected at day 1 (T_1); however, ZO1 and HcRed1-C1 positive cells were found in both transfection setups (data not shown).

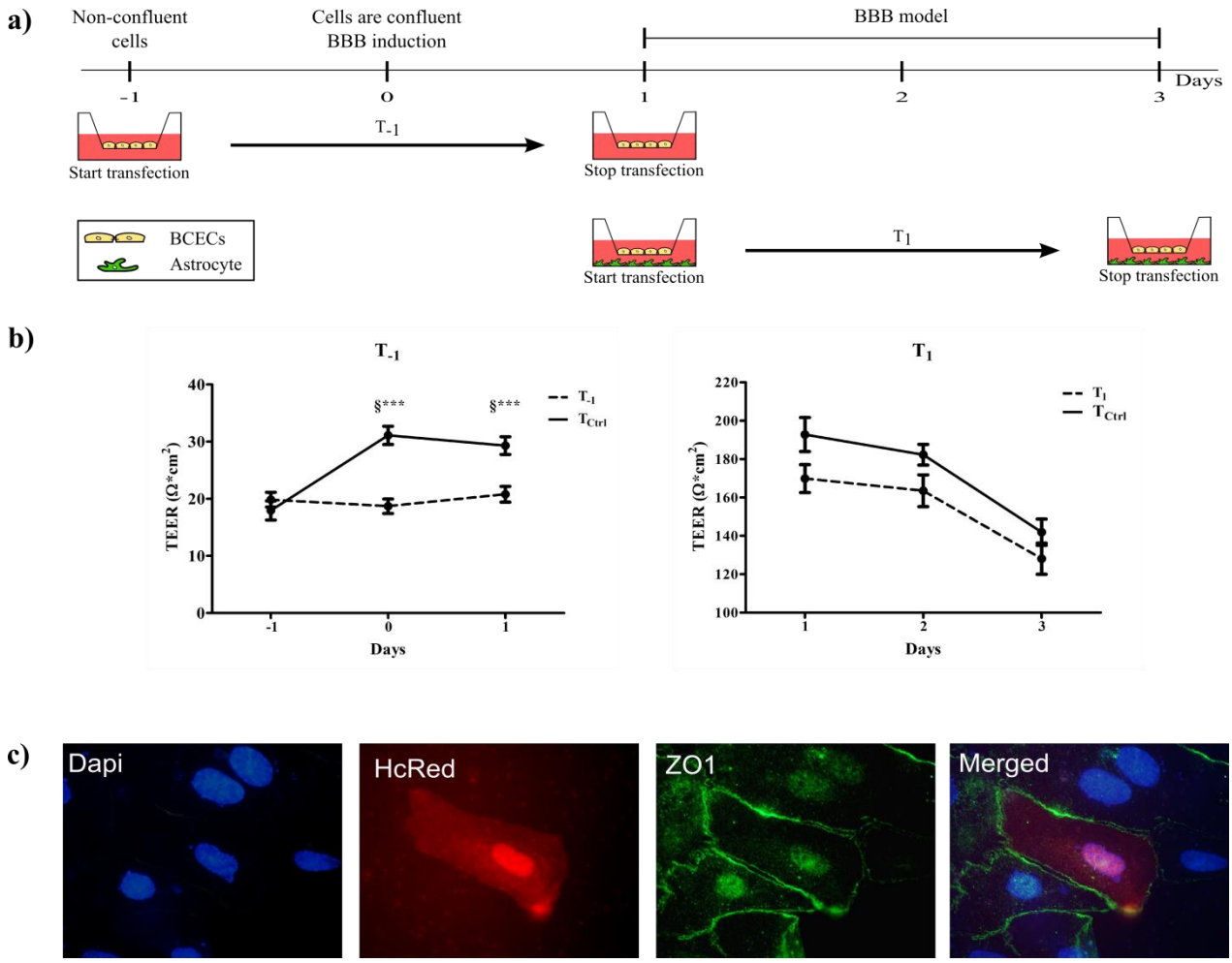

Figure 5. The effect of transfection on the integrity of the brain capillary endothelial cells (BCECs). a) *The experimental design used for in vitro transfection of BCECs. The BCECs (yellow) were isolated on day -3, seeded directly onto the culture inserts and left in puromycin media for two days. To investigate the transfection rate, the BCECs were transfected at two different stages of barrier maturity: T_{-1}) at an immature state, defined by dividing BCECs without barrier properties, and T_1) at a state of maturity defined as the BCECs being confluent and having barrier properties. The barrier properties were induced for T_1 on day 0 by co-culturing with astrocytes (green) in the presence of hydrocortisone, cAMP and RO-201724. BCECs transfected on day -1 (T_{-1}) were never stimulated to increase tight junction formation. On experimental day 1, barrier property was present (TEER above 130 Ω*cm^2) in culture state T_1 and lasted for at least 2 days. The cells were transfected with HcRed1-C1 and TEER was measured daily.* ***b)*** *The integrity of the transfected BCECs (T_{-1} and T_1) (stippled lines) was monitored by measurements of TEER and compared to non-transfected cells (T_{CTRL}) (solid line). Significant differences among the two states and their respective controls were analysed using a 1-way ANOVA with Tukey's multiple comparisons post-hoc test. § defines the significant difference between T_{-1} and T_{CTRL}. No significant difference was found between T_1 and T_{CTRL}. Data are presented as means ± SEM (n=22-34). *** p<0.001.* ***c)*** *To investigate the origin of the HcRed1-C1 positive cells, an immunocytochemical analysis was performed for the tight junction protein ZO1. The cells illustrated here were transfected at day 1 (T_1) and examined 48 hours after transfection. The illustrations depict a BCEC containing both the HcRed1-C1 protein and the ZO1 protein (green). Nuclei are counterstained with DAPI (blue). Magnification: x 1000.*

Correlation between barrier properties and transfection efficiency

The extent of HcRed1-C1 gene expression by BCECs was evaluated by RT-qPCR analysis of RNA extracted from BCECs at the end of transfection. This analysis confirmed HcRed1-C1 gene expression in the two culture conditions (Fig. 6a). The expression of the T_{-1} and T_1 were 3.7 ± 0.8 and 3.7 ± 0.6, respectively (Fig. 6a). No HcRed1-C1 gene expression was found in non-transfected cells (T_{CTRL}). The expression of the claudin-5 gene was, additionally, included in this analysis to simultaneously evaluate the endothelial origin of the transfected cells and the degree of tight junction formation in the different experimental groups. Claudin-5 was present in all three situations confirming the presence of BCECs. No significant differences were found in the expression pattern of claudin-5 among the three groups, although there was a tendency towards claudin-5 being lowest in the immature state (T_{-1}), which correlated with the lower TEER value (Fig. 5b).

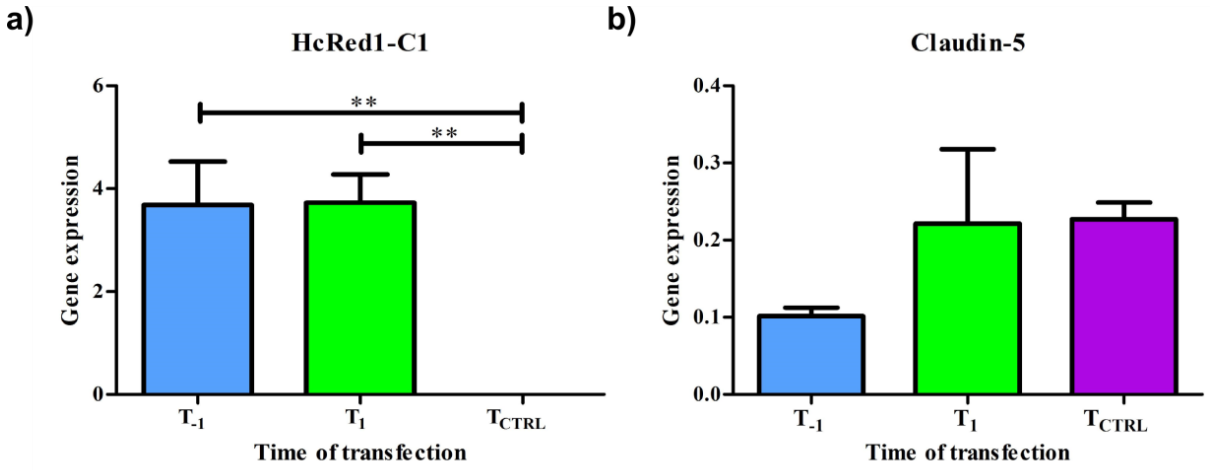

Figure 6. The transfection efficiency of brain capillary endothelial cells (BCECs) at different stages of barrier maturity examined by RT-qPCR. a) *Transfection with red fluorescent protein HcRed1-C1 was performed at the two different states of barrier maturity: T_{-1} (blue) and T_1 (green). The gene expression of the HcRed1-C1 protein was evaluated 48h after transfection. Non-transfected cells (T_{CTRL}) (purple) showed no HcRed1-C1 gene expression. The relative gene expression among the three culture stages was statistically analysed using 1-way ANOVA with Tukey's multiple comparisons post hoc test. Data are presented as sample means ± SEM (n = 6-8). **p<0.01.* ***b)*** *The gene expression of claudin-5 was included to evaluate the origin of the transfected cells and to measure the degree of tight junction formation at the various stages of barrier maturity. No significant differences were found in the expression pattern of claudin-5 among the three groups.*

The transfection efficacy was furthermore analysed by flow cytometry (Fig. 7b). The BCECs were transfected with the red fluorescence HcRed1-C1 protein at the two different stages of barrier maturity for 48 hours and the cells were detached from the culture insert, fixed and the percentages of HcRed1-C1 positive cells were counted using flow cytometry. Unlabelled cells were used to assess the auto fluorescence and to ensure less than 1 % of the HcRed1-C1 positive cells were false positive. A transfection efficiency of approximately 4 % in was found both in the immature dividing state (T_{-1}) and in the mature state (T_1).

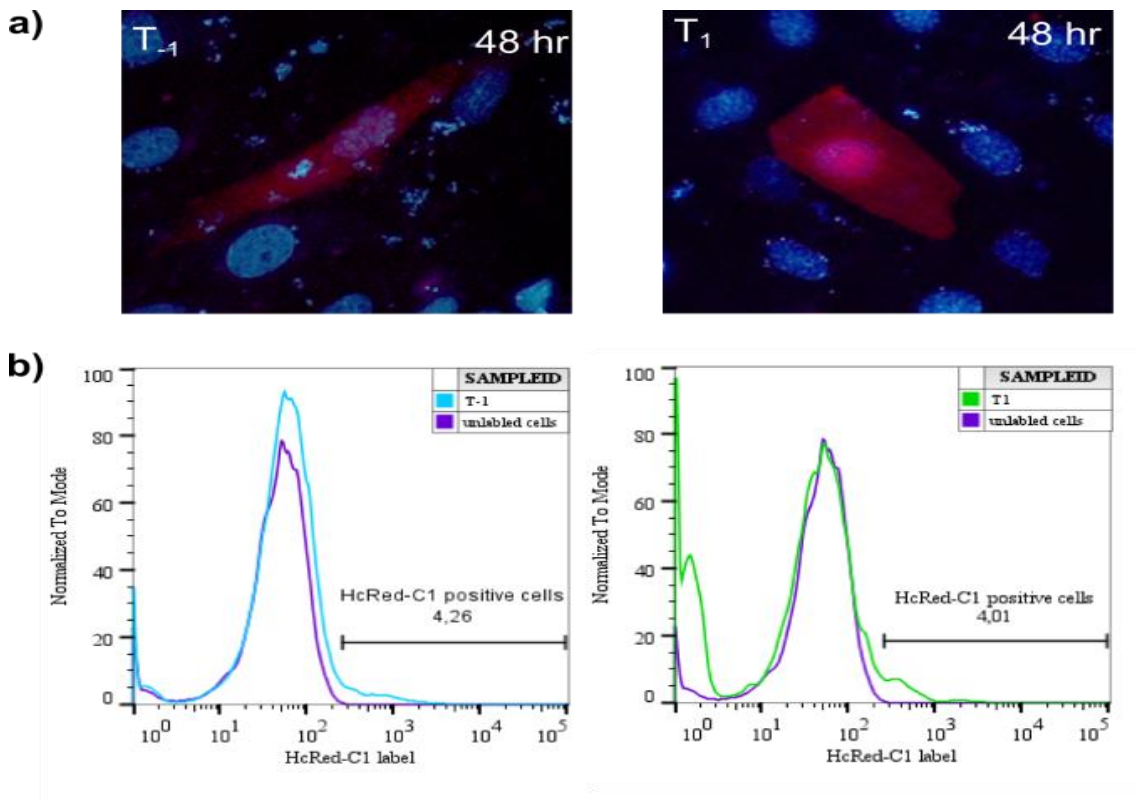

Figure 7. The transfection efficiency of brain capillary endothelial cells (BCECs) at different stages of barrier maturity examined by flow cytometry. a) *Expression of the HcRed1-C1 fluorescence protein as seen in BCECs 48 hours after transfection. HcRed1-C1 positive cells were expressed both stages of barrier maturity. The HcRed1-C1 protein is distributed to both the cell nucleus and cytoplasm. All images are in 400x magnification.* ***b)*** *The transfection efficacy of BCECs transfected at day -1 (T_{-1}) (blue) and 1 (T_1) (green) was assessed by flow cytometry. Results were analysed using the FlowJo V10 software. The cells were gated using forward and side scatter to eliminate cell debri. Additionally, the cells were corrected for auto fluorescence using unlabelled BCECs (purple) to ensure that less than 1 % of the HcRed1-C1 positive cells were false positive. A transfection efficacy of about 4 % was found in both the immature highly diving stage (T_{-1}) and in the mature non-dividing stage (T_1).*

DISCUSSION

Establishment of an *in vitro* BBB model

In this study we successfully isolated primary rat BCECs, pericytes and astrocytes to establish three different *in vitro* BBB models. The BCECs were cultured in a monolayer on culture inserts, and the effects of co-culturing the BCECs with astrocytes and pericytes were analysed based on the TEER values, and gene expression profiles using a variety of BBB specific or otherwise important proteins. The passive permeability was, additionally, analysed in relation to TEER and clearly demonstrated that TEER values above 130 $\Omega*cm^2$ were sufficient to obtain a low permeability. As the TEER values never exceeded 130 $\Omega*cm^2$, BCECs in monoculture did not reach the demands for a tight *in vitro* BBB model [66]. Astrocytes had the highest impact on BCECs as only a minor, non-significant effect was seen when culturing the BCECs in the presence of both astrocytes and pericytes with regard to TEER values. Astrocytes and pericytes were also able to increase the expression of claudin-5, PECAM-1 and ABCG2 by BCECs, while other proteins expressed by BCECs were unaffected when co-cultured with astrocytes and pericytes.

It is generally accepted that astrocyte-endothelial interactions are important for establishment and regulation of the BBB properties of BCECs *in vitro* [2, 3, 6], as this mimicking of the *in vivo* situation was shown to recapitulate many features of the BBB *in vivo* like a dramatic increase in TEER due to increased expression of tight junction proteins [68, 143, 176], a lowering in the passive permeability [143] and up regulation of many important nutrient transporters [67, 120]. Efforts have also been made to develop *in vitro* BBB models based on immortalised cell lines. However, the models never reach a TEER value close to the one obtained with primary cells, indicating that immortalised cell lines are difficult to culture *in vitro* with respect to maintaining *in vivo* characteristics, even in the presence of co-cultured cells, e.g. astrocytes or pericytes [78, 109, 181].

Pericytes has also been shown to induce the BBB phenotype in BCECs cultured *in vitro* [78, 143, 144], although the data of the present study did not find evidence that pericytes, in addition to astrocytes, to form a triple culture significantly improved the BBB characteristics of the BCECs (Figs. 2 and 3). That BCECs did not statistically increase their TEER under the influence of pericytes in the triple culture could reflect the differential state of the pericytes. The expression of α-SMA by pericytes affect the BBB properties of the BCECs, as α-SMA positive pericytes are unable to increase TEER, whereas the reverse applies to α-SMA negative pericytes [193]. When grown in monoculture pericytes differentiate into α-SMA negative state when exposed to bFGF but becomes α-SMA expressing in the presence of transforming growth factor β (TGFβ) [193]. We did not investigate whether the pericytes differentiated into α-SMA negative pericytes or remained α-SMA positive, when cultured together with astrocytes and BCECs. When culturing the pericytes together with BCECs and astrocytes in the triple culture, they invariably get exposed to both bFGF and TGFβ that are secreted by astrocytes [2, 62, 188, 195]. Moreover, bFGF is added to the cell culture media

used for culturing the BCECs. Possibly, the influence of TGFβ secreted by the astrocytes drives the pericytes towards the α-SMA expressing state that subsequently leads to a less BBB inductive function.

Transfection of BCECs in primary culture

Based on the co-culture conditions, we aimed to optimise settings for introducing new genetic material into primary BCECs at different states of barrier maturity, which enabled us to monitor the correlation between cell division, barrier maturity and transfection efficiency. Cells undergoing mitosis are more prone to gene therapy than non-dividing cells [196]. However, the majority of BCECs are post-mitotic *in vivo* [174], which makes it difficult to introduce expression of new genetic material [28]. This problem could have been overcome by using a viral vector, since these have mechanisms for nuclear internalisation [28]. Despite their high efficiency for cellular insertion of genetic material including cells of the CNS [22, 39, 157, 158], viral vectors may also exert some unwanted effect that mainly relate to safety issues [42]. We, therefore, aimed to investigate the transfection efficiency of BCECs taking a non-viral approach in using Turbofect, which is a cationic polymer that forms stable and positively charged complexes with DNA. According to the manufacturer, Turbofect has a low cytotoxicity, is independent of serum free conditions during transfection and is considered a suitable vector for transfection of primary cells and cells generally considered difficult to transfect.

We investigated the cell division profile of BCECs when they were seeded directly onto the culture insert. The cells were highly dividing during their immature state (T_{-1}) and non-diving in their mature stage (T_1). HcRed1-C1 positive cells were found in both stages of barrier maturity, hence indicating that successful transfection of the BCECs was not dependent on cell division. The highest transfection efficacy was expected in the immature state (T_{-1}) where the cells were undergoing cell division. We also investigated the gene expression profile of the HcRed1-C1 gene and counted the percentages of HcRed1-C1 positive cell in both stages of barrier maturities. Surprisingly, no differences were found in the gene expression profile of the HcRed-C1 gene between the immature, highly dividing state of the T_{-1} group, and the mature state without cell division (T_1). Additionally, no difference was found in the percentage of transfected cells, which in both cases were around 4 %.

We observed a minor, yet significant, decrease in TEER during the transfection period of the T_{-1} stage, indicating that the Turbofect-DNA complex might be slightly toxic to the cells during situations of lower cell density. The most interesting observation was, however, that BCECs grown in the mature state of the BBB were able to take up the HcRed1-C1 gene material and transcribe it without influencing the tightness of the cell layer. This highly suggests that transfection seems independent of cell division and that transfection of non-dividing cells with barrier properties is as effective as transfection of dividing cells without barrier properties. Previous transfection studies on BCECs were performed in monocultures without polarised conditions [89, 96, 194, 219]. Therefore, the entry point of the genetic material is difficult to analyse. In the

present study, we performed transfection under polarised conditions by adding the genetic material to luminal side, corresponding to the blood side *in vivo.* Theoretically, the genetic material could pass though the tight junction complexes and enter the BCECs via the abluminal side. This option was not likely in the present study when considering the size of the plasmid-Turbofect complex and the high expression of tight junction protein. We did, however, analyse for HcRed-C1 expression in astrocytes present on the abluminal side of the BCECs by fluorescence microscopy and found no expression within the astrocytes, indicating that passage to the abluminal side by the plasmid-Turbofect complex was absent (data not shown).

Cytoplasmic delivery may only be one of several obstacles with the design of drug carriers. The ultimate challenge might be the entry of genetic material into the nuclear envelope. Molecules smaller than approximately 40 kDa are able to diffuse passively into the nucleus via the nuclear pore complexes, while macromolecules larger than 60 kDa requires a nuclear localisation sequence. The size of plasmid DNA makes it unlikely that nuclear entry occurs through passive diffusion [104]. The transfection efficacy has been reported to be higher in mitotic cells than in quiescent counterparts [28, 59, 196], which has led to the idea that plasmid DNA enters the nucleus during the disassembly of the nuclear envelope during mitotic cell division [104, 196]. Studies investigating the correlation between mitotic activity and transfection efficacy revealed that transfection was probable even when cells were arrested in the G1phase [28, 59, 196], which indicates that plasmid DNA might permeate the nuclear pore complexes by a mechanism that resembles the active transport of polypeptides larger than 60 kDa [103, 104]. Therefore, the transfection of BCECs, when they enter their quiescent stage, may relate to this active transport of plasmid DNA though the nuclear pore complex in a cell cycle stage not directly related to cell division.

The strategy of manipulating BCECs into synthesising therapeutic proteins proved feasible both *in vitro* and *in vivo* [88, 89, 194]. Many therapeutic polypeptides have been acknowledged for their neuroprotective and neurodegenerative effects [21, 100], and, therefore, prospects of using BCECs as protein factories to ease the transport of proteins into the brain without having to pass the BBB is substantial [111]. The present study is to our knowledge the first to demonstrate non-viral gene therapy to BCECs in cultures with defined BBB properties, which opens for future studies on how non-viral gene therapy to BCECs can lead to protein secretion with the perspective of enabling therapeutic protein to target neurons inside the CNS otherwise inhibited to access due to the restraints of the BBB. This therapeutic strategy could be beneficial in the treatment of inherited diseases affecting the CNS like lysosomal storage diseases and classical neurodegenerative disorders like Parkinson's and Huntington's diseases, as these disease could benefit from an increased availability inside the brain of enzymes and growth factors both proteins in nature [111].

In conclusion, we have constructed an *in vitro* model of the BBB that resembles the *in vivo* situation. Non-viral transfection enabled de novo gene expression even in conditions with low mitotic activity, hence simulating the *in vivo* condition. Transfection of BCECs was independent of cell division and as effective as

transfection of dividing cells without barrier properties. Noteworthy, transfection did not disrupt the integrity of the BBB judged from the conserved expression of tight junction proteins. The data suggest that non-viral gene therapy transforming BCECs into protein factories might denote a suitable strategy to enable support of otherwise non-permeable proteins in conditions with CNS disorders.

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Study II:

Transfection of Brain Capillary Endothelial Cells for Protein Synthesis and Secretion: Evaluation on the Strategy for Drug Delivery to the Brain

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TRANSFECTION OF BRAIN CAPILLARY ENDOTHELIAL CELLS FOR PROTEIN SYNTHESIS AND SECRETION: EVALUATION ON THE STRATEGY FOR DRUG DELIVERY TO THE BRAIN

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Running title: Gene therapy at the blood-brain barrier

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Abbreviations:

BBB, blood-brain barrier; BCECs, Brain capillary endothelial cells; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; BSA, Bovine serum albumin; CNS; central nervous system; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; EPO, Erythropoietin; FITC, Fluorescein isothiocyanate; GDNF, glial cell-derived neurotrophic factor; GH1, Growth hormone 1; HBMEC, human brain microvascular endothelial cells; MBEC4, mouse brain capillary endothelial cell line; PBS, phosphate buffered saline; RBE4s, rat brain endothelial cell line 4; RBECs, Rat brain endothelial cells; TMB, 3,3',5,5' Tetramethylbenzidine;

ABSTRACT

The potential for treatment of chronic disorders affecting the central nervous system (CNS) is complicated by the inability of several drugs to cross the blood-brain barrier (BBB). Non-viral gene therapy applied to brain capillary endothelial cells (BCECs) denotes a novel approach to overcome the restraints in this passage, as turning BCECs into recombinant protein factories by transfection could result in protein secretion into the brain. The aim of the present study was to investigate the possibility of transfecting BCECs for recombinant protein synthesis. Gene expression of recombinant proteins with neuroprotective potential was enabled in primary rat brain endothelial cells (RBECs). Their expression pattern was compared with those of the immortalised rat brain endothelial cell line (RBE4s), and the cervix cancer cell line (HeLa) cells using RT-qPCR analyses. The evidence for protein synthesis and secretion was obtained by detection of FLAG tagged peptides fused to the C-terminal of any of the three recombinant proteins; growth factor (GH1), erythropoietin (EPO) and brain derived neurotrophic factor (BDNF). The study opens for knowledge on how non-viral gene therapy to BCECs can lead to protein secretion with the perspective of enabling therapeutic protein to target neurons inside the CNS otherwise inhibited to access due to the restraints of the BBB. This therapeutic strategy could be beneficial in treatment of neurological diseases and classical neurodegenerative disorders.

Keywords Blood-brain barrier, endothelium, gene therapy, primary culture

INTRODUCTION

Disorders affecting the central nervous system (CNS) like neurodegenerative disorders, tumours and infections are becoming more prevalent with great demands for development of new pharmaceutics, but the treatment is complicated because of the inability of most drugs to cross the blood-brain barrier (BBB), which explains why more than 98 % of drug candidates for treatment of CNS disorders never make it to the clinic [154, 163, 192].

The BBB is a dynamic physical and biological barrier between the bloodstream and the CNS. It controls the passage of endogenous and exogenous substances into and out of the CNS. The BBB is formed by brain capillary endothelial cells (BCECs) lining the cerebral microvasculature and non-fenestrated capillaries constitute its structural basis [1, 5, 6].

Attempts to deliver therapeutic drugs like exogenous neuroprotective proteins or their encoding genes to the CNS by intraparenchymal injection are risky, highly invasive and will not enable homogenous delivery throughout the brain. Disrupting the BBB using osmotic or pharmacologic approaches does not only allow for specific entry of the drug compound but also for components of the blood plasma of potential to harm the microenvironment of the brain [163, 192]. Therefore, it is highly relevant to develop methodologies that allows for non-invasive routes for drug delivery. The brain capillary endothelium is considered a target for gene therapy, because these endothelial cells were shown to secrete proteins into the brain parenchyma subsequent to non-viral transfection or viral transduction [88, 194].

Neurotrophins and growth factors are implicated in a variety of neurological disorders [171]. A general feature of many neurological disorders is the loss of specific populations of neurons. Several studies have reported on the potential of neurotrophins and growth factors to protect diseased or injured neurons from dying, induce neuronal sprouting and increase neuronal metabolism and function [13, 146, 207]. Some of these proteins include the growth hormone 1 (GH1), brain derived neurotrophic factor (BDNF) and erythropoietin (EPO). They are all believed to be useful as therapeutic agents in a wide variety of the neurologic disorders. Over the past decade, many therapeutic agents have moved from tissue culture studies and animal models into clinical trials using the gene therapy delivery approach to treat neurodegenerative diseases like Parkinson's and Alzheimer's disease [60, 69, 115, 116, 199]. The basic strategy of these clinical trials is to restore normal neurotrophin signalling. One proposed role of the neurotrophins and growth factors in neurodegenerative disorders originates from the neurotropic hypothesis, which states "that developing neurons compete with each other for a limited supply of growth factors generated by target tissue" [46, 171]. Neurons that successfully bind these limited growth factors will live, while the remaining will die. Therefore, therapeutic strategies need to restore the balance of neurotropic signalling either through replacement of growth factors or by modulating their signalling pathways [171].

The aim of this study is to establish a method to deliver secretory neuroprotective proteins across the BBB by transferring their gene into BCECs. Primary isolated rat brain endothelial cells (RBECs) were transfected with cDNA encoding one of the three recombinant proteins; GH1, EPO and BDNF, using the commercially available transfection agent Turbofect™. The expression and secretion of the recombinant proteins were subsequently analysed. The mode of gene expression and protein translation and secretion by RBECs of the three therapeutic proteins is examined *in vitro* and compared to the expression patterns found in the immortalised rat brain endothelial cell line (RBE4s) and the cervix cancer cell line (HeLa).

MATERIALS AND METHODS

Materials

pCMV6-Entry BDNF, pCMV6-Entry EPO, pCMV6-Entry GH1plasmids (Cat. No RC217190, RC21075 and RC215644, respectively), the Anti-DDK Magnetic Immunoprecipitation kit (Cat. No. AR100024) from Origene and the Macherey Nagel NucleoBond®Xtra Midi EF plasmid DNA purification kit (Cat. No. 740410) was purchased from AH diagnostics (Aarhus, Denmark, DK). The following reagents were purchased from Life Technology (Naerum, Denmark, DK): HAM's-F10 (Cat. No. 41550), Alpha-MEM (Cat. No. 32561), DMEM/F-12 (Cat. No. 31331), RPMI 1640 (Cat. No. 61870-010), fetal calf serum (Cat. No. 10270), penicillin G sodium, streptomycin sulphate (Cat. No. 15140) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cat. No. A11034). Bovine collagen type I (Cat. No 354231) and FACS 7 colour beads (Cat. No. 335775) were purchased from BD Bioscience (Albertslund, Denmark, DK). The following reagents were purchased from Sigma-Aldrich (Brondby, Denmark, DK): Competent CG5 Escherichia coli strain (Cat. No. G3169), collagen type IV (Cat. No. C5533), fibronectin (Cat. No. F1141), heparin (Cat. No. H3149), puromycin (Cat. No. P8833), rabbit anti-FLAG M2 antibody (Cat. No. F7425), Fluorescein isothiocyanate (FITC) conjugated mouse anti-FLAG M2 antibody (Cat. No. F4049), Peroxidase conjugated anti-FLAG M2 antibody, (Cat. No. A8592), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No. D9542) and 3,3',5,5' Tetramethylbenzidine (TMB) (Cat. No. T0565). Insulin transferrin sodium selenite (Cat. No. 11074547001) and basic fibroblast growth factor (bFGF) (Cat. No. 1363697) were purchased from Roche (Hvidovre, Denmark, DK). Bovine serum albumin (BSA) (Cat. No. EQBAH62) was purchased from Europa Bioproducts (Cambridge, United Kingdom, UK). Plasma derived bovine serum (Cat. No. 60-00-810) was purchased from First Link (Wolverhampton, United Kingdom, UK). Gentamicin sulphate (Cat. No. 17-518Z) was purchased from Lonza Copenhagen (Vallensbaek Strand, Denmark, DK). Fluorescence mounting media (Cat. No S3023) were purchased from DAKO (Glostrup, Denmark, DK). Turbofect™ (Cat. No. R0531), phosphate buffered saline (PBS) (Cat. No SH3025802) and all reagents for qPCR were obtained from Thermo Scientific, except primers that were synthesised by TAG Copenhagen (Frederiksberg, Denmark, DK). Amicon®Ultra-15 Centrifugal filter devices (Cat. No. UFC901024) were obtained from Merk Milipore (Hellerup, Denmark, Dk).

Plasmid DNA

The human Myc-DDK-tagged Open Reading Frame (ORF) clones pCMV6-Entry GH1, pCMV6-Entry EPO and pCMV6-Entry BDNF plasmids were propagated in a competent CG5 Escherichia coli (E-coli) strain by heat shock and purified with ion exchange chromatography with the NucleoBond®Xtra Midi EF plasmid DNA purification kit according to the manufacture's protocol.

Cell cultures

Primary cultures of RBECs were prepared from 2-3 week old Sprague Dawley rats as described previously [33, 143]. RBECs were maintained in DMEM/F12 supplemented with 10 % plasma derived bovine serum, heparin, insulin, transferrin, sodium, selenite, 10 µg/ml gentamicin sulphate and 1 ng/µl bFGF. 4 µg/ml puromycin was, additionally, added to the culturing medium for the first three days of culturing. All surface areas for culturing RBECs were collagen IV-fibronectin coated. RBECs were isolated 3 days prior to the experiment.

Immortalised RBE4s were cultured in medium containing 50 % HAM's F-10 and 50 % Alpha-MEM with Glutamax-1 supplemented with 10 % Fetal Calf Serum, 100 U/mL Penicillin G Sodium, 100 µg/mL Streptomycin Sulphate, 300 µg/mL Geneticin sulphate and 1 ng/µl bFGF. All surfaces for culturing RBE4s were coated with 3.0 mg/ml bovine collagen type I in 0.012 M HCL.

Cervix cancer cells (HeLa) were cultured in RPMI 1640 supplemented with 10 % fetal calf serum, 100 U/mL Penicillin G Sodium and 100 µg/mL Streptomycin Sulphate. All cells were cultured in an incubator with humidified 5 % CO_2 / 95 % air at 37 °C.

***In vitro* transfection**

All three cell types were seeded in six well plates at a density of 30.000 cell/cm^2 and left to adhere overnight. For each well in a six well plate 2 µg plasmid DNA encoding either GH1, EPO or BDNF were mixed with 200 µl serum free medium and 4 µl of the commercially available transfection agent Turbofect™. The solution was incubated for 20 min at room temperature, to enable the complexes to form, before being added in droplets dispersed throughout the well. The cells were incubated with the Turbofect-plasmid solution for 48 hours in an incubator with humidified 5 % CO_2 / 95 % air at 37 °C.

The pCMV6-Entry vector used for all three plasmids has a C-terminal Myc-DDK tag (equal to FLAG, a trademark of SIGMA-Aldrich). Therefore, expression of all the plasmids results in recombinant proteins with a short hydrophilic 8-amino acid peptide (N- Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys -C) most likely located on the surface of the proteins, where it is easily accessible to anti-FLAG antibodies. Because of the small size of the FLAG peptide, it was not expected to affect other epitopes or domains, or to alter the intracellular

transport and secretion of the recombinant proteins. Therefore, the FLAG tag was used as the primary target for detecting the recombinant proteins.

Gene expression analysis

Cells transfected in six well plates were used for RT-qPCR analysis. RNA was extracted using the GeneJet RNA purification kit according to the manufactures protocol. A total of six to nine RNA samples were obtained from all three cell types transfected with each of the three different plasmids. Each RNA sample corresponded to two wells in a six well plate. RNA samples were treated with DNase I enzyme to remove genomic DNA contamination. 100 ng of each DNA-free RNA sample were used as template for RT-qPCR. cDNA synthesis was carried out using the Thermo Scientific Maxima H Minus First Strand cDNA Synthesis Kit. To assess the expression profile of the transfected cells quantitative PCR were performed using primers specific for the three plasmids (for additional information on primers see Table 1). β-Actin was used as a housekeeping control gene for normalisation purpose. RBECs and RBE4s were normalised to rat β-actin, while HeLa cells were normalised to human β-actin. 0.5 µl cDNA and 10 pmol of each primer were used together with Luminaris Colour Probe qPCR Master Mix. Non-transfected cells, non-reverse transcribed RNA and water served as negative controls. Quantitative PCR was performed using the Stratagene Mx3000P™ QPCR system (Agilent Technologies, Horsholm, Denmark, DK). PCR conditions were 95 °C for 10 min, 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The relative expression of mRNA was calculated according to Pfaffl [169] and analysed in the GraphPad Prism 5.0 software using a 1-way ANOVA with Tukey's multiple comparisons post hoc test.

***Table 1**: primer sequences used for RT-qPCR analysis*

Primer	**Reference sequence**	**Forward primer**	**Reverse primer**
GH1	RC215644 NM_000515	AACTACGGGCTGCTCTACTGCTTCA	ATCCTCTTCAGAGATGAGTTTCTGCTCG
EPO	RC210775 NM_000799.2	GGAGGCCGAGAATATCACGACGGGC	CCTGCCAGACTTCTACGGCCTGCTG
BDNF	RC217190 NM_170735	GAACTCCCACTGCCGAACTACCCAG	GCGTACGCGTTCTTCCCCTTTTAATG
β-actin rat	NM_031144.3	CCTCTGAACCCTAAGGCCAACCGTGAA	AGTGGTACGACCAGAGGCATACAGGG
β-actin human	NM_001101.3	CCGCCGCCAGCTCACCAT	GCCCCACGATGGAGGGGAAG

Immunocytochemistry

Cells used for immunocytochemistry were seeded on coverslips in a 24 well plate at a cell density of 30.000 cells/cm^2 and left to adhere overnight. Transfection was performed as described above, however, in order to scale the experiment down to a 24 well plate, only 1 µg plasmid DNA was mixed in 100 µl serum free medium together with 2 µl Turbofect™ pr. well. After 48 hours of transfection, the cells were washed in 0.1 M PBS, pH 7.4 and fixed for 10 min in 4 % paraformaldehyde at room temperature. The cells were permeabilised and blocked for unspecific binding of primary antibody using PBS supplemented with 3 % BSA and 0.2 % Triton-X-100 for 30 min. All incubations were performed at room temperature with mild agitation. Primary rabbit anti-FLAG M2 antibody was used in a dilution of 1:500 in PBS supplemented with 3 % BSA and 0.2 % Triton-X-100. The cells were incubated with primary antibodies for one hour. Alexa Fluor 488-conjugated goat anti IgG antibodies was used in a dilution of 1:500 in PBS supplemented with 3 % BSA and 0.2 % Triton-X-100 and incubated for 30 min. Non-transfected cells were used as control for non-specific binding of primary antibodies. Nuclei were counterstained with DAPI. The cells were mounted on glass slides with fluorescent mounting media and examined in a fluorescence microscope (Axiovert 200, Carl Zeiss, Germany). Captured images were corrected for brightness and contrast in Adobe Photoshop C2 or ImageJ.

FACS

The transfection efficiency was analysed using FACS analysis. Cells used for FACS were seeded in six well plates with a cell density of 30.000 cells/cm^2 and left to adhere overnight. Transfection was performed as described above. After 48 hours of transfection, the cells were washed in 0.1 M PBS, pH 7.4 and detached from the culture wells by trypsin and fixated for 10 min in 4 % paraformaldehyde at room temperature. Cells from three wells in a six well plate were pooled together. The cells were blocked for non-specific binding of primary antibody using PBS supplemented with 3 % BSA and 0.2 % Triton-X-100 for one hour at 37 °C. The cells were then added with mouse anti-FLAG M2 antibody covalently conjugated to FITC in a concentration of 1:500 for RBECs and HeLa and 1:1000 for RBE4s. The cells were incubated with the antibody at room temperature for one hour, and finally washed twice in PBS. Transfection efficiency was analysed using the FACS Canto™ flow cytometer (BD bioscience, Albertslund, Denmark, DK), which prior to the flow cytometic analysis was calibrated using FACS 7 colour beads. The cells were gated using forward and side scatters to eliminate cell debris and approximately 60.000 cells were analysed in each sample. Non-labelled cells were used a control for auto fluorescence and non-transfected cells were used as a control for non-specific binding of the antibody. FLAG positive cells were gated based on the auto fluorescence from non-transfected cells. The results were analysed using the FlowJo V7.6.5 software (TreeStar, Ashland, OR, USA).

Protein secretion analysis

Cells transfected in six well plates were used for the protein secretion analysis. 48 hours after transfection, the medium from the transfected cells were collected and stored at -80 °C. Prior to use the cell medium was thawed and concentrated using the Amicon®Ultra -15 Centrifugal filter devices to decrease the volume of the cell medium from 120 ml to 2 ml. Additionally, the transfected cells were washed twice in PBS and lysed in RIPA buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Ingepal, 1 % sodium deoxycholate, 0.1 % SDS) containing protease inhibitors. Cell lysates were incubated on ice for 20 min and cleared by centrifugation at 14000 x g for 10 min. The cell lysate supernatant was collected and stored at -80 °C until further use.

The FLAG tagged recombinant proteins were collected by immunoprecipitation using the anti-DDK magnetic immunoprecipitation kit according to the manufactures protocol. In brief, pre-washed anti-DDK magnetic beads were incubated overnight with either the cell lysate or the concentrated cell medium at 4 °C with agitation. Medium from non-transfected cells and lysis buffer were added as negative controls, while a BSA-DDK protein control was used as a positive control. After overnight incubation the magnetic beads were washed and the FLAG tagged proteins eluted in 2x SDS-PAGE sample buffer after 10 min of boiling (95 °C). The eluted samples were analysed on a 4-12 % Run Blue SDS gel in RUN BLUE running buffer together with the Page Ruler™ Plus prestained Protein ladder. The samples were then transferred to a nitrocellulose membrane by wet blotting for one hour at 100 V. The membrane was blocked in 3 % non-fat milk in PBS for one hour at room temperature, after which the membrane was incubated with Anti-FLAG M2 monoclonal antibody conjugated with peroxidase (1:1000 in blocking buffer) overnight with agitation at 4 °C. Then the membrane was washed three times in PBS and added with TMB solution to visualise the protein bands. Finally, the membrane was washed in double distilled water, dried, photographed and stored in the dark.

RESULTS

Expression of recombinant proteins after transfection

RBECs, RBE4s and HeLa cells were all transfected with plasmids encoding GH1, EPO, or BDNF, using the commercially available transfection agent Turbofect™. The relative gene expression of the recombinant proteins GH1, EPO and BDNF was investigated in RBECs, RBE4s and HeLa cells, after transfection. The primers specific for GH1, EPO and BDNF were all designed to bind within the specific gene sequence and in the FLAG region, ensuring that only the expression of the recombinant proteins were analysed (Table I).

All three cell types were found to express GH1, BDNF and EPO, however, a significantly higher gene expression ($p<0.001$) of any of the three recombinant proteins were observed in HeLa cells compared to that observed in both RBECs and RBE4s (Fig. 1). No significant differences were found in the gene expression levels between RBECs and RBE4s of any of the three recombinant proteins ($p>0.05$). Interestingly, the same expression pattern of GH1, EPO and BDNF were found in all three cell types. The gene expression of EPO was significantly higher than GH1 in RBE4s ($p<0.05$) and significantly higher than both GH1 and EPO in HeLa cells ($p<0.001$). The same tendency was found in RBECs; however, the difference was not statically significant. BDNF showed the lowest expression in all three cell types. Hence, all cells showed the highest expression of EPO and the lowest expression of BDNF. Non-transfected cells showed no gene expression of any of the three proteins (data not shown).

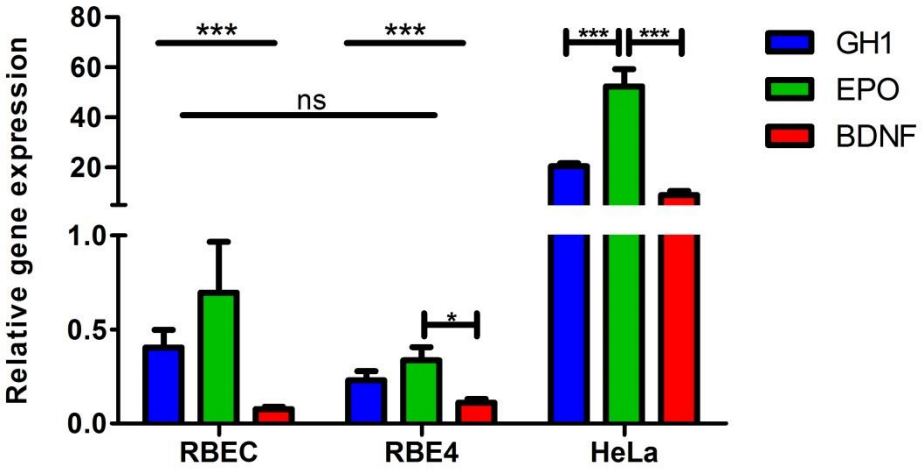

Figure 1. *RBECs, RBE4s and HeLa cells express the recombinant proteins GH1, EPO and BDNF. Gene expression is significantly higher in HeLa cells compared to both RBECs and RBE4s. No significant difference in gene expression was found between RBE4s and RBECs. EPO (green) is generally higher expressed than GH1 (blue) and BDNF (red) in all three cell types. Data were analysed using the GraphPad Prism 5,0 software and the relative expression in each cell type was statistically evaluated using a 1-way ANOVA with Tukey's multiple comparisons post hoc test. Data are presented as means ± SEM (n=6-9). *p<0.05, ***p<0.001 Ns, Non-significant.*

In the protein analysis, investigated by immunocytochemistry, the anti-FLAG antibody labelled any of the three cell types irrespectively of their transfection (Fig. 2). The most frequent expression was found in HeLa cells and in comparison clearly fewer RBECs and RBE4s expressed the three recombinant proteins. The few positively transfected endothelial cells showed a rather low expression of the proteins, compared to HeLa cells. The recombinant proteins were all found within the cytoplasm and a high expression was often observed adjacent the nucleus. This location is possibly corresponding to the site of the rough endoplasmic reticulum and the Golgi apparatus, which are involved in the translation and packaging of proteins and the latter is particularly important in the processing of proteins for secretion. It was not possible to detect binding of anti-FLAG antibodies in the non-transfected cells of any origin.

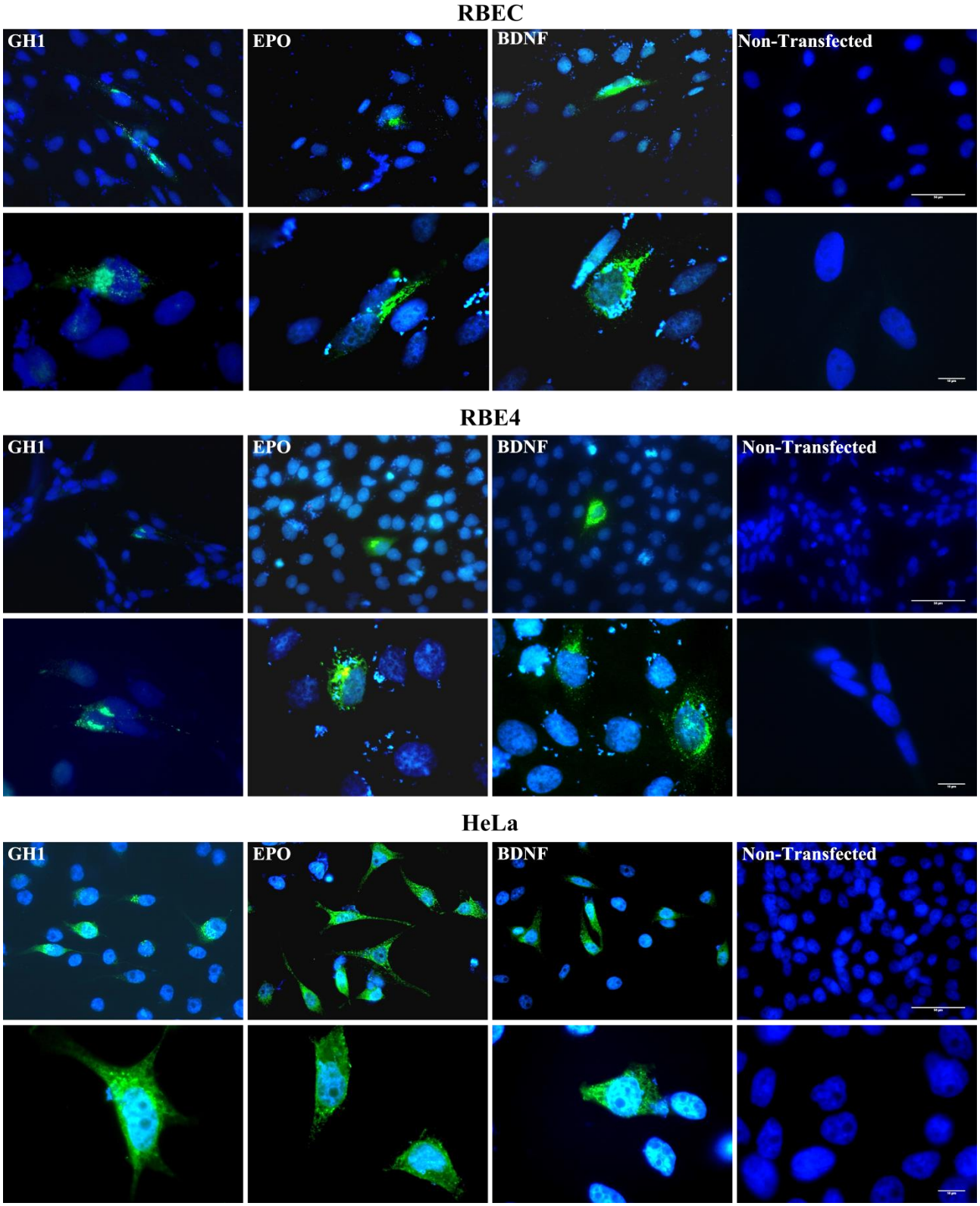

***Figure 2**. RBECs, RBE4s and HeLa cells express the FLAG tagged recombinant proteins GH1, EPO and BDNF. The cells were immunolabled using anti-FLAG primary antibodies and secondary Alexa Fluor 488 antibodies. Cells expressing the FLAG tagged recombinant proteins GH1, BDNF and EPO are all seen in green-face with nuclei counter stained with DAPI (blue). Non-transfected cells were used as a control for non-specific binding of the antibodies and showed no anti-FLAG expression. Scale bars 50µm (top row), 10µm (bottom row)*

A FITC-conjugated anti-FLAG antibody was used in all FACS assays and was used to investigate the percentage of cells expressing the recombinant proteins (Fig. 3). Non-labelled cells were used to monitor the cell auto fluorescence, while binding to non-transfected cells was used to monitor possible non-specific binding of the antibody. It was evident that non-specific binding of the anti-FLAG antibody occurred in the non-transfected cells. Therefore, FLAG positive cells were gated based on emission from the non-transfected cells to ensure less than 1 % false positive emissions occurred.

Some degree of non-specific binding of the antibody was found when analysing the transfection efficiency in RBECs resulting in virtually no evidence for transfection with GH1 and BDNF (Fig. 3). However, a large shift in FITC fluorescence was observed when analysing for EPO transfection in RBECs revealing a transfection efficiency of 8.5 % EPO positive RBECs. The largest degree of non-specific binding by the antibody was seen in RBE4s, in where a clearly visible shift in the fluorescence emitted by FITC was seen between non-labelled RBE4s and non-transfected RBE4s, resulting in virtually no FLAG positive RBE4s. HeLa cells on the other hand exhibited nearly no non-specific binding of the antibody and a high percentage of FLAG positive cells. HeLa cells transfected with EPO revealed a high transfection efficiency of nearly 20 % and the transfection efficiency of GH1 and BDNF positive transfected HeLa cells were found to be approximately 16 % and 13 %, respectively.

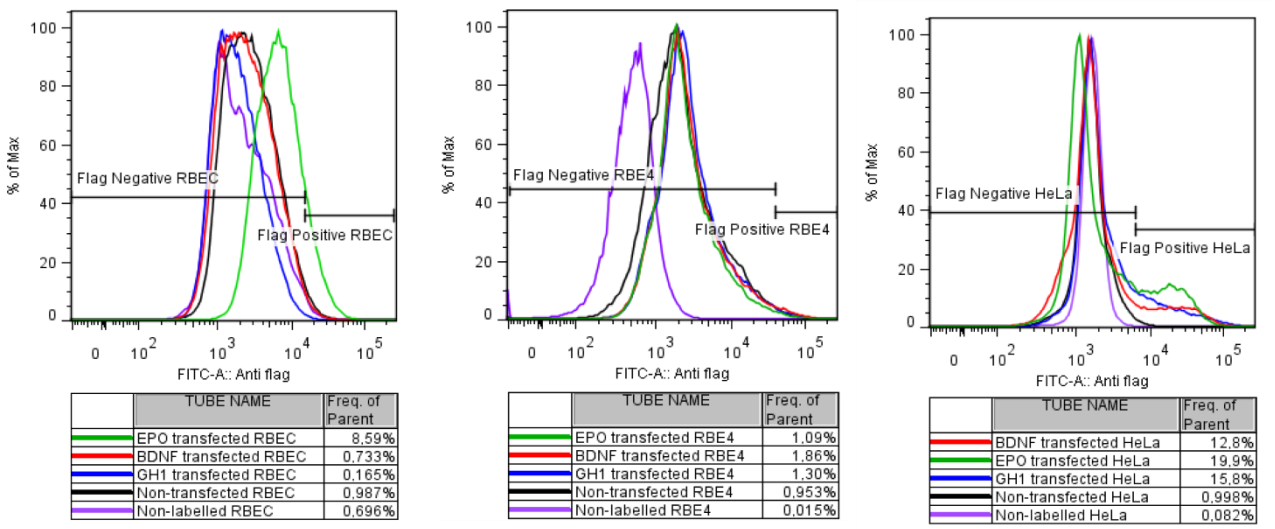

Figure 3. *HeLa cells show higher transfection efficiency of all the three recombinant proteins compared to RBECs and RBE4s. Cells transfected with EPO (green) generally show higher transfection efficiency than GH1 (blue) and BDNF (red). RBE4s showed a high degree of non-specific binding of the anti-FLAG antibodies, which highly influenced the transfection efficiency found in these cells. The transfection efficiency was analysed using FACS 48 hours after transfection. Cells were immunolabled with anti-FLAG FITC conjugated antibodies. Non-labelled cells (purple) were used as a control for auto fluorescence and non-transfected cells (black) were used as a control for non-specific binding of the anti-FLAG antibodies. Cells were examined on BD FACS Canto flow cytometer and analysed with the FlowJo 7.6.5 software. The cells were gated using forward and side scatter to eliminate cell debri and FLAG positive cells were gated based on auto fluorescence/non-specific binding from the non-transfected cells to ensure less than 1 % false positive events occurred.*

Secretion of recombinant proteins after transfection

GH1, EPO and BDNF are naturally occurring proteins, exhibiting specific physiological functions following their secretion from cells. Transfected cells would, therefore, in principle, secrete the recombinant proteins into their surroundings, in this case the cell culture medium. Accordingly, the cell culture medium was collected from the three different cell types and subsequent the FLAG expression of the recombinant proteins were immunoprecipitated and analysed by Western blotting using an anti-FLAG peroxidase conjugated antibody. All three recombinant proteins were found in the cell culture medium extracted from the HeLa cells indicating that the transfected HeLa cells had secreted GH1 (MW 25 kDa), EPO (MW 18kDa) and BDNF (26 kDa) into the culture medium (Fig. 4). Three different protein bands were observed around 27 kDa approximately corresponding to the predicted protein band sizes. In contrast, it was not possible to detect FLAG protein in the culture medium extracted both from RBECs and RBE4s. In an attempt to detect FLAG protein in the cell culture medium from the two endothelial cell types, the medium was collected from more than 60 wells in a 6 well plate each containing 2 ml meaning approximately 120 ml from each transfection experiment were collected. However, in spite of having concentrated the 120 ml to approximately 2 ml, which was examined by Western blotting, it was not possible to detect FLAG proteins from RBECs or RBE4s. Likewise some non-specific binding of the anti-FLAG antibody was seen, suggesting that this antibody displayed some non-specific binding. The specificity of the antibody was further questioned since homogenates of all the transfected cells failed to reveal binding of the anti-FLAG antibodies (data not shown). After blotting the SDS gels were stained with Coomassie Brilliant Blue, to ensure the proteins were successfully blotted to the membrane. The Coomassie stain revealed no proteins bands on the SDS gels after blotting (data not shown).

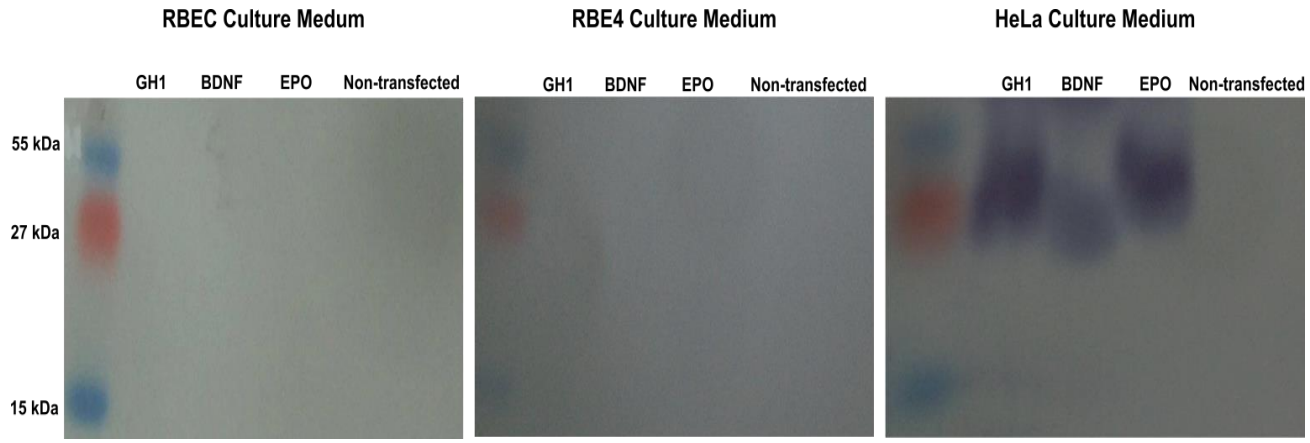

Figure 4. *The recombinant proteins GH1, EPO and BDNF were all secreted into the medium after transfection of HeLa cells. No recombinant proteins were found in the cell culture medium after transfection of RBE4s and RBECs. None of recombinant proteins were observed in the cell homogenates of any of the three cell types (data not shown). Predicted band sizes: GH1 25 kDa, BDNF 26 kDa and EPO 18 kDa. Prestained Protein ladder: 55 kDa, 27 kDa (red) and 15 kDa.*

DISSUSION

We have successfully transfected primary isolated RBECs with plasmids encoding the recombinant proteins GH1, EPO and BDNF, all proteins showing a great neuroprotective potential [13, 36, 141]. We were able to detect gene and protein expression of GH1, BDNF and EPO recombinant proteins in RBECs and RBE4s 48 hours after transfection. We also found evidence for secretion of these proteins in HeLa cells.

Immortalised endothelial cell lines have previously been used for the study of gene therapy to BCECs [82, 83, 88, 89, 194] while only a few studies have used primary isolated endothelial cells [33, 96]. Interestingly, we did not find any significant difference in the transfection potential of the primary endothelial cells, RBECs, compared to the immortalised endothelial cell line, RBE4s, indicating that transfection might be as efficient in these primary cells as in their corresponding immortalised cell line. Additionally, the protein distribution observed in the cell cytoplasm did not reveal any clear differences among the two endothelial cells. Unfortunately, both endothelial cells showed relative low number of transfected cells and a low amount of protein distribution primary located close to the nucleus (Fig. 1).

The largest transfection potential was, not surprisingly, found in HeLa cells. These cells have been extensively used in gene transfection experiments [75, 85, 200, 215], since these cells are easy to transfect and highly susceptible for expressing the newly introduced genes, which were also confirmed in this study as the HeLa cells showed a high degree of transfected cells with a large protein distribution within the cell cytoplasm, very high levels of gene expression of the recombinant proteins and 13-20 % transfection efficiency after transfection. HeLa cells were primarily included in this study as a positive control for the assay, but also as a comparison, in order to better analyse the transfection potential of the primary isolated endothelial cells.

We analysed the percentage of transfected endothelial cells, but were only able to find very low percentages of transfected cells, probably due to a large degree of non-specific binding of the FITC conjugated anti-FLAG antibody. A very large degree of non-specific binding was especially observed when analysing RBE4s. The antibody concentration was, therefore, lowered to half of that used in the other cell types; however, this only resulted in a diminutive decrease in antibody non-specificity, which largely influenced the quality of these results. Less non-specific binding was, however, seen in RBECs, but the percentages of cells expressing GH1 and BDNF were practically non-existent. Cells expressing EPO were on the other hand very high (8.5 %), but still only half of that observed in HeLa cells. We have, previously, reported a transfection efficiency of 4 % after transfection of primary RBECs with a red fluorescence protein [33], indicating that the transfection efficiency with EPO is rather high for this primary cell type. The higher expression of EPO found in RBECs and HeLa cells corresponded well to that observed in the gene expression analyses.

Despite the fact that both gene expression and protein translation within all three cells were detected in all three cell types, we were only able to detect secretion of GH1, BDNF and EPO from HeLa cells. Strangely enough, immunoprecipitation and western blotting of cell homogenates did not detect the presence of any of the three proteins within any of the three cell types, indicating that the assay for protein detecting used in this study is not optimal. The fact that we did not detect any secretion of proteins into the cell culture medium collected from RBE4s and RBECs might be due to the relative low levels of proteins expressed in the endothelial cells after transfection as confirmed with immunocytochemistry, relative gene expression and FACS analysis compared to that observed in HeLa cells. It might, therefore, be necessary to use a more sensitive assay such as ELISA, which have previously been used to detect EPO secretion from genetically modified cells [75, 180].

Thomsen et al. were able to detect protein secretion of GH1 from the immortalised human brain microvascular endothelial cell line (HBMEC) and RBE4s using a similar assay [194]. In this study, however, they used a different transfection agent for nuclear delivery of the plasmid. This difference might have resulted in higher transfection efficiency and, thereby, a higher number of cells secreting the proteins into the cell culture medium. They did, however, compare the transfection agent Pullulan-Spermine to Turbofect™, the transfection agent used in this study, and they estimated the transfection efficiency using the red fluorescence protein HcRed1-C1, and found the two transfection agent to be equally efficient. Thomsen and colleagues were able to report GH1 secretion from both HBMEC and RBE4s. For this purpose they used the Pullulan-Spermine transfection agent. They did, however, not compare the transfection efficiency of Pullulan-Spermine and Turbofect™ in regards to GH1 transfection and secretion. The plasmids encoding the GH1 protein and the HcRed1-C1 protein contains different promoters, which might have an effect on the transfection effiency. The plasmid used in this study is all equal to the GH1 encoding plasmid used by Thomsen et al. Therefore, the lack of secretion from the RBE4 cells in this study compared to the study by Thomsen and colleagues might be due to the different choice of transfection agent.

All three recombinant proteins used in this study were of human origin; however both RBE4s and RBECs, originates from rat. This might have an impact on the translation and secretion of the proteins from the rat endothelial cells, and might also, to some degree, explain the significantly higher expression found in HeLa cells, which are of human origin. It would, therefore, be highly relevant to use plasmid encoding the rat BDNF, EPO and GH1 proteins. The commercially available plasmids are, unfortunately, often designed to encode human proteins. However, this could be overcome by cloning the rat sequence into the plasmid, instead of the human version. It would also be of interest to use human BCECs instead of rat derived BCECs. The use of human primary BCECs are, however, difficult due to the lack of material. The advantages of using cells isolated from rats, instead of human cells, is the easy transition from *in vitro* studies to *in vivo* studies.

Jiang et al. were also able to detect secretion of a neuroprotective protein after gene transfection. They transfected a mouse brain capillary endothelial cell line (MBEC4) with a plasmid encoding the mouse glial cell-derived neurotrophic factor (GDNF) using the commercially available transfection agent Lipofectamine [88]. They found a significantly higher secretion of the protein towards the abluminal (brain) side as opposed to the luminal (blood) side, when the cells were cultured under polarised conditions, as in the transwell system. This observation is important since it emphasises that distribution of proteins to the brain after transfection at the luminal side is not an unrealistic drug delivery approach. One might, however, question the integrity of the monolayer used in this study, since the use of immortalised cell lines are, generally, not considered the best cell type for a sufficient *in vitro* BBB model. Additionally, the use of astrocytes, astrocyte-conditioned medium or other tight junction inducing agents is often necessary to stimulate the integrity of the endothelial cell layer [205], of which they used neither [88]. We were, therefore, planning on transfecting our primary isolated RBECs using the *in vitro* BBB model, which we have previously established and characterised [33], in order to study the polarised secretion of the recombinant proteins. Unfortunately, due to the fact that secretion even in non-polarised monolayer was unsuccessful, the approach seems unrealistic at the time being. Jiang et al. used an ELISA assay to detect the secreted protein [88], indicating that this might be an appropriate assay for our future studies on protein secretion.

Several studies have been published on genetically engineering BCECs using both cell lines and primary cells using both a viral and a non-viral approach [82, 83, 88, 96, 127, 194, 219, 220]. Viral vectors have been recognised for their high transfection efficiency compared to non-viral vectors, but at the same time they carry a potential risk of insertional mutagenesis, which in worse cases can activate oncogenes or enable tumour suppressor genes, both of which can lead to cancerous conditions. It might be necessary to use a viral vector for the transfection of BCECs instead of a non-viral vector as this might enhance the transfection efficiency and thereby enhance the amount of secreted proteins. Many different non-viral carriers have been developed in order to enhance the entry of the genetic material into the BCECs. Non-viral vectors are often internalised into the cells via endocytosis [104]. One of the characteristics of BCECs is low endocytotic activity [5], which renders them difficult to transfect. A substitution of the non-viral vector eases the entry into the BCECs, since the viral vectors are not dependent on endocytosis. Instead the viral vector enters the cells via fusion of the envelope with the plasma membrane, a mechanism which is mediated by fusion proteins located on the surface of the virus [83]. Kim et al. have genetically engineering BCECs to overexpress the tissue plasminogen activator, using an adenoviral mediated transfer approach [96]. They observed secretion of tissue plasminogen activator antigen from BCECs into the culture medium six days after transfection. Huang et al. showed that it was possible to safely enhance the transfection efficiency of BCECs by modifying a non-viral vector with viral fusion proteins [83]. Finally, Jiang et al. have found expression of GDNF inside the brain parenchyma *in vivo* after gene transfection of the brain endothelium by

combining the Hemagglutinating virus of Japan to liposomes [88]. They reported elevated levels of GDNF in the brain for up to 12 days after transfection.

In summary, GH1, EPO and BDNF all show great potential as therapeutic agents in the treatment of a range of neurological diseases. Gene therapy to the BCECs would result in delivery of these neuroprotective agents into the brain, and thereby ease the transport of these proteins into the brain. Gene therapy would be beneficial in the treatment of neurodegenerative diseases like, ischemia, depression, Parkinson's and Alzheimer's disease, as these diseases would benefit from the increased availability of these proteins inside the brain. In this study, we were able to show de novo gene and protein expression in primary RBECs to the same degree as in the immortalised RBE4s. More than 8 % of the RBECs were positive for the recombinant protein EPO, which were markedly higher than that observed for BDNF and GH1. Protein secretion from the RBECs were not detected despite high transfection efficiency, however, this might be due to the protein assay used in this study, which might be solved using a more sensitive essay like ELISA.

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Study III:

Iron Transport Mechanisms in Cultured Rat Brain Capillary Endothelial Cells with Confined Blood-Brain Barrier Properties

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The manuscript contains unpublished data

Iron Transport Mechanisms in Cultured Rat Brain Capillary Endothelial Cells with Confined Blood-Brain Barrier Properties

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Running title: Iron transport at the blood-brain barrier

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Abbreviations

α-SMA, alpha-smooth muscle actin; BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter 1; ^{59}Fe, Iron-59 Radionuclide; GFAP, glial fibrillary acidic protein; GPI, glycophosphatidylinositol; hBMVEC, human brain microvascular endothelial cell line; MTP1, metal transporter protein 1; NVU, Neurovascular unit; PBS, phosphate buffered saline; PDGFRβ, platelet derived growth factor receptor β; RBE4, immortalised rat brain endothelial cell line; RBECs, primary rat brain endothelial cells; TEER, trans-endothelial electrical resistance; Tf, Transferrin; TfR, Transferrin receptor; RO-201724, 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one; Steap, six-transmembrane epithelial antigen of prostate.

ABSTRACT

The understanding of the mechanism for transport of iron into the brain through the blood-brain barrier (BBB) remains a controversy mainly disputing whether iron is transported through the BBB by means of transcytosis of iron-containing transferrin or by detachment of iron inside brain capillary endothelial cells (BCECs) followed by divalent metal transporter 1 (DMT1) and ferroportin-mediated efflux of iron into the brain interstitium.

In this study, we aimed to obtain clarity on the mechanisms by which iron is transported across the BBB. We have analysed and compared the mRNA and protein expression of a variety of proteins involved in the transport of iron in both primary isolated rat brain capillary endothelial cells (RBECs) and immortalised rat brain capillary endothelial cell line (RBE4s) grown in mono culture and in an *in vitro* BBB setup. Additionally, the mRNA expression was investigated in isolated brain capillaries from dietary normal, iron deficient and rats reversed from iron deficiency. We performed iron transport studies to analyse the routes by which iron is transported through the RBECs and found that BCECs expressed DMT1 and ferroportin, which provide evidence for DMT1-ferroportin mediated transport of iron at the BBB. Pericytes, unlike astrocytes, form intimate contact with the BCECs and the data presented here also clearly indicates that ceruloplasmin secreted by pericytes could be instrumental for providing the necessary ferrooxidase activity to allow ferrous iron of the BCECs to obtain transport into the brain as ferric iron.

Keywords: *In vitro* blood-brain barrier model, primary culture, iron transport, transferrin receptor 1, divalent metal transporter 1, ferroportin, ceruloplasmin

INTRODUCTION

Iron, the most abundant transition metal in biology, is an essential cofactor for many cellular enzymes. In the brain, iron is involved in neurotransmission, myelination of axons and cell division. Iron shifts between its ferric or oxidative state (Fe^{3+}) and its ferrous or reduced state (Fe^{2+}), which means that it can catalyse biochemical reactions, but also that it can produce toxic oxygen radicals [126, 134]. Extensive research on iron has contributed to an increasing understanding of the molecular machinery involved in maintaining its homeostasis in the mammalian peripheral tissue. The cellular and intercellular iron transport mechanisms in the central nervous system (CNS) are, however, still poorly understood [134]. Accumulating evidence suggests that impaired iron metabolism is an initial cause of neurodegeneration, and several common genetic and sporadic neurodegenerative disorders have been proposed to be associated with dysregulated CNS iron homeostasis [126, 182]. The maintenance of iron homeostasis at the cellular level is crucial for the viability of all cells, since excess of iron can lead to cell death. However, this is especially important in the brain, because these cells lack the ability to regenerate.

For iron to be transported into the brain, it must pass through brain capillary endothelial cells (BCECs) constituting the blood-brain barrier (BBB). The BBB is a unique structure mainly comprised of the BCECs lining the capillary wall. These cells are supported by pericytes and astrocytes and together these cells form the neurovascular unit (NVU). The main function of the BBB is to regulate the flux of substances into the brain and to protect the brain from potentially harmful substances circulating in the brain [5].

In plasma, iron binds to transferrin with a high binding capacity [136]. Transferrin binds iron in its ferric form and is then referred to as holotransferrin. Ferric iron in plasma is almost exclusively bound to transferrin, but in the absence of ferric iron, transferrin is referred to as apotransferrin. In blood, the percentage of transferrin occupied with iron occurs in the range of 30% [27]. In conditions with hemochromatosis, the iron-binding capacity of transferrin in blood plasma is exceeded and so-called non-transferrin bound iron is detectable. However, there is no evidence for accumulation of iron in the brain in hemochromatosis suggesting that non-transferrin bound iron is unable to pass the BBB [17, 130]. Transferrin is not able to enter the brain due to its hydrophilic nature, but the BCECs express receptors for transferrin on the luminal site of the capillary [134]. This is a unique feature of the brain, since the brain is the only organ that expresses these receptors on the endothelial cells constituting the brain capillaries [12, 87]. The transferrin receptor has the highest affinity toward holotransferrin and the relatively high concentration of holotransferrin results in complete saturation of the transferrin receptor by holotransferrin [134]. Therefore, the transferrin receptor is highly responsible for iron transport into the brain.

The mechanisms of iron transport across the BBB have been extensively debated during the past decade. In general two different routes are discussed. The first possibility suggests that holotransferrin binds the

transferrin receptor, which becomes internalised into the cells interior by receptor mediated endocytosis. The complex is endocytosed via clathrin coated pits that result in uncoated endosomes [11]. The pH within the endosomes is very low due to proton pumping ATPase's and at this low pH; the binding of holotransferrin to the transferrin receptor is weakened, resulting in iron release from the transferrin molecule [45, 136]. Within the endosome iron has been suggested to subsequently be transported to the cytosol by means of a divalent metal-ion transporter (DMT1) [61]. However, DMT1 is only able to transport ferrous iron, indicating the presence of ferrireductases like the six-transmembrane epithelial antigen of prostate (Steap) proteins, which is able to reduce the detached ferric iron to ferrous iron [11, 149]. The apotransferrin receptor complex is then recycled to the luminal membrane, where apotransferrin is released from the transferrin receptor [133]. At low pH apotransferrin has a much higher affinity towards the transferrin receptor, and will, therefore, not detach from the receptor until it has reached the cell surface [136]. Ferroportin, also known as metal transport protein 1 (MTP1), is expressed at the surface of cells involved in iron transport, and is able to facilitate efflux of ferrous iron across the cell membrane [7]. Since the majority of iron found in plasma is in its ferric form, there is a need for oxidation of iron. Within the CNS two possible proteins has been suggested to provide this necessary ferrooxidase activity, namely ceruloplasmin and its homolog hephaestin [10, 162, 168]. Astrocytes have been shown to express both a glycophosphatidylinositol (GPI) anchored and soluble form of ceruloplasmin [160].

The other possibility of iron transport into the brain by means of the transferrin receptor is via receptor mediated transcytosis of the transferrin receptor from the luminal side to the abluminal side. This theory mainly evolved due to the observation that BCECs do not express DMT1 [131, 135]. Transcytosis of transferrin has never been observed [43, 135, 190], therefore, this theory states that factors, like citrate, ATP, hydrogen ions and other nucleotides released from astrocytes is responsible for the detachment of iron at the abluminal side [134, 137]. Apotransferrin, as explained earlier, has a higher affinity towards its receptor at lower pH. Therefore, transferrin would be recycled to the plasma, while iron would enter the brain interstitium bound to citrate or transferrin present in the interstitial fluid [134]. Ceruloplasmin present at the abluminal side would be responsible for the regulation of iron transport into the brain, since lack of this protein as in aceruloplasminemia results in increased iron accumulation within the brain [131, 134, 162].

Previous studies on iron transport have mainly been performed *in vivo* [31, 32, 71, 131, 132, 135, 186, 216] or in immortalised BCECs [121-124]. Therefore, the present study set out to investigate the mechanism by which iron is transported across primary isolated BCECs cultured with confined BBB properties. We investigated and compared the expression profile of proteins involved in receptor mediated endocytosis pathway in the immortalised rat brain endothelial cell line (RBE4s) and primary isolated rat brain endothelial cells (RBECs) grown in non-polarised monolayers and in polarised conditions using an *in vitro* BBB model setup. Furthermore, these gene expression analyses were compared to that found in brain capillaries of

normal-fed, iron deficient and iron-reversible rats. Receptor mediated endocytosis and receptor mediated transcytosis were, additionally, examined in RBECs grown in co-culture with astrocytes using radiolabeled iron and fluorescence labelled transferrin. Finally, we show that pericytes are a source of both the GPI anchored and soluble form of ceruloplasmin.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Sigma-Aldrich (Brondby, Denmark, DK): percoll (Cat. No. P1644), collagen type IV (Cat. No. C5533), fibronectin (Cat. No. F1141), poly-L-lysine (Cat. No. P6282), heparin (Cat. No. H3149), puromycin (Cat. No. P8833), hydrocortisone (Cat. No. H4001), CTP-cAMP (Cat. No. C3912), 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one (RO-201724) (Cat. No. B8279), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No. D9542), Rabbit anti-claudin-5 (Cat. No. SAB4200538) and mouse anti-alpha-smooth muscle actin (α-SMA) (Cat. No. A5228). DNase I (Cat. No. 10104159001), collagenase/dispase (Cat. No. 109113), insulin transferrin sodium selenite (Cat. No. 11074547001) and basic fibroblast growth factor (bFGF) (Cat. No. 1363697) were purchased from Roche (Hvidovre, Denmark, DK). The following reagents were purchased from Life Technology (Naerum, Denmark, DK): Alexa Fluor 488-conjugated goat anti-rabbit IgG (Cat. No. A11034), Alexa Fluor 594-conjugated goat anti-mouse IgG (Cat. No. A11032), Texas red conjugated albumin from bovine serum (BSA) (Cat.No. A23017), collagenase II (Cat. No. 17101105), DMEM/F-12 (Cat. No. 31331), DMEM (Cat. No. 21885) and fetal calf serum (Cat. No. 10270). BSA (Cat. No. EQBAH62) was purchased from Europa Bioproducts (Cambridge, United Kingdom, UK). Plasma derived bovine serum (Cat. No. 60-00-810) was purchased from First Link (Wolverhampton, United Kingdom, UK). Rabbit anti-ferroportin (Cat. No. MTP11-S) and rabbit anti-DMT1 (Cat. No NRAMP-22S) was purchased from Alpha diagnostics (Aarhus, Denmark, DK). Mouse anti-transferrin receptor 1 (TfR) (CD71) (Cat. No. MCA155G) from Adb Serotec were purchased from Nordic BioSite ApS (Copenhagen, Denmark, DK). Mouse anti-hephaestin antibody (Cat. No. ab56729) were purchased from Abcam. Rabbit anti-platelet derived growth factor receptor beta (PDGFRβ) (Cat. No SC-432) was purchased from Santa Cruz. Gentamicin Sulfate (Cat. No. 17-518Z) was purchased from Lonza Copenhagen (Vallensbaek Strand, Denmark, DK). Fluorescence mounting media (Cat. No S3023), rabbit anti-ceruloplasmin (Cat. No Q0121) and rabbit anti-glial fibrillary acidic protein (GFAP) (Cat. No. Z0334) were purchased from DAKO (Glostrup, Denmark, DK). Alexa Fluor 488-conjugated rat transferrin (Tf) from Jackson ImmunoResearch was purchased from TriChem APS (Skanderborg, Denmark, DK). Iron-59 Radionuclide (^{59}Fe) (Cat. No. NEZ037001MC) and Ultima Gold™ liquid scintillation cocktail (Cat. No. 6013326) were purchased from Pelkin Elmer (Skovlunde, Denmark, DK). Rat hepcidin (Cat.No PLP-3769-PI) were purchased from Peptides International (Louisville, Kentucky, USA). Phosphate buffered saline (PBS) (Cat. No SH3025802) and all reagents for qPCR were obtained from Thermo Scientific, except primers that were synthesised by TAG Copenhagen (Frederiksberg, Denmark, DK). Hanging Cell culture inserts (Cat. No. Pirp 15R48) were obtained from Merck Milipore (Hellerup, Denmark, DK).

Cell cultures

Primary cultures of RBECs were prepared from two to three week old Sprague Dawley rats [33, 143]. The rats were deeply anesthetised and the forebrains were collected under sterile conditions. The meninges and any visible white matter were carefully removed and the cerebral cortices were cut into small pieces. The tissue was digested in collagenase II and DNase I for 75 min at 37 °C. The pellet was resuspended in 20 % BSA in DMEM-F12 and centrifuged at 1000 G for 20 min. The pellet was further digested in Collagenase/Dispase and DNase I at 37 °C for 50 min. The digested microvessel fragments were separated and collected using a continuous 33 % Percoll gradient and seeded on collagen type IV and fibronectin coated 35 mm plastic dishes. Primary cultures of RBECs were maintained in DMEM/F12 supplemented with 10 % plasma derived bovine serum, bFGF, heparin, insulin, transferrin, sodium, selenite and gentamicin sulphate (10 µg/ml) and cultured in an incubator with humidified 5 % CO_2 / 95 % air at 37 °C. To obtain a pure culture of RBECs, puromycin was added to the cell culture media in a 4 µg/ml concentration for the first two to three days. Immortalised RBE4s were cultured in the same medium as the RBECs.

Primary cultures of pericytes were obtained by prolonged culture of the isolated microvessel fragments. The microvessel fragments were cultured on uncoated dishes in DMEM supplemented with 10 % fetal calf serum and gentamicin sulphate for about 10 days to ensure pericyte proliferation and survival.

Primary cultures of astrocytes were obtained from neonatal Sprague Dawley rat pups [33]. The brains were collected, and pieces of the cerebral cortex were dissociated through a 40 µm nylon strainer in DMEM supplemented with 10 % fetal calf serum and gentamicin sulphate. Dissociated cells were seeded in poly-L-lysine coated cultured flasks for about two weeks until they reached confluence, by which the cells were either frozen or passaged onto poly-L-lysine coated 12 well culture plates.

Construction of *in vitro* BBB models

RBE4s and RBECs were grown in co-culture with primary astrocytes. Astrocytes were seeded in poly-L-lysine coated 12 well plates approximately two weeks prior to the co-culture experiments. RBE4s and RBECs were seeded with a cell density of $1*10^5$ cells/cm^2 onto collagen IV and fibronectin coated 12 well polyethylene terephthalate, 1.0 µm hanging cell culture inserts. The cells were left to adhere for 24 hours before the culture inserts were placed in the 12 well plates containing astrocytes. To further stimulate BBB characteristics of the RBECs and RBE4s, they were treated with 550 nM hydrocortisone, 250 µM cAMP and 17.5 µM RO-201724. Cells used for the gene expression analysis were stimulated with astrocyte conditioned media instead of astrocytes to ensure the RNA was not contaminated by astrocytes.

Trans-endothelial electrical resistance (TEER) measurements

TEER measurements were carried out using a Millicell ERS-2 epithelial Volt-Ohm meter and STX01 Chopstick Electrodes (Millipore, Hellerup Denmark, DK). The TEER values of coated but cell free culture inserts were subtracted from the measured TEER values, and the difference were multiplied with the area of the culture insert (1.12 cm^2). Measured TEER values are given as $\Omega*cm^2$. Data were analysed with the GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA) using a two-way ANOVA with Bonferroni post hoc test.

Animals

Wistar rats aged postnatal (P) days P70 (adult) were examined. The rats were obtained from timed pregnant rats (the day of conception being Embryonic (E) day 0). For Fe deficient studies, the female rats aged postnatal six weeks were fed a low Fe diet with a Fe content of 5.2 mg/kg (Altromin, Ger) for additionally six weeks. They were then measured for their hematocrit, fertilised and maintained on the Fe low diet. For iron-supplementation, the female rats were injected subcutaneously with iron isomaltaside 1000 (PharmaCosmos, DK) at a dose of 80 mg/kg on day E14 but maintained on the iron-low diet. Control rats were maintained on an iron-containing diet with an iron-content of 158 mg/kg (Altromin, Ger). The rats were kept on their respective diets until P21 at where young male rats were separated from their mother and kept on the same diet until euthanized on P70: The rats were deeply anesthetised with a subcutaneous injection of 0.5 ml / 10 g body weight of Hypnorm/Dormicum (Fentanyl/Fluanisone mixed with Midazolam). Subsequently, the chest was opened and blood sampled by heart puncture. The brain, liver and duodenum of the rats were then dissected and frozen or homogenised as described below. The analyses on the duodenal tissue was made on the proximal 10 mm: The proximal stump was cut open with scissors, washed in 0.1 M PBS, pH 7.4 followed by scraping the luminal part with razor blade to isolate a preparation containing the duodenal mucosa.

Relative gene expression analyses

Gene expression analyses were performed on RBECs, RBE4s, pericytes and astrocytes. Endothelial cells used for gene expression analysis were either grown in non-polarised culture conditions or in the *in vitro* BBB setup to ensure polarity of the endothelial cells. RNA was extracted from RBECs two days after isolation, when the cells were approximately 70 % confluent. RBE4s were seeded on collagen IV-fibronectin coated 35 mm dishes at a cell density of 30.000 cells/cm^2 and RNA was extracted from the cells, when they reached approximately 70 % confluence as well. RNA was extracted from RBECs and RBE4s grown in polarised condition after two days of culturing with astrocyte condition media. RNA was extracted from frozen vials of astrocytes and pericytes. Gene expression analysis was, furthermore, analysed on RNA

extracted from isolated brain capillaries, brain stem (total brain), liver and the duodenal mucosa from the normal, iron deficient and iron-reversed rats.

RNA was extracted using the GeneJET RNA Purification Kit and treated with DNase I enzyme according to the manufacture's protocol. 100 ng of each DNA-free RNA sample was used as a template for RT-qPCR. cDNA synthesis was carried out with the RevertAid Premium First Strand cDNA Synthesis Kit. 0.5 µl cDNA and 10 pmol of each primer (Table 1) were used for each qPCR reaction together with the Maxima™ SYBR Green qPCR Master Mix. Each sample was performed in triplicates, and non-reverse transcribed RNA and water served as negative controls. Beta-actin was used as a housekeeping gene for normalisation purpose. RT-qPCR was performed using the Stratagene Mx 3000P^{TM} QPCR System (Agilent Technologies, Horsholm, Denmark, DK). The PCR conditions were 95 °C for 10 min, followed by 40 cycles of: 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The relative quantities of DNA in the analysed samples were calculated by the Pfaffl method [169]. Data were analysed by the GraphPad Prism 5.0 software using a one-way ANOVA with Tukey's multiple comparisons post hoc test (Fig. 1), or a two-way ANOVA with Dunnett's multiple comparison post hoc test (Fig. 7). Primer sequences are found in table I.

Table I. *Primer sequences used for RT-qPCR*

Primer	**Reference sequence**	**Forward primer**	**Reverse primer**
Transferrin receptor 1	NM_022712.1	TGGATCAAGCCAGATCAGCATTCTC	TTTCTTCCTCATCTGCAGCCAGTTT
Steap 2	NM_001107846.1	GCTTCGTTATTTCCGGATTCCCTGA	TCGAGCTTGGATATTGTTGCTGCAT
Steap 3	NM_133314.1	CTCACTCAACTGGAAGGAGTTCAGC	GAATGTGGGTGGCAGGTAGAACTTG
DMT1	NM_013173.2	GCTTGTTGCTGTCTTCCAAGATGTG	TTCCGTTGGAGAACTCACTCATCAC
Ferroportin	NM_133315	CCCTGCTCTGGCTGTAAAAG	GCACAGGTGGGTTCTTGTTC
Ceruloplasmin GPI anchored	NM_ 001270961.1	CAAACGCCTGGAACCTGGTTACTC	ATGTTCCAGGTCATCCTGTAGCTCTG
Ceruloplasmin Soluble	NM_012532.2	GGAATTGGTAATGCCGATGGACAGA	ATTTGTTGCCACTTTGCAGAGTCCT
Hephaestin	NM_133304.1	TAGCTGGCACCTTGATGACAACATT	CAACTCGGGTAAGTTCCCAAAGACA
Hepcidin	NM_053469	GGCAACAGACGAGACAGACT	AACAGAGACCACAGGAGGAA
GFAP	NM_017009.2	ACATCGAGATCGCCACCTACAGGAA	AGGTGGCCTTCTGACACAGATTTGG
α-SMA	NM_031004.2	GAGTGGAGAAGCCCAGCCAGTC	AACAGCCCTGGGAGCATCATCAC
Claudin-5	NM_031701.2	CTACAGGCTCTTGTGAGGACTTGAC	AGTAGGAACTGTTAGCGGCAGTTTG
β-actin	NM_031144.3	CCTCTGAACCCTAAGGCCAACCGTGAA	AGTGGTACGACCAGAGGCATACAGGG

Immunocytochemical analysis

Cells were rinsed in 0.1 M PBS, pH 7.4, fixed in 4 % paraformaldehyde for 10 min. The cells were incubated with 3 % BSA, 0.2 % Triton-X-100 in PBS for 30 min at room temperature to block for non-specific binding of primary antibodies. All incubations were done at room temperature with moderate agitation. The cells were incubated with primary antibodies raised against claudin-5, α-SMA, PDGFRβ, GFAP, TfR 1, DMT1, ferroportin, hephaestin and ceruloplasmin. The primary antibodies were all used in a dilution of 1:500 in 3 % BSA, 0.2 % Triton-X-100 in 0.1 M PBS and incubated for one hour. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibodies were used in a dilution of 1:500 in 3 % BSA, 0.2 % Triton-X-100 in 0.1 M PBS and incubated for one hour. Nuclei were stained with DAPI. The cells were mounted on glass slides with fluorescent mounting medium, and examined in a fluorescence microscope (Axiovert 2000, Carl-Zeiss, Germany). Captured images were corrected for brightness and contrast using Adobe Photoshop C2. For confocal analysis the cells were examined under a 100x Water objective (NA 1.4) in a spinning disk CSU-X unit (Yokogawa, Japan) using an EMCCD iXon Ultra Camera (Andor, Ireland) both mounted on an Olympus IX83 inverted microscope. The system was controlled with CellSens software (version 1.11, Olympus, Japan). Captured images were analysed and corrected for brightness and contrast using the Image J software.

Transcytosis of transferrin

RBECs were co-cultured with astrocytes and stimulated with 550 nM hydrocortisone, 250µM cAMP and 17.5µM RO-201724 for 24 hours to induce polarised condition with BBB characteristics. Two different studies were performed. 0.2 nM Alexa Fluor 488 labelled Tf and 0.2 nM Texas red labelled BSA were added together to the upper chamber of four culture inserts (n=4). 100 µl samples were collected from the lower chamber after 2, 4, 6, 8 and 24 hours and replaced with 100 µl fresh media. Samples were analysed with the Microplate Readers Infinite M1000 PRO (Tecan, Switzerland). TEER was measured at 0 and 24 hours. Four culture inserts were used as a control for TEER measurements. Data were analysed using the GraphPad Prism 5.0 software using a two-way ANOVA with Bonferroni post hoc test.

Secondly, concentrations of 0.4 nM, 0.2 nM, 0.1 nM, 0.05 nM and 0.025 nM Alexa Fluor 488 labelled Tf and Texas red labelled BSA were added together to the upper luminal chamber of three culture inserts per. concentration (n=3). Samples were collected from the lower abluminal chamber after 30 min and analysed with the Microplate Readers Infinite M1000 PRO. TEER was measured prior to the study. Data were analysed using the GraphPad Prism 5.0 software using a two-way ANOVA with Bonferroni post hoc test. The culture inserts to which 0.2 nM Tf and BSA were added were fixated in 4 % paraformaldehyde and DAPI stained after 4 hours and 24 hours. The culture inserts were cut out with a scalpel and mounted on glass slides with fluorescent mounting medium. The cells were analysed in the confocal microscope as

described previously. Captured images were analysed and corrected for brightness and contrast using the Image J software.

Iron flux

RBECs were co-cultured with astrocytes and stimulated with 550 nM hydrocortisone, 250 µM cAMP and 17.5 µM RO-201724 for 24 hour to induce polarised condition with BBB characteristics. 3 µCi ^{59}Fe (214 ng) were added to the upper chamber for 24 hours with (n=4) or without (n=4) the influence of 700 nM rat hepcidin. After 24 hour, the cells were rinsed once in PBS and the media was replaced. Samples of 100 µl were collected from the upper and lower chambers at 2, 4, 8 and 24 hours, and replaced with 100 µl fresh media. After 24 hours of incubation with ^{59}Fe, four culture insert (n=4) were collected after being rinsed once in PBS. The remaining culture inserts were rinsed once in PBS and collected after 24 hours of sampling. All samples were added with Ultima Gold Scintillations fluid and counted in a Liquid Scintillation counter.

TEER was measured prior to iron incubation (-24 hours), after 24 hours iron incubation (0 hours) and, finally, after 24 hours of sampling (24 hours). Four culture inserts with (n=4) or without (n=4) the influence of hepcidin were used as control for TEER to ensure the RBECs persisted their integrity throughout the experiment. Two of these culture inserts were after 24 hours with or without the influence of hepcidin fixated in 4 % paraformaldehyde and immunostained for ferroportin, as described previously. RNA was, additionally, extracted from nine culture insert (three culture inserts per RNA sample) (n=3) treated with hepcidin for 24 hours and used for gene expression analysis of ferroportin, as previously described. Data were analysed using the GraphPad Prism 5.0 software using a two-way ANOVA with Bonferroni post hoc test.

RESULTS

The gene expression of molecules related to iron transport is highly influenced by the culturing conditions and by the use of immortalised or primary brain capillary endothelial cells.

RBE4s and RBECs were cultured under non-polarised condition, during which the cells were in a highly dividing state (mitosis) and their gene expression of molecules related to iron transport were compared to RBE4s and RBECs cultured under polarised conditions under influence of astrocyte conditioned medium (co-culture) (Fig. 1). Gene expression of all nine investigated proteins were observed in both cell types, and the expression were found to be highly influenced by the culturing conditions and varied significantly between the immortalised cell line, RBE4s and the primary isolated, RBECs. In general, the genes encoding the molecules involved in iron transport, except the ferrireductases Steap proteins and hephaestin, were higher expressed in the RBECs. The expression of the transferrin receptor was found to decrease when the cells reached their polarised state. Low expression of Steap 2 was observed in both cell types; however, the highest expression of both Steap 2 and 3 was found in polarised RBE4s. The expression of DMT1 was found to increase when the endothelial cells reached their polarised state and the same expression pattern was seen for ferroportin, with the highest expression found in RBECs. Polarised RBECs showed the highest expression of hepcidin, GPI anchored and the soluble form of ceruloplasmin. The dominant form of ceruloplasmin expressed by the endothelial cells seems to be the soluble form. Expression of hephaestin was found to increase when the endothelial cells were polarised and co cultured with astrocytes, however, the highest expression was found in polarised RBE4s.

Pericytes and astrocytes, together with endothelial cells, constitute the NVU. Pericytes and astrocytes are in close contact with the endothelial cell, and might, therefore, assist in the regulation of iron transport to the brain, by being a source of the ferrooxidases ceruloplasmin and hephaestin. Primary isolated pericytes and astrocytes were first of all analysed for their cell purity (Fig. 2). Pericytes were found to primarily express the pericyte marker α-SMA and showed practically no expression of the astrocyte marker, GFAP. The astrocyte culture is characterised as a mixed glia culture, primarily consisting of astrocytes [33] and was found to primarily express GFAP. Some degree of pericyte contamination within the mixed glia culture was observed, however, estimated to be insignificant. Pericytes were found to express ceruloplasmin both in soluble and GPI anchored form to the same degree as the astrocytes. Again the soluble form of ceruloplasmin seems to be the dominant form expressed by these two cell types. Additionally, pericytes and astrocytes are also both a source of hephaestin, however, pericytes show a higher expression than astrocytes. When comparing the gene expression of these ferrooxidases to the expression observed in endothelial cells (Fig. 1), the pericytes and astrocytes seem to be the primary source of ferrooxidases within the brain parenchyma. Finally, pericytes and astrocytes are also able to regulate the expression of ferroportin, since

these cells were found to express hepcidin, and this expression were also higher in pericytes and astrocytes compared to that observed in the endothelial cells.

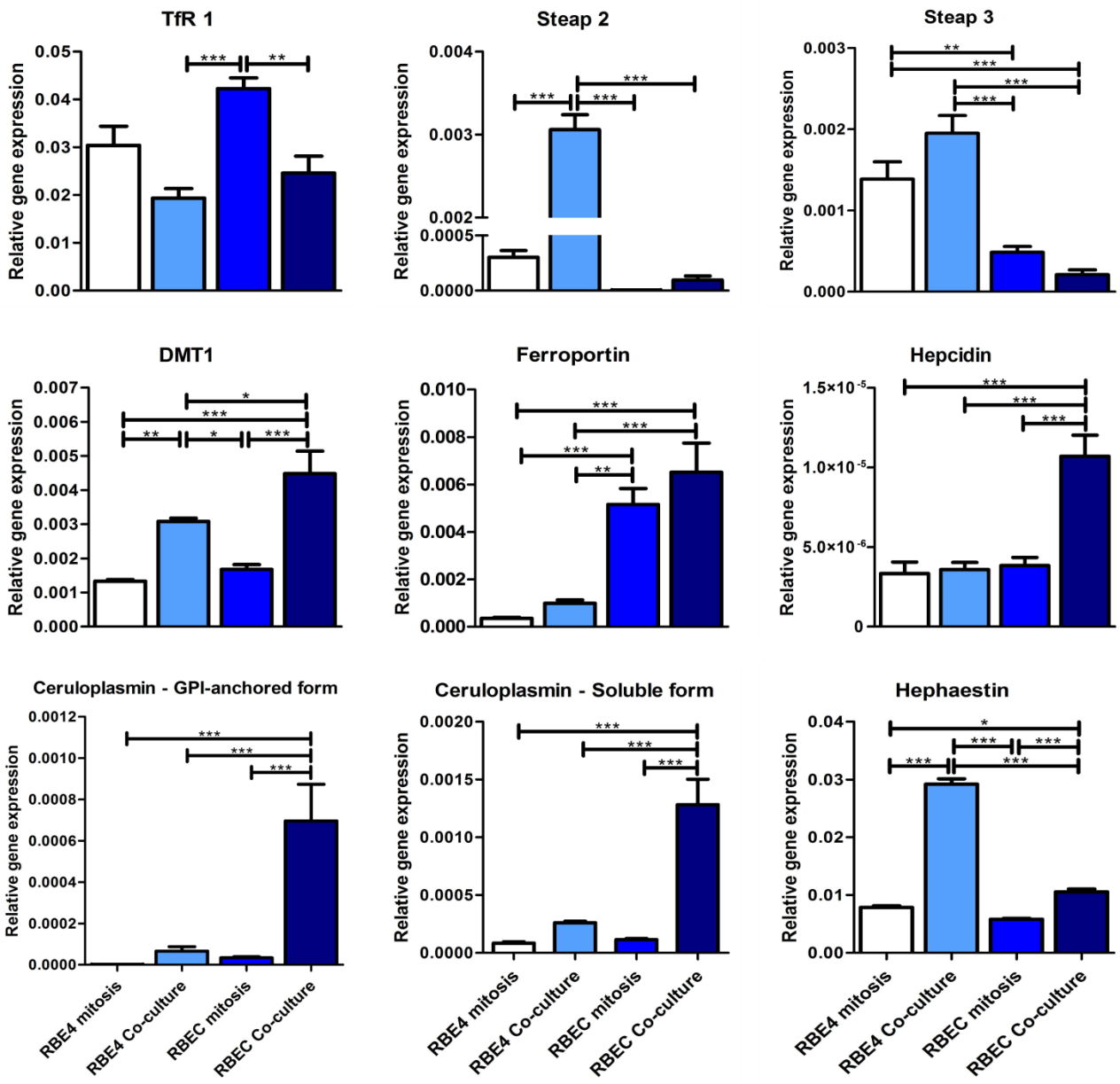

Figure 1. *Gene expression analyses for molecules related to iron transport in brain capillary endothelial cells: transferrin receptor (TfR1), Steap 2, Steap 3, divalent metal transporter I (DMT1), ferroportin, hepcidin, ceruloplasmin and hephaestin. RBE4s and RBECs were examined during a non-polarised division phase ("mitosis") and at a polarised state under the influence of astrocyte conditioned medium ("co-culture"). Note the difference with principally higher expression level of many genes except Steap 2, Steap 3 and hephaestin in differentiated RBECs. Data were analysed using the GraphPad Prism 5.0 software and the relative expression in each cell type were statistically analysed using a one-way ANOVA with Tukey's multiple comparisons post hoc test. Data are presented as means ± SEM (n=6). *p<0.05, **P<0.01, ***p<0.001.*

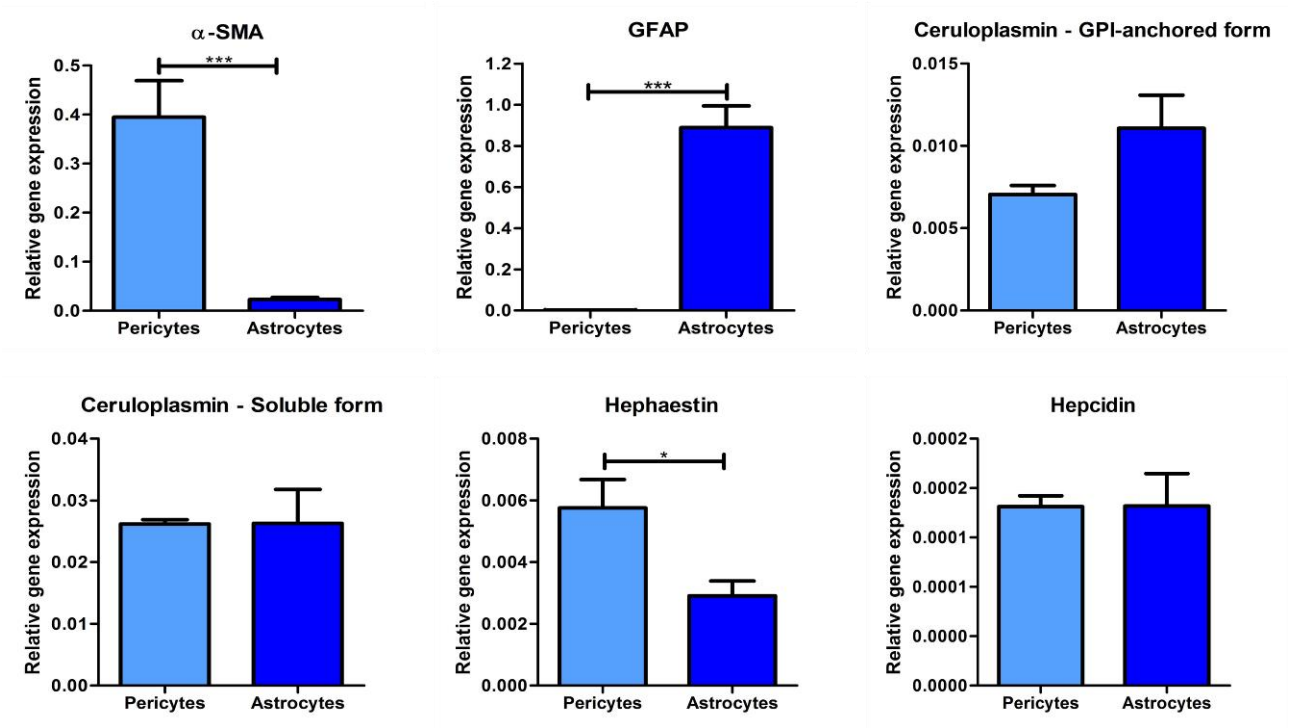

Figure 2. *The role of pericytes and astrocytes in iron transport into the brain parenchyma was analysed by their expression of ferrooxidase proteins and hepcidin. The purity of the isolated pericytes and astrocytes cultures were analysed and found to be high. Pericytes, like astrocytes, express ceruloplasmin and hephaestin and might, therefore, provide the ferrooxidase activity needed for ferroportin-mediated iron release into the brain. Pericytes and astrocytes are also able to regulate the expression of ferroportin by means of hepcidin. Data were analysed using the GraphPad Prism 5.0 software and the relative expression in each cell type were statistically analysed using a one-way ANOVA with Tukey's multiple comparisons post hoc test. Data are presented as means ± SEM (n=6). *p<0.05, ***p<0.001.*

Expression of molecules related to iron transport were confirmed with immunocytochemical staining of the cultured cells.

RBECs and RBE4s were cultured in polarised co-culture condition under the influence of astrocytes and were immunostained for their expression of molecules related to iron transport. Before the immunocytochemical staining, the cell integrity of the endothelial cell layers was analysed by TEER measurements. Mean TEER for RBE4s was 6.1 ± 0.9 Ω*cm^2 and 165 ± 4 Ω*cm^2 for RBECs. RBE4s did, therefore, not show any BBB properties, despite being cultured in the *in vitro* BBB setup. This was, furthermore, confirmed by their expression of the tight junction protein claudin-5 (Fig. 3). RBECs showed consistent expression of claudin-5 at the cell-cell junctions, while this polarised pattern was not observed in RBE4s. Additionally, RBE4s had a tendency to grown in multiple layers, which were not the case for the RBECs.

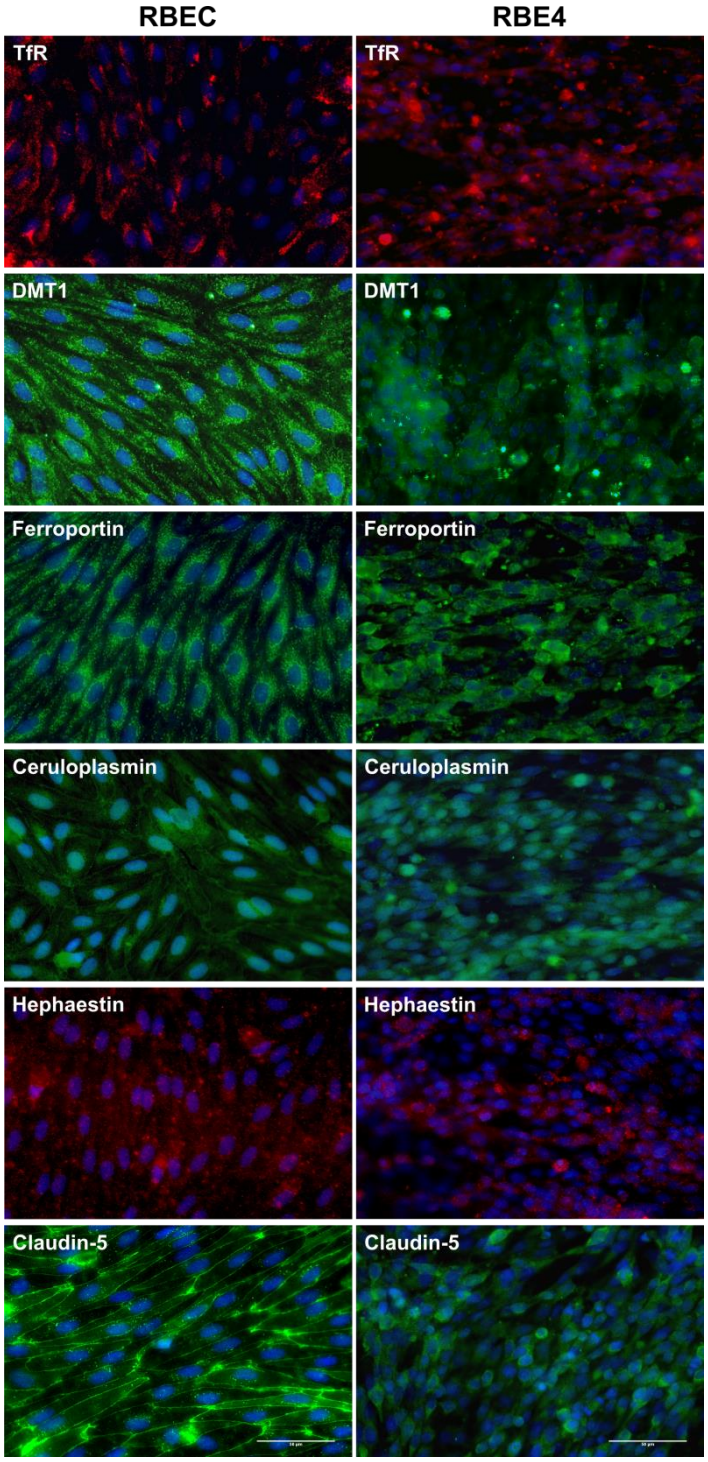

Figure 3. *Immunocytochemistry showing molecules related to iron transport in brain capillary endothelial cells (left RBECs, right RBE4s); transferrin receptor (TfR), divalent metal transporter I (DMT1), ferroportin, ceruloplasmin and hephaestin. RBECs and RBE4s were examined at the polarised stage under the influence of astrocyte conditioned media. All proteins studied are expressed by both RBECs and RBE4s. The RBECs had reached TEER value above 160 Ω*cm^2, which resulted in a fine consistent expression of claudin-5 at the cell-cell junctions. RBE4s never reached TEER values above 10 Ω*cm^2, which is clearly visible in their lack of claudin-5 expression at the cell-cell junction. Scale bar 50 µm.*

Both cell types showed protein expression of the transferrin receptor, DMT1, ferroportin, ceruloplasmin and hephaestin (Fig. 3). The expression of transferrin receptor, DMT1 and ferroportin were primarily seen in the cell cytoplasm, while ceruloplasmin was primarily expressed at the cell membrane. The expression of hephaestin was quit low, however, it seem to mainly distribute to the cell cytoplasm. In order to estimate the cellular distribution of the transferrin receptor, DMT1 and ferroportin within the RBECs, these cells were examined under a confocal microscope (Fig. 4). However, RBECs are very small and thin making it difficult to estimate, whether the protein is located primarily at the luminal or abluminal membrane.

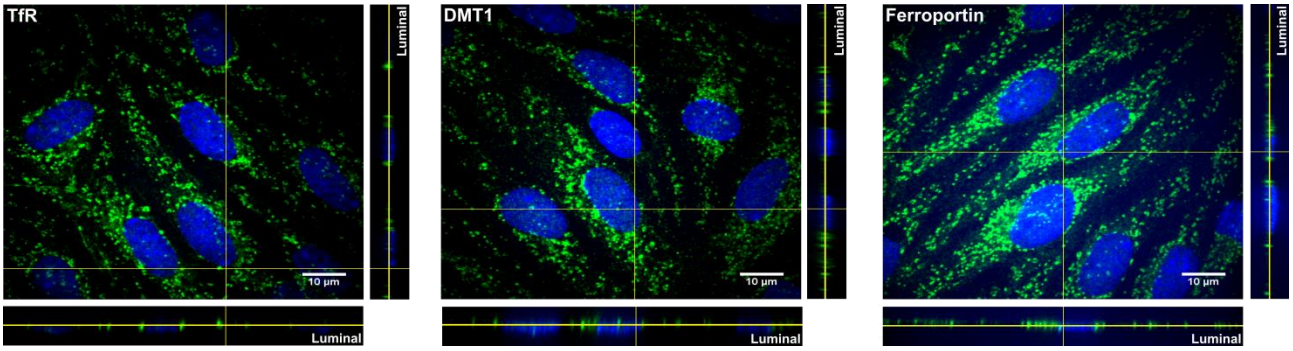

Figure 4. *Rat brain endothelial cells (RBECs) co-cultured with astrocytes were immunostained for their cellular distribution of transferrin receptor (TfR), divalent metal transporter 1 (DMT1) and ferroportin. The cells were examined using a confocal microscope and corrected for brightness and contrast using the ImageJ software. Analysed proteins are seen in green, while the nucleus is counterstained in blue. Scale bar 10 µm.*

Astrocytes and pericytes were, additionally, analysed for their protein expression of cell specific markers and ferrooxidases (Fig. 5). Astrocytes express the cell specific marker, GFAP, but also ceruloplasmin and hephaestin. Pericytes are often divided into two subcategories, one group being α-SMA positive and another being PDGFRβ positive [193]. We found a mixture of both subcategories in our cultured pericytes. They were also found to express ceruloplasmin and to some degree hephaestin.

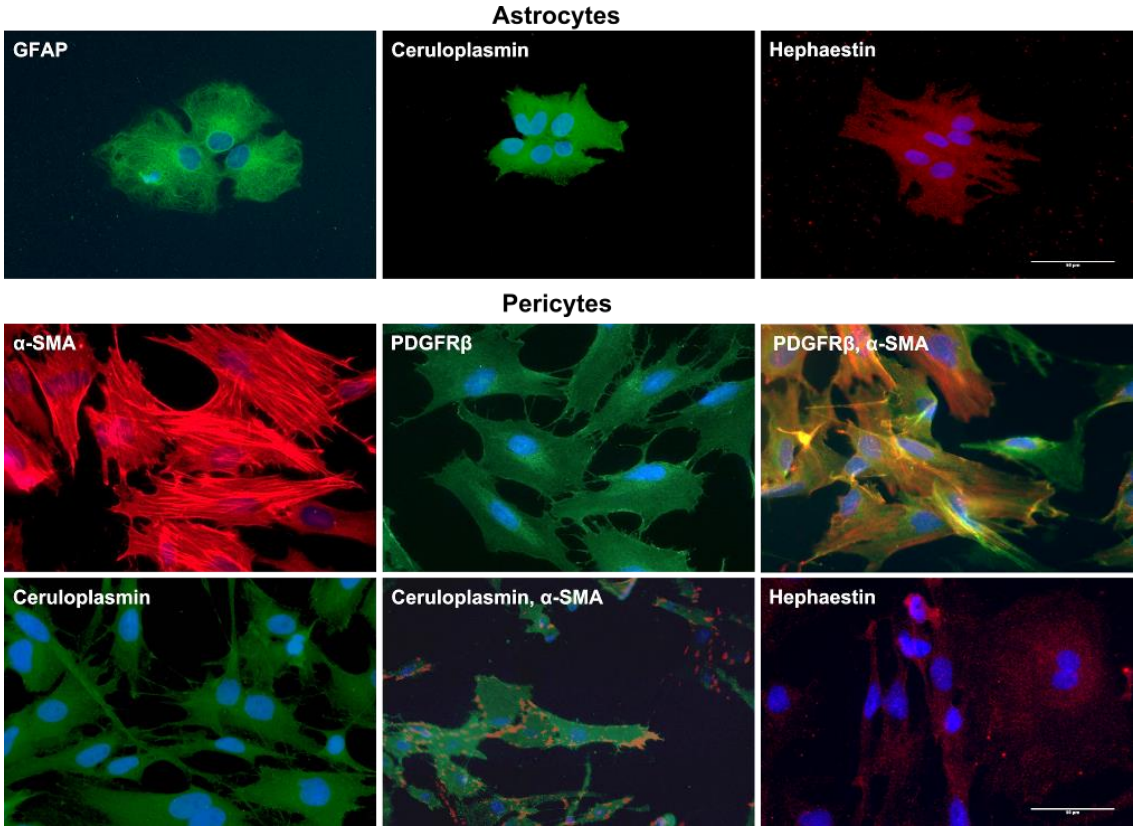

Figure 5. *Immunocytochemistry showing molecules related to iron transport in astrocytes (top) and pericytes (bottom). Both pericytes and astrocytes express cell specific markers, GFAP (astrocytes), α-SMA and PDGFRβ (pericytes). Protein expression of ceruloplasmin and hephaestin were, additionally, found in both pericytes and astrocytes. Scale bar 50 µm.*

Capillaries *in vivo* also express molecules related to iron and they are regulated as a consequence of nutritional levels of iron.

Brain capillaries were isolated from normal-fed, iron deficient and iron-reversed rats and used for gene expression analysis of iron transport related molecules. This expression was compared to that found in total brain, liver and epithelial cells of the duodenum (duodenal mucosa) from the same animals (Fig. 6). The isolated brain capillaries might be a mixture of all cells of the NVU. Therefore, the expression of claudin-5 (BCECs marker), α-SMA (pericyte marker) and GFAP (astrocyte marker) were investigated in these capillaries and compared to the expression found in pure cultures of BCECs, pericytes and astrocytes (Fig. 6). The isolated capillaries were predominantly found to be a mixture of BCECs and pericytes, which is consistent with the close association of these two cell types at the capillaries. In contrast only a small degree of GFAP expression was observed.

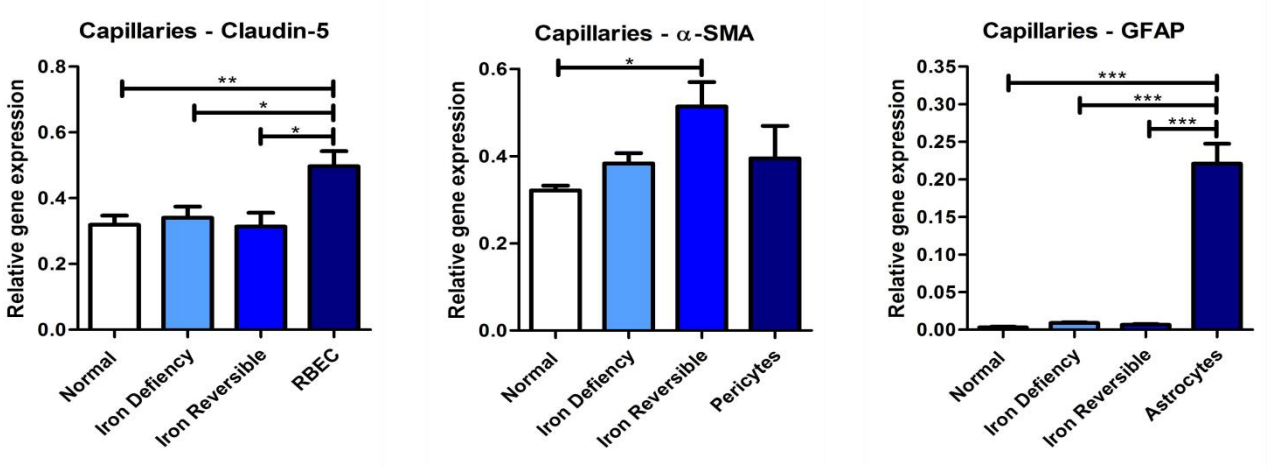

Figure 6. *Capillaries were isolated from normal, iron deficient and iron-reversible rats, and their gene expression of neurovascular unit (NVU) markers claudin-5 (endothelial), α-SMA (pericyte) and GFAP (astrocytes) were compared to cultured primary cells, in order to evaluate the purity of the capillaries. The capillary fragments were found to consist primarily of endothelial cells and pericytes. Data were analysed using the GraphPad Prism 5.0 software and the relative expression in each cell type was statistically analysed using a one-way ANOVA with Tukey's multiple comparisons post hoc test. Data are presented as means ± SEM (n=6-9). *p<0.05, **p<0.01, ***p<0.001.*

The capillaries were, furthermore, found to express all the proteins involved in iron transport, which were also the case in total brain. Some of these proteins were up or down regulated as a consequence of altered iron level in the animals diet. In the capillaries, Steap 2 and ceruloplasmin were significantly upregulated, while Steap 3 and ferroportin were significantly down regulated during the iron deficiency state. Steap 2 expression was not detected in the liver or the duodenal mucosa. The general expression of iron transport related proteins were much lower in the brain compared to the liver and duodenal mucosa, and altered iron levels does not seem to influence the expression of the molecules related to iron transport in the brain as much as that observed in the liver and the duodenal mucosa. The liver is the main source of ceruloplasmin and it seems to mainly express the soluble form of ceruloplasmin.

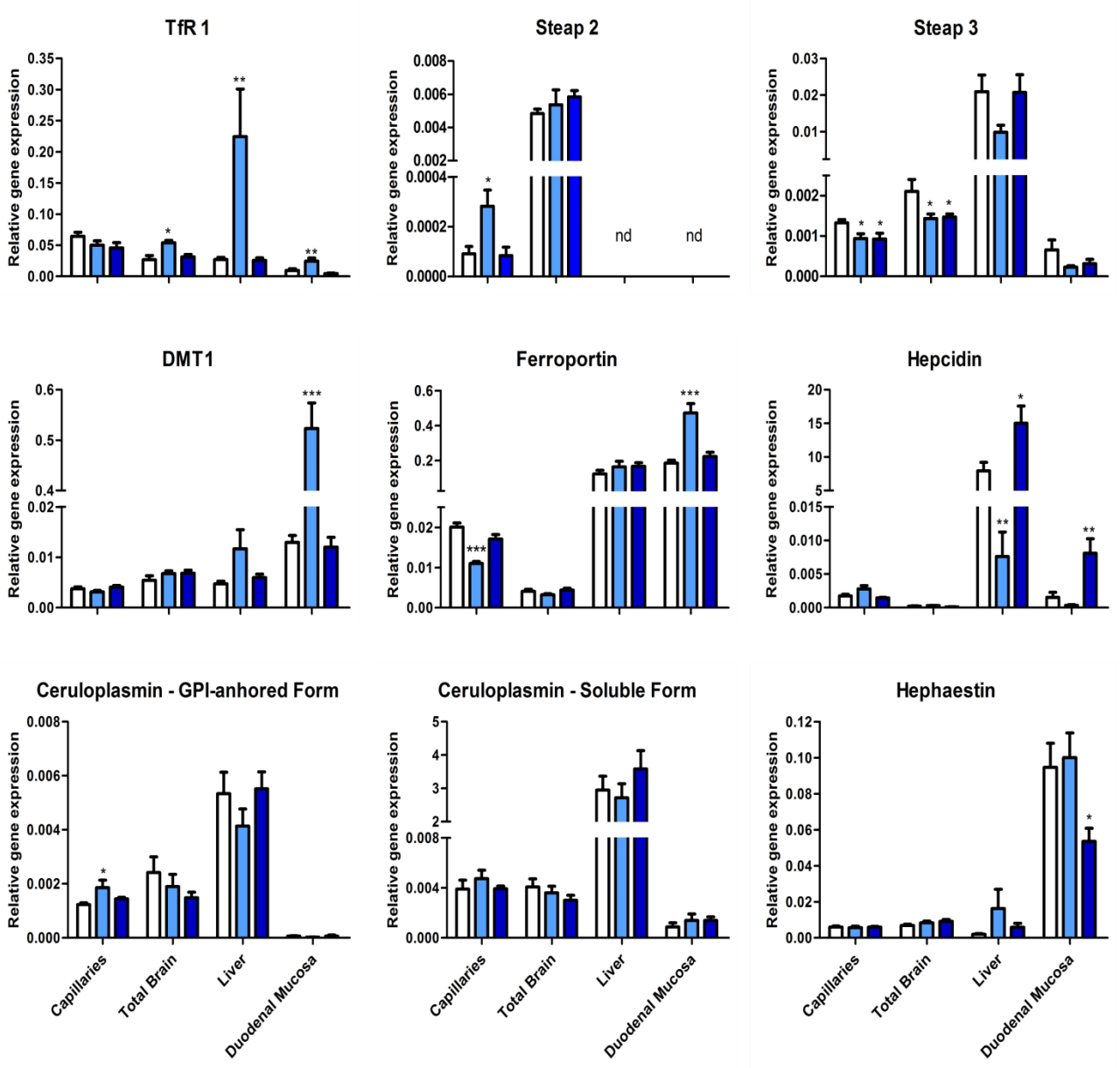

Figure 7. *Gene expression analyses for molecules related to iron transport: transferrin receptor (TfR), Steap 2, Steap 3, divalent metal transporter I (DMT1), ferroportin, hepcidin, ceruloplasmin and hephaestin. Gene expression was analysed in purified brain capillaries, total brain, liver and duodenal mucosa from normal-fed (white), iron-deficient (light blue) and iron-reversed rats (dark blue). Note that transcripts are detected for all genes in the brain capillaries, and these are regulated as a consequence of iron changes. n.d., not determined. Data were analysed using the GraphPad Prism 5.0 software and the relative expression of iron defient and iron-reversible in each type of tissue were statistically compared to the expression found in normal-fed animals using a one-way ANOVA with Dunnett multiple comparison post hoc test. Data are presented as means ± SEM (n=7-9). *p<0.05, **p<0.0, ***p<0.001.*

Transport of transferrin and iron across polarised RBECs with confined BBB properties.

Transcytosis of transferrin was investigated in a RBECs cultured in co-culture with astrocytes. Transferrin and BSA were both added to the upper luminal chamber. BSA was used as a non-specific fluid face marker in the experiment. 24 hours of sampling from the abluminal compartment did not reveal any significant differences in the transport of transferrin and BSA. TEER measurements were carried out throughout the experiment and were found to increase from 196 ± 4.1Ω*cm^2 to 255 ± 6.3 Ω*cm^2 for the control and 169.7 ± 3.7 Ω*cm^2 to 242.4 ± 4.5 Ω*cm^2 for transferrin and BSA. Transferrin and BSA were found to be readily detected within the cell cytoplasm of BCEC after 24 hours of exposure. The transport of transferrin and BSA to the abluminal compartment seems to be independent of the concentration in the luminal compartment after 30 min of exposure. Some degree of non-specificity within the measurements was, however, observed with the Alexa Fluor 488 labelled transferrin. After 30 min of transport the media in the upper chamber was removed and the remaining amount of transferrin and BSA was analysed. The results showed that more transferrin remained in the luminal compartment than was added in the first place. This was, however, only the case for transferrin. Culture inserts were terminated after four and 24 hours and the cellular distribution of transferrin and BSA were analysed by confocal microscopy. Traces of both transferrin and BSA were found in both the luminal and abluminal site of the RBECs after both 4 and 24 hours. However, the RBECs are very small and thin and again it is difficult to see the exact cellular location within the cell.

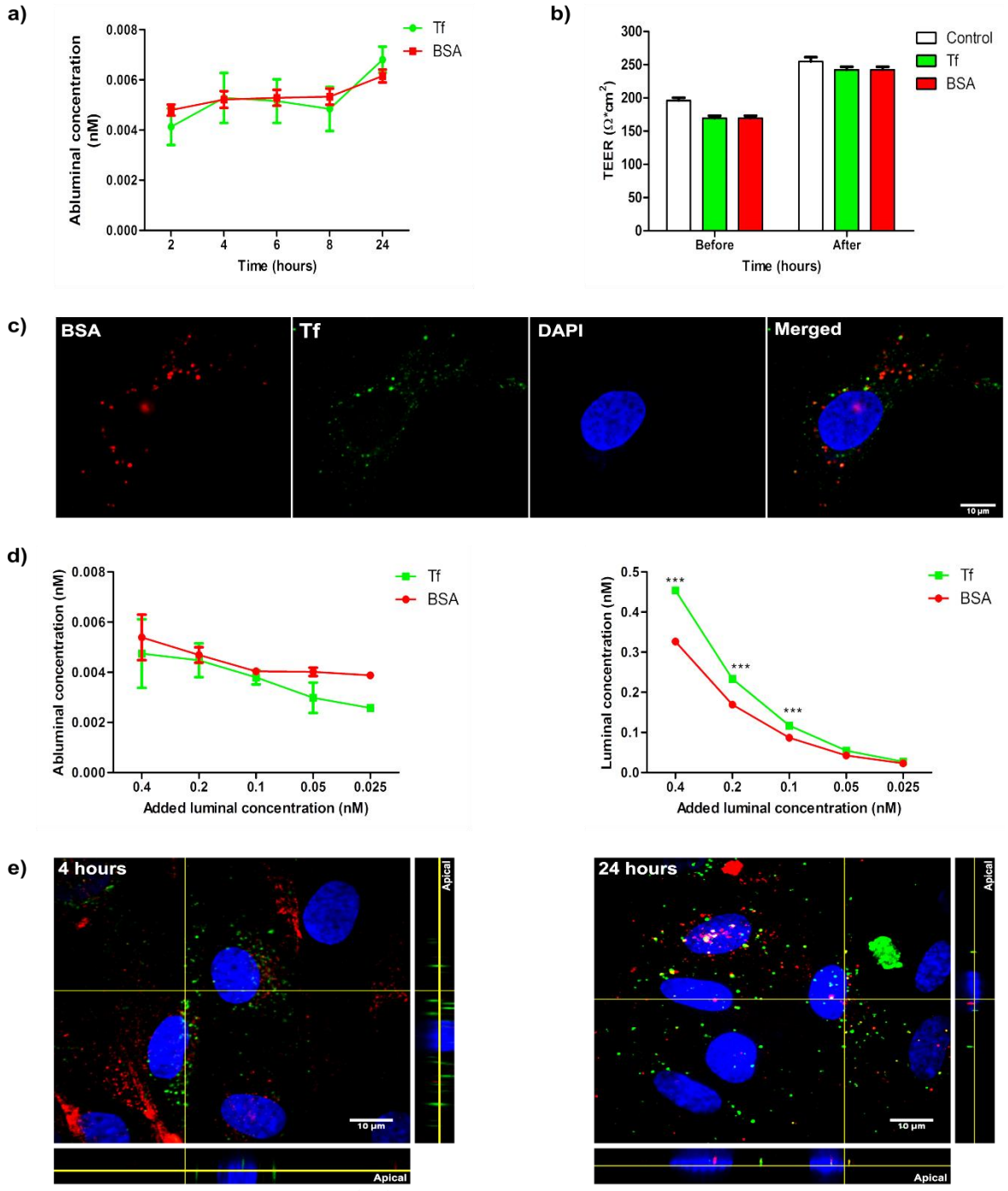

Figure 8. *Transport of transferrin (Tf) through cultured primary rat brain endothelial cells (RBECs).* ***a)*** *24 hours sampling from the abluminal compartment reviled no significant differences between the abluminal transport of Tf and bovine serum albumin (BSA), which acted as a non-specific fluid face marker in the experiment.* ***b****) The integrity of the RBECs persisted throughout the experiment with TEER values above 150 Ω*cm^2.* ***c)*** *The distribution of Tf (green) and BSA (red) was readily detected in the RBECs.* ***d)*** *The transport of Tf and BSA from the luminal to the abluminal compartment after 30 min was independent of concentration. However, some sensitivity issues were observed with the fluorescence measurement in the luminal chamber, especially at the higher concentrations.* ***e)*** *The cellular distributions of Tf (green) and BSA (red) were investigated after 4 (left) and 24 hours (right). Data were analysed using the GraphPad Prism 5.0 software and were statistically analysed using a two-way ANOVA with Bonferroni post hoc tests. Data are presented as means ± SEM (n=3-4) ***p<0.001.*

RBECs co-cultured with astrocytes were, additionally, exposed to ^{59}Fe for 24 hours in the presence or absence of hepcidin, to investigate the cells ability to pump iron out of the cell cytoplasm, which would correspond to the presence of a functional metal transporter protein, like ferroportin (Fig. 9). The cells were incubated with 214 ng ^{59}Fe for 24 hours with or without the addition of hepcidin. After 24 hours, the ^{59}Fe content within the luminal and abluminal compartments was analysed (Fig. 9a). The ^{59}Fe level within the abluminal compartment had increased to 3.3 ± 0.2 ng and 3.1 ± 0.15 ng with or without the presence of hepcidin, respectively. Hence the abluminal transport of iron was not influenced by hepcidin. The luminal concentration decreased to 186 ± 3.2 ng without hepcidin and 179.1 ± 4 ng with hepcidin. Again hepcidin induced no difference between the two groups. TEER measurements were carried out to investigate impact of hepcidin on the integrity of the cell layer during the experiments (Fig. 9b). No difference was seen and TEER remained high throughout the experiment with the lowest TEER values of 148.2 ± 5.4 $\Omega*cm^2$ and the highest TEER values of 177.2 ±2.1 $\Omega*cm^2$. The significantly lower concentration of ^{59}Fe found in the abluminal compartments is also an indication that the integrity of the cell layer were intact. After incubating the cells for 24 hours with ^{59}Fe, the cells were washed and fresh media without ^{59}Fe were added. Hepcidin was still added to some of the culture inserts. During the next 24 hours, samples were collected from the luminal and abluminal compartment (Fig. 9c). The cells were found to be able to remove ^{59}Fe from the cell cytoplasm and the efflux direction was predominantly to the luminal side. Again no effect of hepcidin was seen on the cells ability to transport ^{59}Fe out of the cytoplasm. After 24 hours of sampling the iron load within the cells were analysed, and compared to the amount found within the cells after 24 hours of ^{59}Fe incubation (Fig. 9d). Again no significant differences were seen, however, there was a tendency towards a higher concentration of ^{59}Fe within the cells subjected to hepcidin, indicating that the cells ability to pump ^{59}Fe out of the cytoplasm were somewhat compromised. Hepcidin should be able to regulate the expression of ferroportin. Therefore, both the transcriptional and post translational effects of hepcidin were analysed by RT-qPCR and immunocytochemical staining's, respectively. The gene expression of ferroportin was not altered as a consequence of hepcidin treatment (Fig. 9e). Additionally, no changes in the immunocytochemical staining for ferroportin in the RBECs after 24 hours of hepcidin treatment were observed (Fig. 9f).

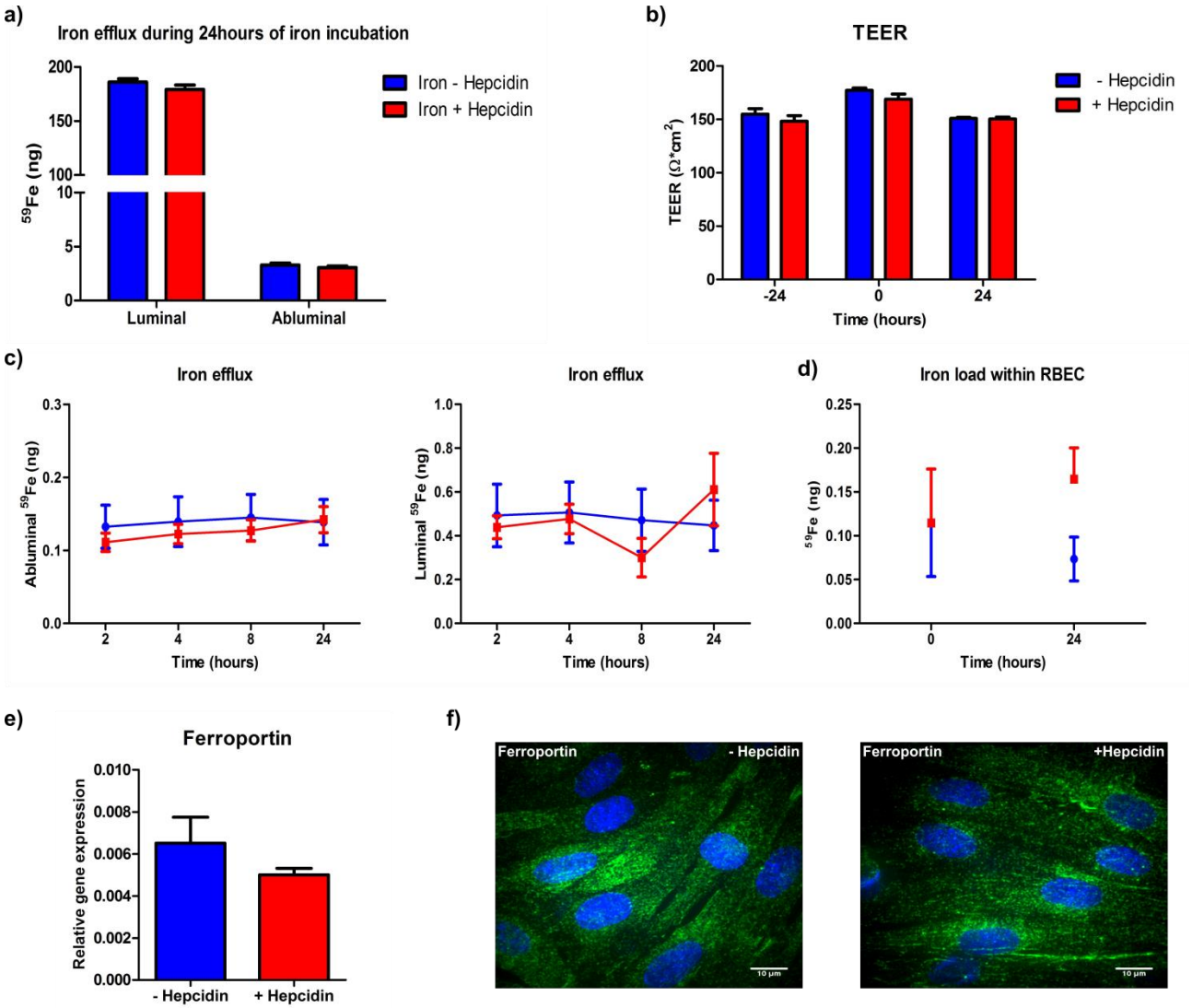

Figure 9. *^{59}Fe efflux under the influence of hepcidin was investigated in primary rat brain endothelial cells (RBECs) co-cultured with astrocytes.* ***a)*** *RBECs were added with ^{59}Fe in the upper luminal chamber for 24 hours with (red) or without (blue) hepcidin. After 24 hours of ^{59}Fe incubation, the luminal and abluminal concentrations were analysed.* ***b)*** *TEER remained high throughout the experiment and was not influenced by hepcidin.* ***c)*** *After 24 hours, the cells were washed and the media replaced with fresh media containing no ^{59}Fe, however, some cells were still under the influence of hepcidin. The media from the luminal and abluminal compartments were sampled at various time intervals* ***d)*** *^{59}Fe content within the cells was, additionally, analysed.* ***e-f)*** *Finally, the regulatory effects of hepcidin on both the gene and protein expression of ferroportin were analysed. The data generally show that RBECs seem to have a functional ferroportin responsible for the efflux of ^{59}Fe across the cell membrane. Hepcidin did not seem to have any effect on the function of ferroportin. Data were analysed using the GraphPad Prism 5.0 software and the data were statistically analysed using a 2-way ANOVA with Bonferroni post hoc test (a-d) and an unpaired t-test (e). Data are presented as means ± SEM (n=3). No significant differences were detected.*

DISCUSSION

The transport of iron into the brain remains debated. In this study, we have investigated the expression of several molecules related to iron transport in BCECs. Previous studies on expression of molecules related to iron transport to the brain have been investigated *in vivo* [25, 71, 131, 132, 135, 216] or by using immortalised BCECs grown in non-polarised conditions [122-124] or in polarised conditions [121]. This study, therefore, set out to examine if the polarisation of the endothelial cells had any impact on the expression of essential molecules related to iron transport. Additionally, we investigated the gene expression of these molecules in both immortalised RBE4s and primary isolated RBECs. Both cell types were found to express all nine proteins involved in iron transport to the brain. However, a large variability in the gene expression of all nine investigated proteins was observed dependent on the culture conditions and the cell type. In general, the gene expression increased when the cells were cultured in an *in vitro* BBB setup. The expression of the molecules related to iron transport were confirmed *in vivo*, since the expression of all the proteins were, additionally, found in isolated brain capillaries.

The expression of the transferrin receptor 1 was found to be down regulated, once the cells were grown in polarised conditions. This observation is consistent with previous observations that the transferrin receptor is higher expressed by proliferating cells compared to non-proliferating cells [133]. The transferrin receptor has also been shown to be regulated by iron supply [133], which was also confirmed in this study. Increased expression of the transferrin receptor during iron deficiency was found in total brain, liver and duodenal mucosa. However, no difference was found in the brain capillaries. The transferrin receptor was found to be mainly expressed in the cytosol of RBECs, corresponding to an endocytosis and recycling pathway of this receptor within the cytosol of the BCECs. Confocal images of transferrin could not determine whether the transferrin receptor is endocytosed or transcytosed across the RBECs, due to the small size of these cells. The observed staining pattern obtained with the anti-transferrin receptor antibody is, however, consistent with that reported by Yang and colleagues [216].

Previously, the expression of DMT1 by BCECs has been extensively debated. Several *in vivo* studies have not been able to detect the expression of DMT1 in the brain capillaries [131, 135], while other have shown its expression both *in vivo* [31, 32, 186] and in cultured immortalised BCECs [123]. DMT1 expression were observed in both RBE4s and RBECs, confirmed by both gene and protein expression analysis. DMT1 expression was, additionally, found in isolated capillaries. Immunocytochemical staining of DMT1 revealed staining that distributed in a punctative manner within the cell cytoplasm. This corresponds to the immunohistochemical staining previously found in neurons [135]. The present study used one of the same antibodies, which have previously failed to detect DMT1 in BCECs *in vivo* [135].

In order for DMT1 to be able to pump ferrous iron out of the endosome, there is a need for ferrireductases. Steap 2 and 3 have been suggested for this function within the brain. Expression of both Steap 2 and 3 were confirmed in RBE4s, RBECs and in isolated brain capillaries. However, the expression of Steap 2 seems to be low, indicating other ferrireductases might be present in the brain as well. The fact that we did not observe Steap 2 expression in the liver or duodenum also emphasises the existence of other important ferrireductases. The ferrireductase duodenal cytochrome b (Dcytb) have been found in both the liver and in immortalised human brain microvessel endothelial cells (hBMVEC) [123]. This enzyme might together with Steap 2 and 3 be responsible for the reduction of ferric iron to ferrous iron within the endosome, in both the brain and peripheral tissue.

Gene and protein expression of ferroportin were, additionally, found within the cultured endothelial cells and in isolated brain capillaries. This corresponds to previous observations [121, 122, 124, 208, 216]. Immunocytochemical staining of ferroportin was, however, expected to be observed within the cell membrane, since this protein is responsible for the transport of iron out of the cell cytoplasm. We observed immunocytochemical staining for ferroportin in the same punctate manner as that observed for DMT1. Therefore, we investigated the cellular distribution of ferroportin in RBECs by confocal microscopy. However, due to the small size of the RBECs we were not able to determine whether the ferroportin mainly distributed to the cell membrane. However, the punctative expression seems to correspond well to that observed by Yang and colleagues using primary RBECs [216] despite the use of two different anti-ferroportin antibodies.

Two different theories have evolved in order to explain the mechanism by which iron is transported into the brain. In this study, we report that BCECs express all the relevant proteins needed for iron uptake by the transferrin receptor, endocytosis, ferrireductase activity, DMT1 mediated translocation of iron across the endosomal membrane and finally ferroportin mediated transport out of the cell cytoplasm. However, in order to convert ferric iron to ferrous iron without the formation of toxic radicals, a ferrooxidase activity is needed within the brain parenchyma. BCECs and astrocytes have previously been shown to supply this enzymatic activity [121, 122, 160], however, we speculated whether pericytes, as the closest neighbouring cell to the BCECs, would also contribute with ferrooxidase activity. Therefore, we investigated the expression of both forms of ceruloplasmin and the ceruloplasmin homolog hephaestin within the pericytes and compared this expression to that found in astrocytes. Pericytes, like astrocytes, were found to express both forms of ceruloplasmin and hephaestin, strongly suggesting that these cells also play an important role in the regulation of iron transport into the brain. The expression of the ferrooxidases was also found to be highest in the pericytes and astrocytes as compared to the BCECs, and this might, additionally, indicate that these cells are the primary sources of the ferrooxidase activity within the brain. Patel and colleagues have reported that the primary from of ceruloplasmin expressed by the liver is the soluble form, while the primary form

expressed in the brain is the GPI anchored form [161]. The gene expression analyses performed in this study confirmed that the soluble form is the main form of ceruloplasmin expressed by the liver; however, we also found the soluble form to be highly expressed in the brain primarily by the pericytes and astrocytes. This observation was confirmed in the gene expression analyses performed on isolated brain capillaries and total brain. Gene expression and protein expression analysis are, however, very different and, therefore, not directly comparable. Protein analysis for ceruloplasmin, additionally, confirmed the expression of ceruloplasmin by BCECs, pericytes and astrocytes. In all cell types ceruloplasmin was predominantly found at the cell membrane corresponding to the location of the GPI anchored form of ceruloplasmin. Some staining was, however, also observed in the cytoplasm, probably to some degree corresponding to the soluble form of ceruloplasmin. Without the proper protein analysis, we can, however, not determine the predominant protein form of ceruloplasmin within the brain; however, both forms seem to be expressed by BCECs, pericytes and astrocytes.

Hephaestin was also found to be expressed in the brain by BCECs, pericytes and astrocytes. Immunocytochemical staining confirmed this expression. Again the expression of hephaestin was expected to be similar to that of ceruloplasmin; however, this was not the case. Hephaestin was found to be primarily located within the cytoplasm in the same punctative manner as DMT1 and ferroportin. However, the staining is very similar to that reported previously [216]. In contrast the staining is not directly comparable to those found by McCarthy and colleagues [122] where hephaestin was primarily found at the cell membrane. Yang and colleagues [216] only observed a faint staining for hephaestin in pericytes when using the same protocol for isolation of pericytes and the same anti-hephaestin antibody, as used in this study. Additionally, they observed gene expression of hephaestin by pericytes, but the expression found by BCECs were, however, higher than that observed in the pericytes, which is consistent with the observation presented in this study.

Functional studies on iron transport were, additionally, performed on polarised RBECs grown in co-culture with astrocytes. Transcytosis of transferrin was investigated by comparing its transport to the non-specific fluid phase marker BSA. No difference was found in the abluminal transport of transferrin and BSA and the transport seems to be independent of concentration, indicating that transferrin is not undergoing transcytosis across the BBB. Previous studies have also failed to show transcytosis of transferrin across the BBB [43, 190]. The transferrin receptor has a high affinity towards apotransferrin at low pH [136], indicating that iron is probably released at low pH and transported into the brain in the absence of transferrin, which instead is redirected to the luminal side bound to the transferrin receptor. Confocal images of BCECs exposed to transferrin and BSA for 4 and 24 hours both showed a distribution of both transferrin and BSA at the luminal and abluminal membrane. The exact cellular location of the two proteins were, however, not possible to determine due to the small size of the RBECs.

The fluorescence assay had some challenges in regards to sensitivity. This was especially observed when analysing the media from the luminal compartment to which a known concentration of transferrin and BSA were added. The results showed that the media from the luminal compartment contained a higher concentration of the Alexa Fluor 488 conjugated transferrin than that added. This was especially a challenge when using high concentrations of Alexa Fluor 488 conjugated transferrin. The same tendency was not observed with the Texas red labelled BSA. Therefore, the experiment needs to be repeated several times to make sure the transport of BSA and transferrin remains insignificant. However, the Alexa Fluor 488 sensitivity often resulted in a higher concentration. Therefore, correction for the insensitivity would result in an even lower transport of transferrin across the BBB compared to BSA.

Iron accumulation within the RBECs and their ability to efflux iron across the cell membrane were, additionally, analysed in polarised RBECs with confined BBB properties. RBECs were found to be able to transport iron out of the cell cytosol, primarily directed to the luminal compartment. This observation has also been reported by McCarthy and colleagues [121] using a co-culture model of immortalised hBMVEC and C6 glioma cells. They showed that the direction of iron efflux was regulated by the presence of C6 glioma cells, probably by their expression and secretion of the GPI anchored and soluble form of ceruloplasmin. Iron efflux was primarily directed towards the abluminal compartment when the BCECs were cultured in a contact co-culture with the C6 glioma cells, but not when the hBMVEC were cultured alone or in a non-contact co culture with the C6 glioma cells. In the present study astrocytes were cultured in non-contact with the RBECs. Therefore, the luminal directed iron efflux observed in this study corresponds well to that observed by McCarthy and colleagues. It would, however, be interesting to see if primary isolated astrocytes had the same effect on the direction of iron efflux if the astrocytes were cultured in contact co-culture with RBECs.

Hepcidin regulates the post transcriptional expression of ferroportin by binding and inducing the internalisation and subsequent degradation of ferroportin. McCarthy and colleagues were, additionally, able to demonstrate that an exogenous hepcidin concentration of 200 nM was able to induce the internalisation and degradation of ferroportin within their hBMECs, seen as a loss of immunocytochemical staining for ferroportin [121]. We were not able to show any significant effect on iron efflux after the addition of 700 nM exogenous hepcidin. Additionally, we examined both the transcriptional and post translational effect of hepcidin on ferroportin, and again observed no difference. This might, therefore, indicate that the function of ferroportin was not compromised by the addition of hepcidin, but still indicate that the RBECs express a functional metal transporter protein, that are able to transport iron across the cell membrane and either back to the circulation or into the brain parenchyma. However, due to the lack of ferroportin degradation by hepcidin the experiment should be repeated and it might be necessary to investigate the effects of different concentration and incubations periods of hepcidin on the post translational expression of ferroportin.

In summary, we were able to show that polarised primary isolated RBECs express all the necessary protein for ferrous iron to be taken up by the transferrin receptor 1, endocytosed, reduced to ferric iron by means of ferrireductases, translocated to the cytosol by DMT1 and pumped out of the cytosol by ferroportin. Pericytes, in addition to RBECs and astrocytes were shown to express ceruloplasmin and hephaestin, and are thereby able to facilitate ferrooxidase activity needed for ferroportin-mediated iron release in the brain parenchyma.

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Discussion

The main objective of this thesis has been to isolate primary BCECs, astrocytes and pericytes from rat brains in order to establish various primary culture models of the BBB. Three types of *in vitro* BBB models were characterised and the most appropriate model was chosen to investigate the possibility of genetically modifying BCECs with defined BBB properties to express and secrete recombinant proteins after non-viral gene therapy. The *in vitro* BBB models were, additionally, used to study the expression of proteins related to iron transport at the BBB and the other cells of the NVU. Finally, the mechanisms by which iron transport is regulated at the BBB were investigated in order to fully elucidate the properties of using the transferrin receptor as a BBB target.

Establishment and characterisation of *in vitro* BBB models

Primary RBECs, pericytes and astrocytes were successfully isolated from rat brains. The three isolated cell types were successfully cultured *in vitro* and were found to be very pure. High purity of the RBECs was obtained by culturing the cells for 2-3 days in puromycin media, a substance that takes advantage of the RBECs high expression of efflux transporter. On the contrary, astrocytes were characterised as a mixed glia culture, mainly consisting of astrocytes. Pericytes were found to be a mixed culture of alpha smooth muscle actin (α-SMA) positive, α-SMA negative and platelet derived growth factor receptor beta (PDGFRβ) positive cells. The isolated cells were used to establish a mono culture of RBECs, a co-culture of RBECs and astrocytes and a triple culture model of all three cell types. The co-culture and triple culture models were found to be superior to the mono culture with respect to TEER and permeability, clearly demonstrating the influence of astrocytes and pericytes in restoring the *in vivo* characteristics of the RBECs when cultured *in vitro.* In the literature astrocytes have clearly been characterised as the most important cell in relation to inducing and maintaining the barrier properties of BCECs [3, 49, 68, 79]. However, more recently the role of pericytes has been discussed [102]. In several studies pericytes, when cultured together with astrocytes, have been shown to induce the barrier properties of the BCECs even further of that seen with astrocytes alone [53, 78, 143, 144]. Even though the triple culture setup have the highest similarity to the *in vivo* situation, this study did not find any significant difference in TEER between the co- and triple culture model, indicating that pericytes were not able to further induce the barrier characteristics of the endothelial cells. Recently, it has become evident that pericytes exhibit a pluripotent phenotype [55, 56] and are able to differentiate *in vitro* as a consequence of the different stimulus obtained by factors secreted by other cells or factors added to the culture medium [193]. Therefore, the lack of significantly higher TEER values with the triple culture model might be due to the differentiation state of the pericytes.

α-SMA positive pericytes are unable to induce barrier properties while α-SMA negative pericytes have significant barrier inductive properties [193]. Pericytes cultured alone were found to be a mixture of α-SMA positive, α-SMA negative and PDGFRβ positive cells. In culture with both RBECs and astrocytes, the pericytes are stimulated by a variety of factors that can stimulate the differentiation of these cells. The triple culture model showed a large variability during the collection of data. This might be due to the limited control of pericyte differentiation, when these are cultured together with RBECs and astrocytes. Different factors have been shown to induce the differentiation of the pericytes either toward the α-SMA positive or negative state. Among these, bFGF and TGFβ might have played a role in this study. bFGF promotes the α-SMA negative, while TGFβ induces the α-SMA positive state [193]. When cultured alone the pericytes were to our knowledge, not subjected to any of these factors, and were found to be primarily α-SMA positive. When cultured together with both astrocytes and RBECs, they were under the influence of both factors, since these are both factors secreted by astrocytes [2, 62, 188, 195]. bFGF was, additionally, added to the cell culture media during co-culturing. The differentiation state of the pericytes was, therefore, difficult to control and might have resulted in different differentiation of pericytes from experiment to experiments, resulting in the large variability in the TEER values obtained with the triple cultures. The final mean of 24 different culture inserts resulted in no significant difference from the co-culture setup, which in general showed more consistent TEER values from experiment to experiment. Additionally, when comparing TEER values to the apparent permeability of mannitol, it was clear that the permeability to mannitol did not decrease further as a response to higher TEER values. As soon as the RBECs layer exhibited TEER values above approximately 130 $\Omega*cm^2$, no significant difference was seen in the permeability to mannitol. This is in correspondence with that previously reported by Gaillard and colleagues [66].

Taking together these results indicate that the co-culture model was the most suitable model to fulfil the aims of the remaining studies. However, it will be interesting to analyse the exact differentiation state of the pericytes when these are cultured with RBECs and astrocytes. It will also be interesting to examine the factors triggering the differentiations of the pericytes, in order to better understand and control the differentiation state of the pericytes, when these are cultured together with RBECs and astrocytes.

Gene therapy at the BBB

One strategy to overcome the impermeability of the BBB is gene therapy. The gene therapy strategy of turning BCECs into protein factories that secrete recombinant proteins of therapeutic potential could be beneficial in many neurodegenerative diseases. In this thesis the BCECs were manipulated to produce GH1, EPO and BDNF, but theoretically it should be possible to manipulate the BCECs into producing and secreting any protein of interest. However, for this strategy to prove feasible *in vivo* some issues had to be

investigated. First previous studies have primarily used immortalised cell lines without no seemingly barrier properties [82, 88, 194]. Additionally, cell division has also been positively related to the transfection efficacy of non-viral gene therapy [28, 139], which would make this strategy useless *in vivo*, since these cells do not undergo cell division at the mature state [174]. Therefore, it was necessary to investigate whether non-mitotic BCECs with defined barrier properties would be receptive for delivery of genetic material by a non-viral carrier, and subsequently process the encoded protein, without compromising the barrier properties of the BCECs. For this purpose a plasmid encoding the red fluorescent protein HcRed1-C1 was used. The transfection efficiency of BCECs was found to be independent of active cell division, which suggests that the plasmid DNA does not only enter the nucleus during mitosis. This observation was confirmed by Thomsen and colleagues, who were also able to transfect BCECs in the non-diving state [194]. Additionally, the barrier properties of BCECs, when co-cultured with astrocytes, were not compromised when genetically manipulating the BCECs into producing the proteins, which also indicated that the non-viral DNA carrier complex was non-toxic for the cells. Cells undergoing division did not exhibit seemingly barrier characteristics, primarily due to large gaps between the non-confluent cells that made it impossible for the cells to make cell-cell contacts. During transfection of the non-confluent cells, TEER was significantly decreased. This was probably due to some toxic effects of the non-viral gene carrier complex. The cell density of the actively dividing cells compared to the non-dividing cells was of course fewer. Therefore, the gene carrier complex, cell density radius was not the same between the two groups. Some optimisation of this radius might decrease the toxic effect seen with the actively dividing cells.

Previous studies have shown that immortalised BCECs are able to secrete GH1 [194], GDNF [88] and Fibroblast growth factor [90] after genetic manipulation of the BCECs using a non-viral gene carrier. Therefore, a second important question needs to be considered. Is it possible to genetically manipulate primary isolated BCECs into secreting the encoded protein to its surroundings? Additionally, it was interesting to establish whether primarily isolated BCECs could be as effectively manipulated as the immortalised cell lines. The transfection and secretion ability of immortalised RBE4s and primary RBECs were investigated. RBECs and RBE4s were transfected with GH1, EPO and BDNF, and gene expression analyses revealed a higher expression level of EPO in both cell types. Cells transfected with EPO were, furthermore, found to have the highest transfection efficiency. No protein secretion was found in either the immortalised or primary endothelial cells, probably indicating a low secretion activity of these cells, or a low sensitivity of the assay. It was, therefore, not possible to establish whether the primary cells are able to secrete a recombinant protein. Other studies have worked with the same issues and have been able to show secretion of a recombinant protein into the culture media with the use of ELISA [88, 180]. This study based all experiments on the use of anti-FLAG antibodies, however, these were often found to be somewhat unspecific. Therefore, it might be favourable to use protein specific antibodies instead. Additional studies are, therefore, needed in order to conclude on the secretion ability of these cells. Another important issue to

address *in vitro*, before testing the gene therapy strategy *in vivo*, is the main direction of the secreted protein from polarised RBECs with confined barrier properties. The secreted protein should be secreted into the CNS instead of back to the blood circulation; otherwise the strategy will be useless. Jiang and colleagues found secretion of GDNF to both the luminal and abluminal side, although primarily towards the brain side [88]. They confirmed this *in vivo* by showing that GDNF secreted by BCECs resulted in protection of neurons after 6-hydrydopamine induced lesions. This study, therefore, strongly supports the idea of turning BCECs into protein factories that secrete proteins with neuroprotective effect on neurons and other CNS cell types.

Previous studies have shown increased nuclear entrance of plasmid DNA into non-mitotic cells, by coupling a nuclear localisation signal to the DNA plasmid [129, 218]. Two different plasmids were used in this study. The plasmid encoding the HcRed1-C1 protein contains a SV40 sequence, while the plasmid encoding GH1, EPO and BDNF contains a cytomegalovirus sequence instead. Only the SV40 sequence contains the nuclear localisation sequence [41]. It might, therefore, enhance the transfection efficiency of GH1, EPO and BDNF in non-mitotic cells, if the nuclear localisation sequence was cloned into these vectors as well.

In vivo BBB targeting

The gene carrier used in this study was the commercially available transfection agent Turbofect, which can be purchased in two variants, an *in vitro* transfection agent and an *in vivo* transfection agent. Therefore, the transmission from *in vitro* studies to *in vivo* studies is easy when using Turbofect as the gene carrier. Some modifications might, however, be needed before moving into animals. Turbofect is a non-cell specific transfection agent, meaning it can potentially enter every cell it encounters after systemic injection. By injecting the DNA carrier complex into the carotid artery, the brain availability of the gene carrier complex would be dramatically increased. This will, however, not eliminate interaction and transfection of other cells in the body, resulting in unwanted side effects. One approach to avoid this could be to design the gene carrier complex, in such a way that it targets a receptor expressed by the BCECs. The OX26 antibody, which is specific towards the transferrin receptor, has extensively been used for this purpose [64, 71, 105, 132, 156, 221]. This might not completely limit the entrance of the gene carrier complex into other cells of the body, since the transferrin receptor is expressed by other cells as well. However, injection into the carotid artery will possibly increase the brain uptake significantly.

The exact mechanism by which the transferrin receptor transports iron into the brain is not yet fully elucidated. Several studies suggest endocytosis [122, 123, 132], others suggest transcytosis [64, 105, 156] while others suggest both [30]. The exact mechanism is important, since it affects the function of the transferrin receptor as a brain specific target. For the purpose of gene therapy, it is only necessary for the

gene carrier complex to enter the BCECs, while transcytosis of the transferrin receptor it is crucial for complexes designed to deliver drugs into the brain. Therefore, the final part of this thesis, tried to clarify some of the transport mechanisms for iron into the brain using the *in vitro* BBB co-culture model of primary isolated RBECs and astrocytes.

Iron transport into the CNS

The mechanisms of iron transport to the brain have been discussed for many years, and still a lot of unanswered questions remains. For many years observations from *in vivo* experiments played a major role in the knowledge on iron transport. However, the observations were ambiguous, resulting in scientists being divided between two interpretations on the mechanisms of iron transport. The main debate was regarding the expression of DMT1 and ferroportin by the BCECs [134]. DMT1 and ferroportin are essential for the endocytotic pathway. If these proteins are not present the transferrin receptor will have to undergo transcytosis in order to deliver iron to the brain parenchyma.

Since the development of immortalised BCECs and *in vitro* BBB models, several *in vitro* studies have emerged. However, to our knowledge, no studies have investigated the expression of molecules related to iron in primary isolated RBECs cultured with confined BBB properties. The expression patterns of molecules related to iron transport were, therefore, investigated in both immortalised RBE4s, and in RBECs cultured under polarised and non-polarised conditions. These results showed that both cell types expressed DMT1, ferrireductases and ferroportin, indicating that transcytosis of the transferrin receptor is not necessary for the delivery of iron into the brain. This was, furthermore, confirmed with immunocytochemical staining. It was, additionally, showed that the transport of transferrin into the brain was not different from that of BSA, indicating no transcytosis of transferrin into the brain. These results might, however, also suggest that only iron is delivered to the brain, while transferrin remains bound to the transferrin receptor as it recycles to the luminal side. An attempt to demonstrate ferroportin activity within the BCECs, failed due to no effect on iron efflux by the addition of hepcidin, which is known to degrade ferroportin. It was, therefore, not possible to establish if the hepcidin treatment failed due to lack of ferroportin function in the BCECs, or due to lack of hepcidin function. Additional studies are, therefore, needed. In general we were not able to determine the exact pathway by which iron is transported into the brain, however, according to the gene and protein expression analysis both pathways seems to be possible.

Recent studies by McCarthy and colleagues also suggests that iron is transported via the endocytotic pathway, since they have also been able to show gene and protein expression of DMT1, ferrireductase and ferroportin within the BCECs *in vitro* [122, 123]. They have, additionally, showed that BCECs are able to reduce ferrous iron to ferric iron, probably due to activity of ferrireductases Steap 2 and/or Dcytb, occurring

in the endosomes [123]. This reduction was dependent on the binding and endocytosis of holotransferrin to the transferrin receptor. In correspondence with this observation Burdo and colleagues have shown that the transferrin receptor and DMT1 co-localised in isolated brain microvessels, indicating similar trafficking of these two proteins within the capillary and thereby also the presence of DMT1 within the transferrin receptor and ferrireductases containing endosome [31]. McCarthy and colleagues have, furthermore, demonstrated that ferroportin and ferrooxidases like hephaestin and ceruloplasmin is needed for iron efflux from the BCECs [122]. Ferrooxidase activity is necessary for the release of iron from ferroportin [47]. Both BCECs and astrocytes have been shown to provide this function [122, 160]. However, in this thesis it was shown that pericytes are an additional source of ferrooxidase activity. McCarthy and colleagues showed that depleting the ferrooxidase activity by a copper chelating agent BCS leads to reduction in ferroportin levels and a complete inhibition of iron efflux [122]. They were, however, able to restore the iron efflux activity by incubating the cells with pure ceruloplasmin or soluble ceruloplasmin from astrocyte conditioned media. It would be highly relevant to replicate these functional studies performed by McCarthy and colleagues, and thereby establish if the same is true for primary isolated RBECs. Additionally, it would be interesting to see if addition of pericytes and or astrocytes to the BCECs would be able to restore the BCS depleted ferrooxidase activity, which would confirm the observation that pericytes are an additional source of functional ceruloplasmin activity. Finally, McCarthy and colleagues have shown that the addition of hepcidin correlated with decreased iron efflux by the BCECs, indicating the knockdown of ferroportin [121].

Using the co-culture model of hBMVEC and C6 glioma cells, McCarthy and colleagues, furthermore, showed that ferroportin expression was regulated by hepcidin secreted by the C6 glioma cells. The C6 glioma cells were able to influence the main direction of iron efflux. If the C6 glioma cells were in contact with the BCECs the direction was primarily towards the luminal membrane, while the reverse was the case when the C6 glioma cells were cultured in non-contact co-culture with the BCECs [121]. Again it would be very interesting to see if this is also the case in our *in vitro* BBB, since this model is based solely on cells derived from healthy tissue. Additionally, it would be interesting to see if the pericytes are also able to influence the efflux direction of iron.

The experiments performed by McCarthy and colleagues included the use of immortalised hBMVEC. They have mainly cultured the hBMVECs under non-polarised conditions [122, 123], but recently they have used these cells to establish an *in vitro* BBB model with confined BBB properties [121]. The gene expression of the key molecules related to iron transport were here shown to be highly influenced by the culturing conditions and the type of cell used. This study was, however, not able to establish an *in vitro* BBB model with defined BBB characteristics using the RBE4s. It can, therefore, not be excluded that this had a high influence on the expression profile of the RBE4s in co culture. Nevertheless, some genes were regulated in RBE4s as a consequence to the polarisation of these cells, compared to the non-polarised cells. However, the

difference between RBE4s and RBECs in co-culture might not have been so big if RBE4s had the same confined BBB properties as the RBECs. Though, it might be worth to consider the choice of cell and culturing conditions for future studies.

To date no study have been able to exclude one of the two different possibilities for transferrin receptor mediated iron transport to the brain, however, emerging evidence suggest that both possibilities might be a reality. The continuous publications on expression of DMT1, ferrireductases, ferroportin and ferrooxidase activity at the BBB suggest that the transferrin receptor binds holotransferrin and becomes endocytosed into endosomes. Within the endosomes the ferric iron is reduced to its ferrous form by ferrireductases like the Steap proteins or Dcytb, which enable the DMT1 translocation of the ferrous iron into the cell cytoplasm. Within the plasma membrane ferroportin and the ferrooxidases are responsible for the transport and oxidation of iron into the brain. However, studies which have successfully used the transferrin receptor as a target to deliver drug complexes into the brain [64, 105, 156], suggest transcytosis of the transferrin receptor. It has been reported that targeting the transferrin receptor with the OX26 antibody results in accumulation of the antibody within the capillaries, indicating that the transferrin receptor does not undergo transcytosis [132]. Recently, however, is has become clear that this was probably due to high affinity of the antibody towards the receptor. A study comparing the transport of high and low affinity antibodies towards the transferrin receptor were able to show transcytosis of the low affinity antibodies into the brain parenchyma [24, 217].

Conclusions and future perspectives

In this thesis an *in vitro* BBB model based on primary isolated RBECs and astrocytes was found to be the best model to study gene therapy at the BBB and iron transport to the brain. Transfection of non-mitotic RBECs cultured in the *in vitro* BBB model with confined BBB properties was found to be as effective as gene transfection of highly mitotic RBECs. Additionally, the barrier properties were unaffected by the transfection. RBECs were, additionally, successfully transfected to produce the therapeutic proteins GH1, EPO and BDNF; however, it was not possible to detect secretion of the protein into the cell media. Finally, expression of important proteins related to iron transport were found in polarised RBECs suggesting that iron can be transported into the brain by means of DMT1-ferroportin mediated transport. Pericytes, in addition to astrocytes, seems to be a main source of ferrooxidase activity within the brain. The results presented in this thesis have raised the following question that would be highly interesting to answer in the near future.

1. Would it be possible to control the differentiation of the pericytes when these are cultured together with both RBECs and astrocytes in the *in vitro* triple culture model? This would be important in order to ensure a higher consistency with the triple culture model from experiment to experiment, and thereby create an *in vitro* BBB models that highly resembles the *in vivo* situation. This might also make the triple culture model more attractive to use instead of the co-culture model.

2. Would the use of a more sensitive assay like ELISA enable the detection of GH1, EPO and BDNF in the cell culture media following non-viral transfection of the RBECs with plasmid DNA encoding the recombinant proteins? This thesis was unable to establish whether the recombinant proteins were not secreted into the cell culture media or whether it was only secreted in such a low amount making it impossible to detect with the current protein assay. It would also be interesting to see if the substitution of the non-viral gene transfection agent to a viral transfection agent would increase the transfection efficiency to an amount where the protein is easily detected.

3. Is the recombinant proteins secreted towards the luminal or abluminal compartment after gene transfection of polarised RBECs? This is an important question to answer in order to use the BCECs as protein factories that can secrete proteins into the brain. If the transfection of BCECs results in secretion towards the luminal compartment, corresponding to the capillary lumen, the strategy might be less usable *in vivo.*

4. Does the transferrin receptor undergo both transcytosis and endocytosis followed by DMT1-ferroportin mediated transport of iron at the BBB? A lot of questions still remain concerning iron transport at the BBB. This thesis was able to answer only a small fraction of these. It would,

therefore, be highly interesting to make more functional transport studies where only a single step in the iron transport pathway is inhibited at a time. Previous studies have performed many of these studies using immortalised BCECs. However, it would be highly relevant to repeat these studies using the *in vitro* BBB models established in this study.

5. Are pericytes able to provide the necessary ferrooxidase activity needed for efflux of iron out of the cell cytoplasm by means of the iron transporter protein ferroportin? Pericytes were shown to be a source of ceruloplasmin and hephaestin; however, the function of these ferrooxidase proteins was never tested in this thesis. It would, therefore, be highly interesting to see if some of the phenomenon's observed with astrocytes might also apply to pericytes. Additionally, it would be interesting to analyse the effect primary isolated astrocytes and/or pericytes will have on the efflux direction of iron from polarised BCECs.

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