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BLOOD-BRAIN BARRIER-DIRECTED GENE THERAPY AS A STRATEGY TO TREAT NIEMANN-PICK TYPE C2 DISEASE

BY
CHARLOTTE LAURFELT MUNCH RASMUSSEN

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

BLOOD-BRAIN BARRIER-DIRECTED GENE THERAPY AS A STRATEGY TO TREAT NIEMANN-PICK TYPE C2 DISEASE

PHD DISSERTATION

by

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AALBORG UNIVERSITY
DENMARK

Dissertation submitted December 30, 2022

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Professional experience

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Rasmussen CLM, Hede E, Routhe LR, Körbelin J, Helgudottir SS, Thomsen LB, Schwaninger M, Burkhart A, Moos T: A novel strategy for delivering Niemann-Pick type C2 proteins across the blood–brain barrier using the brain endothelial-specific AAV-BR1 virus. *J Neurochem. 2023;164:6-28*

Al Humaidan EL, Pedersen SL, Burkhart A, **Rasmussen CLM**, Moos T, Fuchs P, Fernandes EFA, Ozgür B, Strømgaard K, Bach A, Brodin B, Kristensen M: The Cell-

Penetrating Peptide Tat Facilitates Effective Internalization of PSD-95 Inhibitors Into Blood–Brain Barrier Endothelial Cells but less Efficient Permeation Across the Blood–Brain Barrier *In Vitro* and *In Vivo*. *Front. Drug. Deliv. 2022;2:854703.*

Stocki P, Szary J, **Rasmussen CLM**, Demydchuk M, Northall L, Logan DB, Gauhar A, Thei L, Moos T, Walsh FS, Rutkowski JL: Blood-brain barrier transport using a high affinity, brain-selective VNAR antibody targeting transferrin receptor 1. *FASEB J. 2021;35(2):e21172.*

Jensen VFH, Mølck AM, Bøgh IB, Nowak J, Viuff BM, **Rasmussen CLM**, Pedersen L, Fels JJ, Madsen SH, McGuigan FE, Tveden-Nyborg P, Lykkesfeldt J, Akesson KE: Inner histopathologic changes and disproportionate zone volumes in foetal growth plates following gestational hypoglycaemia in rats. *Sci Rep:2020;10,5609.*

Conference activity

2019	27th Congress of the European Society of Gene and Cell Therapy – poster presentation (Transport of therapeutic proteins across the blood-brain barrier using viral gene therapy)
2018	Society for Neuroscience Annual Meeting 2018 – poster presentation (Targeted delivery to brain endothelial cells and transport across the blood-brain barrier of shark antibodies (vNAR) following intravenous administration in mice)

Funding

2019	Fonden til Lægevidenskabens Fremme 50,000 DKK
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- Laboratory Animal Science Function ABD courses, Master and Ph.D. level, Aarhus University and Southern University of Denmark

ENGLISH SUMMARY

Diseases affecting the brain are challenging to treat due to the presence of the blood-brain barrier (BBB). The BBB is composed of specialized endothelial cells forming tight interconnections, preventing harmful blood-borne substances from entering the brain. Consequently, the BBB also hinders most drugs from reaching the brain parenchyma and thus remains a major obstacle for drug delivery to the central nervous system. For decades, several strategies have been explored to smuggle molecules across the BBB, but a highly specific and efficient drug delivery strategy still needs to be developed. Viral gene therapy at the BBB represents a promising approach for the targeted delivery of molecules to the brain. With gene therapy, it is possible to turn brain endothelial cells (BECs) into protein factories that secrete recombinant proteins in the direction of the brain and the blood. Therefore, BBB-directed gene therapy denotes a possible strategy for treating genetic neurovisceral disorders like the Niemann-Pick type C2 disease (NP-C2), where no cure is available. NP-C2 is caused by a loss-of-function mutation in the lysosomal cholesterol transporter protein NPC2, causing accumulation of cholesterol within the lysosomes of all cells, subsequently resulting in both systemic and neurological symptoms. The disease is progressive, leading to premature death. The BEC-specific adeno-associated virus (AAV-BR1) vector has previously shown great potential in treating neurovascular and neurological disorders in different murine models after intravenous administration and was, therefore, also used in this Ph.D. thesis. Accordingly, this dissertation is dedicated to studying protein delivery across the BBB using the AAV-BR1 vector encoding the *Npc2* gene (AAV-BR1-NPC2).

In the first study, the transduction of BECs was evaluated using an *in vitro* BBB model and healthy BALB/cJRj mice after intravenous injections of the AAV-BR1-NPC2 vectors. Widespread transduction of BECs was evident in the brain of healthy mice, resulting in upregulation of the *Npc2* gene expression. However, not sufficient to detect an increase in the NPC2 protein concentration in the brain or the blood *in vivo*. Transduction of the *in vitro* BBB model resulted in the secretion of recombinant NPC2 proteins to the cell culture media in both the upper and lower chamber, corresponding to the blood and the brain, which could reverse the pathological cholesterol storage in NPC2-deficient fibroblasts.

Secondly, the Ph.D. thesis aims to prove the therapeutic potential of the viral gene therapy strategy in a mouse model of the NP-C2. Due to limited data for the NP-C2 mouse model holding a gene trap mutation (*Npc2*$^{Gt(LST105)BygNya}$), a thorough histopathological characterization of this specific murine model was carried out to evaluate the model's translational value to human patients. The hypomorphic NP-C2 mouse model develops similar symptoms seen in patients suffering from NP-C2, including tremors and cerebellar ataxia. The neurovisceral pathology includes severe Purkinje cell degeneration, neuroinflammation, and cholesterol storage in the liver, lung, and spleen. The model is, therefore, valuable for investigating new treatment strategies.

In the last study, the efficiency of the AAV-BR1-NPC2 vector was evaluated in the NP-C2 mouse model with the onset of treatment at six weeks of age. BBB-directed gene therapy using the AAV-BR1 vector could delay the disease progression in NPC2 deficient (NPC2-/-) mice with preservation of Purkinje cells in the cerebellum, resulting in improvement in motor function and disease phenotype compared to untreated NPC2-/- mice. However, no noticeable effect was seen on the visceral pathology, where cholesterol storage in the liver, lung, and spleen was still evident. This challenges the strategy of BBB-directed gene therapy being able to treat the visceral symptoms associated with NP-C2.

In conclusion, systemic administration of the AAV-BR1-NPC2 vector can delay neurodegeneration in the NPC2-/- mice, and thus BBB-directed gene therapy has the potential to treat diseases with neurological involvement.

DANSK RESUME

Behandling af mange sygdomme i hjernen er vanskeliggjort af tilstedeværelsen af blod-hjerne-barrieren (eng. blood-brain barrier (BBB)). BBB, der er lokaliseret i hjernens kapillærer, består af specialiserede endothelceller (eng. brain endothelial cells (BECs)), der er tæt forbundet via såkaldte "tight-junctions" proteiner. Disse særlige karakteristika for BBB medfører at uønskede, skadelige stoffer i blodbanen ikke kan trænge ind i hjernen. Dette resulterer desværre også i, at størstedelen af de lægemidler, der udvikles specifikt mod sygdomme i hjernen, ikke kan passere BBB. I årenes løb er der forsket i mange forskellige behandlingsstrategier til at overkomme BBB problematikken, men på nuværende tidspunkt er der stadig ikke udviklet en specifik og effektiv strategi. Viral genterapi kan dog være løsningen. Ved hjælp af genterapi er det muligt at omdanne BECs til proteinfabrikker, som udskiller terapeutiske proteiner til hhv. blodet og hjernen. Genterapi målrettet BBB kan derfor være effektiv til at behandle neuroviscerale sygdomme som f.eks. Niemann-Picks sygdom type C2 (NP-C2), hvor der endnu ikke findes en effektiv behandling. NP-C2 skyldes en mutation i *Npc2* genet, som medfører en defekt i Niemann-Pick C2 proteinet (NPC2), der er essentiel for transport af kolesterol ud af cellens lysosomer. Som konsekvens ophobes der kolesterol og andre lipider i lysosomerne i alle kroppens celler. Derfor udvikles der både neurologiske samt systemiske symptomer. NP-C2 er progressiv og medfører tidlig død.
Den BEC-specifikke adeno-associeret virus (AAV-BR1) vektor har tidligere vist terapeutisk potentiale i forskellige neurologiske sygdomsmodeller i mus efter intravenøs administration. Afhandlingen har derfor fokuseret på at undersøge transport af proteiner til hjernen vha. AAV-BR1 udtrykkende NPC2 (AAV-BR1-NPC2).

I det første studie, blev transduktion af BECs undersøgt i en *in vitro* BBB model og raske BALB/cJRj mus efter intravenøs injektion af AAV-BR1-NPC2. Transduktionen af BECs var udbredt i hele hjernen i raske mus, hvilke resulterede i en opregulering af *Npc2* genet. Det var dog ikke tilstrækkeligt til at detektere en stigning i NPC2 koncentrationen hverken i blodet eller i hjernen. Transduktion af *in vitro* BBB modellen resulterede i sekretion af rekombinant NPC2 til cellemediet i både øvre og nedre kammer, hvilket tilsvarer hjerne- og blod. NPC2 i cellemediet kunne reversere ophobningen af kolesterol i NPC2 deficiente fibroblaster.

Dernæst skulle den terapeutiske effekt af AAV-BR1-NPC2 vektoren undersøges i en musemodel for NP-C2, som er genetisk induceret (*Npc2*$^{Gt(LST105)BygNya}$). På grund af begrænsede data for denne specifikke model, blev sygdomsmodellen først karakteriseret histopatologisk. Formålet var at vurdere modellens translationelle værdi. NPC2 deficiente (NPC2 -/-) mus udvikler mange af de symptomer, som man ser humant, inklusiv tremor og cerebellar ataksi. Patologien er karakteriseret ved Purkinjecelle degeneration, neuroinflammation samt ophobning af kolesterol i lever, lunge og milt. NP-C2 musemodellen er derfor egnet til at undersøge nye behandlingsstrategier.

I det sidste studie, blev genterapi-strategien undersøgt i NP-C2-modellen. AAV-BR1-NPC2 vektoren blev indgivet intravenøst til 6 uger gamle NPC2-/- mus, hvilket medførte en forsinkelse i sygdomsprogressionen. Bevarelse af Purkinjeceller i cerebellum medførte forbedring af motor funktionen og sygdomsfænotype sammenlignet med ubehandlede NPC2-/- mus. Der var dog ingen umiddelbar effekt på viscerale organer, hvor ophobning af kolesterol i lever, lunge og milt stadig var synlig. Dette udfordrer strategien om, at genterapi målrettet BBB kan behandle visceral patologi associeret med NPC2 mangel.

Systemisk administration af AAV-BR1-NPC2 vektoren kan forsinke neurodegenerationen i NPC2-/- mus og genterapi målrettet BBB har derfor potentialet til at behandle neurologiske sygdomme.

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PREFACE

This Ph.D. thesis: "Blood-brain barrier-directed gene therapy as a strategy to treat Niemann-Pick type C2 disease," has been submitted to the Faculty of Medicine, Aalborg University, Denmark. The work of this dissertation has been performed in the period from September 2017 to December 2022 (including a one-year leave of absence due to work as a laboratory animal veterinarian at the Southern University of Denmark and maternity leave from February 2021 to November 2021). The project was supervised by Associate Professor Louiza Bohn Thomsen, Associate Professor Annette Burkhart Larsen, and senior researcher, Ph.D., Christian Würtz Heegaard. The experimental work was conducted in the Laboratory of Neurobiological Research and Drug Delivery, Department of Health Science and Technology, Aalborg University, Denmark, in the Laboratory for Protein Chemistry at the Department of Molecular Biology and Genetics, Aarhus University, Denmark, and in the animal facilities at Aalborg and Aarhus University, Denmark.

During the Ph.D. period, I have attended courses corresponding to 31 ECTS and been teaching students at the two educations Medicine and Medicine with Industrial Specialization at Aalborg University. These activities correspond to a full year of my Ph.D. study. Furthermore, I have been involved in different research activities concerning drug delivery to the brain. This work has resulted in the following publications: "The Cell-Penetrating Peptide Tat Facilitates Effective Internalization of PSD-95 Inhibitors Into Blood–Brain Barrier Endothelial Cells but less Efficient Permeation Across the Blood–Brain Barrier In Vitro and In Vivo" (Al Humaidan *et al.,* 2022), "Blood-brain barrier transport using a high affinity, brain-selective VNAR antibody targeting transferrin receptor 1" (Stocki *et al.,* 2021), and "CDR3 variants of the TXB2 shuttle with increased TfR1 association rate and enhanced brain penetration" (submitted, Stocki *et al.,* 2022).

The dissertation is based on three original experimental studies, which will be referred to as study I (published), study II (submitted), and study IV (in preparation). The thesis consists of a general introduction encompassing the different topics explored in the manuscripts, the objectives and methods, a summary of results, a joint discussion, and a conclusion and future perspectives. In addition, one review, referred to as study III, is included in the thesis. The review was an essential part of planning study IV. The manuscripts and the review are attached as an appendix.

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LIST OF MANUSCRIPTS

Studies included in this dissertation:

Study I:

A novel strategy for delivering Niemann-Pick type C2 proteins across the blood-brain barrier using the brain endothelial-specific AAV-BR1 virus

Charlotte L. M. Rasmussen, Eva Hede, Lisa Juul Routhe, Jakob Körbelin, Steinunn Sara Helgudottir, Louiza Bohn Thomsen, Markus Schwaninger, Annette Burkhart, Torben Moos

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Study II:

The *Npc2*$^{Gt(LST105)BygNya}$ mouse signifies pathological changes comparable to human Niemann-Pick type C2 disease

Charlotte L.M. Rasmussen, Annette Burkhart, Christian Würtz Heegaard, Louiza Bohn Thomsen, Torben Moos

Manuscript submitted

Study III:

Reporting preclinical gene therapy studies in the field of Niemann-Pick type C disease according to the ARRIVE guidelines: How far are we?

Charlotte L.M. Rasmussen, Annette Burkhart, Torben Moos, Louiza Bohn Thomsen

Review

Study IV:

The brain-specific AAV-BR1 vector delays the disease progression in a mouse model of Niemann-Pick type C2 disease

Charlotte L.M. Rasmussen, Christian Würtz Heegaard, Maj Schneider Thomsen, Eva Hede, Bartosz Laczek, Jakob Körbelin, Louiza Bohn Thomsen, Markus Schwaninger, Annette Burkhart, Torben Moos

Manuscript

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AAV-BR1	The brain endothelial cell-specific AAV2-based capsid variant
BBB	Blood-brain barrier
BECs	Brain endothelial cells
CNS	Central nervous system
eGFP	Enhanced green fluorescent protein
LSD	Lysosomal storage disorder
LY6A	Lymphocyte antigen 6 complex
NP-C	Niemann-Pick type C disease
NP-C1	Niemann-Pick type C1 disease
NP-C2	Niemann-Pick type C2 disease
NPC1	Niemann-Pick C1 protein
NPC2	Niemann-Pick C2 protein
rAAV	Recombinant adeno-associated virus
rpm	Revolution per minute
TfR	Transferrin receptor
VNAR	Variable domain of new antigen receptors

CHAPTER 1. INTRODUCTION

Worldwide, the burden of neurological diseases has increased considerably over the past 25 years (1). Unfortunately, the treatment of diseases affecting the brain is highly challenging due to the presence of the restrictive blood-brain barrier (BBB). The BBB is a thin cellular layer situated at the interface of blood and brain and is composed of non-fenestrated brain endothelial cells (BECs), which are in close contact with pericytes and astrocytes, two cell types believed to support and maintain the barrier properties of the BECs (2,3). The primary function of the BBB is to maintain homeostasis in the brain and protect the brain from neurotoxic blood-borne substances, but as a result the barrier also limits the passage of potential effective therapeutics to the central nervous system (CNS) (4,5).

Despite several different treatment strategies have been investigated to circumvent the BBB, the main issue concerning drug delivery to the brain is still the lack of specificity and achieving therapeutic levels in the brain parenchyma. There is thus a medical need for new treatment options, and here gene therapy represents a promising approach (6–10). In the past, the issues regarding the BBB have been overcome by injecting the vector directly into the brain. This approach is, however, considered highly invasive, associated with several risks, and leads to an uneven distribution of the vector within the brain (6,11–13). It is, therefore, important to develop noninvasive administration routes for delivering genetic material throughout the brain parenchyma (11,14). The BBB is an interesting target for gene therapy since neurons are rarely more than 8-20 μm from a brain capillary, meaning that the secreted therapeutic proteins become available throughout the brain parenchyma with a minimum need for diffusion inside the brain (15). The principle behind gene therapy is to transform cells into protein factories by genetically modifying them to secrete recombinant protein to their surroundings. Previous *in vitro* studies have shown that when genetically modifying the BBB to secrete a protein it will result in a bidirectional secretion pathway, in which the recombinant protein becomes available both inside the brain parenchyma but also in the blood (11,14,16,17). This has the potential for proteins to enter the brain and enable protein delivery to cells elsewhere in the body, which is particularly relevant in diseases characterized by a global lack of proteins as seen in hereditary metabolic disorders, e.g., lysosomal storage disorders (LSDs). The main focus of this Ph.D. thesis will therefore be to study viral gene therapy as a strategy to overcome the BBB in healthy mice and a mouse model of the LSD Niemann-Pick type C2 disease (NP-C2).

1.1. THE BLOOD-BRAIN BARRIER

To maintain homeostasis of the CNS, which is essential for neuronal function in the brain parenchyma, the presence of the highly selective BBB is crucial. The BBB not only protects the brain from the entry of harmful substances circulating in the blood

but also regulates the intracerebral environment by controlling the influx and efflux of biological substances including supplying the brain with indispensable nutrients (18,19). These specific properties are induced and maintained by the interaction between BECs, astrocyte endfeet, pericytes, and neuronal terminations. These cells are also referred to as the neurovascular unit (Fig. 1) (3,15).

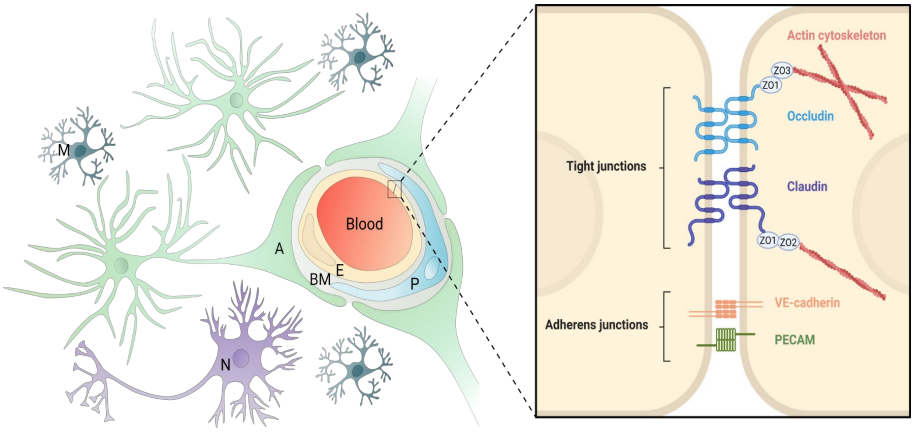

Figure 1. Schematic illustration of the cellular components of the blood-brain barrier (BBB) and the molecular structure of the tight-junction complexes. The BBB is formed by specialized capillary endothelial cells (E), which are surrounded by pericytes (P) embedded in the basement membrane (BM) and astrocytic endfeet (A). Both neurons (N) and microglia (M) are found in close vicinity of the BBB. The brain endothelial cells are connected by tight junction proteins. ZO: zonulae occludens, PECAM: platelet/endothelial cell adhesion molecule, VE-caderin: vascular endothelial cadherin. Created with Inkscape and BioRender.com.

The BECs are distinguishable from endothelial cells lining peripheral blood vessels with the expression of tight junction proteins, absence of fenestration, and limited vesicular transport (20,21). The tight interconnections between BECs include adherence junctions and tight junctions (Fig. 1). Adherence junctions formed by vascular-endothelial cadherin (VE-cadherin) and platelet/endothelial cell adhesion molecule (PECAM) provide structural support by holding the BECs together, and are required for the formation of tight junctions (2). The tight junctions connect the plasma membranes of neighboring endothelial cells and are linked to the cytoskeleton by the cytoplasmic scaffold proteins zonulae occludentes (ZO-1-3). Tight junctions are primarily formed by integral membrane proteins such as claudin and occludin as well as junctional adhesion molecules (JAMs) (Fig. 1) (2). This junctional complex prevents the paracellular transport of molecules into the brain resulting in high transendothelial electrical resistance (TEER) (22,23). In addition, the movement of hydrophilic and charged molecules across the BBB is further complicated by the highly negatively charged glycocalyx located on the luminal surface of the BECs (3).

Astrocytes, which are the most abundant cells in the CNS, form end-feet processes that almost completely cover the brain capillaries, and these play a vital role in the induction and maintenance of the barrier function (19,24). Astrocytes are involved in different physiological and biochemical processes in the brain including regulation of cerebral blood flow and neuronal activity, they supply essential nutrients and growth factors to neurons, and last but not least they are also important immune regulators (25). Another cell type supporting the integrity of the BBB is the pericytes. Pericytes are embedded in the basement membrane and are distributed along the endothelial cells, covering approximately 70 % of the brain capillaries (26). Pericytes are critical for BBB formation during development by regulating the formation of tight junction proteins in the BECs (27).

Microglia, the immune cells of the brain, can also be included as a part of the neurovascular unit. These are found in close vicinity to the BBB and during pathological conditions, activated microglial cells secrete a cascade of inflammatory cytokines, which results in a compromised BBB integrity (22,28). Furthermore, new research has indicated that microglia contribute to the function and structure of brain capillaries during non-pathological conditions (29).

1.1.1. TRANSPORT AT THE BLOOD-BRAIN BARRIER

The brain has a high metabolic demand, but due to the low permeability of the BBB, the supply of nutrients to the brain is dependent on various transport systems (Fig. 2) (22). Some solutes can diffuse passively across the barrier such as small lipophilic and gaseous molecules (e.g., O_2, CO_2), while other molecules require active transport which includes carrier-mediated transport, receptor-mediated transport, and adsorptive-mediated transport. The BECs, therefore, express different receptors and transport molecules on the luminal and abluminal surfaces (Fig. 2). Carrier transporters facilitate the influx of glucose, amino acids, hormones, and vitamins to the brain parenchyma, whereas the transport of macromolecules, such as insulin, lipoproteins, and transferrin, requires binding to specific receptors for mediated uptake into the brain (22,30). The transcytosis of transferrin to the brain is, however, considered negligible (31,32). In opposite hereto, the adsorptive mediated transport is induced in a non-specific manner, where the interaction between positively charged cargos and the negatively charged surface of the BECs triggers transcytosis of various cationic proteins like avidin and cell-penetrating peptides (33–35).

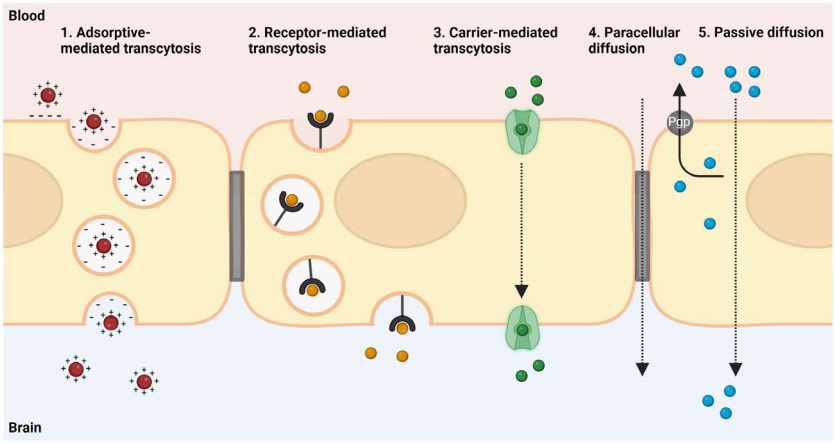

Figure 2. Schematic illustration of the different transport systems at the blood-brain barrier. 1) Adsorptive-mediated transcytosis: Transport of positively charged molecules across the brain endothelial cells (BECs) via non-specific active transport. 2) Receptor-mediated transcytosis: Transport of proteins such as insulin through a specific receptor presented at the luminal surface of the BECs. 3) Carrier-mediated transcytosis: Influx of, e.g., glucose and amino acids through specific membrane carriers. 4) Paracellular diffusion: Transport of small water-soluble molecules, such as alcohol, through tight junctions. 5) Passive diffusion: Transport of small lipid-soluble molecules through the cell membrane of BECs. Active efflux carriers such as the P-glycoprotein (Pgp) can interrupt the diffusion of some of these molecules by pumping them back into the blood. Adapted from (15). Created with BioRender.com.

Another important transport system expressed at the luminal membrane of the BECs is the active efflux transporters, including the P-glycoprotein, a member of the ATP-binding cassette (ABC) transporter family. Their main function is to pump solutes from the endothelium back into the circulation, thereby reducing the entry of potentially harmful substances (Fig. 2) (19). Altogether, these transport systems are crucial for securing proper neuronal function by tightly regulating the microenvironment of the brain. On the other hand, these mechanisms are some of the major challenges in drug delivery to the brain. Therefore, the special characteristics of the BBB need to be taken into consideration in the development of new treatment strategies for neurological diseases (2,30).

1.2. DRUG DELIVERY TO THE BRAIN

Due to the structural and functional characteristics of the BECs and with neurons localized less than 25 μm from the capillaries, the BECs are a favored target for drug delivery to the brain (2,36). Unfortunately, 98 % of all small-molecule drugs, as well as all macromolecules such as antibodies and recombinant proteins, cannot cross the BBB, which challenges the development of new therapeutics for neurological diseases (37). There are some physicochemical parameters affecting the BBB permeability of a drug: molecular weight, lipophilicity, hydrogen bonds, polar surface area, and charge of the molecule. These are referred to as Lipinski's "rule of five" (38). In general, a small molecule can cross the BBB if the molecular weight is < 450 Da, although the permeability decreases 100-fold when the weight increase from 200 to 450 Da (39,40). In addition, a molecule must be lipophilic to diffuse passively across the hydrophobic phospholipid bilayer of the endothelial cell membrane. Therefore, an increase in the number of hydrogen bonds decreases the passive diffusion across the BBB (37). Finally, highly charged molecules and a polar surface area of > 70 Ångström reduce the BBB penetrance (41,42). Even though the rule of five is considered when designing a drug for the brain, less than 2 % of all small-molecule drugs have these above-mentioned properties. Furthermore, the physicochemical properties necessary for BBB penetrance can result in other issues related to pharmacokinetics, e.g., increased protein binding or unspecific accumulation in peripheral tissue, thereby increasing the risk of toxicity (43,44). Altogether, this limits the success of drug delivery to the brain and receiving therapeutic concentrations within the brain.

1.2.1. TREATMENT STRATEGIES

Due to the aforementioned challenges with BBB penetrance, different invasive and non-invasive strategies for drug delivery to the brain have been explored. One strategy includes bypassing the BBB, e.g., by intracerebroventricular or intraparenchymal injections. However, this strategy only allows for local brain tissue exposure due to the limited diffusion of drugs in the extracellular space of the CNS (13,45). Furthermore, the injections are also associated with the risk of severe complications such as infections and hemorrhages in the brain (6,46,47). Alternatively, transient disruption of the BBB allows for passive diffusion of drugs otherwise unable to cross the BBB. One example of this strategy is focused ultrasound in combination with circulating microbubbles (48). By injection of microbubbles, the energy required to open the BBB is less than using focused ultrasound alone, resulting in a reduced risk of tissue damage (49,50). In addition, this method has been proven reversible after approximately four hours, establishing a therapeutic window for delivering drugs to the CNS (51). Furthermore, it shows potential in the treatment of, e.g., brain tumors and mouse models of Alzheimer's disease (52–55). Still, the safety of this method is debatable (49,50,56,57).

Finally, utilizing the existing BEC transport systems, previously mentioned, has been thoroughly investigated as a strategy for delivering therapeutics to the brain. One approach focuses on the adsorptive mediated transcytosis pathway where binding of positively charged molecules to the negatively charged surface of BECs allows for transport across the BBB. This strategy includes, e.g., non-viral gene therapy (see the section "gene therapy") and cell-penetrating peptides (33,35). However, high off-target distribution challenges the efficiency of this strategy (35). Both the carrier- as well as the receptor-mediated transport systems allow for unique opportunities for drug delivery to the brain, which is why targeting these endogenous molecules expressed on the luminal side of the BECs has been used as a strategy to transport therapeutics across the BBB (36). One example of this approach is the pro-drug used for the treatment of Parkinson's disease, L-DOPA, which is a substrate for the L-type amino acid transporter 1 expressed on the BECs. The binding of L-DOPA to the L-type amino acid transporter 1 facilitates the carrier-mediated influx of the pro-drug to the brain (58). If a molecule does not fulfill the criteria previously mentioned for BBB permeability or has an affinity for a receptor system at the BECs, the drug designated for brain delivery can be coupled to a vehicle system targeting the BBB, which then ferries the drug across the BBB. One of the most widely studied targets at the BBB is the transferrin receptor (TfR) 1 since it is highly expressed by the BECs, but not by endothelial cells in other tissues (59,60). Antibodies directed against the TfR1 (e.g., OX26 anti-rat TfR antibody and Ri7 anti-mouse TfR antibody) have been thoroughly investigated as a therapeutic option either alone or attached to drugs, liposomes, or nanoparticles (36,59,61,62). Unfortunately, the majority of systemically administered antibodies will be confined within the BECs resulting in brain concentration as low as 0.1 % of the peripherally injected dose (63). Therefore, the antibodies must be administered at high doses, increasing the risk of off-target toxicity (64). Severe adverse effects resulting in increased mortality have also been observed in mice after intravenous administration of Ri7-functionalized liposomes, probably related to anemia caused by hemolysis of reticulocytes (61). Together, these findings limit their use, and no formulations to date have reached the clinical stage of drug development (30,36,65). To circumvent some of these disadvantages of the aforementioned anti-TfR1 antibodies, the variable domain of new antigen receptors (VNARs) derived from single domain antibodies found in the shark, has been investigated (65). The brain-selective VNAR fragment TXB2 is a promising candidate for brain drug delivery due to the availability to cross the BBB, the favorable safety profile, and cross-species binding properties (65). Further studies are needed to investigate the VNAR's potential in treating brain diseases. The last drug delivery strategy using a vehicle system that will be discussed in this thesis is gene therapy.

1.3. GENE THERAPY

Another promising drug delivery strategy for brain diseases is gene therapy. Gene therapy relies on the delivery of genetic material ("transgene") into the cell to substitute absent or defective genes or silence unwanted gene expression and thus reverse the disease phenotype (66,67). The successful delivery of the transgene to the

target cell critically depends on the vector system. Therefore, the vector needs to fulfill specific requirements, which are of even higher demand when used for treating CNS diseases. First, the vector needs a loading capacity large enough to deliver the specific gene product. Secondly, to increase the translation to human trials, the production of vectors has to be scalable and of low cost. If administered systemically, the vector must be stable in the blood, thus avoiding degradation by, e.g., serum endonucleases. In addition, the vector should have low immunogenicity and cytotoxicity. It is especially important that the vector does not integrate into the host genome otherwise there is a risk of insertional mutagenesis. Finally, the vector has to be specific to the target cell, enable efficient gene delivery, and allow for a prolonged expression of the transgene (14,67–69).

Different vector systems are available for delivering transgenes, and they are classified as either of non-viral or viral origin. Examples of non-viral vectors include cationic polymers, lipids, and peptides (70,71). Non-viral gene therapy for CNS diseases utilizes the drug delivery strategy targeting endogenous BBB transport systems, as previously mentioned. A well-studied approach is the Trojan horse technology. One example includes encapsulating plasmid DNA by PEGylated liposomes where, e.g., transferrin-receptor-specific antibodies are conjugated to the surface of the liposomes (72–75). The approach suggests that upon binding to the designated receptor of BECs, the complex will undergo receptor-mediated transcytosis, and the liposomes carrying the plasmid DNA will be ferried across the BBB. However, one study shows no evidence for transcytosis of TfR-targeted liposomes in the brains of rats after intravenous injections (59), whereas others indicate that by lowering the affinity or using bi-specific antibodies, the brain uptake of the nanoparticles can be improved (76,77). Thus several aspects of the TfR-specific antibodies need to be considered, and the intracellular fate of the cargo is still debated (31,78). Another concern is the risk of off-target accumulation. Several studies investigating the Trojan horse liposome strategy independent of species show a high accumulation of the complexes in the spleen and liver (59,61,72,75). Consequently, the therapeutic effect on the brain is challenged, which was also seen in a study investigating this approach in a mouse model of the neurovisceral Niemann-Pick type C1 disease (NP-C1) (72). Intravenous injections with Trojan horse liposomes in diseased mice could not treat the neurodegeneration, and there was no improvement in survival after using non-viral gene therapy (72). Unfortunately, finding a carrier with high specificity for the BBB is an ongoing problem for developing new treatment strategies for brain disease. Another challenge with non-viral gene therapy is that the expression of the transgene is transient, and weekly administrations are therefore required, which potentially can increase the risk of triggering an immune response (79).

Even though non-viral vectors fulfill several of the requirements stated earlier, such as low immunogenicity, large loading capacity, and low-cost production, which are easy to upscale, one of the major disadvantages is the low transfection efficiency (14,17,67). Non-viral vectors must overcome different extracellular and intracellular barriers for the successful delivery of the DNA to the nucleus, with a risk of

degradation on the way. Contrary to viral vectors, they do not possess natural strategies for overcoming these barriers, why their efficiency is limited (Fig. 3) (67).

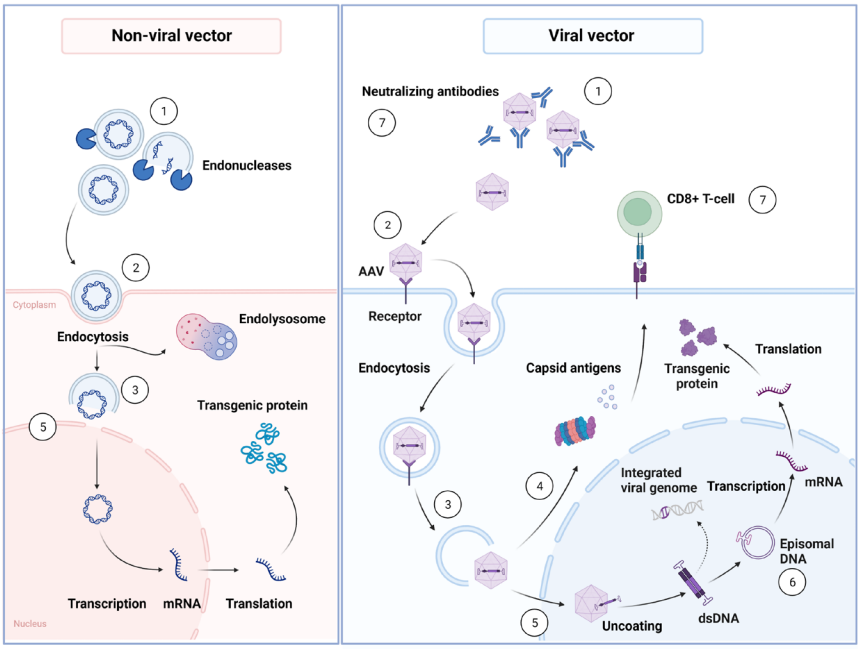

Figure 3. Challenges in the delivery of genetic material using either non-viral or viral gene carriers after systemic administration. The vector carrying the foreign genetic material has to overcome several critical events on its way to the nucleus for successful gene delivery. (1) The first critical event is the risk of degradation in the blood due to, e.g., serum endonucleases or neutralizing antibodies. (2) Next the vector has to be internalized by the cell. Non-viral vectors often have a positively charged surface, which can interact with the negatively charged surface of the cell resulting in endocytosis of the carrier. Other strategies for cellular uptake include conjugating receptor-specific antibodies to the surface of the vector. Adeno-associated virus (AAV) is composed of a protein capsid, which binds to glycosylated receptors on the surface of the host cell, triggering endocytosis of the viral carrier. (3) As both non-viral and viral vectors undergo endocytosis, the next critical step is endosomal escape, necessary to avoid degradation in lysosomes. The capsid of AAV undergoes pH-dependent structural changes necessary for the following cellular transport and transduction. Endosomal escape is especially challenging for non-viral vectors, and a large portion of these vectors will be degraded in the lysosomes. (4) However, AAVs can undergo proteasomal proteolysis resulting in the degradation of the virus. (5) Delivery of DNA to the nucleus for transcription depends on nuclear trafficking. This is one of the major challenges for non-viral vectors as they do not possess a natural mechanism for entering the nucleus compared to viral vectors. Furthermore, some non-viral carriers are depending on cell division for delivering transgenes to the nucleus. AAV enters the nucleus through the nuclear pore complex, where DNA is released, transcribed to mRNA, and translated to proteins. (6) The AAV genome can persist as episomal DNA in the nucleus or be integrated into the host genome on rare occasions. (7) Vector immunogenicity is a risk when working with viral vectors. Both neutralizing antibodies and cytotoxic $CD8^+$ T cell response can induce an immune response resulting in degradation of the AAV capsid or

eliminate the transduced cell, both resulting in lower transduction efficiency. Modified from (69,80,81). Created with BioRender.com.

More efficient and specific drug delivery strategies are still needed for brain diseases. Therefore, in recent years more focus has been pointed toward viral vectors, taking advantage of the viruses' natural ability to bypass cellular membranes and deliver genetic material to cells (17). Viral gene therapy is based on the recombinant virus, where disease-causing parts of the viral genome are replaced by the transgene of interest and a promoter driving the gene expression in the cell (82,83). Subsequently, recombinant viruses are unable to replicate on their own, which is a normal part of the pathogenicity of viruses. However, they are still capable of delivering genes to host cells and reaching a high transduction efficiency (84). One main concern regarding using viral vectors for gene therapy is the risk of genotoxicity due to insertional mutagenesis (Fig. 3). Integration of transgene into the host genome can activate, e.g., oncogenes resulting in cancer development (85–87). Furthermore, the viral capsid can be recognized by the immune system, thus stimulating an immune response leading to the degradation of the virus, including the transgene, which consequently limits the transduction efficiency (Fig. 3) (81). However, the safety and efficiency depend on the type of viral vector. Most recombinant viral vectors for targeting the CNS are based upon adenovirus, adeno-associated virus (AAV), retrovirus, or herpes simplex virus (67,83). Due to their favorable safety profile and high transduction efficiency, AAVs have emerged as the preferred candidate for gene therapy (7,81,88,89). The viral vector used in this Ph.D. thesis is also based on an AAV, and the next section will exclusively focus on AAVs and their potential in treating CNS diseases.

1.3.1. ADENO-ASSOCIATED VIRUS

More than 50 years ago, the first AAVs were discovered by Bob Atchison during the preparation of adenovirus in the laboratory (90). These new viruses were only capable of replicating in the presence of a helper virus, e.g., adenovirus, and thus were classified as dependoparvovirus. Since the first discovery in 1965, AAVs have been thoroughly studied (80,83), and in 2012 the first recombinant AAV (rAAV) were approved by the European Medicines Agency for the treatment of lipoprotein lipase deficiency (91). At present, rAAVs still show great potential in both pre-clinical and clinical trials due to their safe and effective gene delivery (92,93), however much remains to be learned.

AAVs are small, non-enveloped viruses (~26 nm in diameter) with a broad host range, including both humans and non-human primates, but are non-pathogenic. The viruses are composed of an icosahedral protein capsid with a single-stranded DNA genome of 4.7kb (Fig. 4) (80,84). The viral genome consists of three genes; Rep (replication) gene, necessary for viral replication and packaging, Cap (capsid) gene, encoding the capsid proteins, which protect the viral genome and are responsible for cell binding, and Aap (assembly activating proteins), essential for the capsid assembly (Fig. 4). The

genome is flanked by two inverted terminal repeats, which function as a packaging and replication signal (80,83,92).

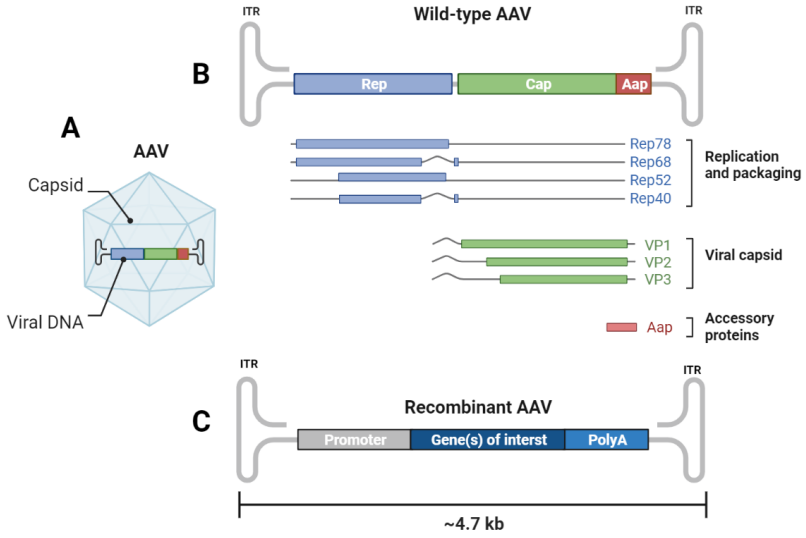

Figure 4. Overview of the adeno-associated virus (AAV) characteristic. A) AAV are non-enveloped and consists of single-stranded DNA of 4.7 kb in length. **B)** The genome contains three genes: Rep, Cap, and Aap flanked by inverted terminal repeats (ITR). The Rep gene encodes four proteins, Rep78, Rep68, Rep52, and Rep40, important for replication, transcription, and packaging. The Cap gene encodes three viral capsid proteins: VP1, VP2, and VP3, forming the icosahedral capsid of ~26 nm. The Aap encodes assembly proteins essential for the assembly of the capsid. **C)** In recombinant AAV (rAAV), the viral genome is replaced with an expression cassette comprising a promoter, the gene(s) of interest, and a termination signal (e.g., polyadenylation sequence (PolyA)). Only the viral ITR remains in the rAAV. The rAAV genome is packed into the AAV capsid important for tissue tropism. The promoter can either be tissue-specific or ubiquitous and drives the transgene expression. Thus the design of the expression cassette is important for transduction efficiency and tissue specificity. Modified and inspired by (79,80,92). Created with Biorender.com.

In the absence of a helper virus, the virus is incapable of replicating, and thus the genome of wild-type AAVs can be latently expressed in humans by integrating into the humane genomic locus, termed AAVS1, however, this phenomenon is greatly reduced when using rAAVs (80,94). This is due to the removal of virus-specific genes in the rAAV vectors. The rep and cap genes are replaced by the gene(s) of interest. It is only the inverted terminal repeats that are retained, which as mentioned previously, are necessary for providing packaging signals during, e.g., the vector production (81).

Removing these viral genes subsequently lowers the risk of viral genome insertion and reduces the AAVs' immunogenicity, increasing the clinical potential. However, it has been stressed that pre-existing neutralizing antibodies against AAVs in both human and animal models can prevent successful transduction (68,95,96). Pre-existing neutralizing antibodies against AAVs are a common finding in healthy individuals due to previous infections with wild-type AAVs. Additionally, similarities in the capsids between serotypes can result in cross-reactive neutralizing antibodies against rAAVs, consequently challenging the therapeutic potential of these vectors in patients (95,97,98). To avoid immune-mediated toxicity, anti-AAV neutralizing antibody titers above a specified threshold exclude subjects from enrollment in clinical trials where the rAAV vectors are administered intravenously (81,92). Pre-existing immunity against AAVs is a major concern, and neutralizing antibodies is an important factor to consider in the pre-clinical assessment of new gene therapy strategies. Moreover, it has been proposed that neutralizing antibodies can challenge the re-administration of viral vectors (99,100). This is probably not an issue with AAVs due to the long-term transgene expression observed in several animal species, including mice (99,101), dogs (102), non-human primates (103), and primates (104), with the longest transgene expression reported to be over 15 years in primates after intracerebroventricular injections of AAVs (104).

The simple genomes of AAVs make them ideal for experimental manipulation and easily adaptable for multiple purposes (83,92). On the other hand, this is also a limitation when using AAVs for gene delivery. The small genome size (4.7 kb) results in a small packaging capacity. This challenges the design of the transgene expression cassette. Thus both the therapeutic gene sequence and the regulatory elements, e.g., promoter or polyadenylation signal must be considered in the production of the rAAVs (Fig. 4) (80,105). Consequently, small and eventually less effective promoters are in some cases chosen to accommodate the packaging of large gene sequences encoding the therapeutic protein of interest. Despite this, AAVs have shown potential in various diseases, including neurodegenerative disorders (106–109).

Several different AAV serotypes have been identified, with at least 12 natural described; AAV1-12 (110). All share the same properties, however, they show variable tissue tropism resulting in different organ distribution after intravenous injections (84,101). AAVs transduction efficiency depends on the cell uptake and thereafter the downstream events for delivery of the transgene to the nucleus (Fig. 3). The interactions between the protein capsid and receptors on the target cell surface determine the cellular uptake. It is presumed that AAV serotypes recognize different glycoprotein receptors, which can explain the diverse tissue- and cell tropism seen among the different serotypes, e.g., AAV2 binds to heparan sulfate proteoglycans (80,83,111). The many available serotypes further increase the potential of AAVs as gene therapy carriers (112). However, the different tissue tropism is very important to consider from therapeutic perspectives, where high specificity and low off-target toxicity is the major goal for delivering genes to the brain. Many serotypes have tropism for several organs, and the most common organ transduced is the liver (101). To circumvent the liver, many pre-clinical (89,104,113,114) and clinical trials

(79,100,108,115) using AAVs inject the vector directly into the brain. However, as mentioned for drug distribution after intracerebral injections, the viral distribution in the brain parenchyma is likewise limited, and the transduction of brain cells is only localized near the injection site (13,116). The local transgene expression limits the clinical application in diseases with widespread neurodegeneration.

One serotype showing great potential in pre-clinical and clinical trials for CNS gene therapy is the AAV9 (7,89,117), which can cross the BBB and transduce both neurons and astrocytes (118). The exact mechanism for crossing is, however, not known (119), but it allows for the non-invasive delivery of genes to the brain, which is highly needed (10,120). On the other hand, the broad tissue tropism seen with the AAV9 increases the risk of off-target accumulation. A study investigating the tissue tropism of serotype AAV1-9 in mice after intravenous injections of AAVs encoding the luciferase transgene under the cytomegalovirus promotor found that AAV9 resulted in widespread luciferase expression in nearly all organs accessed (101). Furthermore, luciferase activity was mainly found in the liver. Consequently, the levels of luciferase protein and genome copy number were limited in the brain (101). Due to low brain accumulation, higher doses are needed when administering AAV9s systemically, resulting in high transgene expression in peripheral tissue, which increases the risk of, e.g., hepatotoxicity. This has also been emphasized in several studies in mice where hepatocellular carcinoma has developed after rAAV gene therapy (85,121–123). In contrast, genotoxicity has not been observed in long-term studies in dogs (102), non-human primates (103,124), and humans (72,81,106,124). However, higher administered doses also increase the risk of adverse immune responses (81,125). Together with the risk of genotoxicity, these are still important considerations when investigating systemically administered rAAVs for future use in humans.

In the last decade, exploring new capsid variations that can increase the specificity of the virus and increase the transduction efficiency necessary for treating neurological disorders has evolved (6,7). In the following section, examples of these modified viral vectors will be provided, with the main focus on the BEC-specific AAV-BR1 vector used in this Ph.D.-thesis (6).

1.3.2. ADENO-ASSOCIATED VIRUS CAPSID ENGINEERING

Vectors based on AAVs are of great importance due to the aforementioned favorable safety profile, transduction efficiency, and long-lasting transgene expression. However, the issues concerning low specificity for the CNS have resulted in the development of new capsid variations of AAVs, which have shown superior effects compared to the wild-type AAVs (6,7,126,127).

Newly engineered AAVs using a cell-type-specific capsid selection method called CREATE (Cre Recombinase-based targeted evolution) *in vivo* resulted in the identification of the novel AAV-PHP.B and AAV-PHP.eB capsids, differing from

AAV9 by a heptamer amino acid insertion in the capsid sequence (126,128,129). The novel AAV-PHP.B capsid family has a higher CNS transduction efficiency compared to rAAV9 after intravenous injections in C57BL/6J mice, probably due to their improved ability to cross the BBB (126,129). The AAV-PHP.B have tropism for both astrocytes and neurons with widespread transduction in the brain, including cells in the cortex, hippocampus, striatum, and cerebellum (126,128). In addition, the AAV-PHP.B and AAV-PHP.eB have lower transduction of the liver compared to the AAV9 (126,130). Thus, the tropism can be directed to be more efficient depending on the amino acid modification (128). Despite these promising results, the viral vectors with a neurotrophic capsid have some limitations. The AAV-PHP.B capsid family is found to be non-permissive in the CNS of several mouse strains including the BALB/cJ, C3H/HeJ, and NOD/ShiLtJ mice (7,126,130,131). It has been emphasized that the interaction between the neurotrophic capsid and the lymphocyte antigen 6 complex (LY6A) expressed on BECs is essential for the ability of the vectors to cross the BBB (130,132,133). The presence of single-nucleotide polymorphisms (SNPs) can affect the *Ly6a* gene function and/or the expression at the BBB. Unfortunately, in approximately 50 % of mouse strains, the genotype is described as restrictive, limiting the interaction of the capsid with the LY6A protein, consequently reducing the transduction efficiency in these strains (7,126,132). In addition, the transduction efficiency of AAV-PHP.B was comparable to the AAV9 in non-human primates, probably due to the absence of LY6A (126,131,134). These findings are very interesting for future studying of, e.g., proteins on the BBB which can direct CNS delivery (131), however, the species-specific transduction efficiency seen with the AAV-PHP.B vectors makes the translation to humans challenging (132).

The need to find an efficient vector for CNS gene therapy after systemic delivery is still ongoing. However, a completely different approach can be the solution. Instead of crossing the impermeable BBB, targeting the BECs could be the aim of future gene therapy for brain diseases (6,127).

1.3.3. THE BRAIN ENDOTHELIAL CELL-SPECIFIC AAV-BR1 VECTOR

The screening of an AAV2 peptide library in FVB/N mice resulted in the generation of the AAV2-derived vector variant with a mutated capsid, referred AAV-BR1, with high specificity for the BECs after intravenous injection (6). Screening of random AAV display peptide libraries *in vivo* is an important approach for selecting targeting ligands, as this allows for a systematic investigation of peptides within the structural constraints of the AAV capsid (6). Other approaches for manipulating the capsid include the insertion of phage-selected peptides into the receptor-binding sites. The targeting properties of these peptides can, however, easily change when they are transferred into the protein context of the viral capsid (6). Thus the approach used by Körbelin and colleagues is based on the intravenous injection of an AAV2 library displaying seven random amino-acids insertions on the capsid protein in mice. Vectors homing to the brain were used for the following selection process, which was

repeated five times in total. The AAV-BR1 displaying the NRGTEWD peptide was found to be the most brain-specific vector and used for further investigation (6). Thus, the capsid of AAV-BR1 differs only by seven consecutive amino acids compared to rAAV2. However, a 650-fold increase in the transgene expression in the brain was seen when using the AAV-BR1 vector. Additionally, the transgene expression was persistent and still present in the brain after 660 days in mice. In contrast, no or only limited transgene expression was observed in off-target tissue, e.g., the liver, indicating a favorable safety profile (6). This has been supported by histopathological evaluation of peripheral tissue in C57BL/6 mice 11 months after the intravenous administration of AAV-BR1 vectors, where no evidence for the development of hepatocellular carcinoma was seen (135), which, as previously described, has been a major concern when using AAVs in gene therapy.

The specific receptor for AAV-BR1, and thus the reason for the high specificity for BECs, is currently unknown. As mentioned previously, AAV2 is known to bind to heparan sulfate proteoglycans (111). Due to a change in the peptide-binding-motif of the AAV-BR1 capsid, it has possibly changed the preference to another cell surface target, which can also explain the lower accumulation of AAV-BR1 in heparin-producing organs, e.g., the liver following systemic administration (6,125). Compared to the liver, the transgene expression was 1,000-fold stronger in the brain (6). In addition, the co-injection of the AAV-BR1 capsid peptide with AAV-BR1-GFP or AAV2-GFP vectors did not change the fluorescent signal of the AAV2. In contrast, the signal from the AAV-BR1 was significantly reduced, indicating that the AAV-BR1 capsid peptide does not compete with the binding site for AAV2 but only the AAV-BR1 (125). Thus, they probably recognize different binding sites. Neither way, the type I transmembrane protein KIAA0319L is essential for the cellular uptake of different serotypes of AAV and could therefore be involved in the endocytosis of the AAV-BR1 vector as well (6,136).

The therapeutic potential of the AAV-BR1 vector has been emphasized in different mouse models, including incontinentia pigmenti (6,135), Sandhoff disease (137), and Allan-Herndon-Dudley syndrome (138) after systemic administration of the vector. Furthermore, the potential of retinal delivery has also been investigated with promising results (125,139), indicating a broad therapeutic potential. Other advantages of the AAV-BR1 vectors are the lower dose needed for the transduction of BECs ($1.8x10^{11}$ genomic particles/mouse) (6) compared to, e.g., AAV9 ($1.3\text{-}1.8x10^{12}$ genomic particles/mouse) (7,10) when administered systemically for delivering genes to the CNS. The lower dosing regimen for AAV-BR1 decreases the risk of off-target toxicity further (6,135,137).

AAV-BR1 has been used in FVB/N and C57BL/6 mice (6,8,125,127,135,137–140). Therefore, the translation to other mouse strains needs to be investigated further to verify whether the AAV-BR1 transduction efficiency is limited to specific strains. In addition, based on previous knowledge that some AAVs show species-specific tropism, it remains to be investigated whether the specificity and efficiency of AAV-

BR1 seen in mice can be translated to larger animal models and finally to clinical settings (135).

1.3.4. THE BLOOD-BRAIN BARRIER-DIRECTED GENE THERAPY STRATEGY

Previous *in vitro* studies investigating gene therapy at the BBB have shown that genetically modifying BECs results in a bi-directional secretion pathway (11,14,17). Subsequently, recombinant proteins will be delivered into the brain parenchyma and the blood. This approach has, therefore, the potential of treating diseases with both visceral and neurological involvement seen in, e.g., LSDs. This particular strategy will be investigated *in vivo* in this Ph.D. thesis.

The last part of the introduction will provide a brief overview of LSDs, and describe the NP-C2 used for investigating the BBB-directed gene therapy strategy.

1.4. LYSOSOMAL STORAGE DISORDERS

LSDs are a heterogeneous group of more than 70 inherited metabolic diseases, which collectively have an incidence of 1 in 5.000 live births (141). LSDs are caused by deficiency or dysfunction in enzymes or proteins involved in lysosomal degradation and transport of waste products, resulting in the accumulation of storage material within the lysosomes. These diseases are, therefore, multisystemic, and more than two-thirds of LSDs have neurological involvement with progressive neurodegeneration, underlining the vulnerability of the brain due to lysosomal dysfunction (141–143). This challenges the treatment of LSDs since current treatment options are ineffective in treating neurological symptoms due to the impermeable BBB (142). Most LSDs are monogenic, and these diseases are, therefore, interesting candidates for gene therapy. One example of a monogenic LSD involving peripheral organs and the brain is the NP-C2 (144). NP-C2 is therefore interesting for investigating BBB-directed gene therapy intending to treat both visceral and neurological symptoms.

1.4.1. NIEMANN-PICK TYPE C2 DISEASE

Niemann-Pick type C disease (NP-C) is a rare autosomal recessive neurovisceral disorder with an incidence between 1:90,000 to 120,000 live births depending on the country (145–147). The disease is characterized by the accumulation of cholesterol and other lipids in the lysosomes due to mutations in either the *Npc1* or *Npc2* gene. More than 470 mutations in the *Npc1* gene (accounting for 95 % of the cases) and approximately 27 in the *Npc2* gene (5% of the cases) have been described (148). Thus,

NP-C is subdivided according to the gene affected into NP-C1 or NP-C2 (145). The severity of the diseases is correlated with the type of mutation, e.g., the nonsense mutation E20X, the most common mutation in the *Npc2* gene, results in a truncated Niemann-Pick C2 protein (NPC2) with a severe clinical phenotype (145,149,150). The *Npc1* gene encodes the large 13-domain transmembrane glycoprotein Niemann-Pick C1 (NPC1) located mainly in the late endosomes, whereas the *Npc2* gene encodes the small soluble NPC2 (132 amino acids) found in the lumen of the lysosomes. Both proteins are involved in the transport of cholesterol out of the lysosomes, functioning cooperatively. NPC2 binds free cholesterol within the lysosomes and transfers it to the N-terminal domain of NPC1, which contains a sterol-binding site, subsequently mediating cholesterol transport to the endoplasmic reticulum and plasma membranes (151–154). The NPC2-NPC1 interaction is necessary for the lysosomal export of cholesterol. This has been emphasized by the comparison of single NPC1 and NPC2 mutant mice with double mutant mice, where no phenotypic differences were observed (155). Thus loss-of-function mutations in either of these two proteins result in the accumulation of cholesterol and other lipids in the late endosomes/lysosomes, consequently impairing cholesterol homeostasis, which is critical for normal function, especially in the brain (156). Yet, the precise mechanism for intracellular cholesterol sorting and hence the functional role of NPC1 and NPC2 is still under investigation (151,157).

The disease course of NP-C1 and NP-C2 resemble each other due to the proteins' closely related function, but the clinical manifestation in patients are diverse with varying onset, progression, and lifespan. Therefore, NP-C patients are classified according to the age at neurological onset, which also correlates with the severity of the disease; 1) perinatal, 2) early infantile, 3) late infantile, 4) juvenile, and 5) adult onset. The juvenile-onset represents the classic form of NP-C (145,146,158). For an overview of the clinical spectrum, see Figure 5.

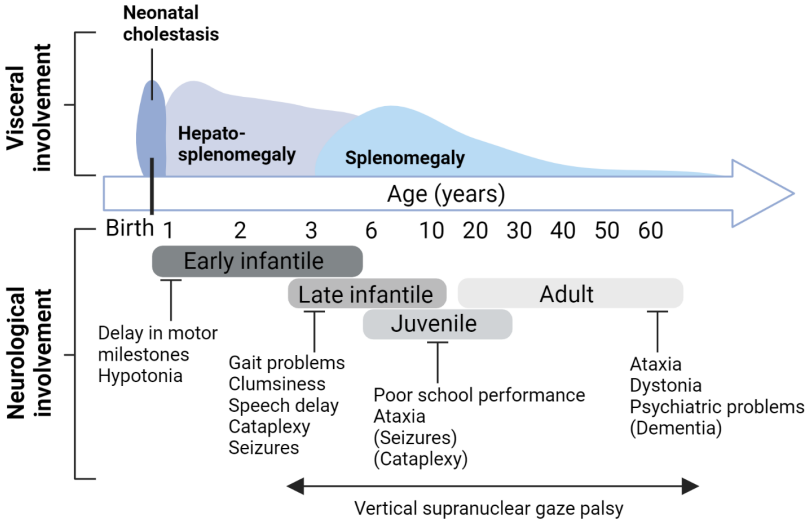

Figure 5. Schematic overview of the clinical manifestation of Niemann-Pick type C disease. Perinatal onset (< 2 months): Patens are presenting with fetal ascites or hydrops, hepatosplenomegaly, and cholestatic icterus, which in severe cases worsen, resulting in death before 6 months of age. Early infantile-onset (2 months to 2 years): Isolated hepatosplenomegaly with the development of neurological symptoms at 1-2 years of age. Patients die before 5 years of age. Late infantile-onset (2-6 years): Isolated hepatosplenomegaly with development of neurological symptoms at 3-5 years of age, as motor impairment worsens, patients develop progressive ataxia, dysphagia, and dysarthria. Juvenile-onset (6-15 years): Isolated splenomegaly, severe motor, and cognitive impairment. The lifespan is variable. Adult-onset (> 15 years): visceral symptoms are often not present. All patients (with few exceptions) develop neurological symptoms. Modified from (145,159). Created with BioRender.com.

The most common visceral manifestation is (hepato)splenomegaly, whereas the neurological symptoms typically consist of ataxia, cataplexy, dysarthria, dysphagia, vertical supranuclear gaze palsy, cognitive impairment, and dementia. Except for the perinatal onset, the systemic disease is seldom severe, and most patients die due to the progressive nature of the neurodegeneration between 10-25 years of age (145,148). NP-C is suspected when both systemic, neurological, and psychiatric symptoms are present. However, due to the clinical heterogeneity, the diagnosis is often delayed or undiagnosed, especially in cases with isolated splenomegaly or psychiatric cases (160,161). The diagnosis can be confirmed with a combination of biochemical and genetic analyses. The first important diagnostic tool includes the detection of disease biomarkers in plasma, which includes cholesterol oxidation products, e.g., cholestane-3β,5α,6β-triol, and bile acids metabolites, e.g., 3β,5α,6β-trihydroxy-cholanic acid. These biomarkers are, however, not solely present in NP-C patients; therefore genetic analysis, e.g., DNA sequencing, is necessary for confirming the diagnosis (148,158,162). The identification of the genetic mutation is mandatory in all

diagnostic cases due to the knowledge about the genotype-phenotype correlation described earlier (158).

Pathology

Cholesterol accumulation in lysosomes of all cells is a pathological hallmark of NP-C. However, the impaired intracellular cholesterol transport results in secondary alterations in several other lipids, such as sphingolipids (sphingomyelin and sphingosine), gangliosides (GM2 and GM3), and glycolipids (glucosylceramide) (148,160). It has been emphasized that cholesterol and sphingosine primarily dominate in visceral tissue, whereas glycosphingolipid storage predominates in the brain (163–166). It is, however, still debatable which lipids are the primary cause of pathology seen in the brain (167–169). Argues against glycosphingolipids being the primary cause of brain pathology were proposed in a study using an NP-C mouse model devoid of CNS ganglioside accumulation. The absence of ganglioside did not impact the disease phenotype or improve the pathology in the brain (170). In addition, it has been found that the cholesterol levels in visceral organs increase with age, whereas the brain cholesterol levels decrease, probably due to the reduction in myelin caused by severe progressive neurodegeneration (171,172), which corresponds with the fact that myelin contains approximately 75 % of the cholesterol in the brain (173). Thus, severe demyelination is associated with lower cholesterol levels.

Cholesterol is essential for the structure and function of membranes and is a precursor of many biological molecules. Hence, the intracellular localization and levels of cholesterol require strict regulation. Therefore, dysregulation of cholesterol homeostasis can easily be related to the neurodegenerative condition in human NP-C (167,174). The pathology of NP-C is probably a combination of the accumulation, mislocalization, and consequently deprivation of lipids (148). First, due to the loss of NPC2, the cholesterol accumulates within the lysosomes, and thus the transport of cholesterol from the lysosomes to plasma membranes is impaired (175). Secondly, the delivery of lipids to the endoplasmatic reticulum is lacking, leading to a reduction in cholesterol in the particular organelle. Finally, as a compensatory mechanism, the sterol regulatory element binding proteins (SREBPs) are activated, causing an upregulation in cholesterol synthesis. Consequently, the low-density lipoprotein (LDL) receptors are upregulated, increasing the intracellular uptake of cholesterol, per se escalating the disease (157,176,177). The lipid accumulation and subsequently deficiency of cholesterol and other lipids for other biological processes induce a cascade of pathological events ultimately leading to cell death (148,160).

Another area receiving much attention is why specific brain regions are more affected than others. The cerebellum is especially vulnerable to NPC1 and NPC2 deficiency, and a pathological hallmark is progressive Purkinje cell death (178–180). Several theories for Purkinje cell degeneration have been proposed including autophagy (181,182), oxidative stress (183), apoptosis (184), neuroinflammation (185,186), and necroptosis (187). Despite years of research, the cause of Purkinje cell death is still

unknown, and this area is also beyond the scope of this Ph.D. thesis. There are, however, some clear pathological hallmarks of the disease, evident in both patient and animal models of NP-C: neuronal storage and axonal dystrophy, Purkinje cell degeneration, widespread neuroinflammation, and accumulation of foam cells in visceral organs (178,186,188–192). Even though the clinical manifestation of NP-C1 and NP-C2 are comparable, patients suffering from NP-C2 have been found to have an increased risk of developing severe lung disease characterized by pulmonary alveolar proteinosis (149,191,193,194). Emphasizing that the function of the NPC1 and NPC2 still needs to be fully understood.

As indicated, NP-C is a complex disease with many unresolved questions, and the pathogenesis leading to severe neurodegeneration remains to be fully elucidated. This highlights the continuous need for animal models reflecting heterogeneous pathology seen in patients with NP-C, which hopefully can provide more knowledge regarding the pathophysiology, subsequently leading to new treatment strategies.

Treatment options

Currently, the only approved disease-modifying therapeutic in Europe is the glucosylceramide synthase inhibitor, Miglustat, which can cross the BBB. Miglustat inhibits the enzyme involved in the synthesis of glucosylceramide, important for the biosynthesis of glycosphingolipids, subsequently reducing the storage of these lipids in the lysosomes (148,195–197). Unfortunately, the lag time for diagnosis after clinical onset limits the effect of the Miglustat treatment (196). Since all patients with NP-C die prematurely, and many patients have the onset of the disease in childhood, it stresses the importance of developing new effective treatment strategies (10,198).

The soluble nature of the NPC2 allows for secretion and endocytosis by adjacent cells via the mannose-6-phosphate-receptor (199), subsequently rendering NPC2 a suitable candidate, e.g., for enzyme replacement therapy. The enzyme replacement strategy has previously been investigated in a mouse model of NP-C2 with the potential to treat visceral symptoms (200). However, they found no neurological improvement after intravenous administration of bovine NPC2, indicating that NPC2 is unable to cross the BBB, which is often a rate-limiting problem (37,200). Based on the current knowledge, the therapy needed for treating the devastating NP-C2 in humans will ideally be one that can enable the transport of the NPC2 across the BBB while also treating the visceral pathology (148,195).

Thus with the great potential of the AAV-BR1 in treating neurological diseases in pre-clinical models, this Ph.D. project will investigate whether transduction of the BECs using the BEC-specific AAV-BR1 vector can result in the secretion of the NPC2 to the blood and the brain, allowing for cross-correction within both visceral and brain tissue (Fig. 6).

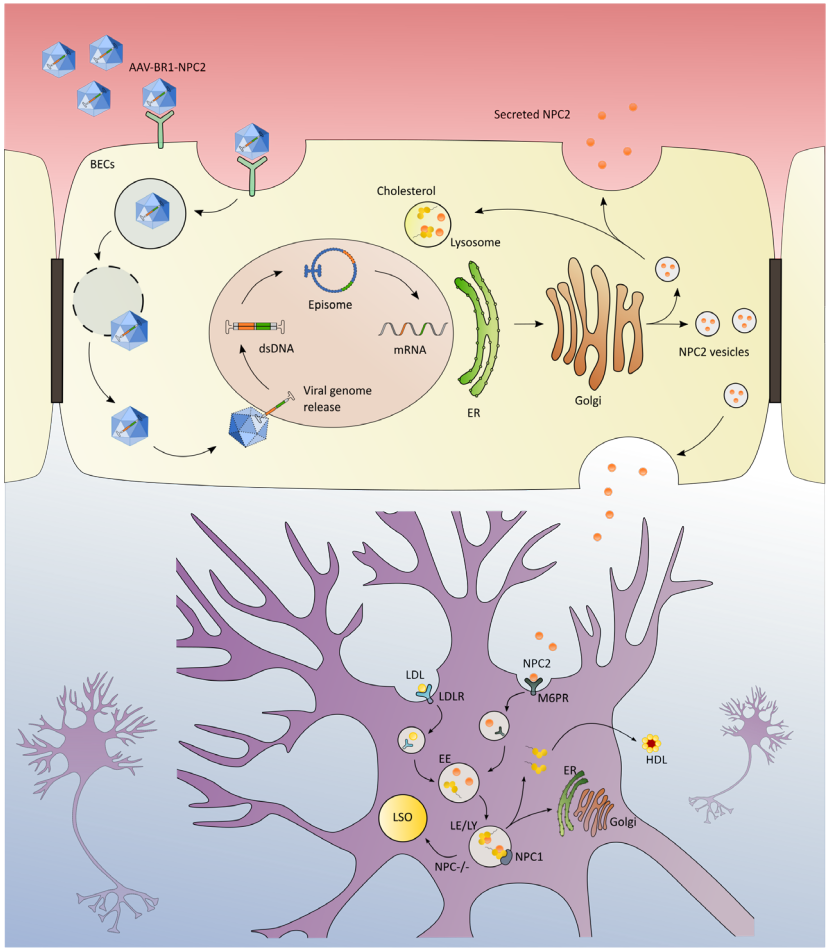

Figure 6. Schematic illustration of the hypothesis of this Ph.D.-thesis. By genetically modifying brain endothelial cells (BECs) using the BEC-specific adeno-associated virus (AAV-BR1) vector, secretion of the Niemann-Pick C2 protein (NPC2) should be towards the blood and the brain resulting in cross-correction of non-transduced cells in both visceral and brain tissue, e.g., neurons. Normally, low-density lipoprotein (LDL) is taken up via the low-density lipoprotein receptor (LDLR). LDL is hydrolyzed to cholesterol inside the endosome, NPC2 binds cholesterol in the late endosome/lysosome (LE/LY), subsequently transferring cholesterol to the membrane-bound NPC1 protein, which facilitates the transport of cholesterol to the endoplasmatic reticulum (ER), plasma membranes or released as high-density lipoprotein (HDL). NPC2 deficient cells lack functional NPC2, resulting in impairment of the cholesterol trafficking within the cell, consequently leading to accumulation of cholesterol within the lysosomes (lysosomal storage organelles (LSO)). However, recombinant NPC2 can be taken up by NPC2 deficient cells via the mannose-6-phosphate receptor (M6PR) and carried to endosomes, subsequently restoring the cholesterol transport. The recombinant NPC2 can also be distributed within the transduced cell, repairing the cholesterol storage in BECs. Modified from (17,201). Created with Inkscape.

CHAPTER 2. OBJECTIVES

In previous non-viral gene therapy studies *in vitro*, it has been shown that genetic modification of BECs results in a bidirectional secretion of the recombinant protein (11,14,17), enabling protein delivery to both the brain and the blood. Gene therapy at the BBB has, therefore, the potential to treat diseases characterized by a global lack of proteins, as seen in the cholesterol storage disorder NP-C2.

The objective of this Ph.D. thesis was to further investigate the potential of this strategy both *in vitro* and *in vivo* using the BEC-specific AAV-BR1 vector, which has previously been proven effective in treating different neurological diseases (6,135,137,138). Furthermore, the therapeutic effect of the AAV-BR1 will be investigated in a mouse model of NP-C2 suffering from both neurological and visceral pathology.

The overall hypothesis of this Ph.D. dissertation is that BBB-directed gene therapy using the BEC-specific AAV-BR1-NPC2 results in the secretion of NPC2 from the BECs to the blood and the brain, subsequently treating both visceral and neurological symptoms in a mouse model of NP-C2.

To address the overall objective of this Ph.D. thesis, four studies with the following aims have been conducted:

Study I: Investigate the strategy of using AAV-BR1 gene therapy at the BBB to induce the secretion of a therapeutic protein *in vitro* and *in vivo* in healthy mice.

Study II: Performing a histopathological characterizing the NP-C2 mouse model holding the LST105 mutation to evaluate the translational value of this specific mouse model. Two different age groups were included to assess the disease progression from before the onset of neurological symptoms to the end stage of the disease.

Study III: Review of the literature in the field of gene therapy for NP-C, with a focus on the ARRIVE guidelines, which were essential for the planning of study IV.

Study IV: To investigate the therapeutic potential of the AAV-BR1-NPC2 vector in treating neurological and visceral pathology in the NP-C2 mouse model described in study II.

CHAPTER 3. METHODS

This chapter describes some of the considerations regarding planning the animal studies included in this dissertation. A description of the exact experimental procedures, including ex vivo biochemical analysis, is provided in the methods section of publication I and study II and IV.

3.1. ETHICAL CONSIDERATIONS

All animal experiments and the breeding of the genetically modified NPC2 deficient mouse model was approved by the Danish Animal Experimentation Council under the Danish Ministry of Food, Fisheries, and Agriculture (licenses #2018-15-0201-01467 and #2019-15-0202-00056 (breeding approval)). The animal studies were carried out at the animal facilities in Aalborg or Aarhus University by licensed staff.

Furthermore, the Danish Working Environment Authority has approved the research project involving the genetically modified virus AAV-BR1 encoding the *Npc2* gene (AAV-BR1-NPC2).

3.2. ANIMAL MODELS

Animal models resembling the heterogeneous pathology in NP-C2 are invaluable for understanding the pathophysiology and development of new therapeutics for this fatal disease (202). While several genetically modified mouse models (155,180,203–207) and two different spontaneous feline models (208,209) are available for investigating NP-C1, only a few animal models are described for NP-C2, including NPC2 deficient zebrafish (210), one spontaneous feline model (211), and two hypomorphic mouse models of NP-C2 (155,200). The majority of studies in NP-C2 are based on the *Npc2*^{tm1Plob} mouse model (155,195,207,212–215) using gene targeting for establishing the model (155). However, only sparse information is available for the *Npc2*$^{Gt(LST105)BygNya}$ mouse model (200,213). The *Npc2*$^{Gt(LST105)BygNya}$ mouse model (200) was used in study II and IV due to the accessibility at the animal facility at Aarhus University. The NP-C2 mouse model holding the LST105 gene trap mutation will be referred to as NPC2-/- in this Ph.D. thesis. The gene trap mutation results in a fusion protein including the first 27 amino acids of the NPC2 encoded by the first exon of the *Npc2* gene (200). The NPC2-/- mice recapitulate many NP-C2 hallmarks seen in patients, e.g., cholesterol storage in visceral and brain tissue, accumulation of foam cells in the liver, lung, and spleen, and development of tremor and ataxic gait. Furthermore, Purkinje cell loss and neuroinflammation were found in the cerebellum (200). However, the description of the pathological findings in the brain is limited. Therefore, phenotypic and histopathological characterization of the NPC2-/- mice

were carried out in study II to provide more knowledge regarding the pathology and disease progression of the NPC2 deficient mouse model and the translatability to human NP-C2.

It is well-known that the genetic background in mouse models can impact outcome measures (126,131,206,207,216,217). Therefore, the BALB/cJRj mouse strain was used in study I since the NP-C2 mouse model (study II and IV) was established on this specific background strain. Furthermore, the primary mouse brain endothelial cells were isolated from BALB/cJRj mice and used for the *in vitro* BBB model (study I and IV).

3.3. BREEDING OF NPC2-/- MICE

The work with the NPC2-/- mice included rederivation of the mouse strain due to relocation to a new animal facility with higher health status. The NPC2-/- mice were rederived using embryos from BALB/cJRj (Janvier Labs) and semen collected from heterozygous NPC2+/- mice. For the breeding of NPC2-/- mice, two NPC2+/- females mice and one NPC2+/- male mouse were used. The choice for continuous trio breeding was based on the previous knowledge that approximately every 6-7 mice are born with the NPC2-/- genotype (200). Thus, rapid colony expansion was needed to provide sufficient offspring for study II and IV (218), together with taking advantage of communal nesting to provide more robust offspring (219,220).

3.4. STUDY DESIGN

Planning, conducting, and reporting data from animal studies in this Ph.D. thesis has been based on the ARRIVE (221) and PREPARE (222) guidelines. In addition, a statistical protocol was prepared before conducting study IV, where the outcome measures and statistical analysis for each parameter were described. Finally, an essential part of planning study IV included reviewing the literature on gene therapy for NP-C (study III).

Due to the explorative nature of study I, where the aim was to evaluate the tissue distribution of the AAV-BR1 vector in healthy BALB/cJRj mice and investigate whether it was possible to induce bi-directional secretion of NPC2 from the BECs, the number of animals included was based on the literature (201). Other CNS studies evaluating biodistribution, transduction efficiency, and/or gene copy number of AAVs used three (89,195), five (6,137), six (7), or 9-11 (135) mice/group. Seven mice/group was included in study I, as I also wanted to compare NPC2 mRNA levels and NPC2 concentrations in plasma in control and AAV-BR1-NPC2 injected mice (223).

For study IV *a priori* sample size calculation was performed (221). Development of tremors and ataxic gait in NP-C2 are correlated with the progressive Purkinje cell loss

in the cerebellum, consequently affecting the ability to accomplish behavioral tasks involving locomotor activity (178,182,195,224). Therefore, the primary outcome measure was rotarod performance, which is an important method for evaluating the therapeutic effect of AAV-BR1 gene therapy on cerebellar pathology (225,226).

The effect size was calculated by the G*Power software (version 3.1.9.2) using extracted data from the literature (mean ± SD from untreated vs. treated NP-C mice) due to limited data on the NPC2-/- mouse model included in this Ph.D. project. When looking at other studies investigating the efficiency of AAVs in NP-C mouse models on motor function (evaluated by rotarod or balance beam test), the estimated effect size was between 1.5-2.2 (195,227). Therefore, the magnitude of effect should be comparable with these studies, and not less. Thus, the sample size in study IV was calculated with a predefined effect size of 1.5, a power of 80 %, and a significance level of 0.05, giving nine animals/group. The sample size was corrected for the expected 10 % loss of mice (228). Subsequently, ten mice were included in each experimental group. All experimental groups included both female and male mice due to previously observed differences in the phenotype between male and female NP-C mice (195,224,229).

The NPC2-/- and NPC2+/+ (wild-type) mice were born at different time points during the study period. Thus the study was conducted in cohorts. Whenever possible, all three experimental groups were included in each cohort, and all three experimental groups were represented in each cage. However, in some weeks, only one NPC2-/- mouse, or one female and one male NPC2-/- was born, challenging the strategy by including all three experimental groups in each cohort or each cage. All experiments were carried out from May to September (2022), meaning that all mice included in study IV were housed in the same IVC rack at some point during the experiment. All handling and behavior analyses were conducted randomly at the same time of the day (8-11 am).

In study I, II, and IV, a thorough examination of brain sections was included. For identification of the neuroanatomical areas, the Allen Brain Reference Atlas of the adult mouse brain was used (230).

3.5. BEHAVIOR ANALYSIS

In study IV, an accelerating rotarod test and a composite phenotype score were included to evaluate the efficacy of the AAV-BR1 gene therapy on cerebellar disease progression. The accelerating rotarod protocol was adapted from (231). However, the final training protocol was based on pilot studies in wild-type mice conducted in our animal facilities. It was experienced that one day with habituation to the rotarod apparatus (4 revolutions per minute (rpm) for 4 minutes), and one day with habituation to the higher speed (10 rpm for 2 minutes), was necessary for implementing the accelerating protocol. Finally, two consecutive training days were included to allow the mice to habituate to the specific test protocol. Each training and test session

included three trials. No significant differences were observed between NPC2-/- mice and NPC2+/+ mice concerning the learning of the test protocol (Fig. 7). In addition, the rotarod data from male and female mice were pooled in study IV as no differences between sexes were observed within the experimental group for all time points assessed.

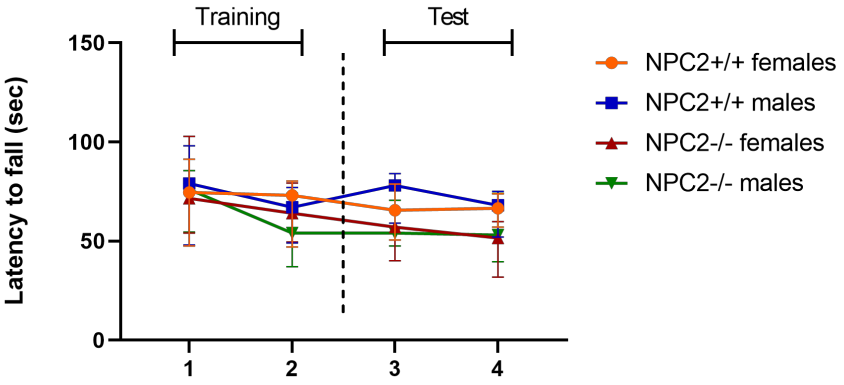

Figure 7. Rotarod performance in NPC2-/- and NPC2+/+ mice during training and test sessions. Before the first test session, all mice were habituated to the accelerating rotarod protocol (4-40 rpm over 5 minutes) for two consecutive days with three trials per day. The latency to fall was recorded, and the average of the three trials was used for the statistical analysis. After the training session, the first test was conducted at five weeks of age. The number on the x-axis refers to 1) training session 1, 2) training session 2, 3) five weeks test, and 4) six weeks test (before the onset of treatment). No significant difference was seen during the training and the first two test sessions between NPC2+/+ females, NPC2+/+ males, NPC2-/- females, and NPC2-/- males, analyzed with repeated measure two-way ANOVA (F[3,32] = 0.911, p = 0.447). The total numbers of mice included in each group were; NPC2+/+ females n = 8 mice, NPC2+/+ males n = 7 mice, NPC2-/- females n = 15, NPC2-/- males n = 9.

To further evaluate the effect of viral gene therapy, a composite phenotype score was included (adapted from (232–235)). The mice were assessed using the following seven parameters: grooming, kyphosis, tremor, ledge test, gait analysis, hindlimb clasping, and explorative behavior (activity level). The ideal combination of parameters depends on the specific disease (233). As all the abovementioned measures previously have been used in assessing disease phenotype in mouse models of NP-C (7,234,236), they were also included for evaluating disease progression in the NP-C2 mouse model of study IV. The mice were scored before treatment initiation (6 weeks of age), after symptom development (9 weeks of age), and at the end-stage of the disease (12 weeks of age) to follow the disease progression. During the assessment, each mouse was transferred to a new, clean cage without bedding (GM500 IVC cage from Techniplast). The first parameter assessed was the explorative behavior. It can be discussed whether explorative behavior is the right term as NPC2-/- mice receiving a higher score (e.g., score 1: exploring 2-3 corners in 90 sec) was due to locomotor impairment, consequently having a slower movement

in the cage. However, it was still valuable for assessing the motor deficit associated with the disease. The gait, grooming, and kyphosis were also evaluated during this assessment. Finally, the mouse was lifted by the tail to assess hindlimb clasping and then placed on the cage ledge for the ledge test. The last test conducted was the gait analysis using staining of the paws. The results from the gait pattern were included in the composite phenotype score.

CHAPTER 4. RESULTS

4.1. STUDY I

A novel strategy for delivering Niemann-Pick type C2 proteins across the blood-brain barrier using the brain endothelial-specific AAV-BR1 virus

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Abstract

Treating central nervous system (CNS) diseases is complicated by the incapability of numerous therapeutics to cross the blood-brain barrier (BBB), mainly composed of brain endothelial cells (BECs). Genetically modifying BECs into protein factories that supply the CNS with recombinant proteins is a promising approach to overcome this hindrance, especially in genetic diseases, like Niemann Pick disease type C2 (NPC2), where both CNS and peripheral cells are affected. Here we investigated the potential of the brain endothelial cell-specific adeno-associated viral vector (AAV-BR1) encoding NPC2 for expression and secretion from primary BECs cultured in an in vitro BBB model with mixed glial cells, and in healthy BALB/c mice. Transduced primary BECs had significantly increased NPC2 gene expression and secreted NPC2 after viral transduction, which significantly reversed cholesterol deposition in NPC2 deficient fibroblasts. Mice receiving an intravenous injection with AAV-BR1-NCP2-eGFP were sacrificed eight weeks later and examined for its biodistribution and transgene expression of eGFP and NPC2. AAV-BR1-NPC2-eGFP distributed mainly to the brain, lightly to the heart and lung, but did not label other organs including the liver. eGFP expression was primarily found in BECs throughout the brain but occasionally also in neurons suggesting transport of the vector across the BBB, a phenomenon also confirmed in vitro. NPC2 gene expression was upregulated in the brain, and recombinant NPC2 protein expression was observed in both transduced brain capillaries and neurons. Our findings show that AAV-BR1 transduction of BECs is possible and that it may denote a promising strategy for future treatment of NPC2.

4.2. STUDY II

The *Npc2*$^{Gt(LST105)BygNya}$ mouse signifies pathological changes comparable to human Niemann-Pick type C2 disease

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Manuscript submitted

Abstract

Introduction: Niemann-Pick type C2 disease (NP-C2) is a fatal neurovisceral disorder caused by defects in the lysosomal cholesterol transporter protein NPC2. Consequently, cholesterol accumulates within the lysosomes, causing a heterogeneous spectrum of clinical manifestations. Murine models are essential for increasing the understanding of the complex pathology of NP-C2. This study, therefore, aims to describe the neurovisceral pathology in the NPC2 deficient mouse model to evaluate the correlation to the human NP-C2.
Methods: NPC2-/- mice holding the LST105 mutation were used in the present study (*Npc2*$^{Gt(LST105)BygNya}$). Body and organ weight and histopathological evaluations were carried out in six and 12 weeks old NPC2-/- mice, with a special emphasis on neuropathology. Thus, the Purkinje cell marker calbindin, the astrocytic marker GFAP, and the microglia marker IBA1 were included for assessing Purkinje cell degeneration and neuroinflammation, respectively. In addition, the pathology of the liver, lungs, and spleen was assessed using hematoxylin and eosin staining.
Results: Six weeks old pre-symptomatic NPC2-/- mice were found to have splenomegaly and obvious neuropathological changes, especially in the cerebellum, where initial Purkinje cell loss and neuroinflammation were evident. The NPC2-/- mice developed neurological symptoms at eight weeks of age, severely progressing until the end-stage of the disease at 12 weeks. At the end-stage of the disease, NPC2-/- mice are characterized by growth retardation, tremor, cerebellar ataxia, splenomegaly, foam cell accumulation in the lungs, liver, and spleen, brain atrophy, pronounced Purkinje cell degeneration, and severe neuroinflammation.
Conclusion: The *Npc2*$^{Gt(LST105)BygNya}$ mouse model resembles the pathology seen in human NP-C2 and denotes a valuable model for increasing the understanding of the complex disease manifestation and is relevant for testing the efficacies of new treatment strategies.

4.3. STUDY III

Reporting preclinical gene therapy studies in the field of Niemann-Pick type C disease according to the ARRIVE guidelines: How far are we?

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Abstract

The lack of essential information when reporting animal studies causing lower reproducibility has been stressed for decades. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were first published in 2010 to improve reporting of animal research, making in vivo studies more transparent and subsequently improving the scientific quality. Despite an endorsement from the scientific community, there is still a continuous need to improve animal research reporting, which is unfortunately also the case in the field of Niemann-Pick type C disease (NPC). Despite years of research in developing new treatment strategies for NPC, there is still no cure for this fatal lipid storage disorder caused by the loss-of-function mutation in the Npc1 or Npc2 gene. In 2020 an updated version of the ARRIVE guidelines (ARRIVE 2.0) describing the ten most essential items needed as the minimum information to be included in a manuscript were published. Pre-clinical studies investigating the efficiency of gene therapy as a treatment strategy for NPC were reviewed to evaluate the degree of compliance with the ARRIVE guidelines. Unfortunately, none of the reviewed papers fulfilled the minimum information needed to assess the reliability of the findings. Especially information regarding the choice of sample size, randomization, blinding, and statistical methodology was lacking. Hopefully, the newly updated guidelines will help researchers when planning and publishing in vivo experiments in the future. However, more awareness of the importance of including these essential items are needed, both from editors and researcher, for complete endorsement in the scientific community.

4.4. STUDY IV

The brain-specific AAV-BR1 vector delays the disease progression in a mouse model of Niemann-Pick type C2 disease

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Abstract

Introduction: Niemann-Pick type C2 disease (NP-C2) is a rare neurovisceral disorder characterized by lysosomal accumulation of cholesterol and other lipids resulting in a diverse spectrum of clinical manifestations including hepatosplenomegaly, growth and developmental delay, cerebellar ataxia, and dementia. A loss-of-function mutation in the *Npc2* gene causes the disease. At present, no effective treatments are available for this fatal disorder, and the treatment is further complicated by the presence of the blood-brain barrier (BBB). However, genetic modification of brain endothelial cells (BECs) results in the secretion of recombinant proteins towards the blood and the brain. Subsequently, BBB-directed gene therapy represents a promising strategy for overcoming the restrictive BBB. It was, therefore, hypothesized that the BEC-specific adeno-associated virus (AAV-BR1) vector was able to treat both visceral and neurological symptoms in a mouse model of NP-C2 (NPC2-/-).
Methods: NPC2-/- mice received a single intravenous injection of the AAV-BR1 vector encoding the *Npc2* gene (AAV-BR1-NPC2) at six weeks of age. Rotarod performance, composite phenotype score, and body weight were assessed during the study. Post-mortem analysis included quantifying total cholesterol levels and *Npc2* gene expression in visceral organs. Furthermore, the protein expression of NPC2, GFAP, IBA1, and calbindin was evaluated using immunohistochemistry to assess the therapeutic effect on neuropathology.
Results/discussion: Systemic administration of the AAV-BR1-NPC2 vector resulted in widespread transgene expression of NPC2 proteins in the brain, subsequently delaying the neurodegeneration with improvement in motor function and disease phenotype compared to untreated NPC2-/- mice. These findings were correlated with the preservation of cerebellar Purkinje cells, reduced cholesterol storage, and neuroinflammation in the cerebral cortex. However, no improvement in growth retardation or visceral pathology was evident. Thus, The AAV-BR1 vector shows great potential in treating diseases with neurological involvement.

CHAPTER 5. DISCUSSION

This thesis aimed to investigate whether BBB-directed viral gene therapy is a potential strategy to treat the neurovisceral pathology in a mouse model of NP-C2. Treating diseases affecting the brain is highly challenging due to the protective nature of the BBB. Most drug delivery strategies focus on facilitating drugs across the BBB using, e.g., antibody-conjugated nanoparticles (36,61,72,74,77,237,238) or completely bypassing the BBB with intracerebroventricular or intraparenchymal injections (89,113,239–241). However, instead of crossing the almost impermeable BBB, the BBB-directed gene therapy strategy takes advantage of the specificity of the AAV-BR1 vector for the BECs and utilizes the secretory function of these cells with the potential of delivering recombinant NPC2 to the brain and also the blood (6,8,11). This chapter includes a joint discussion based on the findings from study I, II, and IV and the literature with the main focus on the potential of the AAV-BR1 vector in treating NP-C2. Finally, model limitations will be discussed.

5.1. MORE BASIC RESEARCH IS NEEDED TO UNRAVEL THE CLINICAL POTENTIAL OF AAV-BR1

Before testing the BBB-directed gene therapy strategy in NPC2-/- mice, the aim was to verify that the AAV-BR1 was efficient in the BALB/cJRj mouse strain (study I), which was the background strain of the NP-C2 mouse model. Until now, the AAV-BR1 vector has only been investigated in FVB/N and C57BL/6 mice or transgenic mouse models established on these background strains (6,125,127,135,137–140,242). In addition, the AAV-BR1 capsid variant was selected in FVB/N mice (6). Our concern regarding the AAV-BR1 vector being ineffective in BALB/c mice was based on previous studies using the capsid variant AAV-PHP.B, where the transduction efficiency was dependent on mouse strain (126,130,131,243). The AAV-PHP.B were found to be ineffective in the transduction of the CNS in various inbred mouse strains, including BALB/cJ (130). However, in study I, it was found that AAV-BR1 successfully transduced the BECs in healthy BALB/cJRj mice, subsequently increasing the brain *Npc2* gene expression. Surprisingly, when evaluating the transgene expression *in vivo* using bioluminescence imaging, a high luciferase expression was seen in the thoracic cavity, corresponding to the accumulation of AAV-BR1 vector DNA in lung tissue (201). These results were confirmed in study IV when testing the therapeutic potential of the AAV-BR1 vector in the NP-C2 mouse model. Thus in the BALB/cJRj mouse strain, the AAV-BR1 vector specifically targets the brain and lung tissue. The reason for these discrepancies between AAV-BR1 biodistribution in the BALB/c mice and FVB/N and C57BL/6 mice is unknown. As for the neurotrophic capsid variant (e.g., AAV-PHP.B) where its ability to cross the BBB is dependent on the interaction with the LY6A proteins expressed on the BECs (130,132), the explanation could likewise be differences in the protein expression on BECs and endothelial cells in the lung between the different mouse strains. It could

be interesting to investigate this aspect further for assessing the clinical potential of the AAV-BR1 vector, especially since the LY6A protein is absent in primates, limiting the human translation of the AAV-PHP.B (126). In addition, several examples of species-specific differences in the gene expression profile of endothelial cells have been seen between mice and humans, challenging the extrapolating of data from targeted delivery studies in mouse models to clinical trials (244). Furthermore, diseases can also influence the effect of viral vectors. A previous study has found that AAV capsid variants with tropism for the BECs selected in healthy mice differ from those selected in mouse models of LSDs maintained on the same background strain (8). This is probably caused by inflammatory stimuli subsequently changing the expression of surface proteins of the BECs (245,246), adding another hurdle to the development of efficient vector systems for brain diseases. However, the transduction efficiency of AAV-BR1 was comparable in study I and study IV, indicating that the binding of the AAV-BR1 vector to BECs was not affected by the disease state in NPC2-/- mice. Currently, the AAV-BR1 vector has only been investigated in mouse models, thus it is unknown whether the therapeutic potential of the AAV-BR1 can be translated to larger animal species or humans (135). The AAV-BR1 vector has been shown to transduce immortalized human brain endothelial cells *in vitro*. However, the infectivity was similar to rAAV2, most likely reflecting the difference from *in vitro* to *in vivo* (6). During culturing, endothelial cells change their expression profile, which is dependent on contact with, e.g., pericytes and astrocytes, and flow and shear stress (23,247,248). Thus the translation of these *in vitro* findings is probably limited (6). Altogether, this emphasizes that much remains to be learned regarding the gene and protein expression of the BBB, which is important for continuously improving drug delivery strategies for brain disease with higher specificity and efficiency.

In study I, it was found that the AAV-BR1 vector was able to cross the BBB enabling neuronal transduction in several different areas of the mouse brain (201), which has also been described for the AAV9 and the neurotropic capsid variants AAV-PHP.B and AAV-PHP.eB (7,10,126,129). Interestingly, the pattern of neuronal transduction is comparable between the different AAV vectors, where especially the pyramidal neurons in layer V of the cortex, neurons of the hippocampus, and striatum are widely transduced, independent of the treatment route, doses, transgene, and promotor (7,10,89,126,201). The transgene expression in the neurons becomes weaker when evaluating the brain in a rostrocaudal direction (10,89,201). The explanation could be related to the dual receptor binding required for the endocytosis of viruses. The capsid proteins depict the receptor specificity and hence determine the cell tropism (249). The binding to the target cell often requires several receptors/co-receptors, with the primary receptor, e.g., proteoglycan receptors or O- or N-linked sugars, being used by several AAV serotypes and are necessary for attachment. In contrast, the proteinaceous co-receptors differ among the serotypes and are essential for cellular uptake (250). The AAV2 serotype binds to, e.g., the hepatocyte growth factor receptor, the fibroblast growth factor 1 receptor, or the laminin receptor, while the AAV9 binds to the laminin receptor (250–253). It has been emphasized that pyramidal neurons of, e.g., cortical layer V express a complement of surface receptors (126), subsequently allowing for multiple binding sites for a broad spectrum of capsid

variants, resulting in similarities in the neuronal transduction pattern between different AAVs. However, the reason for these similarities in neuronal tropism between the different viral vectors remains to be elucidated, especially due to the considerable variation in other aspects. For example, AAV-BR1 mainly transduces BECs with none or limited distribution to, e.g., the liver (6,135,137,201). In opposite hereto, AAV9 has a broad tissue tropism with widespread transduction of, e.g., hepatocytes (7,101,126). Lastly, the AAV-PHP capsid family does not transduce BECs but has a high transgene expression in the cerebellum with the AAV-PHP.eB transducing more than 75 % of the Purkinje cells (128). However, all of these vectors possess the ability to cross the BBB. It is still unknown what factors determine whether AAV-BR1 vectors undergo endocytosis or transcytosis, subsequently transducing BECs or neurons, respectively (6,88). In addition, the receptor(s) involved in the uptake of the AAV-BR1, making this vector specific for the BECs, also requires further investigation, which will be one step towards unraveling the clinical potential of the BEC-specific vector.

5.2. BBB-DIRECTED GENE THERAPY AS A STRATEGY TO TREAT NIEMANN-PICK TYPE C2 DISEASE

When investigating the potential of AAV-BR1 transduction of the BECs *in vivo* (study I and IV), large variations in the transduction efficiency were found between the mice ranging from sparse transgene expression (enhanced green fluorescent protein (eGFP) or NPC2) to widespread distribution in the brain. Consequently, the therapeutic effect in the NPC2-/- mice varied, and in some of the AAV-BR1-NPC2 treated NPC2-/- mice, the disease severely progressed with ataxia and inability to perform on the rotarod. One could speculate that this large variation is due to neutralizing antibodies, which is considered a major obstacle to the clinical use of AAVs administered systemically (68,95). It has previously been assumed that small laboratory animals are devoid of pre-existing neutralizing antibodies against AAVs. However, a study investigating neutralizing antibodies from, e.g., rodents, found neutralizing antibodies against different AAV serotypes in the sera of naïve mice obtained directly from the vendors (101). Unfortunately, this has not been investigated in our NP-C2 mouse model but would be important in future pre-clinical studies evaluating the therapeutic effect of the AAV-BR1 vector.

Another explanation for the variance seen in the therapeutic effect after intravenous injections of AAV-BR1-NPC2 (study IV) could be the onset of treatment at six weeks of age. In study II when evaluating the brain pathology in six weeks old pre-symptomatic NPC2-/- mice, three out of five NPC2-/- mice already had obvious Purkinje cell loss and widespread neuroinflammation characterized by astrogliosis and reactive microglia. Thus, irreversible pathological damage seen in the cerebellum can limit the effect of the AAV-BR1 gene therapy when initiating treatment at six weeks of age. Due to the heterogeneous clinical manifestation, the disease is often not diagnosed until after the onset of neurological symptoms, which is known to limit the effect of therapeutics (196,254). In study IV, the treatment was initiated before the

onset of neurological symptoms, which is one of the limitations of our study and therefore challenges the translational value of our findings. However, due to the advanced disease state found in some of the six weeks old NPC2-/- mice (study II), the initiation of treatment was probably too late. Subsequently, all NPC2-/- mice developed neurological symptoms characterized by tremors and coordination problems. Despite this, moderate improvement in motor function and disease phenotype was still evident in more than half of the NPC2-/- mice assessed, emphasizing that the AAV-BR1 vector has the potential to delay the neurodegeneration in NP-C2.

The investigation of whether BBB-directed gene therapy is a treatment option for NP-C2 was not straightforward. In study I, the AAV-BR1-NPC2-eGFP vector was used to enable the production of two separate proteins after the transduction of BECs. The presence of eGFP indicated transduction of the specific cell due to intracellular accumulation, whereas NPC2 was destined for transport to the lysosomes or secretion (201). In study IV, the AAV-BR1 only encoded the NPC2. In both studies, tracking the NPC2 secreted from transduced cells was not possible. In addition, the ability of AAV-BR1 to cross the BBB further challenged our strategy as both BECs and neurons were transduced, making it difficult to differentiate the therapeutic effect due to transduction or cross-correction. In a study by Markmann and colleagues, they used the AAVrh.10.mNpc2-HA vector, where the HA-tag was used for post-mortem analysis (195). Thus including a reporter coupled with NPC2 could circumvent these challenges. However, when comparing the neuronal eGFP expression pattern in the brain tissue (study I) with the NPC2 expression in study IV, the neuronal expression of NPC2 was more widespread with high NPC2 expression in the brain stem. In the opposite hereto, none or only limited eGFP expression was found in neurons in this brain area in study I (201). This, therefore, highly suggests cross-correction to neurons after AAV-BR1 transduction.

Interestingly, the total cholesterol level was lower in the spleen and liver of 3/7 AAV-BR1-NPC2-treated NPC2-/- mice assessed. However, when evaluating the *Npc2* gene expression in the splenic tissue, no difference was found between the untreated and AAV-BR1-NPC2-treated NPC2-/- mice. Furthermore, the AAV-BR1 vector distribution is limited or absent in the liver (6,137,201). In addition, the three AAV-BR1-NPC2-treated NPC2-/- mice had a higher transgene expression of NPC2 in the BECs (evaluated with immunohistochemistry), indicating that the effect in visceral organs could be due to the secretion of NPC2 from BECs. However, the visceral effect was limited, and growth retardation and visceral pathology were still evident in the liver, lung, and spleen of NPC2-/- mice receiving viral gene therapy. Thus, the AAV-BR1 vector is efficient for recombinant protein delivery to the brain, subsequently delaying neurodegeneration. However, the secretion of proteins from the BECs is most likely too low to have a therapeutic effect on visceral pathology.

A previous study has evaluated the efficiency of the AAVrh.10 vector, isolated from rhesus monkeys (119), in another mouse model of NP-C2 (*Npc2*^{tm1Plob}) (195). They found a significant improvement in the disease phenotype and an increase in survival

after intracisternal administration of the vector in NPC2-/- mice at six weeks of age (195). However, there are some important differences to consider when comparing these studies. The disease progression of the NP-C2 mouse model holding a gene trap mutation (200) used in the present study is more severe than the *Npc2*^{tm1Plob} mouse model generated using targeted gene mutation (155,195). Also indicated by Hughes and colleagues, it can be even more difficult to compare these studies due to the differences in administration route, vector type, promotor, and humane end-points (89). The invasive nature of the intracisternal route poses a risk of infectious complications, and thus BBB-directed gene therapy is an important, less invasive alternative approach (6,195). Furthermore, the intracisternal-directed approach limits the administration of the viral vectors to brain surfaces exposed to the cerebrospinal fluid (72). However, the intravenous administration of the AAV-BR1 allows for widespread distribution due to the specificity of the BECs. In addition, the AAV-BR1 vector has a broad application potential. For example, the high neuronal expression of recombinant proteins in, e.g., the hippocampus makes the AAV-BR1 vector a potential candidate for future research in the treatment of Alzheimer's disease.

5.3. MODEL LIMITATIONS

Animal models are of great value for increasing knowledge of complex pathological processes and testing new therapeutics for incurable diseases, which is the case of NP-C2. However, one important aspect to consider is the large differences in the lifespan between humans and mice. Consequently, the disease progression of the NPC2-/- mice was more aggressive from the onset of symptoms to reach the end stage of the disease than in humans. Together this limit the therapeutic window for testing treatment strategies in the NPC2-/- mouse model. In study IV, no significant differences were found between untreated and AAV-BR1-NPC2-treated NPC2-/- mice when evaluating the therapeutic effect on motor function and coordination using an accelerating rotarod and composite phenotype score. Due to the severe Purkinje cell degeneration seen in both untreated and AAV-BR1-NPC2-treated NPC2-/- mice, they are severely challenged when performing on the accelerating rotarod; consequently, there is a risk of diminishing the differences between the two experimental groups (255,256). Alternatively, the rotarod with set speed could have been used, previously shown effective when evaluating the AAV-BR1 vector in a mouse model of the LSD Sandhoff disease (137). Untreated NPC2-/- mice had severely poor coordination and fell from the rotating drum even before the start of the trial. On the opposite, the AAV-BR1-NPC2-treated NPC2-/- mice fall off at a speed of approximately 6 rpm. A rotarod with a constant speed could have resulted in a more clear difference in the rotarod performance after AAV-BR1 gene therapy.

In addition, the composite phenotype score used in study IV was based on a scoring scheme ranging from 0-2 (adapted from (232–235). A comprehensive phenotypic assessment of the NP-C1 mouse model has recently been published (236). The phenotype scoring scheme includes hindlimb clasp, grooming, motor function (gait analysis), kyphosis, and the ledge test, with the score ranging from 0-3. By increasing

the scoring scheme from 0-2 to 0-3 for each point assessed, the differences in disease phenotype between untreated and treated NPC2-/- mice would be more visible, increasing the likelihood of finding a significant difference.

Despite these limitations in the study design, the objective was to investigate whether BBB-directed gene therapy was a possible strategy to treat both neurological and visceral pathology in NP-C2. In this case, the NPC2-/- was useful as a proof-of-concept model. In addition, the NP-C2 mouse model is also valuable for studying pathophysiology from single gene alteration, hopefully improving the understanding of the disease and subsequently finding a cure for NP-C2.

CHAPTER 6. CONCLUSION AND FUTURE PERSPECTIVES

With neurons located less than 25 µm from the BECs (2), the gene therapy strategy targeting BECs with subsequent cross-correction of diseased neurons is a promising approach. In this thesis, it was found that the AAV-BR1 vector successfully transduces BECs to produce NPC2, with moderate correction of the brain pathology in NPC2-/- mice, subsequently improving motor function and disease phenotype. However, the BBB-directed gene therapy was unable to reverse the cholesterol storage in visceral organs and growth retardation. These findings emphasize the potential of the AAV-BR1 vector for diseases with neurological involvement. However, there are still some questions regarding the efficiency of AAV-BR1 needed to be addressed:

1. What causes the considerable variation in transduction efficiency resulting in different therapeutic outcomes after AAV-BR1 administration in NPC2-/- mice? Can the presence of pre-existing neutralizing antibodies limit the use of AAV-BR1?

2. Can the effect of the AAV-BR1 capsid variant selected in mice be translated to other species or even humans? Six years after the first publication of the AAV-BR1 (6), it is still unknown what receptor the AAV-BR1 vector binds as well as the mechanism for crossing the BBB. To support the development of future gene therapy for brain diseases in humans, the understanding of the AAV-BR1s specificity for BECs is important.

3. What are the reasons for higher off-target distribution in the lung tissue of BALB/cJRj mice? Could this limit the therapeutic potential of AAV-BR1 in brain diseases, and consequently, is higher doses needed? Dose-response analysis could be necessary for evaluating the effect of dose on brain transduction efficiency, consequently finding the best dosing regime. It is unknown whether this has been carried out previously for the AVV-BR1 vector as no data are available.

Despite years of research in new drug delivery strategies for brain diseases, new aspects of the complex BBB still emerge, emphasizing that basic research is of great importance for increasing the knowledge of the BBB essential for developing new therapeutics for CNS diseases. The BEC-specific AAV-BR1 vector is not only promising for future CNS gene therapy, but also for unraveling disease pathology and increasing the knowledge of the BECs. This has been highlighted by newly published studies on the AAV-BR1 (140,257,258). The AAV-BR1 vector has been used to investigate the effect of different brain endothelial genes by the knock-out or overexpression of specific genes to provide a better understanding of genes involved in BBB integrity (257) and the development of diseases (140,258).

The NP-C2 mouse model used in this thesis is important for increasing the understanding of the pathology of NP-C2. As previously stated, the exact function of NPC2 is not fully understood. To provide more insight into the role of NPC2 and lipid storage in brain pathology, one-half of the brain was collected from NPC2-/-, AAV-BR1-NPC2-treated NPC2-/- and wild-type mice for quantitative mass spectrometry imaging (174), subsequently evaluating the brain lipid profile in NP-C2. When the results of these studies become accessible, hopefully, more insight into the role of cholesterol and other lipids in the progression of NP-C2 will be available.

Viral gene therapy at the BBB has the potential for treating rare genetic diseases with neurological involvement such as NP-C2. However, the challenge of initiating treatment in NP-C2 patients before irreversible neuronal loss remains. More research in early diagnosis is highly needed, as early treatment gives the best clinical outcome (196,259). It has therefore been suggested that NP-C should be included in the newborn screening program, especially after the identification of the sensitive and specific biomarker glycine-conjugated bile acid for the detection of NP-C (148,162,259). However, one of the criteria for including a new disease in the newborn screening program is that an accepted treatment exists (260). The possibility of early detection by newborn screening, and thus initiating treatment before symptom development, renders BBB-directed gene therapy a promising approach for treating the uncurable NP-C2.

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