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**EXPRESSIONAL PREREQUISITES FOR
TARGETED DRUG DELIVERY TO THE
PATHOLOGICAL BRAIN**

**BY
STEINUNN SARA HELGUDÓTTIR**

DISSERTATION SUBMITTED 2021



AALBORG UNIVERSITY
DENMARK

EXPRESSIONAL PREREQUISITES FOR TARGETED DRUG DELIVERY TO THE PATHOLOGICAL BRAIN

By

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List of publications

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Epigenetic regulation of ferroportin expression in the blood-brain barrier

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Hepcidin mediates transcriptional alteration of ferroportin in differentiated neuronal-like PC12 cells subjected to iron challenge

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ENGLISH SUMMARY

Neurodegenerative diseases are becoming more prevalent due to the increased lifespan of the general population as aging is the primary risk factor for the majority of neurodegenerative diseases. The symptoms vary depending on the given neurodegenerative disease, ranging from cognitive decline, learning and memory deficits, hallucinations to motor defects. Unfortunately, there is no cure for any of the devastating neurodegenerative diseases, and most treatment options are symptomatic and do therefore not address the underlying cause of pathology. Furthermore, although many drug candidates have been developed, it has proven more complicated than anticipated to deliver therapeutics to the brain due to restraints of the brain's vasculature. The blood-brain barrier (BBB) is composed of highly specialized brain capillary endothelial cells (BCECs) that are characterized by tight interconnections and low passive permeability. The BBB separates the brain parenchyma from the blood, ensuring that harmful molecules in the bloodstream do not reach the brain, however, it also keeps most macromolecules and therapeutics out of the brain parenchyma as well.

The BCECs express various receptors and transporters on the cell membrane, which allows for active transport of molecules essential for the brain e.g. amino acids, glucose, vitamins, and essential metals. These receptors and transporters can also be utilized to deliver therapeutics across the barrier via specific targeting of molecular constructs. Hence, by conjugating drugs or drug-encapsulated nanoparticles to molecules that specifically interact with a receptor or transporter selectively expressed on the luminal side of BCECs, uptake and transport of such constructs across the BBB can be increased substantially compared to injection of non-targeted drugs.

The expression of nutrient receptors and transporters suitable as drug delivery targets may, however, be altered due to inflammation and other pathological factors associated with neurodegenerative diseases. However, the alteration in expression levels at the BBB are poorly understood. Possible alterations of these targets may occur not only at the transcriptional level, but also by epigenetic modifications.

In this dissertation, the expression of three suitable targets for drug delivery, Cluster of Differentiation 98 Heavy Chain (CD98hc), glucose transporter 1 (Glut1), and transferrin receptor 1 (TfR1), were investigated in models of brain pathology. Furthermore, the possibility of regulating their expression using epigenetic inducer, valproic acid, was explored.

It was found that it was indeed possible to epigenetically upregulate BCECs expression of receptors and transporters, however the increased expression did not increase the transport across the barrier of targeted drug-carriers.

Neuroinflammation is a common feature in neurodegenerative diseases. However, little research has been carried out investigating the transcriptional alterations of these targets during neuroinflammation. The expression of CD98hc, Glut1, and TfR1 by the BCECs during neuroinflammation was further investigated in experimental lipopolysaccharide (LPS) models both *in vivo* and *in vitro*. The latter was employed in order to optimize the identification of early molecular alterations during neuroinflammation by constructing a new *in vitro* BBB model using BCECs and mixed glial cells that displayed prominent secretion of proinflammatory cytokines without completely demolishing the integrity of the barrier. Collectively, the data from acute and persistent inflammation induced by LPS did not influence the expression of CD98hc, Glut1 and TfR1 in BCECs, which was contrasted by marked increases in expression of ICAM-1 and VCAM-1.

In conclusion, the results presented in this dissertation provide new insight into the prospective of using epigenetic agents for enhancement of the expression of drug delivery targets on the BCECs. The dissertation also highlights the importance of considering transcriptional alterations in expression of surface receptors and nutrient transporters on BCECs in response to neuroinflammation, a common feature of neurodegenerative diseases, which could influence the transport of targeted therapeutics.

DANSK RESUME

Da aldring er den største risikofaktor for udvikling af neurodegenerative sygdomme, bliver disse sygdomme mere udbredte hos hele befolkningen i takt med at den gennemsnitlige levealder stiger. Symptomerne varierer afhængigt af, hvilken neurodegenerativ sygdom patienten rammes af, men inkluderer ofte kognitive vanskeligheder, indlæringsbesvær, hukommelsestab, hallucinationer og motoriske defekter. Der findes desværre ingen helbredende behandling, og de fleste behandlingsmuligheder er symptomatiske og afhjælper derfor ikke den underliggende patologiske årsag. Selvom der udvikles mange lægemiddelkandidater, har det vist sig at være mere kompliceret end forventet at levere behandling effektivt til hjernen. Årsagen er blod-hjernebarrieren, som består af højt specialiserede endotelceller, som karakteriseres ved meget tætte intercellulære forankringer og lav passiv permeabilitet. Blod-hjernebarrieren adskiller hjernen fra blodet for at sikre, at skadelige molekyler som cirkulerer i blodbanen ikke når frem til hjernevævet, men samtidigt holder den også de fleste makromolekyler og lægemidler ude.

Grundet den begrænsede passive permeabilitet igennem blod-hjernebarrieren skal de fleste næringsstoffer aktivt transporteres ved hjælp af specifikke transportmolekyler. Transportmolekylerne eller targets, som inkluderer receptorer og transporters, kan også anvendes til at levere lægemidler over barrieren ved at målrette lægemidlet mod disse targets. Ved at konjugere lægemidler eller indkapslede nanopartikler til molekyler, som specifikt interagerer med en receptor, der selektivt udtrykkes på den lumenale side af endotelcellen, kan optagelse og transport over BBB forøges.

Ekspressionen af disse targets kan ændres ved inflammation, eller andre patologiske faktorer forbundet med neurodegenerative forandringer, som kunne ændre ekspressionen i blod-hjernebarrieren, men det fænomen er endnu ikke fuldt klarlagt. Transkriptionelle ændringer kan bl.a. skyldes epigenetiske modifikationer.

I denne afhandling er ekspressionen af tre targets, CD98hc, Glut1 og TfR1, ved blod-hjernebarrieren blevet undersøgt i patologiske modeller. Det er ligeledes undersøgt om det er muligt at regulere ekspressionen af CD98hc, Glut1 og TfR1 på baggrund af epigenetiske mekanismer. Ved hjælp af histon-deacetylase hæmmeren valporate var det muligt at øge blod-hjernebarrierens ekspression af targets, men denne opregulering i endotelcellerne var ikke ensbetydende med højere transport over barrieren.

Neurodegenerative sygdomme ledsages ofte af en tilstand med neuroinflammation, men der er udført meget lidt forskning, der undersøger de transkriptionelle ændringer af de førnævnte targets ved neuroinflammation. Neuroinflammation blev derfor

induceret ved hjælp af lipopolysaccharid både *in vivo* og *in vitro* for at undersøge om ekspressionen af CD98c, Glut1 eller TfR ændres under neuroinflammatoriske forhold. For bedre at kunne identificere tidlige molekulære ændringer blev der etableret en alternativ inflammatorisk *in vitro* blod-hjernebarriere model, som viste høj sekretion af inflammatoriske cytokiner uden ledsagende ødelæggelse af barriereintegriteten. Data fra akut og vedvarende inflammation induceret af LPS, viser stabil ekspression af CD98hc, Glut1 og TfR1 i endotelcellerne, men markante stigninger i ekspressionen af ICAM-1 og VCAM.

Resultaterne præsenteret i denne afhandling giver ny indsigt i potentialet af epigenetiske modulatorer til at øge ekspressionen af targets på blod-hjernebarrierens endotelceller. Afhandlingen fremhæver ligeledes vigtigheden af de transkriptionelle ændringer på blod-hjernebarrierens endotelceller som konsekvens af neurodegenerative sygdomme ledsaget af en neuroinflammatorisk tilstand, hvilket ultimativt vil kunne påvirke transporten af målrettede lægemidler til hjernen.

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LIST OF MANUSCRIPTS

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Epigenetic regulation of ferroportin in primary cultures of the rat blood-brain barrier

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Study II

In preparation

Epigenetic induction of transferrin receptor expression on brain capillary endothelial cells

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Study III

In preparation

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Study IV

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Neuroinflammation-induced expressional alterations of targets for drug delivery

Helgudóttir. S.S., Routhe. L, Rasmussen. C, Thomsen. M. S, Moos. T.

Other activities

Hepcidin Mediates Transcriptional Changes in Ferroportin mRNA in Differentiated Neuronal-Like PC12 Cells Subjected to Iron Challenge

Steinunn S. Helgudóttir, Jacek Lichota, Annette Burkhart, Torben Moos

Molecular Neurobiology (2019) 56, pages 2362–2374

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
A β	Amyloid beta
AJs	Adherence junctions
APP	Amyloid precursor protein
α SMA	Alpha-smooth muscle actin
BBB	Blood-brain barrier
BCECs	Brain capillary endothelial cells
BDNF	Brain-derived neurotrophic factor
BM	Basement membrane
CD11b	Macrophage antigen complex 1
CD98hc	Cluster of Differentiation 98 Heavy Chain
CMT	Carrier mediated transport
CNS	Central nervous system
CSF	Cerebrospinal fluid barrier
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DMT1	Divalent metal transporter 1
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-Linked Immunosorbent Assay
GFAP	Glial fibrillary acidic protein
GLUT1	Glucose transporter 1
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
ICAM-1	Intercellular Adhesion Molecule 1
ID	Injected dose
IgG	Immunoglobulin G
IL	Interleukin
i.p	Intra peritoneal
i.v	Intravenous
LAT1	L-type amino acid transporter 1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
mRNA	Messenger RNA
MS	Multiple Sclerosis

NFκB	Nuclear factor kappa-light-chain-enhancer of activated Bcells
NVU	Neurovascular unit
PD	Parkinson's disease
PDGF-β	Platelet derived growth factor beta
PDGFRβ	Platelet derived growth factor receptor beta
PEG	Polyethylene glycol
RMT	Receptor mediated transport
SB	Sodium butyrate
TEER	Transendothelial electrical resistance
TfR1	Transferrin receptor 1
TGFβ	Transforming growth factor
TJs	Tight junctions
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion molecules
VPA	Valproic acid
ZO	Zonula occludens

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CHAPTER 1. INTRODUCTION

Neurodegenerative diseases of the central nervous system (CNS) are becoming increasingly common due to the prolonged lifespan of the general population (1). Even though many different neurodegenerative diseases exist, some common characteristics are progressive cellular dysfunction of neurons in the brain or spinal cord, involving cell death in specific areas of the CNS (2). Neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD) and Multiple Sclerosis (MS). Currently, there is no cure for any of the aforementioned diseases, and the diagnosis is devastating to patients and their families. With the increasing prevalence of neurodegenerative diseases, it is vital to develop better treatment options. Many novel drug candidates have been created in hopes of treating neurodegenerative diseases, but none have evolved to be among the current treatment options. Unfortunately, it is much more complicated to treat brain diseases than disorders elsewhere in the body due to the blood-brain barrier (BBB). As a result, delivering therapeutics to the bloodstream and hoping that the drug will reach the brain is extremely inefficient (3). The BBB keeps harmful agents from entering the brain parenchyma (4), but at the same time it hinders the transport of therapeutics through the BBB in sufficient amounts to elicit a pharmacological response (5). Several strategies have been explored in order to deliver macromolecules across the BBB. Despite the effort, a highly specific and efficient drug delivery strategy has not been developed. One of the biggest hurdles is unspecific targeting, which results in poor accumulation of a drug within the CNS, which is then not efficacious in slowing cognitive decline (6).

Targeting of the BBB can be obtained by adding specific ligands on the surface of a drug that will then bind to a receptor or nutrient transporter that is highly or selectively expressed on the brain endothelium. However, limited information and understanding of how the expression of BBB specific transporters alters during neurodegeneration remains a challenge (7). Furthermore, most studies that investigate drug delivery through the BBB are conducted on a healthy and intact BBB, neglecting the matter of aging and inflammation associated with neurodegenerative diseases (8). Inflammation, whether it originates from the periphery or from the CNS itself, can affect the expression of transporters located on the brain capillary endothelial cells (BCECs) (9) and the same can be said about aging (10). These transcriptional alterations of receptors and nutrient transporters due to various environmental influences are not well understood, but there are some indications that they might be due to epigenetic modifications. However, possible epigenetic regulation of transporters on the BCECs surface in response to neurodegenerative pathology has not been comprehensively investigated.

The expression and availability of surface targets on the BCECs in a healthy and pathological BBB will therefore be discussed in the current thesis where the aim of the PhD project are to:

- Investigate specific targets of the BBB with focus on transcriptional and translational expressional changes *in vitro* and *in vivo* following experimental inflammation.
- Investigate whether it is possible to upregulate expression of drug delivery targets at the BBB by inducing epigenetic modifications.

1.1. THE BLOOD-BRAIN BARRIER

The brain requires a highly balanced and stable microenvironment to ensure normal function of neurons and their finely tuned electrical and chemical signaling (11). The main protective strategy against influx of toxins, pathogens, and other harmful molecules to the brain is denoted by the vascular barrier system, consisting of the BBB and the blood-cerebrospinal fluid barrier (4,12,13). The BBB will be the main focus of the thesis and the blood-cerebrospinal fluid barrier will therefore not be discussed further. The BBB itself consists of BCECs that form tight junctions between each other (14). Pericytes surround the BCECs, and are embedded in the basement membrane (BM)(14). The last cellular layer that helps regulate the function of the BBB is astrocytes, which cover the BM almost completely by their endfeet (14,15). In order to develop new ways of delivering therapeutics to the brain, the structure and function of the BBB must be understood in detail. The following sections will cover the structure and function of the BBB focusing on the cells associated with BBB integrity.

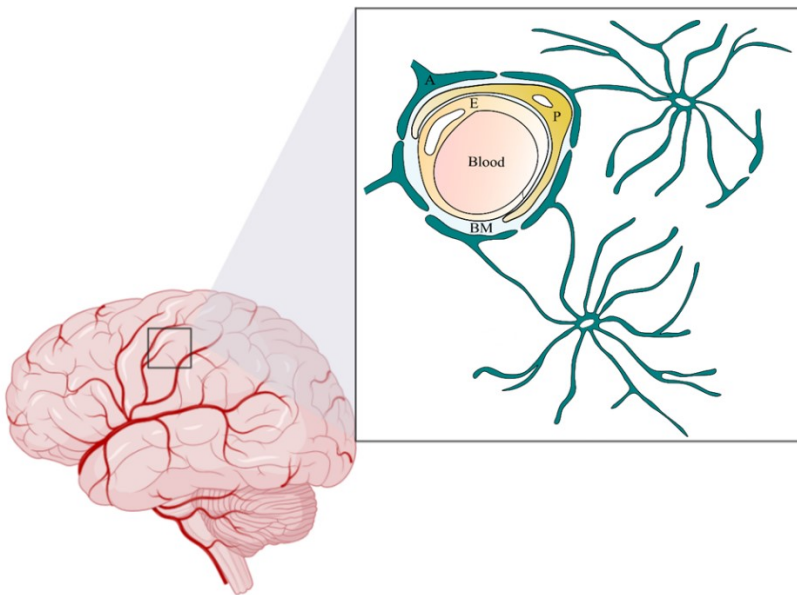


Figure 1: Schematic illustration of the cellular content that supports the blood-brain barrier (BBB). The endothelial cells (E) make up the BBB that is supported by pericytes (P) and astrocytes (A). The basement membrane (BM) surrounds the pericytes. Illustration is a modified version from Maj Schneider Thomsen (16).

BRAIN CAPILLARY ENDOTHELIAL CELLS

The brain's microvasculature is comprised of a monolayer of non-fenestrated and highly specialized BCECs, which are closely associated with pericytes and astrocytes that support and maintain the barrier integrity (13,14). The luminal surface of BCECs is covered by glycocalyx, which are glycoproteins that can prevent some interactions between BCECs and larger molecules (17). The abluminal surface of the BCECs is covered by the BM, which is only 20 nm thick and made up of structural proteins such as laminins, collagens and proteoglycans (15,17).

A key feature of the BCECs is the combination of physical- and molecular barrier properties (18). The physical barrier is due to tight junction (TJ) protein complexes, which are the basis for low paracellular transport and transcytosis (18–20). These protein complexes are occludin and claudin-1, -3, -5, -12, where claudin-5 is the most abundant (19). They are then connected to cytoplasmic scaffold proteins zonula occludens 1-3 (ZO1-3) and cingulins, which bind to the actin cytoskeleton. Junctional adhesion molecules are transmembrane proteins that interact with TJ and participate in restricting paracellular transport (21). Various adherent junctional proteins, such as vascular endothelial-cadherin, vascellin-1 and platelet endothelial cell adhesion molecule-1, also help regulate the BBB integrity (3,22).

Due to the low permeability of the barrier as well as low levels of transcytosis, most nutrients need to be actively transported. Various transporters are therefore expressed on the BCECs to ensure sufficient supply of hydrophilic nutrients and metabolites (23). This is a very energy demanding task and the BCECs do therefore contain more mitochondria than any other endothelial cells (18). The high amount of mitochondria is crucial for generating sufficient adenosine triphosphate to drive the ion gradient that is critical for transport of molecules from blood to brain, or waste products from brain to blood (18). BCECs express two distinct types of transporters: efflux transporters, and nutrient transporters (18). Some substances enter the BBB by passive diffusion such as lipophilic molecules, oxygen (O_2), carbon dioxide (CO_2), as well as some hydrophilic molecules smaller than 400 Da that contain fewer than nine hydrogen bonds. Even so, not all small lipophilic molecules diffuse through the BCECs (3,24). This is due to the expression of efflux transporters, such as multidrug resistance protein and P-glycoprotein that can transport the molecules up their concentration gradients back to the bloodstream (14,25). These efflux transporters are usually located at the luminal surface of the BCECs and make up the molecular barrier section of the BBB (18).

PERICYTES

The cells in closest proximity to BCECs are pericytes, which are multi-functional, polymorphic mural cells that are present along the walls of capillaries, along arterioles and post-capillary venules (26). Pericytes are crucial for development of the BBB (27,28) and continue to be of importance throughout the lifespan (27,29,30). They support BBB function, stabilize newly formed capillaries, regulate immune cell entry to the brain, aid in scar formation, and may even regulate cerebral blood flow (26,31,32). Pericytes are also able to determine the number of BCECs tight junctions and control the polarization of astrocytic end-feet (29,33). Pericytes are separated from the BCECs by the BM (18,30,34–36) but extend long cellular processes to the BCECs and they are therefore directly linked through so-called peg-and-socket interactions, which is crucial for BBB integrity (37,38). Pericytes and endothelial cell cross talk is not well understood. However, it is known that platelet-derived growth factor beta (PDGF- β) is secreted by BCECs, which binds to platelet-derived growth factor receptor beta (PDGFR β) on pericytes and initiates various signal transduction pathways that regulate pericytes recruitment and proliferation (30,39). PDGFR β has recently been suggested to protect the BBB in mice following a stroke (40). Additionally, pericytes seem to control the cell cycle of BCECs and contribute to the formation of the BM (41).

Pericyte coverage seems to be greatly associated with the permeability of the BBB as well as neurodegeneration, where the permeability becomes compromised in different brain regions (42,43). Even though the BBB gets referred to as a whole, various studies have shown that permeability for certain molecules varies between brain regions that cannot be explained by increased expression of receptors (42,44,45). Due to aging, the initial decrease in BBB integrity occurs in the hippocampus, which has been correlated with pericyte injury in the area (42,46). How reduced coverage or injury to pericytes affects the permeability of the BCECs seems to be related to increased transcytosis as pericyte deficient mice displayed increased water content, albumin, immunoglobulin G (IgG), 70 kDa dextran and uptake of macrovesicles in the brain, without degradation of tight junction complexes (29). Interestingly, the BCECs were, in some areas of the brain such as the midbrain, able to maintain low transcytosis despite decreased pericyte coverage (42). One possible explanation is the heterogeneity of pericytes and their localization in the brain (42). Pericytes in the forebrain originate from the neuroectoderm whereas pericyte located in the midbrain originate from the mesoderm (42), so their function may differ. Another possibility is that pericyte coverage is not the sole regulator of BBB permeability (42).

Due to the aforementioned heterogeneity in terms of origin and function of pericytes, they express different cellular markers (47). Some pericytes share expression of markers with smooth muscle cells, which are present in the wall of arteries and

arterioles (33). These entail contractile proteins such as alpha-smooth muscle actin (α -SMA), tropomyosin and myosin (31,48). In order to identify pericytes, commonly used markers include α -SMA, PDGFR β and CD13 (33,47). These are expressed at various differential state of the pericytes, as well as in response to stimulation (49) and can therefore provide information on the location and function of the pericyte.

ASTROCYTES

The next cellular layer that supports the BCECs is the astrocytes. Astrocytes are heterogenic, star-shaped and multifunctional cells that are considered to be the most abundant glia cell type (18). Astrocytic endfeet cover the BM almost completely (14,15) and can control CNS blood flow by releasing molecular mediators such as prostaglandins, nitric oxide, and arachidonic acid (50,51). Besides covering the BM, astrocytes are also functionally connected to each other and oligodendrocytes through gap junctions (52,53). In addition, they extend their processes to neurons and can thereby regulate the neuronal activity and participate in synaptic cleft clearing of neurotransmitters (50,54). This close connection is essential for astrocytic supply of antioxidants, growth factors and other essential nutrients to the neurons. (55,56). Another important function of astrocytes, that protects the metabolically sensitive neurons, is supplying energy. Astrocytes take up glucose, transform it to lactate or store it as glycogen (57). Astrocytes are the main storage site of glycogen granules in the brain, which they can transform to lactate and secrete to neurons during hypoglycemia or high neuronal activity (58). When glucose storage is low, astrocytes are also able to induce expression of glucose transporter 1 (GLUT1) on BCECs (59), which increases transport of glucose through the BBB.

Astrocytes are important immune regulators within the brain as they are able to secrete various inflammatory mediators when activated. They can become activated by oxidative stress, pro-inflammatory cytokine release from adjacent cells, damage-associated molecular patterns, pathogen associated patterns, or endotoxins (50,60,61). Reactive astrogliosis is the response of astrocytes that is seen in many neurodegenerative diseases (62). It entails changes in transcriptional regulation, morphological and physiological alteration resulting in gain of new functions or loss of homeostatic ones (63). It is a defense mechanism aiming to limit tissue damage and restoring CNS homeostasis mainly in response to BBB disruption (63). However, chronic astrogliosis leads to inhibition of neural plasticity (62). Astrocytes can exert either pro- or anti-inflammatory functions, dependent on stimuli (64). Pro-inflammatory astrocytes will release pro-inflammatory interleukins (IL) e.g. IL-1 β , IL-6 as well as tumor necrosis factor alpha (TNF- α), whereas an anti-inflammatory astrocyte will release e.g. IL-10 and transforming growth factor beta (TGF- β) (65,66). Nonetheless, the phenotype of reactive astrocyte and thereby the secreted cytokines

can vary depending on the type of insult or inflammatory stimuli (67).

Astrocytes can be identified by their structure and expression of certain markers (68). Expression of glia fibrillary acid protein (GFAP) has been a popular astrocyte marker for many years. Astrocytes increase their expression of GFAP when reactive, but GFAP is also expressed in non-reactive astrocytes *in vivo* (56). GFAP content of astrocytes varies between brain regions, where cortical, thalamic or striatal astrocytes express more GFAP without being more reactive than astrocytes elsewhere in the brain (63). GFAP has been extensively investigated, and expression of GFAP is not essential for appearance and function of astrocytes in an healthy brain (69), but it is crucial for reactive astrogliosis and scar formation (70). Other popular molecular markers used to identify astrocytes are glutamine synthetase and S100 β (56,71).

The complicated nature of the ever-changing BBB is reflected in the complex interaction between its cellular components. Even though the BBB refers to the tightly connected BCECs, the regulation of the BBB is highly dependent on pericytes and astrocytes as the integrity of the barrier is regulated by coverage and secretion of various molecular mediators.

1.2. TRANSPORT AT THE BLOOD-BRAIN BARRIER

Due to the BBB, numerous molecules must be actively transported to the brain in order to deliver the crucial nutrition and ions. Transport through BBB can be classified into five main categories: passive diffusion, paracellular diffusion, carrier mediated transport, receptor mediated transcytosis, and adsorptive mediated transport (see Figure 2) (14,22). Each of the aforementioned will be briefly mentioned in the following section, but the focus will be on carrier mediated transport and receptor mediated transport as these are of great interest for drug delivery. Furthermore, a few receptors that are of importance for the current thesis will be discussed in details.

Transcellular passage of non-polar, small (<400 Da) lipophilic molecules or gasses such as O₂ or CO₂ can be achieved by passive diffusion across the BCECs following their concentration gradient (see Figure 2). (3,24). Furthermore, a few small polar, hydrophilic molecules such as water, alcohol, nicotine, and morphine can pass the BCECs by paracellular diffusion through the TJs (13,14,72) (see Figure 2).

Adsorptive mediated transcytosis is utilized by molecules that are positively charged and can interact with binding sites on the negatively charged cell surface of the BCECs (73,74). That binding will induce endocytosis and subsequent transcytosis (72,75). This route does not depend on specific matching receptors and ligands like receptor-mediated transport. Molecules transported via this route are cationised albumin, avidin, and various cell-penetrating peptides (14).

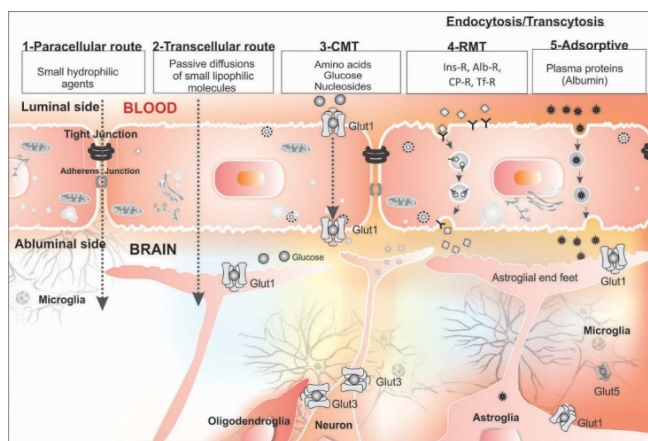


Figure 2: Transport mechanisms at the blood-brain barrier. The paracellular route can be taken by small hydrophilic molecules, whereas small lipophilic molecules utilize the transcellular route. Carrier mediated transport (CMT) is dependent on solute carrier proteins and is the route glucose and amino acids take. Receptor mediated transport (RMT) requires a specific receptor on the luminal side of the brain capillary endothelial cell to be present. Adsorptive mediated transport is obtainable for positively charged molecules. Figure is from Barar et al. (76)

CARRIER MEDIATED TRANSPORT

Specific transport systems supply the brain with adequate nutrition which is regulated by the metabolic needs of the brain (22). Small molecules, such as glucose (77), amino acids (78), vitamins (79), and hormones (80) utilize carrier-mediated transport (CMT), that requires a solute carrier protein to be present (see Figure 2). Essential amino acids are transported through the L-type amino acid transporter 1 (LAT1) or LAT2, and cationic amino acids are transported through the cationic amino acid transporter 1 and 3 (81). Another example of an important transporter is the Major facilitator superfamily domain-containing protein 2a that is a lysophosphatidylcholine bound fatty acids transporter for fatty acids such as omega-3 fatty acid docosahexaenoic acid (82,83) which is expressed selectively and exclusively by the BCECs. Even nucleotides and bases necessary for DNA and RNA synthesis are transported through the BCECs by this pathway via Concentrative nucleoside transporter 2 and Equilibrative nucleoside transporter 1 and 2 (81,84).

Many transporters can be found on the BCECs that are essential for nutritional supply to the brain. However, in the following sections two nutrient transporters, GLUT1 and Cluster of Differentiation 98 Heavy Chain (CD98hc), will be highlighted, due to their importance in the thesis' studies.

GLUT1

Glucose is the main energy source of the brain and is primarily transported across the BBB by GLUT1 (*Slc2a1*) (77,85). GLUT1 is a 12 transmembrane-spanning α -helices (86), that functions as a uniporter transporting glucose down the concentration gradient. As glucose concentration is lower in the brain compared to blood plasma, glucose will be transporter from blood to brain (81). Expression of this transporter is particularly high in the BCECs compared to endothelial cells elsewhere in the body (87) and it is notably one of the most abundantly expressed proteins in BCECs (88). GLUT1 is expressed on both luminal and abluminal membranes (89) of the BCECs and the level of expression is regulated in accordance to the brains metabolic needs (90) (see Figure 3). However, there is generally a higher expression on the luminal side (91). GLUT1 displays the highest expressional level of solute carriers in human BCECs (92) and its expression seems to be both transcriptional and post-transcriptionally regulated (86,93,94). Due to its high expression in the BCECs, GLUT1 has been seen as a potentially suitable target for drug delivery.

CD98hc

CD98hc (*SLC3A2*) is an intracellular amino acid transporter and integrin signaling enhancer that belongs to the solute carrier-mediated transporter family (95). It forms a heteromeric amino acid transporter with e.g. LAT1 (96,97) that transports large

neutral amino acids and various drugs such as L-dopa and valproic acid (VPA) across the BBB (97,98). CD98hc knockout fibroblasts were unable to survive in standardized cell culture due to ferroptosis and it was established that CD98hc was crucial to control oxidative stress and reactive oxygen species (99). Whether CD98hc contributes to similar mechanisms in the BCECs is unknown. CD98hc may also help regulate glucose metabolism through interaction with GLUT1 (100). A great deal of the research on CD98hc has been conducted in connection with cancer. It has been suggested as a therapeutic target as cancer cells upregulated the expression of CD98hc (101) and the level of CD98hc expression correlates with progressive or metastatic tumors (102,103). Exactly why cancer cells upregulated their expression of CD98hc is controversial but it could be due to metabolic demands of the tumor (101). CD98hc has furthermore been suggested as a target to inhibit T-cells in autoimmune diseases as loss of CD98hc prevents disease development in MS (104).

RECEPTOR-MEDIATED TRANSCYTOSIS

Receptor-mediated transcytosis (RMT) is needed when transporting macromolecules across the BBB (105). This type of transport requires a specific receptor to be present at the luminal side of the BCECs (see Figure 2). In theory, binding of a ligand to its cognate receptor will trigger an invagination of the plasma membrane, forming a vesicle containing the ligand and receptor (106). The vesicle is then transported across the cytoplasm to be exocytosed on the abluminal side where the receptor and macromolecule will separate (14). However, there is some disagreements on exactly how RMT occurs. Currently, receptor-mediated transport is the most promising approach for drug delivery to the brain. Among the macromolecules that are possibly transported by RMT are: transferrin that is transported with transferrin receptor 1 (TfR1), melanotransferrin transported with melanotransferrin receptor, amyloid- β transported with receptor for advanced glycation end products (107), and insulin transported with insulin receptor (14). The following section will focus on transferrin receptor 1 (TfR1) due to its application in the thesis' studies.

TfR1

TfR1 (*TFRC*) is a transmembrane glycoprotein that consists of two identical subunits of 90 kDa in humans (108). It promotes uptake of transferrin bound iron and is expressed on the surface of most cells (108). TfR1 binds to transferrin, which is one of the most abundant plasma proteins found in humans. Only a third of the transferrin molecules found in plasma are saturated with iron (called holo-transferrin), where the remainder functions as a buffer should the iron concentration increase, thereby preventing the toxic accumulation of non-transferrin bound iron (108). Iron has an essential part in vital physiological functions such as DNA synthesis and repair,

oxygen transport and cell division (109–114). Iron can exist in two forms in the blood, as ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), and its activity in biochemical reaction is dependent on the electron transfer (109,114). Iron can participate in redox reactions, also known as Fenton reaction, when it reacts with hydrogen peroxide generating toxic free radicals that can cause damage to lipids, proteins and DNA (108,114). Therefore, to limit free extracellular iron it is bound to transferrin that carries iron in its oxidized and non-toxic form (108,115). Excess intracellular iron that is not essential for metabolic purposes is oxidized and stored in the iron storage protein, ferritin (108,116).

The exact mechanism of how transferrin-bound iron is transported across the BBB remains a source of uncertainty with various hypotheses prevailing, including transport via endocytotic or transcytotic pathways (117). The two transport pathway hypotheses are based on the presence or absence of divalent metal transporter 1 (DMT1) in BCECs, but conflicting results have been reported on this matter (117). In both pathways, iron import is initiated by binding of holo-transferrin to TfR1, which is located on the luminal membrane of the BCECs. In the transcytosis pathway, the holo-transferrin-TfR1 complex is transported via endosomes through the BCECs and released directly into the brain. In the endocytosis pathway, the holo-transferrin-TfR1 complex is internalized within the endosome and ferrous iron is then released through DMT1 (116). Iron is subsequently exported from the BCECs into the brain parenchyma through the only known iron exporter ferroportin (12,110,117,118). Accumulating evidence suggests that the transferrin molecule and the TfR1 do not transcytose into the brain, and that iron is released from its binding to transferrin inside the endosome (108,116).

1.3. STRATEGIES TO TARGET THE BLOOD-BRAIN BARRIER

It is important to develop new and more efficient strategies to deliver therapeutics to the brain as the BBB hinders transport of most macromolecules. At this given moment, not a single recombinant protein that crosses the intact BBB is approved for treatment of neurodegenerative diseases (5). Pharmaceutical companies have been developing therapeutics for treating brain disorders for decades, and many of these have been promising in preclinical models but failed in clinical trials (108). A potential explanation is that drug development within pharmaceutical companies has failed to ensure adequate drug delivery of potentially novel drugs (5). This is a concerning fact given that the prevalence of neurodegenerative diseases increase every year.

The many challenges of delivering drugs to the brain include: modification of the drug in the bloodstream by enzymes and other proteins, instability of the drug, low transport to the brain as it often accumulates in lungs or liver, highly selective BBB and limited specific transporters unique to the BCECs that can be used as targets (7). Almost all macromolecular drugs and 98% of small molecular drugs (<400 Da) are unable to cross the BBB (7). There are some physical and chemical properties that favor transport of small molecules across the BBB. Lipinski originally described these for transport across the gastrointestinal barrier, known as Lipinski's "rule of five" (119). However, Goldberg has modified the original "rule of five" to better fit the BBB (120). There he states that molecules should: have a weight under 400 Da, have a calculated logP less than five, a fewer than three hydrogen bonds and less than seven hydrogen bond donors (92,120). Around 70% of all FDA approved CNS drugs fit the model described above (92,121) but even though a drug fulfills all the aforementioned requirements does not guarantee BBB passage (92). Even though the BBB does not deny all larger molecules entry to the brain, only around 0.1-0.2% of the drug serum concentration is likely to be transported via nonspecific transcytosis (122). Multiple strategies have therefore been generated in order to deliver therapeutics to the brain. Targeted drug delivery systems usually consists of three entities. The first one is the cargo, which is the therapeutic part and can consist of small molecules, nucleic acids, peptides, antibodies and more. The second is the targeting ligand, which can be any molecule that will bind the target receptor or transporter on the target organ. The third is a linker between the ligand and cargo (123).

The broad field of targeted delivery will not be discussed in detail, as it is beyond the scope and aims of the thesis. The following section will focus on strategies that have utilized ligands that bind to TfR1, GLUT1 and CD98hc due to their application in the thesis' studies.

TARGETING THE TRANSFERRIN RECEPTOR, GLUT1 AND CD98HC

Three molecular targets that are enriched on the BCECs of the BBB were chosen for further investigation in this dissertation (95). The validity of the aforementioned targets in regards to drug delivery will be discussed in the following section.

TfR1 is among the most studied receptors for BBB targeting with publications dating back to 1984 (124), but results have been conflicting (3). The reason for TfR1 popularity as a drug target on BCECs is because TfR1 is highly specific for BCECs as it is not found on other endothelial cells in the body (124). This could lead to preferential accumulation of TfR1-targeted molecules in the brain (108). Multiple drug conjugates targeting the TfR1 have been created as well as different nanocarriers e.g. liposomes, polymers, and gold nanoparticles (108). Furthermore, various radiolabeled antibodies targeting TfR1 have been shown to accumulate in the brain parenchyma (108). A popular approach for drug delivery is the 'Trojan horse' method where therapeutics are fused to a transport vector that consist of either peptide or monoclonal antibody (mAb) that binds to a specific receptor on the BCECs. The Trojan horse will upon binding, theoretically, undergo receptor mediated transcytosis through the BBB (15,125). The Trojan horse method targeting the TfR1 was first achieved in the 1990s, where methotrexate was linked to the rat specific OX26. Both the antibody and methotrexate bound to the BCECs, and the data even suggested that the antibody-drug conjugate crossed the BBB (126). This study proved that small molecular drugs could be transported through the BBB using TfR1 targeting. The downside was that only 0.3% fraction of injected dose (ID) of methotrexate was transported to the brain parenchyma. In regards to transporting larger molecular drugs, nerve growth factor linked to OX26 was able to penetrate the BBB (127) and even prevent degeneration in a rat Huntington disease model (128) Later, OX26 mAb linked to brain derived neurotrophic factor (BDNF) provided neuroprotective effect in the hippocampus after ischemia in rats (129). Similarly, OX26 linked to BDNF was able to reduce stroke volume due to the targeting system being able to deliver BDNF through the BBB (130).

After discovering that the OX26 antibody was inefficient in mice, the 8D3 and RI7-217 mAb targeted to the mouse TfR1 were studied. The 8D3 displayed higher uptake in the mouse brain with around 3.1% ID compared to 1.6 % ID for Ri7-217 (131). The Ri7 mAb, was, however, more selective for the brain as it was not taken up by the liver (131).

Multiple groups have questioned the efficiency of this transport system as the antibodies often remain in the BCECs and were therefore not transported further into the brain parenchyma (108,132). Fortunately, recent studies show that therapeutic antibodies reach greater brain exposure when the affinity to TfR1 is lowered, as it

enables dissociation of the antibody from the TfR1 within the endosome (133,134). How the Ri7 mAb performs in a dysfunctional BBB, which is a common feature of neurodegenerative diseases, has been investigated recently. There was no difference in the expression of TfR1 in isolated microvessels from postmortem parietal cortex samples of individuals with or without AD. The same was found in isolated murine brain microvessels from 12- and 18-month old NonTg and 3xTg AD mice, and the uptake of the Ri7 mAb was also similar (135). This emphasizes the validity of Ri7 as a transport system not only in healthy subjects, but also neurodegeneration.

Another transporter that can be used as a target is GLUT1, which is thought to mediate transport through carrier-mediated pathway that does not rely on a specific receptor but a solute carrier protein. A great example of this type of transport is the transport of L-dopa, the precursor of dopamine. It was discovered that L-dopa was able to cross the BBB after systemic administration (136) and later revealed that it was transported through carrier mediated transport via LAT1 (5,137,138). Unfortunately, as the conversion of L-dopa to dopamine does not only occur in the brain, the chronic treatment can result in excessive dopamine in the periphery (139). Therefore, derivatives of dopamine and L-Dopa have been linked to glucose at the C-3 position. As the dopamine derivatives were more active in suppressing symptoms in rats, it was suggested that the glycosyl conjugation allowed the drug to cross the BBB, possibly through GLUT1 (139), which was confirmed a few years later (140). Multiple drugs have been conjugated with glucose in hopes of targeting GLUT1 for BBB penetration. An example is D-glucose conjugates of 7-chlorokynurenic acid, which is used to produce antidepressant effects in animal models of depression but is unable to cross the BBB. The drug conjugate was able to cross the BBB and GLUT1 was suggested to be the route of transport (141). GLUT1 does not only affect transport of conjugates using glucose, as mannose-conjugated liposomes displayed significantly higher brain fluorescence in mice (142). Similarly, BDNF containing liposomes with mannose and cell penetrating peptides as ligands displayed a significantly higher astrocyte and neuron penetration than unmodified liposomes reaching 7% transport of ID (143). Exactly how these liposomes are transported is unknown, but the addition of a ligand that targets to GLUT1 has proven quite efficient.

It was recently discovered by transcriptomic and proteomic profiling, that CD98hc is enriched in the BBB (95,144). As it is newly identified as a potential target for BBB, there is very little literature on the subject. However, antibodies against CD98hc did accumulate in the brain of mice reaching 80-90 times higher levels than the control IgG and even 4 to 5 times higher level than the TfR1 targeted antibodies (95,145). A bispecific antibody binding CD98hc and β -secretase 1, the enzyme that cleaves amyloid precursor protein (APP) and thus increases A β plaques formation in AD,

reduced the level of A β plaques by 30-45% when compared to IgG treated mice (95). These results make CD98hc an interesting target to investigate.

These three targets will be investigated further in the thesis' studies. TfR1 is the most studied target on the BBB where multiple different ligands have been utilized in various animal models. GLUT1 is an interesting target due to its abundance in the BBB, and has proven efficient in facilitating transport of therapeutics in relatively high concentrations. At last, CD98hc is a newly identified target for drug delivery that needs further investigation.

1.4. THE PATHOLOGICAL BLOOD-BRAIN BARRIER

Dysfunction of the BBB is associated with both onset and progression of various disorders e.g. AD (146), stroke (147), MS (148), traumatic brain injury (149), and amyotrophic lateral sclerosis (150). The pathology of these disorders vary, but there is a common detrimental effect, which is inflammation. Inflammation in the BBB can be caused by systemic inflammation that subsequently affects the BCECs, which can mediate changes in pericytes, astrocytes, and even neurons (151). Low grade systemic inflammation, defined as slightly increased level of C-Reactive Protein (152), affects around 40% of people residing in Western countries (153). This low grade inflammation causes damage to the BBB (154) and it is therefore extremely relevant to understand the effect of continuous systemic inflammation on the BBB. The second origin of inflammation in the BBB is neuroinflammation, which is the inflammatory response within the brain and spinal cord in response to injury, trauma, autoimmunity or infection (151). Neuroinflammation is primarily driven by microglia that reside within the brain, which is further amplified by astrocytes (17,155) and comprises response from both the innate and adaptive immune system (156).

Pathophysiological factors, such as inflammation, increase the permeability of the BBB which can cause barrier disruption (157). The term disruption of the BBB is a broad term, and the alterations of the BBB can therefore be categorized into disruptive and non-disruptive changes (158). In non-disruptive changes, the TJs are intact and the alterations of the BBB are functional and usually occur at a molecular level. This can include cytokine production by BCECs, up- or down regulation of various transporters, and modulation of astrocyte function (158). Disruptive alterations are visible at the histological level and include changes in tight junction arrangement, degradation of the glycocalyx, endothelial damage, and increased vesicular transport (158). The dynamic BBB is not likely to be permanently disrupted early in the course of neurodegenerative diseases (159). Hence, it is important to understand the subtle, early, non-disruptive changes as well as mild disruptive alterations that happen in the BBB. It is believed that inflammation typically precedes the degeneration in neurodegenerative diseases such as AD, PD, and MS, which can then progress independently of inflammation (155,160). The effect of inflammation on disease progression and the BBB in two very different neurodegenerative diseases, MS and AD, will be briefly discussed in the following sections.

MS is a neurodegenerative disease characterized by autoimmune attacks on the myelin sheath that result in multifocal lesions throughout the CNS (161,162). What sets MS apart from other neurodegenerative diseases is that it usually affects young adults with an onset between 20 years and 40 years of age (162) and is currently the second largest cause of disability in young adults (163,164). MS is more frequent in women than

men, and more common in regions of northern Europe than elsewhere (165,166). The breakdown of the BBB is a key event that is present early on in the pathogenesis of MS (167). However, barrier leakage is usually only present in new lesions, and not in older lesions (168), suggesting that the BBB restores its integrity. The pathophysiology of MS is initiated by increased migration of autoreactive lymphocytes across the altered BBB, which then activate local glia cells to release pro-inflammatory cytokines. The immune system fails to suppress inflammation, which enables various immune cells such as autoreactive lymphocytes, monocytes, and macrophages to migrate across the BBB and attack myelinated axons (161,169). The endothelial cells have an active role in leukocyte adherence and migration due to their expression of various adherence molecules, Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular cell adhesion molecules (VCAM), and are therefore of great importance in initiating the immune response in MS (170). The multistep cascade of leukocyte trafficking into CNS has been well studied and the so-called intraluminal crawling step is dependent on ICAM-1, and the transendothelial migration is dependent on ICAM-1 and VCAM (171). The resulting inflammation leads to additional migration into the brain of other cell types of the specific and innate immune system, including monocytes and macrophages (169). Healthy BCECs exhibit low leukocyte adhesion molecule expression compared to endothelial cells elsewhere in the body, but increase the expression during neuroinflammation (88).

AD is a progressive neurodegenerative disorder that is characterized by cognitive impairment, which can include memory loss, language deterioration, and emotional instability (146). AD is also the most common form of dementia (172) and epidemiologists fear that the number of new cases will double every 20 years (173). The etiology of Alzheimer's disease is complex and still not fully understood (174), but has been characterized by accumulation of A β plaques and neurofibrillary tangles (156). The majority of AD cases is sporadic where early onset usually occurs after the age of 65 (175). Autosomal dominant familial AD is thought to account for 1 in 200 cases and usually presents before the age of 65. The patients usually carry a mutation in prenilin 1, prenilin 2 or APP gene which all increase the production of A β (175). However, the accumulation of A β alone seems insufficient to generate symptoms (174,176). The pathology of AD is highly affected by chronic neuroinflammation that is driven by microglia and further enhanced by astrocytes (177). In AD, microglia accumulate around A β plaques, attain activated morphology and bind to A β plaques via CD36, toll-like receptor (TLR) 4 and TLR6. This will increase their secretion of proinflammatory cytokines and chemokines (178,179). Microglia activation can consequently promote astrogliosis (63), where astrocytes become activated in response to stimuli. The hypertrophic reactive astrocytes will then accumulate around A β plaques and escalate scar formation (179,180). The activation of microglia

and astrocytes is therefore the main driver of neuroinflammation in AD, which contributes to disease progression and severity (179). High cytokine levels have been associated with more severe disease progression. As an example have high levels of TNF- α in the cerebrospinal fluid (CSF) of patients with mild cognitive impairment shown to increase their risk for conversion to the dementia stage of the disease (181). Increased expression of adhesion molecules can, similarly to MS, affect the neuroinflammation in AD. Expression of both ICAM-1 and VCAM is increased in the brains of Arc/SweA β and 5xFAD mouse models of AD (177,182,183). Similarly, both ICAM-1 and VCAM are increased in the CSF of AD patients (184).

Even though MS and AD have different pathologies and etiologies, neuroinflammation has a severe effect on the disease progression. Both diseases present with increased microglial and astrocyte activation, increased cytokine secretion and higher adhesion molecule expression by the BCECs. The aforementioned factors are also present in other neuroinflammatory diseases such as PD and Huntington's disease (185). The broad detrimental effect of neuroinflammation should therefore not be overlooked.

1.5. EPIGENETIC REGULATION AT THE BLOOD-BRAIN BARRIER

Epigenetics is the study of the cells transcriptome rather than the DNA itself, and has been defined as heritable changes in gene expression that are stable between cell divisions and even between generations without involving changes in the DNA sequence (186,187). All cells in a given organism contain the same genetic material, but the biology and function of cells and tissues vary due to a variation in the transcriptome (186,188). In other words, epigenetic factors regulate how and when genes are expressed (186,189,190). These epigenetic changes are dynamic, which may make it possible to alter a disease state by manipulating the expression of receptors, molecules or enzymes that are involved in the pathogenesis or a response to a given stimuli (186,187).

Epigenetic regulation can occur on the DNA itself, or on histones as post-transcriptional regulation, which include acetylation, methylation, phosphorylation (191). DNA methylation is a rigid form for transcriptional silencing that results in a long term alteration of the gene expression, and is currently the best characterized covalent modification of DNA (192–194). Actively transcribed genes do therefore contain low DNA methylation percentage and vice versa (188,194). DNA methylation is the addition of a methyl group to the 5-carbon position of cytosine catalyzed by methyltransferases (188,194,195). A more flexible form of transcriptional regulation are post-translational histone modifications where histone H3 is modified by acetylation, methylation, or phosphorylation (196). Histone acetylation impacts the folding of histones, where hypoacetylation driven by histone deacetylase forms a highly compact chromatin and hyperacetylation disrupts nucleosome folding, thereby increasing the accessibility to binding of transcriptional factors resulting in enhanced transcription (197). There are multiple approved HDAC inhibitors that promote acetylation of histones and consequently transcription of multiple genes (198), one of which is VPA (199). VPA is a FDA approved anti-epileptic drug that can be used as a mood stabilizer, and to treat both migraine and psychiatric disorders (200). It is generally well-tolerated and has sparked interest as treatment in other areas. The effect of VPA on the brain is versatile as it can increase the activity of the inhibitory neurotransmitter Gamma-Aminobutyric acid (194,201). In addition, VPA prevents BBB disruption following a subarachnoid hemorrhage (202), lessens BBB disruption after transient focal cerebral ischemia (194,203), and reduces neuroinflammatory cytokines in late stages of AD Tg6779 murine model (204). VPA has especially gained popularity in traumatic brain injury research where its neuroprotective properties are credited upregulation of several genes within certain pathways (194,205–207).

Epigenetic alterations can occur throughout the entire lifespan, and there is even signs of some regional heterogeneity present in the BCECs of the CNS, where some regions such as choroid plexus, pineal gland, and median eminence of the hypothalamus have fenestrated capillaries that lack BBB properties (17,18).

The BBB has been shown to be affected by simple things such as exercise and diet (17). A high-fat diet has been shown to increase BBB permeability, neuro-inflammation, and production of reactive oxygen species in mice (208). Furthermore, a mouse model of diet-induced type II diabetes displayed an increase in BBB permeability (209) and so did a study carried out on insulin-resistant mice (210). The lifestyle of an animal can affect the function of the BBB, which has been shown to upregulate ketone transporters in hibernating animals in order to survive (211). All of these alterations of the BBB due to various stimuli may be epigenetically regulated. GLUT1 has been shown to be epigenetically upregulated in the BBB during fasting mice via histone modifications (212). This was the only published data, to the author's best knowledge, on epigenetic regulation of GLUT1 in the BBB to date, but there are some other implications for epigenetic regulation. As an example, GLUT1 expression is higher in adult mice brain compared with neonatal brain (213), which could indicate a layer of epigenetic regulation dependent on age. Similar results were found in isolated brain capillaries from newborn rats compared to 56 day postnatal rats (214). Conversely, GLUT1 is downregulated in patients with AD in multiple studies (215–217). Recent study on mice has shown that endothelial-specific loss of GLUT1 leads to progressive neuronal loss and CNS inflammation (213), which are both hallmarks of neurodegenerative diseases.

Alteration in the epigenetic landscape of adhesion molecules of BCECs have been found in response to inflammation in various neurodegenerative diseases. Hypermethylation of ICAM-1 was found in MS patients during the remission phase in cell free plasma DNA (218). That means, that ICAM-1 expression is inhibited through increased methylation following a relapse phase (196,218). Similarly, both ICAM-1 and VCAM expression in rodent model of stroke seems to be correlated with the activity of histone lysine methylases and demethylases (219).

Unfortunately, very few published studies have investigated the epigenetic regulation of various transporters in the BBB during neuroinflammation or other pathology related to neurodegenerative diseases. In order to achieve greater accumulation of therapeutics in the brain, it is crucial to understand the dynamic alteration in BCECs surface transporters in response to various stimuli. Downregulation of otherwise suitable targets, in response to e.g. inflammation, could affect the transport of an otherwise potent drug.

CHAPTER 2. MODELSYSTEMS AND METHODS

2.1. LIPOPOLYSACCHARIDE RODENT MODELS

Multiple rodent models have been generated to investigate the complex nature of systemic inflammation (220). These models can be divided to three main groups: exogenous administration of endotoxins such as lipopolysaccharide (LPS), exogenous administration of viable pathogens such as *Escherichia coli*, and disruption of endogenous protective barrier e.g. with cecal ligation. A clear advantage of using LPS is that it can in addition to causing systemic inflammation also induce neuroinflammation (221–225) and the focus will therefore be on LPS in the following sections.

INFLAMMATION AND NEUROINFLAMMATION USING LPS

The LPS models are an important and widely used animal model for neuroinflammation as this systemically induced inflammation is able to induce cytokine production in the brain (221). LPS is an endotoxin that is found in the outer membrane of gram-negative bacteria that binds to TLR4 with the help of LPS binding protein and the endotoxin receptor CD14 (226). TLR4 is expressed in immune cells, such as monocytes and macrophages, but also in endothelial cells and astrocytes (226–229). Binding will initiate the production of pro-inflammatory cytokines, including IL-1 β , IL-6 and TNF- α (230). As LPS does not pass the BBB, the inflammatory effect is thought to be mediated to the brain by one of these potential routes: through activation of the blood-cerebrospinal fluid barrier in choroid plexus, by active transport of pro-inflammatory cytokines through the BBB or direct activation of BCECs that express TLR4. Even though the exact mechanism of how systemic LPS creates neuroinflammation remains to be elucidated, the active transport of pro-inflammatory cytokines from the periphery has been shown to mediate neuroinflammation, and secretion of TNF- α seems to be crucial (231,232). Even just a single intra peritoneal (i.p) injection of LPS resulted in elevated TNF- α levels within the brain, and remained high for 10 months whereas the systemic inflammation was cleared within a week (231). Why pro-inflammatory cytokines are found in the brain so long after the systemic inflammation has been cleared is unknown.

PARAMETERS THAT INFLUENCE THE OUTCOME OF THE LPS MODEL

There are multiple discrepancies between results from various laboratories to which inflammatory cytokines are produced in the periphery upon LPS stimulation. This can be explained by the numerous experimental details that vary between laboratories e.g. the strain and age of mice, gender, administration route, concentration and type of LPS, as well as time of sacrifice after last LPS injection (221,225).

Multiple strains of mice have been used when inducing inflammation with LPS. Female BALB/c and C57BL/6 mice have been investigated in context of which inflammatory cytokines peritoneal macrophages produce upon intraperitoneal (i.p) injections of LPS (233). Cytokine measurements exhibited that C57BL/6 macrophages produced more IL-17, IL-10 and interferon-gamma whereas BALB/c macrophages produced more TGF- β and IL-4 (233). Another study found that the inflammatory response was higher in male BALB/c mice than in male C57BL/6 mice (234).

Gender and age of mice can also have an effect on how the animals respond to LPS injections (235,236). It has been demonstrated that male mice generally respond more severely than female mice after LPS injections. Males were immobile 24 hours after LPS injections and displayed depressive like behavior, whereas the females displayed sickness like behavior. Moreover, the males had significant hippocampal apoptosis and various markers of oxidative stress increased (237). The females, however, increased their expression of antioxidant metallothionein (237) which could have protective properties. Another study found that only male mice increased their TNF- α brain expression after LPS treatment (232). It has even been reported that exposure to inflammation intrauterine causes different reactions between male and female fetuses, where male offspring display higher cytokine response with increased IL-1 β and TNF- α whereas females did not (238). There are many possible explanations to the gender-based differences in immune response and the answer is likely complicated and multi-factored (237,239,240). Additionally, adult mice have been shown to increase their pro-inflammatory cytokine production more than pubertal mice, where pubertal mice secreted more anti-inflammatory cytokines after acute LPS treatment (241). It has been demonstrated that microglia response increases during aging in wild-type C57BL/6 mice after LPS injection in the hippocampus (242). Similarly, another study has shown that older mice (22 months old) increase their expression of TNF- α , IL-1 β , and IL-10 protein levels in plasma more than younger mice (6 months old) but the younger C57BL/6 mice displayed higher levels of the aforementioned cytokines in the brain after a single dose of maximally tolerated dose of LPS (243).

LPS can be isolated from various bacterial strains, which can cause the immune system to act differently dependent on the signal transduction pathways that are

activated (244). Strains that are often used include *Escherichia coli*, *Salmonella typhimurium*, *S. minnesota*, and *Neisseria meningitidis* (244). This can explain the difference in the cytokine profile measured between laboratories, even though the concentration of LPS and duration of stimuli was the same. How LPS is administered can also influence the results. LPS can be administered systematically i.v, i.p, as well as intracranial (245,246). In general, i.v and i.p injections produce similar inflammatory responses (247–249). When administering a single dose of LPS systematically, the concentrations vary from 0.33 mg/kg to 10 mg/kg (250–252). 20 mg/kg is a lethal dose for BALB/c mice (253) and concentrations between 15 mg/kg and 27 mg/kg are lethal in C57BL/6 mice (254–256). Additionally, LPS has been administered multiple times, even multiple times a day in some models with two or three doses within 24 hours (250). LPS has, furthermore, been administered daily for a week, where the doses are generally between 0.25 mg/kg (257,258) and 1.5 mg/kg (259). When looking at messenger RNA (mRNA) expression of various genes, the time of sacrifice after administration of LPS can determine whether you find an up- or downregulation of the target gene. One study using C57BL/6 mice found that TNF- α was upregulated in the hippocampus 3 hours after i.p administration of LPS, but returned to baseline after 6 hours.

The objective of the LPS animal study was to investigate the effect of systemic inflammation and neuroinflammation on the expression of both adhesion molecules as well as receptors and nutrient transporters present on the BCECs suitable for targeted drug delivery. It has been shown that systemic inflammation, provoked by i.p injections of LPS for seven consecutive days where the mice were sacrificed a week after last injection, can induce an increase in multiple cytokines within the brain (258). The same authors displayed a clear increase in activation of microglia in the hippocampus as well as cognitive impairment in the LPS treated animals (258). Due to the general difference in reported results when inducing inflammation or neuroinflammation with LPS, we chose to replicate the aforementioned study in hopes of recreating the neuroinflammatory environment.

2.2. *IN VITRO* MODELS OF THE BLOOD-BRAIN BARRIER

Due to the complexity of studying various molecular interactions in the BBB *in vivo*, multiple *in vitro* models have been constructed to study cellular interaction, molecular alterations in response to insult, and transport of numerous molecules. Many *in vitro* BBB models have been developed using primary cells isolated from multiple species, including porcine, mouse, rat, and primate, as well as cell line models, including the immortalized bEND3 and HBMEC cell models (260). Primary BBB models of rat and mouse origin are well characterized, easy to obtain and commonly the first choice for pre-clinical studies (260).

An optimal *in vitro* BBB model would replicate all aspects of the human brain endothelium, but such a model has not been generated simply because cells that have been taken out of their natural microenvironment and cultured in relative simple media will behave differently (4). Among basic characteristics an BBB *in vitro* model is required to comprise are low passive paracellular permeability, high expression of tight junction proteins and functional transporters, and maybe most importantly the barrier needs to be reproducible with similar characteristics (261). Immortalized cell cultures are often utilized as they are more easily obtainable than primary cells (262). Nevertheless, immortalized cell lines, as well as all other cells, are known to lose many of their *in vivo* characteristics when cultured for multiple passages (261). Primary BBB models are therefore an optimal choice as they resemble the *in vivo* condition more (260).

A popular setup for an artificial BBB is the so-called Transwell system, which can be created using both primary and immortalized cells. A Transwell system creates a diffusion system that consists of a semipermeable membrane separating the vascular side from the parenchymal side in an upper and lower compartment (263). There are multiple ways of setting up a co-culture Transwell system. BCECs are grown in a culture insert and make up the luminal side of the barrier model (see Figure 3). Glia cells that can consist of astrocytes, pericytes or mixed glia, are then cultured in the bottom of the well and make up the abluminal side of the barrier (see Figure 3). It is possible to establish the barrier as so-called contact or non-contact cultures, which refers to whether the glia cells are in direct contact with the BCECs on the other side of the filter insert, or if they are cultured in the bottom of the well. (264). As mentioned earlier, it is important for the model to display low paracellular transport. The Transendothelial Electrical Resistance (TEER) is a method of assessing the electrical resistance across a cellular monolayer, which indicates the paracellular water flow and pore size of tight junctions, thereby evaluating barrier integrity of the Transwell system in real-time without damaging the barrier (265,266). Astrocytes increase the TEER when grown in co-culture with BCECs (see Figure 3), as they induce TJ protein expression through secretion of neurotrophic factors such as Glia-cell derived

neurotrophic factor, basic fibroblast growth factor, and transforming growth factor beta 1 (13,267,268). Due to the clear advantages of incorporating astrocytes or mixed glia in the Transwell system, the current thesis will utilize a non-contact co-culture model containing mixed glia cells for its studies (see Figure 3).

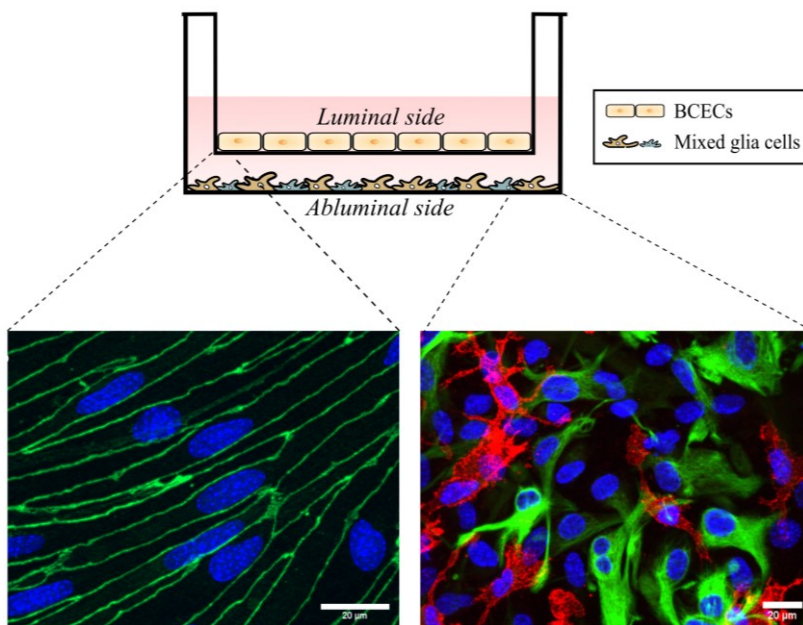


Figure 3: Transwell system displaying a non-contact co-culture system where brain capillary endothelial cells (BCECs) are cultured on hanging inserts, making up the luminal side of the barrier. The mixed glia cells are cultured in the bottom of the well, making up the abluminal side of the barrier. The immunocytochemistry illustrations display BCECs (on the left) stained with ZO-1 and Dapi, and the mixed glia culture (on the right) stained for GFAP to identify astrocytes and Cd11b to identify microglia, as well as Dapi. The scale bar is 20 μm .

As the most common feature of neurodegenerative diseases is neuroinflammation (269), it would be beneficial to have an *in vitro* model that simulates many of the characteristics, such as activation of microglia and astrocytes, increased cytokine secretion from glia and alteration of adhesion molecules on the BCECs. However, the complex nature of neuroinflammation has not yet been simulated sufficiently *in vitro*. There are multiple primary cell inflammatory *in vitro* models utilizing LPS as a stimulant. However, LPS is usually added directly to the BCECs whilst the barrier is assembled (270–274) and as BCECs are more sensitive to LPS stimulation than astrocytes (275), this will result in a disruptive BBB. LPS stimulated BCECs will lose expression of TJs, the glycocalyx will degrade, and the paracellular permeability will

increase (158,275,276). Astrocytes and other microglia respond differently to LPS stimuli than BCECs, which include increased expression of pro-inflammatory cytokines (158,277,278). LPS itself does not take any part in neuroinflammation, and it is therefore not optimal to stimulate the BCECs with LPS directly. LPS induces a broad immunological response, which can serve as a model for neuroinflammation. Others have stimulated BCECs, astrocytes or mixed glia with a specific cytokine (279), but that produces a limited inflammatory response that is an oversimplification of the complicated nature of neuroinflammation. An improved neuroinflammatory *in vitro* model is therefore needed in order to investigate molecular alterations on a cellular level.

CHAPTER 3. THESIS OBJECTIVES

The objectives of this dissertation have been to understand how the expression of the promising molecular drug targets TfR1, Glut1, and CD98hc, by BCECs can be affected by systemic inflammation and neuroinflammation, as well as to investigate whether their epigenetic manipulation is achievable. In order to accomplish this, four different studies were conducted. Study I was a “proof of concept” for study II, both focusing on epigenetic manipulation of different molecules related to iron transport at the BBB, i.e. ferroportin and TfR1. Study III focused on creating an *in vitro* BBB model that simulated neuroinflammation, whereas study IV compares the effects of neuroinflammation on the expression of the molecular targets CD98hc, Glut1 and TfR1 *in vivo* and *in vitro*.

Study I:

Aim: To investigate if epigenetic regulation of ferroportin enables increased iron efflux in cultured BCECs.

Objectives: To treat primary rat BCECs cultures with two different histone deacetylase inhibitors (HDACi) to enhance ferroportin expression. To analyze the expression of ferroportin at the transcriptional, translational and functional level.

Study II:

Aim: To investigate whether HDACi epigenetically increases the expression of TfR1, Glut1, and CD98hc in BCECs.

Objectives: To study the expression of TfR1, Glut1, and CD98hc by mouse BCECs *in vitro* and *in vivo* following HDACi treatment. Furthermore to investigate if HDACi treatment can increase the surface availability of TfR1 and possibly leading to increased *in vivo* uptake at the BBB of anti-TfR (Ri7)-conjugated gold nanoparticles.

Study III:

Aim: To investigate if an *in vitro* model consisting of primary mouse BCECs and mixed glia can be modified to allow reactive glia to subsequently influence the BBB.

Objectives: To establish an *in vitro* model of the BBB where inflammation is induced in mixed glia cells using LPS. How the reactive mixed glia affect the BCECs will subsequently be investigated.

Study IV:

Aim: To investigate possible changes in expression of the suitable drug targets TfR1, Glut1, and CD98hc by the BCECs *in vivo* and *in vitro* following inflammatory stimuli.

Objectives: C57BL/6JRj mice will be injected with LPS in a different dose regiment to obtain neuroinflammatory changes in the brain with the presence of activated microglia, possible migration of macrophages into the brain, as well as activated BCECs seen by increased expression of the cell adhesion molecules ICAM-1 and VCAM. The expression of drug delivery targets by the BCECs will be investigated following acute inflammation *in vitro*, and more persistent inflammation *in vivo*.

CHAPTER 4. METHODS AND RESULTS

4.1. STUDY I

Epigenetic regulation of ferroportin in primary cultures of the rat blood-brain barrier

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Abstract

*Ferroportin plays an essential role for iron transport through the blood-brain barrier (BBB), which is formed by brain capillary endothelial cells (BCECs). To maintain the integrity of the BBB, the BCECs gain support from pericytes and astrocytes, which together with neurons form the neurovascular unit (NVU). The objectives of the present study were to investigate ferroportin (Fpn) expression in primary cells of the NVU and to determine if Fpn expression is epigenetically regulated. Primary rat BCECs, pericytes, astrocytes, and neurons all expressed ferroportin mRNA and protein at varying levels, with BCECs exhibiting the highest expression of Fpn, peaking when co-cultured, but examined separately, from astrocytes. Conversely, Fpn expression was lowest in isolated astrocytes, which correlated with high DNA methylation in their *Slc40a1* promoter. To provide further evidence for epigenetic regulation, mono-cultured BCECs, pericytes, and astrocytes were treated with the histone deacetylase inhibitors valproic acid (VPA) and sodium butyrate (SB), which significantly increased Fpn and ferroportin protein in BCECs and pericytes. Furthermore, ⁵⁹Fe export from BCECs was elevated after treatment with VPA. In conclusion, we present first time evidence stating that Fpn expression is epigenetically regulated in BCECs, which may have implications for inadvertent pharmacological induction of iron transport through the BBB.*

4.2. STUDY II

Epigenetic induction of transferrin-receptor expression on brain capillary endothelial cells

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The transferrin receptor plays an essential role for iron transport through the blood-brain barrier (BBB) formed by brain capillary endothelial cells (BCECs). To maintain the integrity of the BBB, the BCECs gain support from pericytes and astrocytes, which together with neurons form the neurovascular unit (NVU). The objectives of the present study were to investigate the expression of transferrin receptor (TfR), Cluster of Differentiation 98 Heavy Chain (CD98hc) and glucose transporter 1 (Glut1) in BCECs and to determine if their expression is epigenetically regulated. The expression of these targets were investigated both in vitro and in vivo following treatment with the histone deacetylase inhibitor (HDACi) valproic acid (VPA). Mice were injected with VPA followed by analysis of isolated brain capillaries, and the capillary depleted brain samples, which revealed expressional increase in mRNA and protein content. To evaluate the surface availability of the epigenetically enriched TfR and the ability to target it for transport of large molecules, anti-TfR (Ri7)-conjugated gold nanoparticles were studied for uptake and transport into the brain following intravenous injection in VPA treated mice. Estimating the antibody uptake by measure of gold using ICP-MS revealed equal uptake in brains of VPA and un-injected mice, which indicates that the increase in overall TfR protein expression in BCECs consists mainly of increased intracellular TfR pool.

4.3. STUDY III

A novel neuroinflammatory blood-brain barrier model using primary mouse brain capillary endothelial cells

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Abstract

Neuroinflammation is a common feature in neurodegenerative diseases and the main reason for detrimental alteration in the blood-brain barrier (BBB). However, no standardized neuroinflammatory in vitro model that preserves the integrity of the BBB has been established. Therefore, we have generated a novel transwell system, where only the mixed glia cultures were stimulated with lipopolysaccharide (LPS) for three hours, after which the LPS was removed and the BBB model established. The brain capillary endothelial cells (BCECs) were, therefore, only affected by cytokines and other inflammatory factors released from the stimulated mixed glia culture. The expression and secretion of various cytokines from mixed glia were measured using RT-qPCR and Meso Scale Discovery analysis. The effect of the inflammatory stimuli on BCECs was investigated by measuring the integrity of the BCECs by evaluating alterations in the transendothelial and paracellular resistance. Immunocytochemistry was utilized to investigate the composition of the mixed glia culture as well as the BCECs tight junction protein arrangement and expression of adhesion molecules Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular cell adhesion protein 1 (VCAM). Glia cells showed significant upregulation of mRNA expression of interleukin 6 (IL-6), interleukin 1 β (IL-1 β), and Tumor necrosis factor α (TNF- α). Secretion of proinflammatory cytokines was significantly higher following LPS stimulation and the morphology of astrocytes changed to a more reactive form. The expression of adhesion molecules ICAM-1 and VCAM increased in the BCECs following co-culture with LPS stimulated mixed glia cells, and the arrangement of tight junction molecules was altered. In conclusion, the model displays neuroinflammation and activation of glia cells that endures even after stimuli is removed and is therefore suitable for investigating molecular alterations at the BBB during neuroinflammation.

4.4. STUDY IV

Neuroinflammation-induced expressional alterations of targets for targeted drug delivery

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Abstract

A common feature among neurodegenerative diseases, neuroinflammation denotes the main reason for detrimental alterations of the blood-brain barrier (BBB) integrity. The BBB keeps harmful substances out of the brain but simultaneously hinders the transport of therapeutics to the brain parenchyma. The presently available portfolio of small, synthetic drugs do not fulfill the demands for treatment of neurodegenerative disorders, which have justified research in transport of large molecules of biological nature to access the brain. The transport of the latter across the BBB is clearly enhanced when simultaneously targeting receptors or transporters expressed at the BBB. In vivo and in vitro studies were performed to study the impact of proinflammatory stimuli on the expression of targetable molecules on brain capillary endothelial cells (BCECs). Mice were injected intraperitoneally with 0.75 mg/kg or 1.25 mg/kg lipopolysaccharides (LPS) for seven consecutive days, and euthanized either 24 hours later or 7 days after last injection. Meso Scale Discovery analysis was carried out on plasma and brain homogenate to measure the cytokine levels of ten pro- and anti-inflammatory cytokines. To further evaluate neuroinflammatory effects, brains were stained for microglia marker CD11b, adhesion molecules Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular cell adhesion molecules 1 (VCAM1). Furthermore, a non-contact co-cultured BBB Transwell system was prepared, where mixed glia cells were stimulated with LPS. The expression of Cluster of Differentiation 98 Heavy Chain (CD98hc), Glucose transporter 1 (Glut1) and Transferrin receptor 1 (TfR) was investigated in vivo and in vitro using immunolabeling. LPS-treated mice increased their expression of IL-1 β and IL-10 in plasma, and IL-1 β , IL-2 and KC/GRO in brain. The animals did show clear signs of neuroinflammation with increased CD11b, ICAM-1 and VCAM immunoreactivity. However, there was not seen a drastic downregulation of CD98hc, Glut1 or TfR in response to neuroinflammation.

CHAPTER 5. DISCUSSION

This chapter will discuss the findings of Study I-IV in context of existing literature. Contrary to the discussions presented in each of the individual studies, the focus here will be on discussing the findings as a whole. The first part of the thesis (Study I and II) investigated whether it was possible to use epigenetics to upregulate the expression and surface availability of suitable targets for drug delivery in young and healthy mice. Study I served primarily as a simplified “proof of concept” for study II, investigating the expression of the only iron exporter ferroportin *in vitro* (194). The second part of the thesis (Study III and IV) focusses on the neuroinflammation that is present in neurodegenerative diseases, and how it could affect the expression of suitable drug targets on the BCECs. Due to a lack of suitable *in vitro* model for neuroinflammation, Study III was devoted to establishing such a model and study IV investigated how expression of targets on the BCECs altered during neuroinflammation both *in vitro* and *in vivo*.

The dissertation discussion will be divided into two parts reflecting the two different topics of the thesis, the first focusing on targeted delivery and epigenetics, and the second on neuroinflammation.

5.1. IS TARGETED DRUG DELIVERY NECESSARY TO OBTAIN BLOOD-BRAIN BARRIER TRANSPORT IN ORDER TO TREAT NEURODEGENERATIVE DISEASES?

When looking at the limited progress in treating neurodegenerative diseases over the last decades it is hard not to be discouraged. Fortunately, there has been some success in treatment of neurodegenerative diseases. MS stands out in that regard, as multiple different therapeutics have been successful in slowing down the disease progression. Even though MS is categorized as a neurodegenerative disease, the treatment approach is actually not confined to the brain. The effective strategies include: immune modification in the periphery by retaining naive memory T cells in the lymph nodes or lymphatic tissues with fingolimod, reducing the number of cytotoxic T cells in the blood with dimethyl fumarate or depleting circulating autoreactive B and T lymphocytes with cladribine (280,281). Other approaches focus on inhibiting the leukocyte migration into the brain by blocking interaction with VCAM on BCECs with e.g. natalizumab (282,283). All of these treatment options are therefore not dependent on transport across the BBB.

When it comes to developing effective therapies for neurodegenerative diseases that are reliant on transport to the brain parenchyma, no major breakthrough has been achieved. It is surprising that after decades of research and hundreds of clinical trials, there still is no approved treatment that can successfully slow down the progression of AD. Only 170 drugs are currently in development for AD, which may seem like a lot, but compared to 433 for diabetes and 6833 for malignant neoplasms, once perspective might change (284). It is maybe less surprising when the estimated failure rate of 99.6% in AD drug development is taken into account (284). But why do promising therapeutics that yield great efficacy in preclinical models fail to provide therapeutic effect in clinical trials? There are many possible reasons, but one could be that most of the drugs did not provide sufficient transport into the brain parenchyma. Delivering targeted therapeutics to the brain is no easy task as transporting the drug to the BCECs is just the first step. Successful drug delivery of therapeutics need to fulfill two criteria: they need to be efficiently targeted and delivered to the pathological site within the brain, and then be able to release the therapeutic drug in appropriate amount to elicit a pharmacological response without significant side effects (285).

It is therefore critical to obtain better understanding of how a drug is transported through the BCECs and into the brain parenchyma, as well as how the drug will reach the pathological site. But why is it so difficult to achieve? The problems start within the blood circulation as therapeutics are usually designed to be administered i.v. This can trigger immune activation to the foreign object. Liposomes are a great example of this. To combat this immune activation, liposomes can be conjugated with polyethylene glycol (PEG) to increase stability and prolong circulation time but to researchers concern, multiple studies have reported unexpected immune responses even with PEG conjugation. It is important to note that immune reactions can happen whether the liposome is targeted or not (286). Another problem is off-target accumulation of therapeutics after systemic administration, which has been the biggest problem in passive drug delivery, causing toxicity and lower efficacy of the drug (123). Unfortunately, targeted therapy does not completely eliminate this problem as the drug can accumulate in organs that express the receptor for the ligand that has been utilized for the targeting. If we take the TfR1 as an example, it is true that the BCECs are the only endothelial cell within the body that expresses TfR, but multiple other cell types, such as erythrocytes and macrophages, do also express the receptor. This can cause the accumulation in liver, spleen, kidney heart and lungs (287).

Even if the drug surpasses these obstacles and actually binds to the ligand on the BCEC, there is no guarantee that the drug will be transported into and across the BCEC. It seems to be of great importance how the ligand binds to a receptor (288,289). Lowering the affinity of TfR-targeted antibodies seems to increase the

uptake (288), but whether this factor is specific to TfR-targeted antibodies is unknown. After the ligand of the drug complex has bound to the receptor on the BCECs, the strength of the ligand-receptor complex, the avidity, can highly affect transport (290,291). Even if all of these aforementioned pitfalls are evaded, when utilizing carrier-mediated transcytosis and receptor mediated transcytosis, which are vesicular routes, the drug or nanoparticle must escape lysosomal degradation within the BCECs to provide a therapeutic effect (14). And at last, as if the road hasn't been long enough, the drug needs to be transported out of the BCECs and to the pathological site.

Non-targeted transport of otherwise promising drugs has not been prosperous in the past, thus it is time to take a step back and investigate the target molecules better, from its expressional pattern to how it interacts with and transport therapeutics.

The industry has taken notice, and pharmaceutical companies have started to develop targeted drug delivery systems. Here some of the most promising candidates will briefly be discussed.

Roche was the first to enter the clinical phase using a brain delivery system, or their so-called "Brain Shuttle", delivering anti-A β mAb through targeting the TfR1. Their preclinical research displayed an uptake of 2-3% of the ID where protein concentrations was 9 times higher in AD mice compared to wild type mice (292). The initial trial RO 7126209 is completed (293) where the second study has begun on participants with prodromal, mild or moderate AD (294).

Denali Therapeutics was founded in 2015 with the main purpose of designing therapeutics for neurodegenerative diseases that were able to pass the BBB. At that time, they received the largest initial funding for a biotech company ever, 217 million dollars. They have designed a transport vehicle that consisted of Fc fragment that binds to TfR1, which proved efficient in lowering the levels of amyloid protein in mice and monkeys when delivering an antibody against β -secretase 1. Furthermore, in a mouse model of Hunter syndrome the transport vehicle delivered 20 times the amount the therapeutic enzyme compared to when the enzyme was injected alone (295–297). Denali has progressed to early clinical testing of their enzyme transport vehicle, DNL310, which delivers recombinant iduronate 2-sulfatase enzyme that is deficient in Hunter syndrome (298).

It will be interesting to follow the progress of these delivery systems that up until now, have only utilized TfR1 as a target. However, it may not be the best target for all neurodegenerative diseases.

5.2. EPIGENETIC REGULATION OF BRAIN CAPILLARY ENDOTHELIAL CELLS TRANSPORTERS

The focus of the current thesis was only on a small part of the drug delivery equation, which is how targets on the BCECs are expressed in healthy animals and during neuroinflammation, and whether expression can be altered using epigenetics. The latter has not been investigated before from a drug delivery point of view. Obviously, choosing the right target is not going to solve all problems with drug delivery, but understanding how a target molecule is expressed by the BCECs in response to a disease state, or being able to influence the expression is a step in the right direction.

Alterations in the epigenome are related to cognitive decline, neuropsychiatric disorders and neurodegenerative diseases (299). The use of drugs that target the epigenetic mechanisms have therefore emerged as new therapeutic opportunities. The gene expression will be influenced by how tightly packed chromatin is on histones. Increased acetylation will disrupt the folding of nucleosome and increase the accessibility of transcriptional factors (196,197). This can be manipulated with medications capable of inhibiting enzymes that are important in the acetylation and deacetylation process. VPA inhibits histone deacetylase and the deacetylation process, which enhances gene transcription (199). HDAC inhibitors, such as VPA, do modulate the gene expression of multiple genes, which represents a promising approach when targeting multifactorial diseases such as cancer and AD. The possibility of modulating two or more targets that are involved in a disease could result in a synergistic positive effect (299). HDACs have therefore emerged as potential therapeutic targets for neurodegenerative diseases. The use of HDAC inhibitors in preclinical treatment of Tg2576 model of AD was able to reverse cognitive deficits in mice by inducing the expression of genes related to synaptic transmission (300). HDAC inhibitors have, furthermore, been used for cancer therapy as they display a broad anti-tumor activity. However, they also increase the expression of ABC efflux transporters on the tumor cells, making them more resistant to multiple drugs (301–303). The broad effect of HDAC inhibitors is therefore not always beneficial.

In regards to the BBB, it has been demonstrated that HDAC inhibitors are able to upregulate protein expression, as inhibition of HDAC increases the expression of tight junction proteins and reduces the transendothelial permeability in Type 2 diabetes mice (304). This was also demonstrated *in vitro* in Manuscript I (194), where we were able to upregulate the iron exporter ferroportin significantly in primary rat BCECs. Looking at these findings, it was evident that this could be a promising prospect for delivering more therapeutics via certain receptor or transporter. As the maximum transport capacity of a drug complex is dependent on the surface availability of a receptor or transporter (92), it was interesting to see if it was possible to upregulate

the expression of prominent brain drug delivery targets, including TfR1, Glut1, and CD98hc, *in vitro* and *in vivo*. Only *TfR1* displayed significantly higher mRNA expression *in vitro* after VPA treatment. In order to see how the expression of aforementioned targets was affected in the BCECs *in vivo* following VPA treatment, both male and female mice were treated with VPA. There was no increase in *CD98hc* mRNA expression in the capillary depleted samples. Interestingly, only male mice increased their mRNA expression of *Glut1* following VPA treatment in the capillary depleted samples, whereas the females did not. This raises the question whether there are different expressional regulatory mechanisms for Glut1 in the brain of males and females. This is unquestionably interesting as reduction of Glut1 expression in the BCECs is thought to occur early in the disease course of AD and known to worsen AD pathology, where women are more often affected by AD than men (305,306). TfR1 protein expression was increased *in vivo* in the capillaries following VPA treatment, without any sign of upregulated *TfR1* mRNA in the capillary depleted brain samples. Thus, the upregulation of TfR1 seems to be specific for the BCECs. A possible explanation for why VPA could be specific to BCECs is that the uptake of VPA across the BCECS is slower than the efflux transport of the drug, which prevents VPA from sustaining a therapeutic concentration within the brain parenchyma (98). On the contrary, VPA was able to upregulate *Glut1* expression in male mice, which might suggest that the transcriptional regulation of *Glut1* is highly adaptable and responds to transcriptional signals faster than *TfR1*.

When looking at the increase in TfR1 protein in capillaries, we were unsure if the increased expression was due to an increase in the intracellular pool of TfR1, or if the receptors were available on the BCECs surface. Hence, we investigated the surface availability by measuring the transport of injected anti-TfR gold-conjugated Ri7 antibody to the brain. The transport of the Ri7 antibody was similar between the control animals and the VPA treated animals, which suggests that the quantity of surface TfR1 remains unaltered despite of the overall increase in TfR1 within the BCECs. This indicates that the increased TfR1 protein content in capillaries is mainly due to a larger intracellular TfR1 pool. The exact mechanism of TfR1 trafficking in the BCECs remains unclear, however, the cells iron concentration impacts the concentration of TfR1 and consequently the uptake of transferrin-bound iron. When cellular iron concentrations are low, the iron-regulatory proteins bind to iron responsive elements on the 3' untranslated region on the *TfR1* mRNA, protecting it from degradation. The protein will thereafter be transported to the cell membrane, ensuring sufficient TfR1 availability (307). On the contrary, when intracellular iron concentration is high, no binding will occur leaving the *TfR1* mRNA exposed to degradation (307). In normal circumstances where iron concentration is in equilibrium with the cells requirement, TfR1 protein may not be transported to the cell membrane as there is no need for additional iron import. Hence, increased TfR1 expression

within the BCECs due to VPA treatment does not guarantee that the protein will be transported to the cell membrane.

Of the nutritional transporters and receptors that were investigated for epigenetic regulation, those that were significantly affected at both mRNA and protein levels by VPA treatment were TfR1 and ferroportin. These are both implicated in iron regulation, where TfR1 regulates iron uptake and ferroportin iron export. Iron homeostasis is known to be out of balance in neurodegenerative diseases, where an increase in cerebral iron levels has been implicated in neurodegenerative diseases that present with protein aggregates, which then seem to co-localize with iron (308). Exactly how iron transport at the BBB is affected by VPA treatment in e.g. epilepsy patients is an interesting research question. However, as our results suggest an increase in these proteins can occur without it affecting the surface availability and thereby transport of iron. Thus, in future studies, it is important to increase the luminal surface availability and activity of the receptors for targeting purposes.

5.3. HOW DOES INFLAMMATION INFLUENCE THE EXPRESSION OF DRUG DELIVERY TARGETS?

In addition to investigating whether TfR1, Glut1, and CD98hc could be pharmacologically upregulated using HDAC inhibitors, this dissertation focused on investigating how neuroinflammation affected the expression of these targets. Most drug delivery studies investigate young and healthy animals, neglecting the fact that the population they would like to treat in the future are in fact older and affected by neurodegeneration. There has been an ongoing debate on the extent of BBB disruption in neurodegenerative diseases (309,310). It is generally believed that there is some form of BBB breakdown, even in the early phases of neurodegenerative diseases (311), but the severity of the breakdown and duration is more uncertain. However, it is becoming more apparent that the dynamic BBB is not universally leaky in neurodegenerative diseases.

After establishing an inflammatory BBB model where only mixed glia cells were stimulated with LPS, it displays only minor breakdown of the BBB with 10-30% decrease in TEER, which is lower than other *in vitro* models where LPS affects the BCECs directly (159,312). The inflammatory response was simulated well in our model as the mixed glial culture secreted various cytokines, thereby mimicking the diverse response of neuroinflammation. The exact cytokines that are secreted during neuroinflammation is difficult to confirm, as it could be dependent on the duration of the neuroinflammatory state, type of neurodegenerative disease as well as age and sex of the patient, but TNF- α , IL-6 and IL-1 β seem to play a vital role in neuro-

inflammation (313–315). The heterogeneity of the neuroinflammation in neurodegenerative diseases is thought to be due to the origin of the inflammation itself. In MS, even though residential microglia and astrocytes also produce cytokines, the major producer of inflammatory cytokines within the brain are invading leukocytes, and the changes in the brain tissue that follow are usually more severe and acute compared to AD (313). In AD, where the main producer of inflammatory cytokines that drive neuroinflammation are microglia and astrocytes, it is more unclear whether this secretion is beneficial or detrimental, at least in the beginning of the disease (313). The secretion may be beneficial as a response to a given stimuli, but chronic overproduction of pro-inflammatory cytokines can enhance the degenerative process (313). The broad secretion of inflammatory cytokines in the *in vitro* BBB model did affect the BCECs, not only by slightly decreasing the integrity of the barrier, but also by increasing the expression of the adhesion molecules ICAM-1 and VCAM by the BCECs. The expression of these adhesion molecules is crucial for leukocyte migration into the brain parenchyma in response to neuroinflammatory injury. The novel inflammatory model could therefore be beneficial for investigating paracellular transport of molecules during neuroinflammation, as well as leukocyte migration across the endothelium.

The purpose of establishing a neuroinflammatory *in vitro* BBB model was also to enable the investigation of the BCECs target molecules Tfr1, Glut1, and CD98hc and compare it to the expression in LPS treated mice. The rationale for investigating the availability of these targets on the BCECs following neuroinflammation was to ensure that they remain feasible targets for drug delivery, as neuroinflammation is an inevitable part of neurodegenerative diseases. Reduction in Glut1 expression at the BBB in AD has been reported, thereby questioning its efficacy as a target (11,305). On the other hand, the expression of Tfr1 seems to be stable in mouse models of AD and in patients compared to controls (309). No study has been carried out on the expressional alteration of CD98hc in neurodegenerative diseases. Both Tfr1 and Glut1 expression seemed unaffected by the inflammatory stimuli in the barrier model but the expression of CD98hc was undetectable in the BBB model. It is important to keep in mind that the inflammatory stimuli provided in the BBB *in vitro* model is a short and acute form of inflammation, and can therefore only provide us with information on the short-term effect of neuroinflammation on the aforementioned targets.

In order to see how persistent neuroinflammation, although not completely comparable to the long lasting neuroinflammation in neurodegenerative diseases, impacted the expressional pattern and distribution of Tfr1, Glut1, and CD98hc in mice, they were treated with LPS daily for seven consecutive days. The hypothesis was that animals euthanized 24 hours after last injection would mainly be affected by systemic inflammation, whereas the animals that were euthanized seven days after

last injection would have cleared the systemic inflammation and be left with only neuroinflammation. This was not the case as the animals seemed to be equally as affected systemically one day and seven days post injection. The neuroinflammatory effect was visible both 24 hours and seven days post injection, as demonstrated by increased expression of CD11b, ICAM-1, and VCAM.

The distribution and expression of TfR1, Glut1, and CD98hc was investigated in animals that were euthanized seven days post LPS injection. The expression of CD98hc was stable between control animals and LPS treated animals, however the expression did not seem to be enriched in the vasculature and was universally distributed over the brain parenchyma. When CD98hc was suggested as a potential target in BCECs, it was due to the fact that it was enriched compared to endothelial cells elsewhere in the body (95), but that may not be sufficient to categorize as a great target. Still, *CD98hc* has recently been identified as one of the most abundantly expressed transporter on the BCECs in humans, and that the level of expression is very similar between mouse and human brains. Furthermore, it was demonstrated that the high expression of *CD98hc* transcripts within the mouse brain was mainly in the brain microvessels (316). As this is in opposition to the thesis's results, further investigations should be carried out to clarify this discrepancy.

Glut1 is one of the most abundantly expressed proteins in the BCECs making it an interesting target on the BBB. Glut1 is furthermore expressed on both the luminal and abluminal membrane, which could enhance the continued transport of the targeted molecule (77,85,88–90). Even though Glut1 seems like an optimal target in healthy animals, it may not be ideal in neurodegenerative diseases, as its expression is downregulated in AD (11,90,305). There are multiple pathological aspects in AD that could contribute to the downregulation of Glut1, and neuroinflammation is only one of them. We found that Glut1 was highly expressed in the endothelium *in vivo* and no drastic changes in expression or localization was observed between control animals and the LPS treated animals. However, in order to detect small expressional alterations in any of the targets, a quantitative analysis such as ELISA should be performed.

TfR1 has been extensively studied for targeted BBB delivery. It has furthermore been demonstrated that transport of TfR1-targeted mAbs was not impaired by AD pathology (135). We found that TfR1 was highly expressed in capillaries, and as expected there was also some staining of neurons they also express TfR1 (12,317,318). Similarly to Glut1, no dramatic alteration in the expression was observed.

Future studies could include protein quantification of these drug delivery targets, preferably in older animals, as they could respond quite differently to the inflammatory stimuli.

5.4. UPREGULATION OF OTHER SUITABLE TARGETS IN BRAIN CAPILLARY ENDOTHELIAL CELLS IN RESPONSE TO NEUROINFLAMMATION

The aforementioned targets were identified as suitable targets due to their high or selective expression in the BCECs in healthy animals, but the expression of receptors and nutrient transporters feasible for targeting may be altered due to aging, inflammation, and neurodegenerative pathology. There may also be transporters or receptors on the BCECs that may not seem as suitable targets in a healthy subject, but may be strongly upregulated during neurodegeneration. Although many of the alterations that occur in BCECs in response to inflammation are detrimental, they should not be overlooked in the search for suitable targets for enhanced drug delivery. ICAM-1 and VCAM are great examples, as they are upregulated in BCECs following inflammation, VCAM even more so than ICAM-1 (319). This is demonstrated in our LPS experiment, where LPS treated animals did increase the expression of especially VCAM following inflammatory stimuli, and the expression was still increased seven days after the last injection. Utilizing this fact as a treatment possibility has been performed in Experimental autoimmune encephalomyelitis (EAE) model of MS, where blocking of VCAM delayed disease onset (171). The ultimate proof of the importance of VCAM and the validity of inhibiting the interaction between VCAM and immune cells takes place when using Natalizumab, that efficiently limits the immune cell transport across the BBB, thereby limiting the tissue damage (320). But how can this be translated into BBB targeting? Recently, an inflammatory mouse model of acute brain inflammation induced by TNF- α displayed a 10-fold greater uptake of injected antibodies when targeting VCAM compared to TfR1 and ICAM-1 (321). Likewise, the uptake of anti-VCAM liposomes was 27-fold greater than that of anti-TfR1 liposomes and 8-fold greater than of anti-ICAM liposomes (321). This is just one example of how the alteration in protein expression due to pathology can be utilized, and further demonstrates how important it is to have an all-around understanding of the structural differences and functions of the healthy and inflamed BBB in order to deliver targeted therapeutics successfully to the inflamed brain.

CHAPTER 5. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, the projects of the PhD thesis have provided new insight into the expression of novel drug delivery targets expressed by the BCECs. Although neuroinflammation is implicated in neurodegenerative diseases, little is known about the expressional alteration or translocation of receptors and nutrient transporters that have been assessed as optimal targets for drug delivery. The results presented in the current thesis demonstrate that TfR1 and Glut1 remain stable in their expression during acute neuroinflammation *in vitro* and prolonged neuroinflammation *in vivo*. A novel *in vitro* neuroinflammatory model that preserves the integrity of the barrier was established, which could prove valuable in future studies on drug delivery or cellular transport through the inflamed BBB. Future studies should investigate the distribution of these targets in the BBB in neurodegenerative disease models to comprehend whether the expression is affected. Furthermore, future studies should investigate the potential of dual targeting during neuroinflammation, utilizing e.g. VCAM and TfR1. In addition to valuable insight into the regulation of targets in response to neuroinflammation, the projects of this dissertation have, as the first ones, investigated the potential of epigenetically upregulating molecular targets of interest on the BCECs. Upregulation of both ferroportin and TfR1 mRNA and protein was demonstrated in the BCECs, but further research is needed in order to understand how to increase the luminal surface availability of these for targeting purposes.

Together, these finding may serve as inspiration for investigating how pathology in various neurodegenerative diseases might affect receptors or nutrient transporters chosen for targeted drug delivery in the future, and maybe even for discoveries of new and feasible targets. It is my hope that more research will be carried out utilizing the expressional alterations that consequently follow the pathology of neurodegeneration, and that it will pave the way for new and innovative ways of treating these diseases. Furthermore, there will hopefully be more focus on epigenetic agents and their potential to enhance the expression of genes that may be in deficit in certain neurodegenerative diseases.

REFERENCES

1. Swenson BL, Meyer CF, Bussian TJ, Baker DJ. Senescence in aging and disorders of the central nervous system. Vol. 3, *Translational Medicine of Aging*. KeAi Communications Co.; 2019. p. 17–25.
2. Chi H, Chang HY, Sang TK. Neuronal cell death mechanisms in major neurodegenerative diseases. Vol. 19, *International Journal of Molecular Sciences*. MDPI AG; 2018.
3. Bendix Johnsen K, Moos T. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release*. 2016;222:32–46.
4. Abbott NJ. Blood–brain barrier structure and function and the challenges for CNS drug delivery. *J Inherit Metab Dis*. 2013 May 23;36(3):437–49.
5. Pardridge WM. Blood-Brain Barrier and Delivery of Protein and Gene Therapeutics to Brain. Vol. 11, *Frontiers in Aging Neuroscience*. Frontiers Media S.A.; 2020.
6. Zhang W, Wang W, Yu DX, Xiao Z, He Z. Application of nanodiagnostics and nanotherapy to CNS diseases. *Nanomedicine*. 2018 Sep;13(18):2341–71.
7. Kevadiya BD, Ottemann BM, Thomas M Ben, Mukadam I, Nigam S, McMillan JE, et al. Neurotheranostics as personalized medicines. *Adv Drug Deliv Rev*. 2018;(xxxx).
8. Dong X. Current Strategies for Brain Drug Delivery. *Theranostics*. 2018;8(6):1481–93.
9. Cressman AM, Petrovic V, Piquette-Miller M. Inflammation-mediated changes in drug transporter expression/activity: Implications for therapeutic drug response. Vol. 5, *Expert Review of Clinical Pharmacology*. *Expert Rev Clin Pharmacol*; 2012. p. 69–89.
10. Erdo F, Krajcsi P. Age-related functional and expressional changes in efflux pathways at the blood-brain barrier. Vol. 10, *Frontiers in Aging Neuroscience*. Frontiers Media S.A.; 2019.
11. Keaney J, Campbell M. The dynamic blood-brain barrier. *FEBS J*. 2015 Nov 1;282(21):4067–79.

12. Mills E, Dong X-P, Wang F, Xu H. Mechanisms of brain iron transport: insight into neurodegeneration and CNS disorders. *Future Med Chem.* 2010;2(1):51–64.
13. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. *Nat Rev Neurosci.* 2006;7(1):41–53.
14. Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis.* 2010;37(1):13–25.
15. Pardridge WM. Drug and gene targeting to the brain with molecular Trojan horses. Vol. 1, *Nature Reviews Drug Discovery.* 2002. p. 131–9.
16. Schneider M. The vascular integrity of the brain in chronic neurodegeneration. 2015;
17. Profaci CP, Munji RN, Pulido RS, Daneman R. The blood–brain barrier in health and disease: Important unanswered questions. *J Exp Med.* 2020;217(4):1–16.
18. Daneman R, Prat A. The blood–brain barrier. *Cold Spring Harb Perspect Biol.* 2015 Jan 1;7(1).
19. Greene C, Hanley N, Campbell M. Claudin-5: gatekeeper of neurological function. *Fluids Barriers CNS.* 2019 Jan 29;16(1):3.
20. Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J Cell Biol.* 1967 Jul;34(1):207–17.
21. Jia W, Martin TA, Zhang G, Jiang WG. Junctional adhesion molecules in cerebral endothelial tight junction and brain metastasis. *Anticancer Res.* 2013 Jun;33(6):2353–9.
22. Zlokovic B V. The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. *Neuron.* 2008;57(2):178–201.
23. Mittapalli RK, Manda VK, Adkins CE, Geldenhuys WJ, Lockman PR. Exploiting nutrient transporters at the blood-brain barrier to improve brain distribution of small molecules. Vol. 1, *Therapeutic Delivery.* 2010. p. 775–84.
24. Dyrna F, Hanske S, Krueger M, Bechmann I. The blood-brain barrier. Vol. 8, *Journal of Neuroimmune Pharmacology.* 2013. p. 763–73.
25. Löscher W, Potschka H. Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx.* 2005;2(1):86–98.

26. Attwell D, Mishra A, Hall CN, O'Farrell FM, Dalkara T. What is a pericyte? *J Cereb Blood Flow Metab.* 2016 Feb 1;36(2):451–5.
27. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science (80-).* 1997 Jul 11;277(5323):242–5.
28. Hellström M, Gerhardt H, Kalén M, Li X, Eriksson U, Wolburg H, et al. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J Cell Biol.* 2001 Feb 5;152(3):543–53.
29. Armulik A, Genové G, Mäe M, Nisancioglu MH, Wallgard E, Niaudet C, et al. Pericytes regulate the blood-brain barrier. *Nature.* 2010 Nov 25;468(7323):557–61.
30. Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, et al. Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. *Neuron.* 2010 Nov 4;68(3):409–27.
31. Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, et al. Capillary pericytes regulate cerebral blood flow in health and disease. *Nature.* 2014;508(1):55–60.
32. Khennouf L, Gesslein B, Brazhe A, Oceau JC, Kutuzov N, Khakh BS, et al. Active role of capillary pericytes during stimulation-induced activity and spreading depolarization. *Brain.* 2018 Jul 1;141(7):2032–46.
33. Armulik A, Genové G, Betsholtz C. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. Vol. 21, *Developmental Cell.* 2011. p. 193–215.
34. Allt G, Lawrenson JG. Pericytes: Cell biology and pathology. Vol. 169, *Cells Tissues Organs.* 2001. p. 1–11.
35. Peppiatt CM, Howarth C, Mobbs P, Attwell D. Bidirectional control of CNS capillary diameter by pericytes. *Nature.* 2006 Oct 12;443(7112):700–4.
36. Shimizu F, Sano Y, Saito K, Abe MA, Maeda T, Haruki H, et al. Pericyte-derived glial cell line-derived neurotrophic factor increase the expression of claudin-5 in the blood-brain barrier and the blood-nerve barrier. *Neurochem Res.* 2012 Feb;37(2):401–9.
37. Berthiaume AA, Grant RI, McDowell KP, Underly RG, Hartmann DA, Levy M, et al. Dynamic Remodeling of Pericytes In Vivo Maintains Capillary Coverage in the Adult Mouse Brain. *Cell Rep.* 2018 Jan 2;22(1):8–16.

38. Lendahl U, Nilsson P, Betsholtz C. Emerging links between cerebrovascular and neurodegenerative diseases—a special role for pericytes. *EMBO Rep.* 2019 Nov 5;20(11).
39. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Vol. 97, *Circulation Research*. 2005. p. 512–23.
40. Nguyen QL, Okuno N, Hamashima T, Dang ST, Fujikawa M, Ishii Y, et al. Vascular PDGFR-alpha protects against BBB dysfunction after stroke in mice. *Angiogenesis*. 2020;
41. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol.* 2005 Oct;7(4):452–64.
42. Villaseñ Or R, Kuennecke B, Ozmen L, Ammann M, Kugler C, Grü Ninger F, et al. Region-specific permeability of the blood-brain barrier upon pericyte loss.
43. Miners JS, Schulz I, Love S. Differing associations between A β accumulation, hypoperfusion, blood-brain barrier dysfunction and loss of PDGFRB pericyte marker in the precuneus and parietal white matter in Alzheimer's disease. *J Cereb Blood Flow Metab.* 2018 Jan 1;38(1):103–15.
44. Banks WA, Kastin AJ, Jaspan JB. Regional variation in transport of pancreatic polypeptide across the blood-brain barrier of mice. *Pharmacol Biochem Behav.* 1995;51(1):139–47.
45. Banks WA, Kastin AJ. Differential permeability of the blood-brain barrier to two pancreatic peptides: Insulin and amylin. *Peptides.* 1998;19(5):883–9.
46. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, et al. Blood-Brain barrier breakdown in the aging human hippocampus. *Neuron.* 2015 Jan 21;85(2):296–302.
47. Brown LS, Foster CG, Courtney JM, King NE, Howells DW, Sutherland BA. Pericytes and neurovascular function in the healthy and diseased brain. Vol. 13, *Frontiers in Cellular Neuroscience*. Frontiers Media S.A.; 2019. p. 282.
48. Cai C, Fordsmann JC, Jensen SH, Gesslein B, Lønstrup M, Hald BO, et al. Stimulation-induced increases in cerebral blood flow and local capillary vasoconstriction depend on conducted vascular responses. *Proc Natl Acad Sci U S A.* 2018 Jun 19;115(25):E5796–804.
49. Thomsen LB, Burkhardt A, Moos T. A triple culture model of the blood-brain barrier using porcine brain endothelial cells, astrocytes and pericytes. *PLoS One.* 2015 Aug 4;10(8):e0134765.

50. Marina N, Kasymov V, Ackland GL, Kasparov S, Gourine A V. Astrocytes and brain hypoxia. In: *Advances in Experimental Medicine and Biology*. Springer New York LLC; 2016. p. 201–7.
51. Gordon GRJ, Mulligan SJ, MacVicar BA. Astrocyte control of the cerebrovasculature. Vol. 55, *GLIA*. 2007. p. 1214–21.
52. Orthmann-Murphy JL, Abrams CK, Scherer SS. Gap junctions couple astrocytes and oligodendrocytes. Vol. 35, *Journal of Molecular Neuroscience*. 2008. p. 101–16.
53. Gutiérrez Y, García-Marques J, Liu X, Fortes-Marco L, Sánchez-González R, Giaume C, et al. Sibling astrocytes share preferential coupling via gap junctions. *Glia*. 2019;67(10):1852–8.
54. Halassa MM, Fellin T, Takano H, Dong J-H, Haydon PG. Synaptic islands defined by the territory of a single astrocyte. *J Neurosci*. 2007 Jun 13;27(24):6473–7.
55. Ponath G, Park C, Pitt D. The role of astrocytes in multiple sclerosis. Vol. 9, *Frontiers in Immunology*. Frontiers Media S.A.; 2018.
56. Sofroniew M V, Vinters H V. Astrocytes: biology and pathology. *Acta Neuropathol*. 2010 Jan;119(1):7–35.
57. Benarroch EE. Brain glucose transporters: implications for neurologic disease. *Neurology*. 2014 Apr 15;82(15):1374–9.
58. Brown AM, Ransom BR. Astrocyte glycogen and brain energy metabolism. Vol. 55, *GLIA*. 2007. p. 1263–71.
59. Régina A, Morchoisne S, Borson ND, McCall AL, Drewes LR, Roux F. Factor(s) released by glucose-deprived astrocytes enhance glucose transporter expression and activity in rat brain endothelial cells. *Biochim Biophys Acta - Mol Cell Res*. 2001 Sep 26;1540(3):233–42.
60. Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Vol. 28, *Trends in Immunology*. 2007. p. 138–45.
61. Jensen CJ, Massie A, De Keyser J. Immune players in the CNS: The astrocyte. Vol. 8, *Journal of Neuroimmune Pharmacology*. 2013. p. 824–39.
62. Pekny M, Pekna M. Astrocyte reactivity and reactive astrogliosis: Costs and benefits. *Physiol Rev*. 2014 Oct 1;94(4):1077–98.
63. Escartin C, Galea E, Oamp JP, Petzold GC, Serrano-Pozo A, Steinhamp C, et

- al. Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci* 2021. 2021 Feb 15;1–14.
64. Sofroniew M V. Astrocyte barriers to neurotoxic inflammation. Vol. 16, *Nature Reviews Neuroscience*. Nature Publishing Group; 2015. p. 249–63.
 65. Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol*. 1995 Apr;37(4):424–35.
 66. John GR, Lee SC, Brosnan CF. Cytokines: Powerful regulators of glial cell activation. Vol. 9, *Neuroscientist*. 2003. p. 10–22.
 67. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. *J Neurosci*. 2012 May 2;32(18):6391–410.
 68. Wei DC, Morrison EH. *Histology, Astrocytes*. StatPearls. StatPearls Publishing; 2019.
 69. Pekny M, Levéen P, Pekna M, Eliasson C, Berthold CH, Westermarck B, et al. Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. *EMBO J*. 1995 Apr 18;14(8):1590–8.
 70. Herrmann JE, Imura T, Song B, Qi J, Ao Y, Nguyen TK, et al. STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *J Neurosci*. 2008 Jul 9;28(28):7231–43.
 71. Norenberg MD. The distribution of glutamine synthetase in the rat central nervous system. *J Histochem Cytochem*. 1979 Mar;27(3):756–62.
 72. Fu BM. Transport across the blood-brain barrier. In: *Advances in Experimental Medicine and Biology*. Springer New York LLC; 2018. p. 235–59.
 73. Ramanathan S, Archunan G, Sivakumar M, Tamil Selvan S, Fred AL, Kumar S, et al. Theranostic applications of nanoparticles in neurodegenerative disorders. *Int J Nanomedicine*. 2018;13:5561–76.
 74. Patel MM, Patel BM. Crossing the Blood-Brain Barrier: Recent Advances in Drug Delivery to the Brain. *CNS Drugs*. 2017;31.
 75. Sauer I, Dunay IR, Weisgraber K, Bienert M, Dathe M. An apolipoprotein E-derived peptide mediates uptake of sterically stabilized liposomes into brain capillary endothelial cells. *Biochemistry*. 2005 Feb 15;44(6):2021–9.
 76. Barar J, Rafi MA, Pourseif MM, Omid Y. Blood-brain barrier transport

- machineries and targeted therapy of brain diseases. *BioImpacts*. 2016;6(4):225–48.
77. Simpson IA, Carruthers A, Vannucci SJ. Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J Cereb Blood Flow Metab*. 2007 Nov;27(11):1766–91.
 78. Hawkins RA, O’Kane RL, Simpson IA, Viña JR. Structure of the Blood–Brain Barrier and Its Role in the Transport of Amino Acids. *J Nutr*. 2006 Jan 1;136(1):218S-226S.
 79. Spector R, Johanson CE. Vitamin transport and homeostasis in mammalian brain: Focus on vitamins B and E. Vol. 103, *Journal of Neurochemistry*. 2007. p. 425–38.
 80. Burkhart A, Azizi M, Thomsen MSS, Thomsen LBB, Moos T. Accessing Targeted Nanoparticles to the Brain: The Vascular Route. Vol. 21, *Current medicinal chemistry*. 2014.
 81. Sweeney MD, Zhao Z, Montagne A, Nelson AR, Zlokovic B V. Blood-brain barrier: From physiology to disease and back. *Physiol Rev*. 2019;99(1):21–78.
 82. Nguyen LN, Ma D, Shui G, Wong P, Cazenave-Gassiot A, Zhang X, et al. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. *Nature*. 2014;509(7501):503–6.
 83. Ben-Zvi A, Lacoste B, Kur E, Andreone BJ, Mayshar Y, Yan H, et al. Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature*. 2014 May 22;509(7501):507–11.
 84. Cass CE, Young JD, Baldwin SA. Recent advances in the molecular biology of nucleoside transporters of mammalian cells. *Biochem Cell Biol*. 1998;76(5):761–70.
 85. Pardridge WM. Delivery of Biologics Across the Blood–Brain Barrier with Molecular Trojan Horse Technology. Vol. 31, *BioDrugs*. Springer International Publishing; 2017. p. 503–19.
 86. Patching SG. Glucose Transporters at the Blood-Brain Barrier: Function, Regulation and Gateways for Drug Delivery. Vol. 54, *Molecular Neurobiology*. Humana Press Inc.; 2017. p. 1046–77.
 87. Cornford EM, Hyman S, Swartz BE. The human brain GLUT1 glucose transporter: Ultrastructural localization to the blood-brain barrier endothelia. *J Cereb Blood Flow Metab*. 1994 Jan;14(1):106–12.

88. Daneman R, Zhou L, Agalliu D, Cahoy JD, Kaushal A, Barres BA. The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells. *PLoS One*. 2010 Oct 29;5(10):e13741.
89. Simpson IA, Vannucci SJ, DeJoseph MR, Hawkins RA. Glucose Transporter Asymmetries in the Bovine Blood-Brain Barrier. *J Biol Chem*. 2001 Apr 20;276(16):12725–9.
90. Guo X, Geng M, Du G. Glucose transporter 1, distribution in the brain and in neural disorders: its relationship with transport of neuroactive drugs through the blood-brain barrier. *Biochem Genet*. 2005 Apr;43(3–4):175–87.
91. Moura RP, Martins C, Pinto S, Sousa F, Sarmento B. Blood-brain barrier receptors and transporters: an insight on their function and how to exploit them through nanotechnology. Vol. 16, *Expert Opinion on Drug Delivery*. Taylor and Francis Ltd; 2019. p. 271–85.
92. Helms HCC, Kristensen M, Saaby L, Fricker G, Brodin B. Drug Delivery Strategies to Overcome the Blood–Brain Barrier (BBB). In: *Handbook of experimental pharmacology*. Handb Exp Pharmacol; 2020.
93. McGowan KM, Long SD, Pekala PH. Glucose transporter gene expression: Regulation of transcription and mRNA stability. Vol. 66, *Pharmacology and Therapeutics*. Pharmacol Ther; 1995. p. 465–505.
94. Dwyer KJ, Boado RJ, Pardridge WM. Cis-element/cytoplasmic protein interaction within the 3'-untranslated region of the GLUT1 glucose transporter mRNA. *J Neurochem*. 1996;66(2):449–58.
95. Zuchero YJY, Chen X, Bien-Ly N, Bumbaca D, Tong RK, Gao X, et al. Discovery of Novel Blood-Brain Barrier Targets to Enhance Brain Uptake of Therapeutic Antibodies. *Neuron*. 2016 Jan 6;89(1):70–82.
96. Cano-Crespo S, Chillarón J, Junza A, Fernández-Miranda G, García J, Polte C, et al. CD98hc (SLC3A2) sustains amino acid and nucleotide availability for cell cycle progression. *Sci Rep*. 2019 Oct 1;9(1):14065.
97. Lee Y, Wiriyasermkul P, Jin C, Quan L, Ohgaki R, Okuda S, et al. Cryo-EM structure of the human L-type amino acid transporter 1 in complex with glycoprotein CD98hc. *Nat Struct Mol Biol*. 2019;26(6):510–7.
98. Gynther M, Peura L, Vernerová M, Leppänen J, Kärkkäinen J, Lehtonen M, et al. Amino Acid Promoieties Alter Valproic Acid Pharmacokinetics and Enable Extended Brain Exposure. *Neurochem Res*. 2016 Oct 1;41(10):2797–809.

99. de la Ballina LR, Cano-Crespo S, González-Muñoz E, Bial S, Estrach S, Cailleteau L, et al. Amino Acid Transport Associated to Cluster of Differentiation 98 Heavy Chain (CD98hc) Is at the Cross-road of Oxidative Stress and Amino Acid Availability. *J Biol Chem.* 2016 Apr 29;291(18):9700–11.
100. Ohno H, Nakatsu Y, Sakoda H, Kushiya A, Ono H, Fujishiro M, et al. 4F2hc stabilizes GLUT1 protein and increases glucose transport activity. *Am J Physiol Cell Physiol.* 2011 May;300(5):C1047-54.
101. Cantor JM, Ginsberg MH. CD98 at the crossroads of adaptive immunity and cancer. *J Cell Sci.* 2012 Mar 15;125(Pt 6):1373–82.
102. Kobayashi K, Ohnishi A, Promsuk J, Shimizu S, Kanai Y, Shiokawa Y, et al. Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma cells. *Neurosurgery.* 2008 Feb;62(2):493–503; discussion 503-4.
103. Kaira K, Ohde Y, Endo M, Nakagawa K, Okumura T, Takahashi T, et al. Expression of 4F2hc (CD98) in pulmonary neuroendocrine tumors. *Oncol Rep.* 2011 Oct;26(4):931–7.
104. Cantor J, Slepak M, Ege N, Chang JT, Ginsberg MH. Loss of T cell CD98 H chain specifically ablates T cell clonal expansion and protects from autoimmunity. *J Immunol.* 2011 Jul 15;187(2):851–60.
105. Patel MM, Bfiadada S V, Amin AF. Getting into the Brain: Approaches to Enhance Brain Drug Delivery Mayur. *CNS Drugs.* 2009;35–59.
106. Pulgar VM. Transcytosis to cross the blood brain barrier, new advancements and challenges. *Front Neurosci.* 2019;13(JAN).
107. Neuwelt E, Abbott NJ, Abrey L, Banks WA, Blakley B, Davis T, et al. Strategies to advance translational research into brain barriers. Vol. 7, *The Lancet Neurology.* Elsevier; 2008. p. 84–96.
108. Johnsen KB, Burkhart A, Thomsen LB, Andresen TL, Moos T. Targeting the transferrin receptor for brain drug delivery. *Prog Neurobiol.* 2019 Jul 31;101665.
109. Crielaard BJ, Lammers T, Rivella S. Targeting iron metabolism in drug discovery and delivery. *Nat Rev Drug Discov.* 2017;
110. Drakesmith H, Nemeth E, Ganz T. Ironing out Ferroportin. *Cell Metab.* 2015;22(5):777–87.

111. Gulec S, Anderson GJ, Collins JF. Mechanistic and regulatory aspects of intestinal iron absorption. *AJP Gastrointest Liver Physiol.* 2014;307(4):G397–409.
112. Belaidi AA, Bush AI. Iron neurochemistry in Alzheimer’s disease and Parkinson’s disease: targets for therapeutics. *J Neurochem.* 2016;139:179–97.
113. Silva B, Faustino P. An overview of molecular basis of iron metabolism regulation and the associated pathologies. *Biochim Biophys Acta.* 2015;1852(7):1347–59.
114. Duck KA, Connor JR. Iron uptake and transport across physiological barriers. *BioMetals.* 2016;29(4):573–91.
115. Moos T, Morgan EH. Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol.* 2000 Feb;20(1):77–95.
116. Skjørringe T, Burkhart A, Johnsen KB, Moos T. Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology. *Front Mol Neurosci.* 2015 Jan;8:19.
117. Burkhart A, Skjørringe T, Johnsen KB, Siupka P, Thomsen LB, Nielsen MS, et al. Expression of Iron-Related Proteins at the Neurovascular Unit Supports Reduction and Reoxidation of Iron for Transport Through the Blood-Brain Barrier. *Mol Neurobiol.* 2016;53(10):7237–53.
118. Andersen HH, Johnsen KB, Moos T. Iron deposits in the chronically inflamed central nervous system and contributes to neurodegeneration. *Cell Mol Life Sci.* 2014;71(9):1607–22.
119. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Vol. 23, *Advanced Drug Delivery Reviews.* Elsevier Science B.V.; 1997. p. 3–25.
120. Goldberg JS. Low Molecular Weight Opioid Peptide Esters Could be Developed as a New Class of Analgesics. *Perspect Medicin Chem.* 2011 Jan 25;5(5):PMC.S6803.
121. B. Fernandes T, C. F. Segretti M, C. Polli M, Parise-Filho R. Analysis of the Applicability and Use of Lipinski’s Rule for Central Nervous System Drugs. *Lett Drug Des Discov.* 2016 Oct 31;13(10):999–1006.
122. Yu YJ, Watts RJ. Developing Therapeutic Antibodies for Neurodegenerative Disease. Vol. 10, *Neurotherapeutics.* Springer; 2013. p. 459–72.

123. Zhao Z, Ukidve A, Kim J, Mitragotri S. Targeting Strategies for Tissue-Specific Drug Delivery. Vol. 181, Cell. Cell Press; 2020. p. 151–67.
124. Jefferies WA, Brandon MR, Hunt S V., Williams AF, Gatter KC, Mason DY. Transferrin receptor on endothelium of brain capillaries. *Nature*. 1984;312(5990):162–3.
125. Pardridge WM. Molecular Trojan horses for blood-brain barrier drug delivery. Vol. 6, *Current Opinion in Pharmacology*. *Curr Opin Pharmacol*; 2006. p. 494–500.
126. Friden PM, Walus LR, Musso GF, Taylor MA, Malfroy B, Starzyk RM. Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc Natl Acad Sci U S A*. 1991;88(11):4771–5.
127. Friden PM, Walus LR, Watson P, Doctrow SR, Kozarich JW, Bäckman C, et al. Blood-brain barrier penetration and in vivo activity of an NGF conjugate. *Science* (80-). 1993;259(5093):373–7.
128. Kordower JH, Charles V, Bayer R, Bartus RT, Putney S, Walus LR, et al. Intravenous administration of a transferrin receptor antibody-nerve growth factor conjugate prevents the degeneration of cholinergic striatal neurons in a model of Huntington disease. *Proc Natl Acad Sci U S A*. 1994 Sep 13;91(19):9077–80.
129. Wu D, Pardridge WM. Neuroprotection with noninvasive neurotrophin delivery to the brain. *Proc Natl Acad Sci U S A*. 1999 Jan 5;96(1):254–9.
130. Zhang Y, Pardridge WM. Blood-brain barrier targeting of BDNF improves motor function in rats with middle cerebral artery occlusion. *Brain Res*. 2006 Sep 21;1111(1):227–9.
131. Lee HJ, Engelhardt B, Lesley J, Bickel U, Pardridge WM. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. *J Pharmacol Exp Ther*. 2000 Mar;292(3):1048–52.
132. Moos T, Morgan EH. Restricted transport of anti-transferrin receptor antibody (OX26) through the blood-brain barrier in the rat. *J Neurochem*. 2001;79(1):119–29.
133. Hersom M, Helms HC, Pretzer N, Goldeman C, Jensen AI, Severin G, et al. Transferrin receptor expression and role in transendothelial transport of transferrin in cultured brain endothelial monolayers. *Mol Cell Neurosci*. 2016 Oct 1;76:59–67.
134. Yu YJ, Zhang Y, Kenrick M, Hoyte K, Luk W, Lu Y, et al. Boosting brain

- uptake of a therapeutic antibody by reducing its affinity for a transcytosis target. *Sci Transl Med.* 2011 May 25;3(84):84ra44.
135. Bourassa P, Alata W, Tremblay C, Paris-Robidas S, Calon F. Transferrin Receptor-Mediated Uptake at the Blood-Brain Barrier Is Not Impaired by Alzheimer's Disease Neuropathology. *Mol Pharm.* 2019 Feb 4;16(2):583–94.
 136. Davidson L, Lloyd K, Dankova J, Hornykiewicz O. L-DOPA treatment in Parkinson's disease: Effect on dopamine and related substances in discrete brain regions. *Experientia.* 1971 Sep;27(9):1048–9.
 137. Wade LA, Katzman R. Rat brain regional uptake and decarboxylation of l DOPA following carotid injection. *Am J Physiol.* 1975;228(2):352–9.
 138. Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, et al. The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain Res.* 2000 Oct 6;879(1–2):115–21.
 139. Bonina F, Puglia C, Rimoli MG, Melisi D, Boatto G, Nieddu M, et al. Glycosyl Derivatives of Dopamine and l -dopa as Anti-Parkinson Prodrugs: Synthesis, Pharmacological Activity and In Vitro Stability Studies. *J Drug Target.* 2003 Jan;11(1):25–36.
 140. Dalpiaz A, Filosa R, de Caprariis P, Conte G, Bortolotti F, Biondi C, et al. Molecular mechanism involved in the transport of a prodrug dopamine glycosyl conjugate. *Int J Pharm.* 2007 May 4;336(1):133–9.
 141. Battaglia G, La Russa M, Bruno V, Arenare L, Ippolito R, Copani A, et al. Systemically administered D-glucose conjugates of 7-chlorokynurenic acid are centrally available and exert anticonvulsant activity in rodents. *Brain Res.* 2000 Mar 31;860(1–2):149–56.
 142. Du D, Chang N, Sun S, Li M, Yu H, Liu M, et al. The role of glucose transporters in the distribution of p-aminophenyl- α -d-mannopyranoside modified liposomes within mice brain. *J Control Release.* 2014 May 28;182(1):99–110.
 143. Arora S, Sharma D, Singh J. GLUT-1: An Effective Target to Deliver Brain-Derived Neurotrophic Factor Gene across the Blood Brain Barrier. *ACS Chem Neurosci.* 2020 Jun 3;11(11):1620–33.
 144. Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, et al. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J Neurochem.* 2011;117(2):333–45.
 145. Kingwell K. Drug delivery: New targets for drug delivery across the BBB.

- Vol. 15, *Nature Reviews Drug Discovery*. Nature Publishing Group; 2016. p. 84.
146. Cai Z, Qiao PF, Wan CQ, Cai M, Zhou NK, Li Q. Role of Blood-Brain Barrier in Alzheimer's Disease. Vol. 63, *Journal of Alzheimer's Disease*. IOS Press; 2018. p. 1223–34.
147. Jiang X, Andjelkovic A V., Zhu L, Yang T, Bennett MVL, Chen J, et al. Blood-brain barrier dysfunction and recovery after ischemic stroke. Vols. 163–164, *Progress in Neurobiology*. Elsevier Ltd; 2018. p. 144–71.
148. Spencer JJ, Bell JS, DeLuca GC. Vascular pathology in multiple sclerosis: Reframing pathogenesis around the blood-brain barrier. Vol. 89, *Journal of Neurology, Neurosurgery and Psychiatry*. BMJ Publishing Group; 2018. p. 42–52.
149. Bhowmick S, D'Mello V, Caruso D, Wallerstein A, Abdul-Muneer PM. Impairment of pericyte-endothelium crosstalk leads to blood-brain barrier dysfunction following traumatic brain injury. *Exp Neurol*. 2019 Jul 1;317:260–70.
150. Zhong Z, Deane R, Ali Z, Parisi M, Shapovalov Y, O'Banion MK, et al. ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. *Nat Neurosci*. 2008 Apr;11(4):420–2.
151. Abbott NJ. Inflammatory Mediators and Modulation of Blood-Brain Barrier Permeability. Vol. 20, *Cellular and Molecular Neurobiology*. 2000.
152. Dinh KM, Kaspersen KA, Mikkelsen S, Pedersen OB, Petersen MS, Thøner LW, et al. Low-grade inflammation is negatively associated with physical Health-Related Quality of Life in healthy individuals: Results from the Danish Blood donor Study (DBDS). *PLoS One*. 2019 Mar 1;14(3).
153. Małkiewicz MA, Szarmach A, Sabisz A, Cubała WJ, Szurowska E, Winklewski PJ. Blood-brain barrier permeability and physical exercise. Vol. 16, *Journal of Neuroinflammation*. BioMed Central Ltd.; 2019.
154. Rönnbäck C, Hansson E. The importance and control of low-grade inflammation due to damage of cellular barrier systems that may lead to systemic inflammation. Vol. 10, *Frontiers in Neurology*. Frontiers Media S.A.; 2019. p. 533.
155. Cervellati C, Trentini A, Pecorelli A, Valacchi G. Inflammation in neurological disorders: the thin boundary between brain and periphery. *Antioxid Redox Signal*. 2020 Mar 6;1–57.

156. Calsolaro V, Edison P. Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Vol. 12, *Alzheimer's and Dementia*. Elsevier Inc.; 2016. p. 719–32.
157. Lochhead JJ, Ronaldson PT, Davis TP. Hypoxic Stress and Inflammatory Pain Disrupt Blood-Brain Barrier Tight Junctions: Implications for Drug Delivery to the Central Nervous System. *AAPS J*. 2017 Jul 1;19(4):910–20.
158. Varatharaj A, Galea I. The blood-brain barrier in systemic inflammation. *Brain Behav Immun*. 2017;60:1–12.
159. Banks WA, Gray AM, Erickson MA, Salameh TS, Damodarasamy M, Sheibani N, et al. Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J Neuroinflammation*. 2015 Nov 25;12(1):223.
160. Ortiz GG, Pacheco-Moisés FP, Bitzer-Quintero OK, Ramírez-Anguiano AC, Flores-Alvarado LJ, Ramírez-Ramírez V, et al. Immunology and oxidative stress in multiple sclerosis: clinical and basic approach. *Clin Dev Immunol*. 2013;2013:708659.
161. Yang L, Tan D, Piao H. Myelin Basic Protein Citrullination in Multiple Sclerosis: A Potential Therapeutic Target for the Pathology. *Neurochem Res*. 2016;
162. Hemmer B, Kerschensteiner M, Korn T. Role of the innate and adaptive immune responses in the course of multiple sclerosis. *Lancet Neurol*. 2015;14(4):406–19.
163. Harris VK, Sadiq SA. Biomarkers of Therapeutic Response in Multiple Sclerosis: Current Status. *Mol Diagn Ther*. 2014;605–17.
164. Liu M, Xiao L, Liu S, Hu Y, Tian J, Gao G, et al. Immunoregulation of myelin-specific CD4+ T cell response by neural stem/progenitor cells: Role of prostaglandin E2. *J Neuroimmunol*. 2013;255(1–2):32–8.
165. Mallucci G, Peruzzotti-Jametti L, Bernstock JD, Pluchino S. The role of immune cells, glia and neurons in white and gray matter pathology in multiple sclerosis. *Prog Neurobiol*. 2015;127–128:1–22.
166. Farias AS, Santos LMB. How can proteomics elucidate the complexity of multiple sclerosis? *Proteomics - Clin Appl*. 2015;9(9–10):844–7.
167. Cramer SP, Modvig S, Simonsen HJ, Frederiksen JL, Larsson HBW. Permeability of the blood-brain barrier predicts conversion from optic neuritis to multiple sclerosis. *Brain*. 2015 Sep;138(Pt 9):2571–83.

168. Bastianello S, Pozzilli C, Bernardi S, Bozzao L, Fantozzi LM, Buttinelli C, et al. Serial study of gadolinium-DTPA MRI enhancement in multiple sclerosis. *Neurology*. 1990 Apr;40(4):591–5.
169. Basivireddy J, Somvanshi RK, Romero IA, Weksler BB, Couraud PO, Oger J, et al. Somatostatin preserved blood brain barrier against cytokine induced alterations: Possible role in multiple sclerosis. *Biochem Pharmacol*. 2013;86(4):497–507.
170. Cook-Mills JM, Deem TL. Active participation of endothelial cells in inflammation. *J Leukoc Biol*. 2005;77(4):487.
171. Wu F, Liu L, Zhou H. Endothelial cell activation in central nervous system inflammation. *J Leukoc Biol*. 2017 May;101(5):1119–32.
172. Medala VK, Gollapelli B, Dewanjee S, Ogunmokun G, Kandimalla R, Vallamkondu J. Mitochondrial dysfunction, mitophagy, and role of dynamin-related protein 1 in Alzheimer’s disease. *J Neurosci Res*. 2021 Jan 19;jnr.24781.
173. Mayeux R, Stern Y. Epidemiology of Alzheimer disease. *Cold Spring Harb Perspect Med*. 2012;2(8).
174. Aisen PS, Cummings J, Jack CR, Morris JC, Sperling R, Frölich L, et al. On the path to 2025: Understanding the Alzheimer’s disease continuum. Vol. 9, *Alzheimer’s Research and Therapy*. BioMed Central Ltd.; 2017. p. 60.
175. Shea YF, Chu LW, Chan AOK, Ha J, Li Y, Song YQ. A systematic review of familial Alzheimer’s disease: Differences in presentation of clinical features among three mutated genes and potential ethnic differences. Vol. 115, *Journal of the Formosan Medical Association*. Elsevier B.V.; 2016. p. 67–75.
176. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer’s disease: Progress and problems on the road to therapeutics. Vol. 297, *Science*. Science; 2002. p. 353–6.
177. Zenaro E, Piacentino G, Constantin G. The blood-brain barrier in Alzheimer’s disease. Vol. 107, *Neurobiology of Disease*. Academic Press Inc.; 2017. p. 41–56.
178. Stewart CR, Stuart LM, Wilkinson K, Van Gils JM, Deng J, Halle A, et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol*. 2010 Feb;11(2):155–61.
179. Heneka MT, Carson MJ, Khoury J El, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer’s disease. Vol. 14, *The Lancet*

- Neurology. Lancet Publishing Group; 2015. p. 388–405.
180. Osborn LM, Kamphuis W, Wadman WJ, Hol EM. Astrogliosis: An integral player in the pathogenesis of Alzheimer’s disease. Vol. 144, Progress in Neurobiology. Elsevier Ltd; 2016. p. 121–41.
 181. Tarkowski E, Andreasen N, Tarkowski A, Blennow K. Intrathecal inflammation precedes development of Alzheimer’s disease. *J Neurol Neurosurg Psychiatry*. 2003 Sep 1;74(9):1200–5.
 182. Zenaro E, Pietronigro E, Bianca V Della, Piacentino G, Marongiu L, Budui S, et al. Neutrophils promote Alzheimer’s disease-like pathology and cognitive decline via LFA-1 integrin. *Nat Med*. 2015 Aug 8;21(8):880–6.
 183. Ferretti MT, Merlini M, Späni C, Gericke C, Schweizer N, Enzmann G, et al. T-cell brain infiltration and immature antigen-presenting cells in transgenic models of Alzheimer’s disease-like cerebral amyloidosis. *Brain Behav Immun*. 2016 May 1;54:211–25.
 184. Janelidze S, Mattsson N, Stomrud E, Lindberg O, Palmqvist S, Zetterberg H, et al. CSF biomarkers of neuroinflammation and cerebrovascular dysfunction in early Alzheimer disease. *Neurology*. 2018 Aug 28;91(9):e867–77.
 185. Stephenson J, Nutma E, van der Valk P, Amor S. Inflammation in CNS neurodegenerative diseases. Vol. 154, Immunology. Blackwell Publishing Ltd; 2018. p. 204–19.
 186. Arrowsmith CH, Bountra C, Fish P V, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov*. 2012;11(5):384–400.
 187. Cavalli G, Heard E. Advances in epigenetics link genetics to the environment and disease. Vol. 571, Nature. Nature Publishing Group; 2019. p. 489–99.
 188. Tough DF, Tak PP, Tarakhovsky A, Prinjha RK. Epigenetic drug discovery: breaking through the immune barrier. *Nat Rev Drug Discov*. 2016;
 189. Qureshi IA, Mehler MF. Advances in epigenetics and epigenomics for neurodegenerative diseases. *Curr Neurol Neurosci Rep*. 2011;11(5):464–73.
 190. Szyf M. Prospects for the development of epigenetic drugs for CNS conditions. *Nat Rev Drug Discov*. 2015;14(7):461–74.
 191. Kim S, Kaang BK. Epigenetic regulation and chromatin remodeling in learning and memory. Vol. 49, Experimental and Molecular Medicine. Nature Publishing Group; 2017. p. 281.

192. Guo JU, Ma DK, Mo H, Ball MP, Jang M-H, Bonaguidi MA, et al. Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nat Neurosci.* 2011;14(10):1345–51.
193. Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature.* 2013;502(7472):472–9.
194. Helgudottir SS, Routh LJ, Burkhart A, Jönsson K, Pedersen IS, Lichota J, et al. Epigenetic Regulation of Ferroportin in Primary Cultures of the Rat Blood-Brain Barrier. *Mol Neurobiol.* 2020;57(8):3526–39.
195. Yao B, Christian KM, He C, Jin P, Ming G, Song H. Epigenetic mechanisms in neurogenesis. *Nat Rev Neurosci.* 2016;17(9):537–49.
196. Hwang JY, Aromolaran KA, Zukin RS. The emerging field of epigenetics in neurodegeneration and neuroprotection. Vol. 18, *Nature Reviews Neuroscience.* Nature Publishing Group; 2017. p. 347–61.
197. Bowman GD, Poirier MG. Post-translational modifications of histones that influence nucleosome dynamics. Vol. 115, *Chemical Reviews.* American Chemical Society; 2015. p. 2274–95.
198. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone deacetylase inhibitors as anticancer drugs. Vol. 18, *International Journal of Molecular Sciences.* MDPI AG; 2017.
199. Bhatti UF, Williams AM, Georgoff PE, Alam HB. The ‘Omics’ of Epigenetic Modulation by Valproic Acid Treatment in Traumatic Brain Injury—What We Know and What the Future Holds. Vol. 13, *Proteomics - Clinical Applications.* Wiley-VCH Verlag; 2019.
200. Ornoy A, Becker M, Weinstein-Fudim L, Ergaz Z. S-adenosine methionine (SAME) and valproic acid (VPA) as epigenetic modulators: Special emphasis on their interactions affecting nervous tissue during pregnancy. Vol. 21, *International Journal of Molecular Sciences.* MDPI AG; 2020.
201. Bertelsen F, Landau AM, Vase KH, Jacobsen J, Scheel-Krüger J, Møller A. Acute in vivo effect of valproic acid on the GABAergic system in rat brain: A [11C]Ro15-4513 microPET study. *Brain Res.* 2018 Feb 1;1680:110–4.
202. Ying G, Jing C, Li J, Wu C, Yan F, Chen J, et al. Neuroprotective Effects of Valproic Acid on Blood-Brain Barrier Disruption and Apoptosis-Related Early Brain Injury in Rats Subjected to Subarachnoid Hemorrhage Are Modulated by Heat Shock Protein 70/Matrix Metalloproteinases and Heat Shock Protein 70/AKT Pathways. *Neurosurgery.* 2016 Aug;79(2):286–95.

203. Wang Z, Leng Y, Tsai L-K, Leeds P, Chuang D-M. Valproic Acid Attenuates Blood–Brain Barrier Disruption in a Rat Model of Transient Focal Cerebral Ischemia: The Roles of HDAC and MMP-9 Inhibition. *J Cereb Blood Flow Metab.* 2011 Jan 27;31(1):52–7.
204. Noh H, Seo H. Age-dependent effects of valproic acid in Alzheimer’s disease (AD) mice are associated with nerve growth factor (NGF) regulation. *Neuroscience.* 2014 Apr 25;266:255–65.
205. Chang P, Williams AM, Bhatti UF, Biesterveld BE, Liu B, Nikolian VC, et al. Valproic Acid and Neural Apoptosis, Inflammation, and Degeneration 30 Days after Traumatic Brain Injury, Hemorrhagic Shock, and Polytrauma in a Swine Model. *J Am Coll Surg.* 2019 Mar 1;228(3):265–75.
206. Nikolian VC, Dekker SE, Bambakidis T, Higgins GA, Denny IS, Georgoff PE, et al. Improvement of Blood-Brain Barrier Integrity in Traumatic Brain Injury and Hemorrhagic Shock Following Treatment With Valproic Acid and Fresh Frozen Plasma. *Crit Care Med.* 2018 Jan;46(1):e59–66.
207. Nikolian VC, Denny IS, Higgins GA, Williams AM, Weykamp M, Georgoff PE, et al. Transcriptomic changes following valproic acid treatment promote neurogenesis and minimize secondary brain injury. In: *Journal of Trauma and Acute Care Surgery.* Lippincott Williams and Wilkins; 2018. p. 459–65.
208. de Aquino CC, Leitão RA, Alves LAO, Coelho-Santos V, Guerrant RL, Ribeiro CF, et al. Effect of hypoproteic and high-fat diets on hippocampal blood-brain barrier permeability and oxidative stress. *Front Nutr.* 2019 Jan 9;5:131.
209. Salameh TS, Mortell WG, Logsdon AF, Butterfield DA, Banks WA. Disruption of the hippocampal and hypothalamic blood-brain barrier in a diet-induced obese model of type II diabetes: prevention and treatment by the mitochondrial carbonic anhydrase inhibitor, topiramate. *Fluids Barriers CNS.* 2019 Jan 8;16(1):1.
210. Yamamoto M, Guo D-H, Hernandez CM, Stranahan AM. Endothelial Adora2a Activation Promotes Blood-Brain Barrier Breakdown and Cognitive Impairment in Mice with Diet-Induced Insulin Resistance. *J Neurosci.* 2019 May 22;39(21):4179–92.
211. Andrews MT, Russeth KP, Drewes LR, Henry P-G. Adaptive mechanisms regulate preferred utilization of ketones in the heart and brain of a hibernating mammal during arousal from torpor. *Am J Physiol Regul Integr Comp Physiol.* 2009 Feb;296(2):R383-93.

212. Tanegashima K, Sato-Miyata Y, Funakoshi M, Nishito Y, Aigaki T, Hara T. Epigenetic regulation of the glucose transporter gene *Slc2a1* by β -hydroxybutyrate underlies preferential glucose supply to the brain of fasted mice. *Genes to Cells*. 2017 Jan 1;22(1):71–83.
213. Veys K, Fan Z, Ghobrial M, Bouché A, García-Caballero M, Vriens K, et al. Role of the GLUT1 Glucose Transporter in Postnatal CNS Angiogenesis and Blood-Brain Barrier Integrity. *Circ Res*. 2020 Jul 31;127(4):466–82.
214. Omori K, Tachikawa M, Hirose S, Taii A, Akanuma S-I, Hosoya K-I, et al. Developmental changes in transporter and receptor protein expression levels at the rat blood-brain barrier based on quantitative targeted absolute proteomics. 2019;
215. Liu Y, Liu F, Iqbal K, Grundke-Iqbal I, Gong CX. Decreased glucose transporters correlate to abnormal hyperphosphorylation of tau in Alzheimer disease. *FEBS Lett*. 2008 Jan 23;582(2):359–64.
216. Mooradian AD, Chung HC, Shah GN. GLUT-1 expression in the cerebra of patients with Alzheimer's disease. *Neurobiol Aging*. 1997 Sep;18(5):469–74.
217. Simpson IA, Chundu KR, Davies-Hill T, Honer WG, Davies P. Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. *Ann Neurol*. 1994;35(5):546–51.
218. Liggett T, Melnikov A, Tilwalli S, Yi Q, Chen H, Replogle C, et al. Methylation patterns of cell-free plasma DNA in relapsing-remitting multiple sclerosis. *J Neurol Sci*. 2010 Mar 15;290(1–2):16–21.
219. Choi JY, Yoon SS, Kim SE, Ahn Jo S. KDM4B histone demethylase and G9a regulate expression of vascular adhesion proteins in cerebral microvessels. *Sci Rep*. 2017 Mar 22;7.
220. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. *J Biomed Sci*. 2017 Aug 24;24(1):60.
221. Nava Catorce M, Gevorkian G. LPS-induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-clinical Assessment of Nutraceuticals. *Curr Neuropharmacol*. 2016 Feb 24;14(2):155–64.
222. Layé S, Parnet P, Goujon E, Dantzer R. Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice. *Mol Brain Res*. 1994;27(1):157–62.
223. Nishioku T, Dohgu S, Takata F, Eto T, Ishikawa N, Kodama KB, et al.

- Detachment of brain pericytes from the basal lamina is involved in disruption of the blood-brain barrier caused by lipopolysaccharide-induced sepsis in mice. *Cell Mol Neurobiol.* 2009 May;29(3):309–16.
224. Czapski GA, Gajkowska B, Strosznajder JB. Systemic administration of lipopolysaccharide induces molecular and morphological alterations in the hippocampus. *Brain Res.* 2010 Oct 14;1356:85–94.
 225. Lopes PC. LPS and neuroinflammation: a matter of timing. *Inflammopharmacology.* 2016 Oct 1;24(5):291–3.
 226. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Vol. 1, *Cellular and Molecular Life Sciences.* Springer Science and Business Media Deutschland GmbH; 2020. p. 3.
 227. Nazem A, Sankowski R, Bacher M, Al-Abed Y. Rodent models of neuroinflammation for Alzheimer’s disease. Vol. 12, *Journal of Neuroinflammation.* BioMed Central Ltd.; 2015. p. 1–15.
 228. Gorina R, Font-Nieves M, Márquez-Kisinousky L, Santalucia T, Planas AM. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. *Glia.* 2011 Feb;59(2):242–55.
 229. Fiebich BL, Batista CRA, Saliba SW, Yousif NM, de Oliveira ACP. Role of microglia TLRs in neurodegeneration. *Front Cell Neurosci.* 2018 Oct 2;12:329.
 230. Cazareth J, Guyon A, Heurteaux C, Chabry J, Petit-Paitel A. Molecular and cellular neuroinflammatory status of mouse brain after systemic lipopolysaccharide challenge: Importance of CCR2/CCL2 signaling. *J Neuroinflammation.* 2014 Jul 28;11(1).
 231. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia.* 2007 Apr 1;55(5):453–62.
 232. Meneses G, Rosetti M, Espinosa A, Florentino A, Bautista M, Díaz G, et al. Recovery from an acute systemic and central LPS-inflammation challenge is affected by mouse sex and genetic background. *PLoS One.* 2018 Aug 1;13(8).
 233. Soudi S, Zavarán-Hosseini A, Hassan ZM, Soleimani M, Adegani FJ, Hashemi SM. Comparative study of the effect of LPS on the function of BALB/c and C57BL/6 peritoneal macrophages. *Cell J.* 2013 Mar;15(1):45–54.

234. Magaña-Guerrero FS, Quiroz-Mercado J, Garfias-Zenteno N, Garfias Y. Comparative analysis of inflammatory response in the BALB/c and C57BL/6 mouse strains in an endotoxin-induced uveitis model. *J Immunol Methods*. 2020 Jan 1;476.
235. Mello BSF, Chaves Filho AJM, Custódio CS, Cordeiro RC, Miyajima F, de Sousa FCF, et al. Sex influences in behavior and brain inflammatory and oxidative alterations in mice submitted to lipopolysaccharide-induced inflammatory model of depression. *J Neuroimmunol*. 2018 Jul 15;320:133–42.
236. Maggioli E, McArthur S, Mauro C, Kieswich J, Kusters DHM, Reutelingsperger CPM, et al. Estrogen protects the blood–brain barrier from inflammation-induced disruption and increased lymphocyte trafficking. *Brain Behav Immun*. 2016 Jan 1;51:212–22.
237. Millett CE, Phillips BE, Saunders EFH. The Sex-specific Effects of LPS on Depressive-like Behavior and Oxidative Stress in the Hippocampus of the Mouse. *Neuroscience*. 2019 Feb 10;399:77–88.
238. Makinson R, Lloyd K, Rayasam A, McKee S, Brown A, Barila G, et al. Intrauterine inflammation induces sex-specific effects on neuroinflammation, white matter, and behavior. *Brain Behav Immun*. 2017 Nov 1;66:277–88.
239. Fish EN. The X-files in immunity: Sex-based differences predispose immune responses. Vol. 8, *Nature Reviews Immunology*. 2008. p. 737–44.
240. Lasselin J, Lekander M, Axelsson J, Karshikoff B. Sex differences in how inflammation affects behavior: What we can learn from experimental inflammatory models in humans. Vol. 50, *Frontiers in Neuroendocrinology*. Academic Press Inc.; 2018. p. 91–106.
241. Cai KC, van Mil S, Murray E, Mallet JF, Matar C, Ismail N. Age and sex differences in immune response following LPS treatment in mice. *Brain Behav Immun*. 2016 Nov 1;58:327–37.
242. Go M, Kou J, Lim JE, Yang J, Fukuchi K ichiro. Microglial response to LPS increases in wild-type mice during aging but diminishes in an Alzheimer’s mouse model: Implication of TLR4 signaling in disease progression. *Biochem Biophys Res Commun*. 2016 Oct 14;479(2):331–7.
243. Mouton PR, Kelley-Bell B, Tweedie D, Spangler EL, Perez E, Carlson OD, et al. The effects of age and lipopolysaccharide (LPS)-mediated peripheral inflammation on numbers of central catecholaminergic neurons. *Neurobiol Aging*. 2012;33(2):423.e27-423.e36.

244. Gaekwad J, Zhang Y, Zhang W, Reeves J, Wolfert MA, Boons GJ. Differential induction of innate immune responses by synthetic lipid derivatives. *J Biol Chem*. 2010 Sep 17;285(38):29375–86.
245. Espinosa-Oliva AM, De Pablos RM, Herrera AJ. Intracranial Injection of LPS in Rat as Animal Model of Neuroinflammation. *Methods Mol Biol*. 2013;1041:295–305.
246. Miyazaki S, Ishikawa F, Fujikawa T, Nagata S, Yamaguchi K. Intraperitoneal injection of lipopolysaccharide induces dynamic migration of Gr-1high polymorphonuclear neutrophils in the murine abdominal cavity. *Clin Diagn Lab Immunol*. 2004 May;11(3):452–7.
247. Somann JP, Wasilczuk KM, Neihouser K V., Sturgis J, Albors GO, Robinson JP, et al. Characterization of plasma cytokine response to intraperitoneally administered LPS & subdiaphragmatic branch vagus nerve stimulation in rat model. Tache Y, editor. *PLoS One*. 2019 Mar 28;14(3):e0214317.
248. Zanetti G, Heumann D, Gérard J, Kohler J, Abbet P, Barras C, et al. Cytokine production after intravenous or peritoneal gram-negative bacterial challenge in mice. Comparative protective efficacy of antibodies to tumor necrosis factor-alpha and to lipopolysaccharide. *J Immunol*. 1992 Mar 15;148(6):1890–7.
249. Copeland S, Shaw Warren H, Lowry SF, Galvano SE, Remick D. Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol*. 2005 Jan 1;12(1):60–7.
250. François A, Terro F, Quellard N, Fernandez B, Chassaing D, Janet T, et al. Impairment of autophagy in the central nervous system during lipopolysaccharide-induced inflammatory stress in mice. *Mol Brain*. 2014 Aug 27;7(1).
251. Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. *FASEB J*. 2005 Aug;19(10):1329–31.
252. Biesmans S, Meert TF, Bouwknecht JA, Acton PD, Davoodi N, De Haes P, et al. Systemic immune activation leads to neuroinflammation and sickness behavior in mice. *Mediators Inflamm*. 2013;2013.
253. Soromou LW, Jiang L, Wei M, Chen N, Huo M, Chu X, et al. Protection of mice against lipopolysaccharide-induced endotoxic shock by pinocembrin is correlated with regulation of cytokine secretion . *J Immunotoxicol*. 2014;11(1):56–61.

254. Fisher AB, Dodia C, Chatterjee S, Feinstein SI. A peptide inhibitor of naph oxidase (Nox2) activation markedly decreases mouse lung injury and mortality following administration of lipopolysaccharide (lps). *Int J Mol Sci*. 2019 May 2;20(10).
255. Li Q, Li L, Fei X, Zhang Y, Qi C, Hua S, et al. Inhibition of autophagy with 3-methyladenine is protective in a lethal model of murine endotoxemia and polymicrobial sepsis. *Innate Immun*. 2018 May 1;24(4):231–9.
256. Dinges MM, Schlievert PM. Comparative analysis of lipopolysaccharide-induced tumor necrosis factor alpha activity in serum and lethality in mice and rabbits pretreated with the staphylococcal superantigen toxic shock syndrome toxin 1. *Infect Immun*. 2001;69(11):7169–72.
257. Ifuku M, Katafuchi T, Mawatari S, Noda M, Miake K, Sugiyama M, et al. Anti-inflammatory/anti-amyloidogenic effects of plasmalogens in lipopolysaccharide-induced neuroinflammation in adult mice. *J Neuroinflammation*. 2012 Aug 13;9.
258. Zhao J, Bi W, Xiao S, Lan X, Cheng X, Zhang J, et al. Neuroinflammation induced by lipopolysaccharide causes cognitive impairment in mice. *Sci Rep*. 2019;9(1):1–12.
259. Wang D, Liu Y, Zhao YR, Zhou JL. Low dose of lipopolysaccharide pretreatment can alleviate the inflammatory response in wound infection mouse model. *Chinese J Traumatol - English Ed*. 2016 Aug 1;19(4):193–8.
260. Helms HC, Abbott J, Burek M, Cecchelli R, Couraud P-O, Deli MA, et al. In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use.
261. Reichel A, Begley DJ, Abbott NJ. An overview of in vitro techniques for blood-brain barrier studies. Vol. 89, *Methods in molecular medicine*. 2003. p. 307–24.
262. Siddharthan V, Kim Y V., Liu S, Kim KS. Human astrocytes/astrocyte-conditioned medium and shear stress enhance the barrier properties of human brain microvascular endothelial cells. *Brain Res*. 2007 May 25;1147(1):39–50.
263. Sivandzade F, Cucullo L. In-vitro blood–brain barrier modeling: A review of modern and fast-advancing technologies. Vol. 38, *Journal of Cerebral Blood Flow and Metabolism*. SAGE Publications Ltd; 2018. p. 1667–81.
264. Thomsen MS, Birkelund S, Burkhart A, Stensballe A, Moos T. Synthesis and deposition of basement membrane proteins by primary brain capillary

- endothelial cells in a murine model of the blood-brain barrier. *J Neurochem*. 2017 Mar 1;140(5):741–54.
265. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER Measurement Techniques for In Vitro Barrier Model Systems. Vol. 20, *Journal of Laboratory Automation*. SAGE Publications Inc.; 2015. p. 107–26.
266. Elbrecht DH, Long CJ, Hickman JJ. Transepithelial/endothelial Electrical Resistance (TEER) theory and applications for microfluidic body-on-a-chip devices. Vol. 1, *J Rare Dis Res Treat*. 2016.
267. Igarashi Y, Utsumi H, Chiba H, Yamada-Sasamori Y, Tobioka H, Kamimura Y, et al. Glial cell line-derived neurotrophic factor induces barrier function of endothelial cells forming the blood-brain barrier. *Biochem Biophys Res Commun*. 1999 Jul 22;261(1):108–12.
268. Merwin JR, Anderson JM, Kocher O, Van Itallie CM, Madri JA. Transforming growth factor beta 1 modulates extracellular matrix organization and cell-cell junctional complex formation during in vitro angiogenesis. *J Cell Physiol*. 1990 Jan;142(1):117–28.
269. Niranjana R. Recent advances in the mechanisms of neuroinflammation and their roles in neurodegeneration. Vol. 120, *Neurochemistry International*. Elsevier Ltd; 2018. p. 13–20.
270. Coisne C, Dehouck L, Faveeuw C, Delplace Y, Miller F, Landry C, et al. Mouse syngenic in vitro blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. *Lab Invest*. 2005;85:734–46.
271. Hultman K, Björklund U, Hansson E, Jern C. Potentiating effect of endothelial cells on astrocytic plasminogen activator inhibitor type-1 gene expression in an in vitro model of the blood-brain barrier. *Neuroscience*. 2010 Mar 17;166(2):408–15.
272. Poetsch V, Neuhaus W, Noe CR. Serum-derived immunoglobulins neutralize adverse effects of amyloid- β peptide on the integrity of a blood-brain barrier in vitro model. *J Alzheimer's Dis*. 2010;21(1):303–14.
273. Haileselassie B, Joshi AU, Minhas PS, Mukherjee R, Andreasson KI, Mochly-Rosen D. Mitochondrial dysfunction mediated through dynamin-related protein 1 (Drp1) propagates impairment in blood brain barrier in septic encephalopathy. *J Neuroinflammation*. 2020 Jan 27;17(1).
274. Alkabi S, Basivireddy J, Zhou L, Roskams J, Rieckmann P, Quandt JA. SPARC expression by cerebral microvascular endothelial cells in vitro and its influence on blood-brain barrier properties. *J Neuroinflammation*. 2016 Aug

31;13(1).

275. Gaillard PJ, De Boer AG, Breimer DD. Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro. *Microvasc Res.* 2003 Jan;65(1):24–31.
276. de Vries HE, Blom-Roosemalen MC, de Boer AG, van Berkel TJ, Breimer DD, Kuiper J. Effect of endotoxin on permeability of bovine cerebral endothelial cell layers in vitro. *J Pharmacol Exp Ther.* 1996 Jun;277(3):1418–23.
277. Tarassishin L, Suh HS, Lee SC. LPS and IL-1 differentially activate mouse and human astrocytes: Role of CD14. *Glia.* 2014;62(6):999–1013.
278. Gynther M, Puris E, Peltokangas S, Auriola S, Kanninen KM, Koistinaho J, et al. Alzheimer’s Disease Phenotype or Inflammatory Insult Does Not Alter Function of L-Type Amino Acid Transporter 1 in Mouse Blood-Brain Barrier and Primary Astrocytes. *Pharm Res.* 2019 Jan 1;36(1):17.
279. De Vries HE, Blom-Roosemalen MCM, Van Oosten M, De Boer AG, Van Berkel TJC, Breimer DD, et al. The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J Neuroimmunol.* 1996;64(1):37–43.
280. Derfuss T, Mehling M, Papadopoulou A, Bar-Or A, Cohen JA, Kappos L. Advances in oral immunomodulating therapies in relapsing multiple sclerosis. Vol. 19, *The Lancet Neurology.* Lancet Publishing Group; 2020. p. 336–47.
281. Jacobs BM, Ammoscato F, Giovannoni G, Baker D, Schmierer K. Cladribine: Mechanisms and mysteries in multiple sclerosis. *J Neurol Neurosurg Psychiatry.* 2018 Jul 10;89(12):1266–71.
282. Hutchinson M. Natalizumab: A new treatment for relapsing remitting multiple sclerosis. Vol. 3, *Therapeutics and Clinical Risk Management.* Dove Press; 2007. p. 259–68.
283. Gholamzad M, Ebtekar M, Ardestani MS, Azimi M, Mahmodi Z, Mousavi MJ, et al. A comprehensive review on the treatment approaches of multiple sclerosis: currently and in the future. Vol. 68, *Inflammation Research.* Birkhauser Verlag AG; 2019. p. 25–38.
284. Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer’s disease drug development pipeline: 2020. *Alzheimer’s Dement Transl Res Clin Interv.* 2020;6(1).
285. Mulvihill JJ, Cunnane EM, Ross AM, Duskey JT, Tosi G, Grabrucker AM. Drug delivery across the blood–brain barrier: recent advances in the use of

- nanocarriers. *Nanomedicine*. 2020 Jan 9;15(2):205–14.
286. Mohamed M, Abu Lila AS, Shimizu T, Alaaeldin E, Hussein A, Sarhan HA, et al. PEGylated liposomes: immunological responses. Vol. 20, *Science and Technology of Advanced Materials*. Taylor and Francis Ltd.; 2019. p. 710–24.
 287. Johnsen KB, Bak M, Melander F, Thomsen MS, Burkhart A, Kempen PJ, et al. Modulating the antibody density changes the uptake and transport at the blood-brain barrier of both transferrin receptor-targeted gold nanoparticles and liposomal cargo. *J Control Release*. 2019 Feb 10;295:237–49.
 288. Johnsen KB, Bak M, Kempen PJ, Melander F, Burkhart A, Thomsen MS, et al. Antibody affinity and valency impact brain uptake of transferrin receptor-targeted gold nanoparticles. *Theranostics*. 2018;8(12):3416–36.
 289. Niewoehner J, Bohrmann B, Collin L, Urich E, Sade H, Maier P, et al. Increased Brain Penetration and Potency of a Therapeutic Antibody Using a Monovalent Molecular Shuttle. *Neuron*. 2014 Jan 8;81(1):49–60.
 290. Klasse PJ. How to assess the binding strength of antibodies elicited by vaccination against HIV and other viruses. Vol. 15, *Expert Review of Vaccines*. Taylor and Francis Ltd; 2016. p. 295–311.
 291. Do TM, Capdevila C, Pradier L, Blanchard V, Lopez-Grancha M, Schussler N, et al. Tetravalent Bispecific Tandem Antibodies Improve Brain Exposure and Efficacy in an Amyloid Transgenic Mouse Model. *Mol Ther - Methods Clin Dev*. 2020 Dec 11;19:58–77.
 292. Hultqvist G, Syvänen S, Fang XT, Lannfelt L, Sehlin D. Bivalent brain shuttle increases antibody uptake by monovalent binding to the transferrin receptor. *Theranostics*. 2017;7(2):308–18.
 293. A Single Ascending Dose Study to Investigate the Safety, Tolerability, Immunogenicity and Pharmacokinetics of Intravenously Administered RO7126209 in Healthy Participants - No Study Results Posted - *ClinicalTrials.gov* [Internet]. [cited 2021 Feb 21]. Available from: <https://clinicaltrials.gov/ct2/show/results/NCT04023994?term=RO+7126209&draw=2&rank=2>
 294. Brainshuttle AD: A Multiple Ascending Dose Study to Investigate the Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of RO7126209 Following Intravenous Infusion in Participants With Prodromal or Mild to Moderate Alzheimer's Disease - Full Text View - *ClinicalTrials.gov* [Internet]. [cited 2021 Feb 21]. Available from: <https://clinicaltrials.gov/ct2/show/NCT04639050?term=RO+7126209&draw>

=2&rank=1

295. Jarvis L. Denali Therapeutics unveils blood-brain barrier delivery system [Internet]. [cited 2021 Feb 21]. Available from: <https://cen.acs.org/biological-chemistry/neuroscience/Denali-Therapeutics-unveils-blood-brain/98/web/2020/05>
296. Ullman JC, Arguello A, Getz JA, Bhalla A, Mahon CS, Wang J, et al. Brain delivery and activity of a lysosomal enzyme using a blood-brain barrier transport vehicle in mice. *Sci Transl Med.* 2020 May 27;12(545).
297. Kariolis MS, Wells RC, Getz JA, Kwan W, Mahon CS, Tong R, et al. Brain delivery of therapeutic proteins using an Fc fragment blood-brain barrier transport vehicle in mice and monkeys. *Sci Transl Med.* 2020 May 27;12(545).
298. A Study of DNL310 in Pediatric Subjects With Hunter Syndrome - Full Text View - ClinicalTrials.gov [Internet]. [cited 2021 Feb 21]. Available from: <https://clinicaltrials.gov/ct2/show/NCT04251026>
299. De Simone A, Milelli A. Histone Deacetylase Inhibitors as Multitarget Ligands: New Players in Alzheimer's Disease Drug Discovery? Vol. 14, *ChemMedChem.* John Wiley and Sons Ltd; 2019. p. 1067–73.
300. Cuadrado-Tejedor M, Garcia-Barroso C, Sánchez-Arias JA, Rabal O, Pérez-González M, Mederos S, et al. A First-in-Class Small-Molecule that Acts as a Dual Inhibitor of HDAC and PDE5 and that Rescues Hippocampal Synaptic Impairment in Alzheimer's Disease Mice. *Neuropsychopharmacology.* 2017 Jan 1;42(2):524–39.
301. You D, Richardson JR, Aleksunes LM. Epigenetic Regulation of Multidrug Resistance Protein 1 and Breast Cancer Resistance Protein Transporters by Histone Deacetylase Inhibition. Vol. 48, *Drug Metabolism and Disposition.* American Society for Pharmacology and Experimental Therapy; 2020. p. 459–80.
302. Ni X, Li L, Pan G. HDAC inhibitor-induced drug resistance involving ATP-binding cassette transporters (Review). *Oncol Lett.* 2015 Feb 1;9(2):515–21.
303. You D, Shin HM, Mosaad F, Richardson JR, Aleksunes LM. Brain region-specific regulation of histone acetylation and efflux transporters in mice. *J Biochem Mol Toxicol.* 2019 Mar 21;e22318.
304. Zhao Q, Zhang F, Yu Z, Guo S, Liu N, Jiang Y, et al. HDAC3 inhibition prevents blood-brain barrier permeability through Nrf2 activation in type 2 diabetes male mice. *J Neuroinflammation.* 2019 May 17;16(1).

305. Winkler EA, Nishida Y, Sagare AP, Rege S V., Bell RD, Perlmutter D, et al. GLUT1 reductions exacerbate Alzheimer's disease vasculo-neuronal dysfunction and degeneration. *Nat Neurosci.* 2015 Apr 28;18(4):521–30.
306. Viña J, Lloret A. Why women have more Alzheimer's disease than men: Gender and mitochondrial toxicity of amyloid- β peptide. Vol. 20, *Journal of Alzheimer's Disease.* IOS Press; 2010.
307. Anderson GJ, Frazer DM. Current understanding of iron homeostasis. In: *American Journal of Clinical Nutrition.* Oxford University Press; 2017. p. 1559S-1566S.
308. Ndayisaba A, Kaindlstorfer C, Wenning GK. Iron in neurodegeneration - Cause or consequence? Vol. 13, *Frontiers in Neuroscience.* Frontiers Media S.A.; 2019. p. 180.
309. Bien-Ly N, Boswell CA, Jeet S, Beach TG, Hoyte K, Luk W, et al. Lack of Widespread BBB Disruption in Alzheimer's Disease Models: Focus on Therapeutic Antibodies. *Neuron.* 2015 Oct 21;88(2):289–97.
310. Zhao Z, Nelson AR, Betsholtz C, Zlokovic B V. Establishment and Dysfunction of the Blood-Brain Barrier. *Cell.* 2015;163(5):1064–78.
311. Lochhead JJ, Yang J, Ronaldson PT, Davis TP. Structure, Function, and Regulation of the Blood-Brain Barrier Tight Junction in Central Nervous System Disorders. Vol. 11, *Frontiers in Physiology.* Frontiers Media S.A.; 2020.
312. Cardoso FL, Kittel Á, Veszelka S, Palmela I, Tóth A, Brites D, et al. Exposure to lipopolysaccharide and/or unconjugated bilirubin impair the integrity and function of brain microvascular endothelial cells. *PLoS One.* 2012 May 7;7(5).
313. Becher B, Spath S, Goverman J. Cytokine networks in neuroinflammation. Vol. 17, *Nature Reviews Immunology.* Nature Publishing Group; 2017. p. 49–59.
314. Chang R, Knox J, Chang J, Derbedrossian A, Vasilevko V, Cribbs D, et al. Blood-Brain Barrier Penetrating Biologic TNF- α Inhibitor for Alzheimer's Disease. *Mol Pharm.* 2017 Jul 3;14(7):2340–9.
315. Okuda Y, Sakoda S, Bernard CCA, Fujimura H, Saeki Y, Kishimoto T, et al. IL-6-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein. *Int Immunol.* 1998;10(5):703–8.

316. Zhang W, Liu QY, Haqqani AS, Leclerc S, Liu Z, Fauteux F, et al. Differential expression of receptors mediating receptor-mediated transcytosis (RMT) in brain microvessels, brain parenchyma and peripheral tissues of the mouse and the human. *Fluids Barriers CNS*. 2020 Jul 22;17(1).
317. Todorich B, Zhang X, Slagle-Webb B, Seaman WE, Connor JR. Tim-2 is the receptor for H-ferritin on oligodendrocytes. *J Neurochem*. 2008;107(6):1495–505.
318. Cheli VT, Correale J, Paez PM, Pasquini JM. Iron Metabolism in Oligodendrocytes and Astrocytes, Implications for Myelination and Remyelination. Vol. 12, *ASN Neuro*. SAGE Publications Inc.; 2020.
319. Cerqueira SR, Ayad NG, Lee JK. Neuroinflammation Treatment via Targeted Delivery of Nanoparticles. Vol. 14, *Frontiers in Cellular Neuroscience*. Frontiers Media S.A.; 2020. p. 329.
320. Miller DH, Khan OA, Sheremata WA, Blumhardt LD, Rice GPA, Libonati MA, et al. A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med*. 2003 Jan 2;348(1):15–23.
321. Marcos-Contreras OA, Greineder CF, Kiseleva RY, Parhiz H, Walsh LR, Zuluaga-Ramirez V, et al. Selective targeting of nanomedicine to inflamed cerebral vasculature to enhance the blood–brain barrier. Vol. 117, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences; 2020. p. 3405–14.

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