Developmental and Regulatory Challenges Regarding AAV-Based Gene Therapy for CNS Disorders

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1. Summary

Disorders of the central nervous system (CNS) have traditionally been difficult to treat. The medical literature has described a plethora of diseases that affect various cell types in different parts of the brain, spinal cord, or eye. Consequently, a large pool of treatment modalities has been developed, ranging from rehabilitative procedures, application of stimuli, or prescription of medicinal products such as small molecules or biologicals. Recently, gene therapy medicinal products (GTMPs), a sub-class of advanced therapy medicinal products (ATMPs), garnered renewed interest and hold promise to change course of some of these diseases.

What is particularly interesting about gene therapy approaches for CNS disorders is the notion that administration induces a long-lasting, if not permanent, treatment effect. This approach is rendered even more attractive when one considers the inaccessibility of the brain (or other parts of the CNS), which is stringently sealed off from surrounding circulation by the blood-brain barrier. Consequently, gene therapy offers the potential to introduce corrective components through a single administration.

Adeno-associated viral vectors (AAVs) are the workhorses of many gene therapy trials worldwide. In comparison to other (viral) delivery vehicles they possess favorable characteristics such as stable host cell transduction, adequate packaging capacity to deliver almost any therapeutic gene, and absence of host genome integration. However, inherent characteristics such as induction of the host immune system pose significant translational impediments. Furthermore, developmental challenges regarding host tissue tropism, route of delivery and production-related issues must be overcome to garner regulatory approval.

To this end, I performed a systematic literature review of AAV-based gene therapy approaches for CNS disorders. This work will introduce and discuss key aspects that drive AAV development, review pre-clinical trials of the past three decades, and highlight current clinical trials that pursue gene therapeutic approaches through AAV delivery. In light of recently approved *in vivo* GTMPs regulatory challenges will also be discussed. Indeed, in its 2025 strategic reflection paper on regulatory science EMA identified the translation of ATMPs into patient treatments an overarching goal that should draw increased attention.

2. Introduction

2.1. Legal Basis for Gene Therapies

In the European Union (EU) gene therapies are considered advanced therapy medicinal products (ATMPs) and are subject to special legislation and guidance. The underlying legal framework will be discussed in the following sections and highlight key aspects that define an ATMP marketing authorization application (MAA).

The Committee for Advanced Therapies (CAT) provides scientific advice to ATMP developers throughout the development process and helps with the classification of such products into three categories. Furthermore, since the development of ATMPs is a costly, time-consuming and resource-intensive process, especially for small and medium-sized enterprises (SMEs), financial incentives have been introduced (see 1.1.1.).

Based on a favorable opinion by the CAT the Committee for Medicinal Products for Human Use (CHMP) adopts a, normally concurring, final opinion which results in the granting of a MA by the European Commission.

2.1.1. Reglulation (EC) 1394/2007 (ATMP Regulation)

The overarching European ATMP Regulation[1] lays the foundation for a marketing authorization of a medicinal product (MP) via the centralized procedure. Article two of the Regulation defines the scope of an ATMP, i.e. (1) gene therapy MP; (2) somatic cell therapy MP; and (3) tissue engineered product. A GTMP contains or consists of a recombinant nucleic acid sequence with the aim to regulate, repair, replace, add or delete a genetic sequence. A sCTMP contains or consists of cells that have been subjected to substantial manipulation in order to achieve therapeutic use by treating, diagnosing or preventing a disease modality. A tissue engineered product contains or consists of cells or tissue(s) that are administered to regenerate, repair or replace a human tissue. Article seventeen of the Regulation defines the process of ATMP classification. This process, however, is optional and was mainly introduced to deal with borderline classifications, e.g. when

[1] Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004, as amended

there is overlap with other areas, such as medical devices. Article sixteen introduces financial incentives. As such, SMEs receive a 90% fee reduction on scientific advice, and larger developers receive a 65% reduction[2].

Recital three of Commission Directive 2009/120/EC[3] highlights the necessity to amend Annex I of Directive 2001/83/EC 'due to scientific and technical progress in the field of advanced therapies'. Consequently, Part IV of Annex I was introduced and provides technical guidance for the contents related to Modules 3-5 of a MAA ATMP dossier. For instance, the concept of a risk-based approach[4] was mentioned. A product-specific risk-analysis might cover the entire development and could evaluate factors such as (1) potential to elicit an immune system response; (2) replication deficiency/competence of viruses; (3) potential for integration of nucleic acid sequences/genes into the genome; (4) duration of functionality; (5) risk of oncogenicity; (6) route of administration; amongst others.

2.1.2. European Guidelines Regarding GTMPs

The overarching European guideline[5], which is currently under revision, provides guidance on the development of (cell based) gene therapies. More specifically, a revised and updated guideline regarding (non-cell based) GTMPs[6] was published in 2018 and provides a thorough description of all necessary steps during product development, i.e. quality aspects, non-clinical and clinical development.

In general, GTMPs are comprised of a delivery vehicle, a so-called vector, containing a genetic sequence, i.e. the therapeutic entity, with the aim of regulating, repairing, replacing, adding or deleting a target genetic sequence within the human body, i.e. *in vivo*. Cells genetically modified via *ex vivo* or *in vitro* gene therapy and which are then infused into a patient are covered by the guideline of genetically modified cells[5]. *In vivo* gene therapy vectors can be separated into three

[2] Explanatory note on general fees payable to the European Medicines Agency as of 1 April 2019
[3] Commission Directive 2009/120/EC of 14 September 2009 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products, as amended
[4] Risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products (CAT/CPWP/686637/2011)
[5] Quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells (CHMP/GTWP/671639/2008)
[6] Quality, preclinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)

groups, i.e. (1) viral vectors; (2) DNA vectors; and (3) bacterial vectors. The former, especially the sub-class of adeno-associated virus (AAV), will be discussed in depth within this thesis, while the latter two will be briefly touched upon in 1.2.1.

Several other guidelines, summarized below in Table 1, provide detailed guidance on different aspects/parts of a dossier regarding gene therapies in general. Those specifically or concomitantly addressing non-cell based GTMPs, i.e. AAVs (among others), are highlighted in grey.

Table 1 European guidelines regarding gene therapies

Guideline	Doc. Ref.	Content	Status	Module/s
Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials	EMA/CAT/852602/2018	Data requirements for clinical trial (CT) applications with ATIMPs	Draft	5
Guideline on safety and efficacy follow-up and risk management of advanced therapy medicinal products	EMEA/149995/2008	Risk Management, Pharmacovigilance	Revision	5
Quality, preclinical and clinical aspects of gene therapy medicinal products	EMA/CAT/80183/2014	Development of non-cell based GTMPs	Adopted	3, 4, 5
Quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells	EMA/CAT/GTWP/671639/2008	Development of cell based GTMPs	Revision	3, 4, 5
Development and manufacture of lentiviral vectors	CHMP/BWP/2458/03	Quality aspects and non-clinical testing	Adopted	3, 4
Non-clinical studies required before first clinical use of gene therapy medicinal products	EMEA/CHMP/GTWP/125459/2006	Non-clinical testing required for first-in-human (FIH) CT	Adopted	4
Non-clinical testing for inadvertent germline transmission of gene transfer vectors	EMEA/273974/2005	Non-clinical risk assessment of germline transmission	Adopted	4
Risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products	EMA/CAT/CPWP/686637/2011	Product-specific risk-analysis	Adopted	3, 4, 5

Guideline	Doc. Ref.	Content	Status	Module/s
Follow-up of patients administered with gene therapy medicinal products	EMEA/CHMP/GTWP/60436/2007	Clinical Follow-up of patients receiving GTMPs and Pharmacovigilance	Adopted	5
Scientific requirements for the environmental risk assessment of gene-therapy medicinal products	EMEA/CHMP/GTWP/125491/2006	Environmental risk assessment in accordance with EU environmental legislation	Adopted	3

2.2. Vectors for Targeted Gene Therapies

Conceptually, gene therapy was already envisaged in the 1940s by Clyde E. Keeler and his short discussion on 'achieving a permanent correction of hereditary diseases' [1]. In general, gene therapies show great promise in long-lived or possibly permanent correction of a disease modality. This is especially attractive for organ systems that are difficult to access or physiologically separated from the systemic environment. One such case is the brain. Direct access represents an invasive and, depending on the brain region to be targeted, complex procedure for treatment. Furthermore, brain tissue is physiologically sealed off from the systemic environment through the blood-brain-barrier (BBB) which prevents passage of large molecules, e.g. therapeutic molecules, into the central nervous system (CNS) aimed at treating neurological disorders.

For a successful gene therapy approach 3 criteria should be met, i.e. (1) identification/presence of a target; (2) a treatment paradigm; and (3) a delivery mechanism. While the former two can be easily conceived, e.g. a genetic mutation within the retina of the eye (i.e. criteria 1) should be corrected with a gene therapy replacing the defective genetic sequence (i.e. criteria 2), the latter part can be achieved through various means. Simple injection of so-called naked DNA (or RNA) is not feasible as DNA or RNA molecules are easily degraded by endonucleases, rendering the therapeutic approach highly ineffective. Also, naked DNA molecules have been shown to be cleared within 10 minutes after *intravenous* (*i.v.*) injection in mice [2]. Furthermore, nucleotide sequences require intrinsic stability and lack lipid bilayer penetrating capacity, i.e. they cannot pass cell membranes on their own. Additionally, nucleotide molecules themselves lack target specificity. As a result, nucleotide sequences have to be transported via a so-called vector, e.g.

via a viral vector as discussed in 1.2.2. Different approaches for (1) protecting the nucleotide sequence and allowing (2) tissue specificity will be presented in the following sections.

2.2.1 Non-Viral Vectors

The use of non-viral vectors has several advantages regarding host/recipient reactions. For instance, injection of a virus can lead to immunotoxicity, cytotoxicity and the risk of viral DNA integration into the host genome which might lead to insertional mutagenesis, e.g. disrupting the function of an otherwise normally operating gene or promoting the expression of oncogenes. Another beneficial feature of non-viral vectors is their limited transduction efficiency, as compared to viral vectors. Consequently, off-target effects, i.e. random transduction of non-targeted cell types, are limited to negligible levels.

Traditionally, the delivery of gene therapies has been achieved by physical methods, such as simple injection with a needle, gene gun, electroporation, sonoporation, photoporation, hydroporation or magnetofection. More recently, chemical carriers, e.g. calcium phosphate, or biodegradable carriers, e.g. lipid nano emulsions/particles or peptide-based vectors, have been employed. Novel approaches, e.g. polymer-based vectors, are being evaluated and further improved. All of these methods are reviewed and summarized in [3].

2.2.2. Viral Vectors

The following sections will provide important information on viral vectors employed for gene therapy. Successful GT approaches encompass numerous aspects, including (1) efficient and stable therapeutic gene expression; (2) high safety profile, e.g. induction of host immune responses, host genome integration and genotoxicity; and (3) ease of production, e.g. sufficient quantities without contaminants (endotoxins) present.

A more thorough and detailed introduction on AAVs is given in 2.1., however, a brief discussion of key criteria is warranted at this place. Non-dividing cells can be stably transduced and the genomic packaging capacity of AAVs allows potential targeting of more than 95% of human genes [4]. Most importantly, widespread application of AAVs in gene therapy approaches is envisaged

due to (almost) complete absence of host genome integration events while responses of the host (patient) immune system have to be controlled, i.e. suppressed, in a clinical treatment setting. While immunosuppression is a key feature in all GT approaches, non-integration of AAVs into the host genome is a decisive feature in its recent and constantly growing success in both pre-clinical and clinical research. Modern production techniques that yield high and endotoxin-free AAV titers are constantly improved and developed in accordance with regulatory agencies around the world.

2.2.2.1. Retroviruses

Retroviruses are single-stranded RNA (ssRNA) viruses with a genome size of ~8 kilobases (kb) encoding structural proteins and enzymes, such as *gag*, *pol*, and *env*. The *gag* gene codes for structural glycoproteins which are required to assemble viral particles. Likewise, *env* codes for membrane-embedded proteins that are responsible for virus attachment to host cells, i.e. an important feature for tissue tropism. The *pol* gene codes for enzymes that are required for replication and integration into the host genome. For instance, reverse transcriptase synthesizes DNA from viral RNA while integrase is responsible for the integration of this reversely transcribed DNA into the host genome. Furthermore, so-called long terminal repeat (LTR) sequences are located at the 5' and 3' extremities of the viral genome, serving as promotor region (5'LTR) and termination site (3'LTR). In addition, a packaging signal, i.e. ψ sequence, lies downstream of the 5'LTR to assure only RNA containing ψ will be packaged into viral particles.

For retroviral gene therapies only so-called cis-acting elements are retained within the viral vector genome. These include the 5' and 3' LTRs as well as the ψ sequence, which are required for integration into the host DNA. Instead of *gag*, *pol*, and *env* genes (which have been removed to render the virus replication-defective) a therapeutic sequence is inserted, enabling the theoretical delivery of an up to 11-12 kb therapeutic gene. To produce therapeutic vector particles, a so-called packaging cell line is required. Structural elements, i.e. *gag* and *env*, as well as necessary enzymes for reverse transcription and integration, i.e. *pol*, are expressed in this packaging cell line along with the therapeutic vector genome to produce viable retroviral (therapeutic) particles.

Retroviral gene therapy achieves long-lasting transgene expression, however, safety issues regarding insertional mutagenesis (e.g. (1) integration of the retroviral genome into a host gene, thereby disrupting its original function; or (2) insertion within a promotor/repressor/enhancer

region of a functional host gene to alter its expression) or immunological responses leading to the elimination of virally transduced cells remain an issue to be addressed. A further complication, especially when treating quiescent cells, i.e. cells not undergoing mitosis, of the CNS is the fact that simple retroviruses require passage through the cell cycle, i.e. they can only target dividing cells.

2.2.2.2. Lentiviruses

Lentiviruses belong to the family of retroviruses. Consequently, they share many similarities to simple retroviruses as described in 1.2.2.1. For instance, they provide similar packaging capacity, i.e. 8 kb ssRNA, and employ the same machinery for integration (*gag*, *pol*, and *env* genes, among others). One major advantage is the capacity to translocate across the intact nuclear pore. Consequently, integration, of a therapeutic gene, into the host genome is not dependent on the cell cycle, which enables the transduction of non-dividing, quiescent cells.

First generation lentiviral vectors contained parts of the HIV genome and envelope proteins from vesicular stomatitis virus (VSV, a rhabdovirus) which allowed transduction of many different cell types. Second generation lentiviral vectors saw the removal of some virulence factors and the most recent third generation employs splitting of the viral genome into separate plasmids, i.e. to avoid generation of replication-competent viruses, and introduced deletions into the 3'LTR to create self-inactivating vectors by disrupting the promotor activity of the LTR. Nevertheless, modern lentiviral vectors still bear the potential for insertional mutagenesis. Properties of lentiviral vector biology as well as their clinical use are reviewed in [5]. As outlined in Table 1 there is a European guideline detailing aspects to take into consideration when developing and manufacturing lentiviral vectors.

2.2.2.3. Adenoviruses

Adenoviruses (Ads) possess a double-stranded DNA (dsDNA) genome with a packaging capacity similar to those of retroviruses, i.e. ~7.5 kb. So far, 57 human-infecting Ad serotypes have been described and in fact, most humans have been infected with one or more serotypes throughout their lives (infections mostly take place in the respiratory system), leading to life-long immunity.

Ads can infect dividing and non-dividing cells and the process of DNA replication is separated by two phases, i.e. an early phase prior to replication which leads to the expression of regulatory proteins (their purpose being to (1) alter the expression of host proteins necessary for DNA synthesis; (2) expression of virus-encoded proteins, e.g. virus encoded DNA polymerase; and (3) avoiding death through inhibiting immune responses); and a late phase after replication which leads to the expression of structural proteins to package synthesized viral DNA into new particles.

Therapeutic Ad vectors are mostly based on serotype 5 (Ad5). First generation therapeutic vectors encompassed deletion of the E1 and E3 early genes which rendered them replication-defective and allowed packaging of ~8 kb foreign (therapeutic) sequence into the viral genome. Second generation vectors saw the deletion of E2 and E4 early genes to increase the size of a therapeutic insert, i.e. ~10.5 kb. Modern, third generation high capacity adenoviral vectors (HCAds) lack all viral genes except the 5' and 3' inverted terminal repeats (ITRs) and the packaging sequence and allow insertion of therapeutic sequences up to 36 kb in length. However, these vectors require the presence of a helper Ad, which is difficult to remove from large-scale vector stocks. One major limitation of Ads is the notion that they trigger strong immune responses upon injection of large titers which are needed for sufficient transgene expression. Nevertheless, this type of viral vector is heavily researched in animal models and found its way into many clinical trials. A more thorough review on Ad biology and development is presented in [6].

2.2.2.4. Herpes Simplex Virus

Herpes simplex viruses (HSV-1 and HSV-2) possess a relatively large dsDNA genome with a packaging capacity ranging from ~20-40 up to 150 kb. They can infect dividing and non-dividing cells and possess a unique feature called viral latency (see below). Immediate-early, early and late proteins are synthesized from its genome and fulfill different functions such as, regulation of viral DNA replication, degradation of host mRNA, and the synthesis of structural proteins followed by lytic release from the host cell.

In the latent state, HSV maintains a pool of quiescent virus residing in ganglia and expressing the so-called latency-associated transcript (LAT) RNA. LAT interferes with/regulates the host's genome and attenuates the immune response, thereby sparing host cells from lysis which in turn

allows recurrent outbreaks of HSV. This property represents an interesting treatment avenue to treat diseases long-term, e.g. neurological disorders.

Two different approaches for developing HSV vectors are currently employed. In the first, minimal HSV vectors, termed HSV amplicons (essentially an amplicon plasmid), allow packaging of up to 150 kb therapeutic sequence. However, they require replication-deficient HSV helper viruses for full-scale production in cells – a process that is difficult to validate and control, and which also yields contamination with helper virus. The second approach encompasses the genetic manipulation of HSVs to render them replication-deficient. This is achieved by deleting certain immediate-early and early genes. A thorough review of HSV vector development, especially for CNS applications, is presented in [7].

3. Adeno-Associated Virus-Based Gene Therapy for CNS Disorders

3.1. Adeno-Associated Viruses

Adeno-associated viruses (AAVs) are single-stranded DNA viruses with a genome size of ~4.8 kb. An interesting feature in their replicative capacity is the dependency on co-infection with other viruses, mostly Ads. In fact, already in the 1960s researchers discovered AAVs in adenoviral preparations [8], [9], hence the name adeno-associated.

The single-stranded DNA genome contains three genes, i.e. *rep* (replication), *cap* (capsid), and *aap* (assembly), that give rise to at least 9 proteins through the use of three promotors, alternative translation start sites and alternative splicing. Furthermore, inverted terminal repeats (ITRs) flank the 5' and 3' ends and are required for proper replication and packaging. Four proteins are responsible for viral genome replication and packaging and are encoded by the *rep* gene, i.e. Rep40, Rep52, Rep68, and Rep78 (the numbers describe their respective molecular mass in kilodalton (kDa)). The outer shell of the virus, the capsid, is composed of VP proteins and is responsible for protection as well as cell-binding and internalization. The *cap* gene drives the synthesis of VP1, VP2, and VP3 proteins (their respective molecular masses being 87, 72, and 62 kDa) which assemble in an icosahedral manner in a molar ratio of 1:1:10. In an alternate reading frame overlapping the *cap* gene the assembly-activating protein (AAP) is synthesized from the *aap* gene. This protein has been proposed to possess a scaffolding function in the capsid assembly process [10].

An exemplary sequence arrangement of AAV serotype 2 is shown in Figure 1.

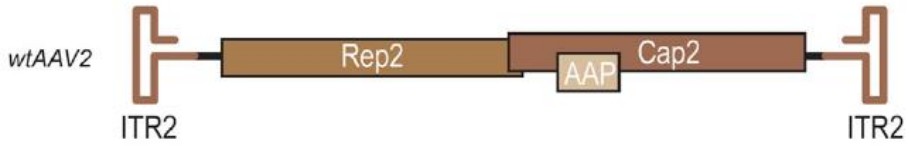

Figure 1 Wild-type adeno-associated virus sequence
Genomic structure of AAV serotype 2 depicts genes responsible for the synthesis of replication (Rep2), capsid (Cap2), and assembly-associated protein (AAP) products. These genomic sequences are flanked by inverted terminal repeats (ITR2) on both sides and form a so-called hairpin structure. For detailed information on the function of protein products refer to main text.
Taken from: Hudry and Vandenberghe, "Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality.," *Neuron*, vol. 101, p. 840, 2019

3.1.1. AAV Serotypes

Based on the initial identification of AAV serotype 2 (AAV2) most research focused on this variant. However, it became evident that a plethora of different AAV serotypes exist and to date, more than 100 specific serotypes have been identified. For the purpose of this thesis, only those variants that are well-studied and deemed applicable to translation into a clinical setting are presented and discussed, i.e. those isolated from human and non-human primates (NHPs).

As an initial step in the viral life-cycle attachment to a host cell membrane and internalization via endocytosis are essential. Indeed, it was shown that the viral capsid proteins utilize different cell surface proteins for attachment, allowing to bind different cell types which is responsible for tissue selectivity (tissue tropism). For the most important AAV serotypes, i.e. AAV1-9, these can be broadly categorized into three groups, as depicted in Table 2. Heparan sulfate proteoglycans (HSPGs) have been identified as the receptors for AAV2 and AAV3. AAV1, AAV4 and AAV5 bind different sialic acid (SA) receptors, while AAV6 binds both HSPG and SA receptors. AAV9 binds galactose and the cellular receptors for AAV7 and AAV8 remain unidentified. Upon cellular attachment, AAVs utilize different co-receptors to mediate internalization via endocytosis. The so-far identified co-receptors for each of the AAV1-9 serotypes are also shown in Table 2.

Table 2 Important AAV serotypes and their Cellular Receptors/Co-receptors ([4], [11]*)*

Serotype	**Cell surface receptor**	**Co-receptor for internalization**	**CNS tissue tropism** [12], [13]
AAV1	SA	unknown	Observed (to variable degrees depending on route of administration, brain region)
AAV2	HSPG	FGFR, HGFR, LR, integrins	
AAV3	HSPG	HGFR, LR	
AAV4	SA	unknown	
AAV5	SA	PDGFR	
AAV6	HSPG and SA	EGFR	
AAV7	unknown	unknown	
AAV8	unknown	LR	
AAV9	Galactose	LR	

FGFR, fibroblast growth factor receptor; HGFR, hepatocyte growth factor receptor, LR, laminin receptor, PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor

It should further be noted that other isolates, e.g. from rhesus macaque, are employed in basic and pre-clinical research. These viruses are termed AAVrh.8 or AAVrh.10. Also, researchers are actively altering serotype properties to enhance tissue tropism. For instance, hybrid AAVs were prepared through packaging the genome of AAV2 into the capsid of either AAV1 or AAV5. These AAV2/1 and AAV2/5 hybrids enhanced neuronal transduction efficiency and revealed brain region-specific cell tropisms [14]. Also, use of mutation libraries led to the development of AAV.PHP.B, which appears to have superior neuronal transduction efficiency [15]. The concept of vector design will further discuss approaches to alter the capsid structure/interaction with the host cell membrane to enhance tissue tropism.

In general, most pre-clinical studies to date have employed rodents, especially mice, as model organisms to assess proof-of-concept and efficacy of AAV gene therapies [16]. Interestingly, a comparative study between human induced pluripotent stem cell-derived neurons and isolated rat cortical neurons found profound differences in tissue tropism amongst various AAV serotypes [17]. To circumvent this obstacle new sources which are antigenically more similar to human cells/tissues, and by inference, possess comparable tissue tropism characteristics are pursued. To this end, porcine-derived AAV isolates [18] might yield promising results in future pre-clinical trials.

3.1.2. Recombinant AAVs (rAAVs) and their Packaging Capacity

In theory, recombinant AAVs (rAAVs) can accommodate a therapeutic sequence up to a length of ~4.7 kb. Only the ITRs have to be maintained for successful viral genome replication and packaging. Upon internalization via endocytosis rAAV capsids are transported into the host cell nucleus through the nuclear pore and release their genome for replication. As noted in 2.1. AAVs possess a ssDNA genome. For successful transcription this single-stranded molecule needs to be converted into the double-stranded version. One possibility to overcome this rate-limiting step in the viral replication process is the use of reversely oriented strands, i.e. plus- and minus-stranded ssDNA molecules, which are packaged into separate capsids and anneal once inside the host cell nucleus. Another possibility is the use of a so-called self-complementary genome. To achieve this, a third ITR is introduced into the viral genome (at equidistant spacing from the flanking ITRs) to

generate a dsDNA molecule, immediately ready for transcription by the host cell machinery. However, a major caveat of this approach is the sequence reduction by ~ 50%.

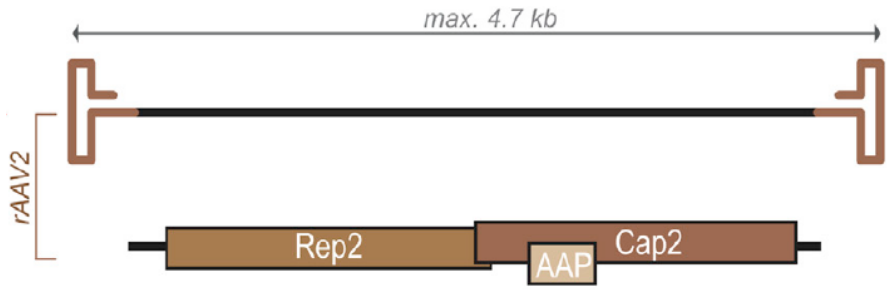

Figure 2 Single-stranded rAAV

A single-stranded rAAV2 molecule in which all viral genes are replaced by a therapeutic sequence (up to a maximum capacity of 4.7 kb; black horizontal line in the drawing on top). Only the ITRs (brown vertical structures in the drawing on top) flanking the genome are maintained. For the successful production of viral particles, e.g. in cell culture, essential viral genes, i.e. Rep2, Cap2, and AAP (bottom drawing) are expressed in trans.

Taken from: Hudry and Vandenberghe, "Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality.," *Neuron*, vol. 101, p. 840, 2019

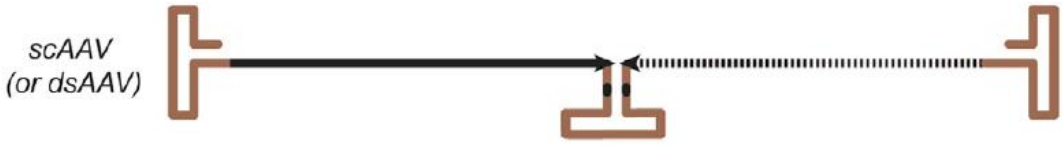

Figure 3 Self-complementary rAAV

A self-complementary AAV (scAAV) employs the use of a third, structurally changed ITR (denoted by black dots in the center ITR). This structure leads to the formation of a double-stranded molecule ready for immediate replication by the host cell. However, the packaging size is reduced in half due to the required presence of the complementary strand (dashed horizontal line).

Taken from: Hudry and Vandenberghe, "Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality.," *Neuron*, vol. 101, p. 840, 2019

In all these cases, the ITRs of the double-stranded molecule undergo circularization and remain, in the majority of cases, episomal, i.e. they do not integrate into the host cell's genome.

One recent communication of long-term follow-up data (between 2 and 10 years) on *intravenous* AAV delivery in dogs to treat hemophilia observed stable transgene expression over several years,

an intended and promising result in AAV gene therapy. Specifically, the target of this pre-clinical study was to treat hemophilia by stable factor VIII (FVIII, an essential blood clotting protein) expression to reduce bleeding episodes. However, after several years some dogs showed elevated levels of FVIII which might also serve as an indicator for the development/presence of tumors. Functional tests and pathological observations concluded that liver function was not altered, or tumors developed. Intriguingly, genomic analyses revealed that 20 tissue samples (from six AAV-treated dogs and 2 naïve controls) contained more than 2,000 unique integration events distributed across the canine genome. Furthermore, some of these integrations were localized close to genes associated with growth control and transformation. While this certainly represents an intriguing finding that warrants further long-term follow-up studies the authors emphasize that (1) there was no evidence for tumorigenesis, and (2) there was no causal link between elevated FVIII and the integration events observed[7,8].

[7] Nguyen et al., Long-Term AAV-Mediated Factor VIII Expression in Nine Hemophilia A Dogs: A 10 Year Follow-up Analysis on Durability, Safety and Vector Integration (accessed 15 February 2020)
[8] https://www.sciencemag.org/news/2020/01/virus-used-gene-therapies-may-pose-cancer-risk-dog-study-hints# (accessed 15 February 2020)

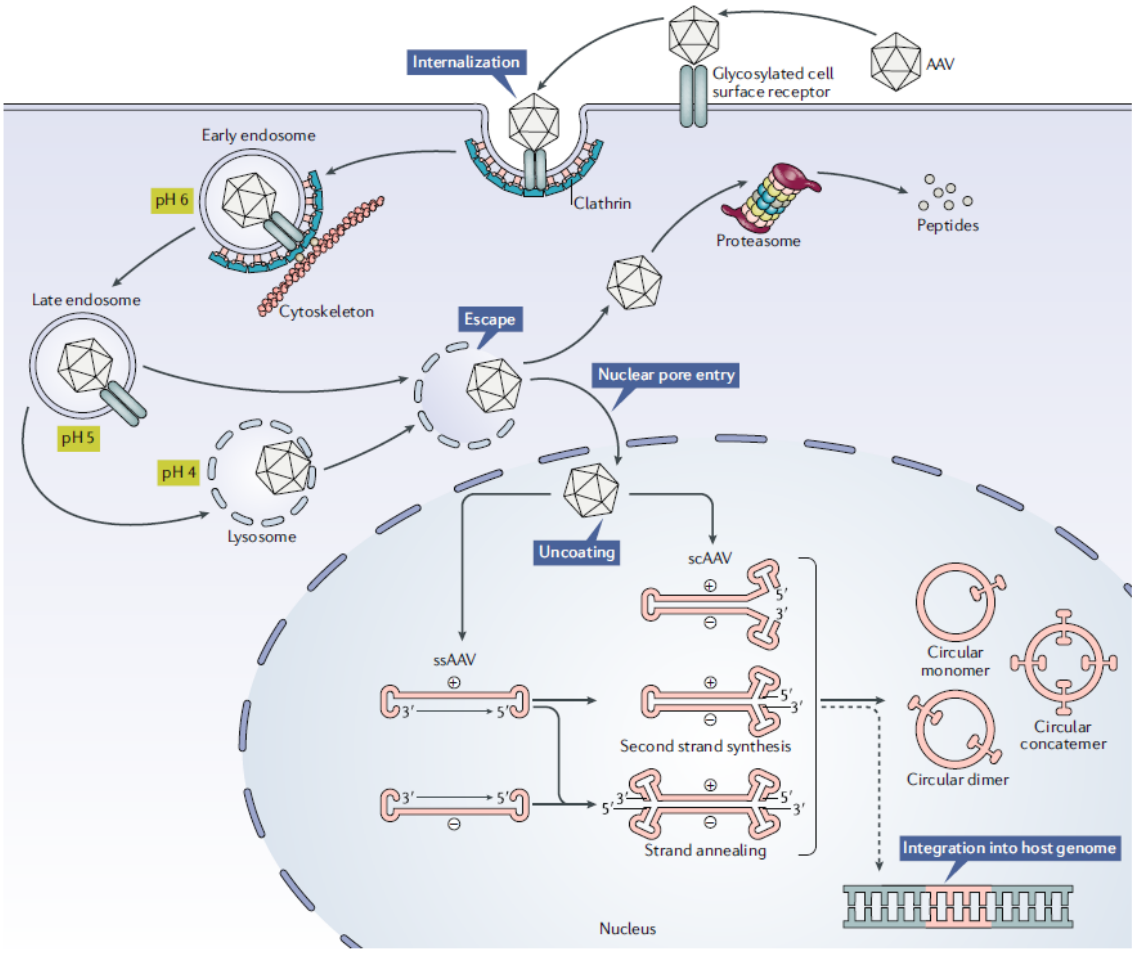

Figure 4 Pathway for rAAV transduction

The viral capsid interacts with host cell membrane receptors/co-receptors which eventually leads to virus internalization via endocytosis. Upon escape from the endosome/lysosome the viral capsid translocates into the host cell nucleus through nuclear pores where the genome will be uncoated. In the case of ssAAVs, either (1) the second strand will have to be synthesized for viral replication, or (2) separately transduced viral genomes carrying reversely oriented ssDNA strands (plus and minus) will anneal through base-pairing. These two approaches allow delivery of a ~4.7 kb therapeutic sequence. Another possibility is the use of scAAVs which have been engineered to form dsDNA molecules. One limitation of this approach is the reduction in packaging capacity (reduced by 50%). Through molecular interactions at the ITRs the resulting dsDNA molecules will circularize and remain episomal. However, a slight chance of host genome integration is possible and has, in fact, been observed.

Taken from: Wang et al., "Adeno-associated virus vector as a platform for gene therapy delivery.," *Nat. Rev. Drug Discov.*, vol. 18, no. 5, p. 360, 2019

Production of therapeutic viral particles requires successful replication and packaging through the utilization of the host cell machinery and the presence of helper viruses, e.g. HSV or Ad. Consequently, the manufacturing process of rAAVs is highly complex and under constant development. Especially production scale-up, viral yield purification and process characterization

are challenges that render the entire process a major focus for clinical development and marketing departments.

3.1.3. AAV Vector Design Approaches & Strategies

The following sections will highlight approaches and strategies that are currently employed to generate rAAVs with enhanced tissue and cell type-specific tropism. Viral biology allows (1) structural changes to the capsid, (2) design of the vector genome, and (3) regulation of (therapeutic) gene expression.

3.1.3.1. rAAV Capsid Development

The development and design of novel or enhanced capsid variants can be classified into four distinct approaches. The methods have constantly progressed, based on technical advancements and most recently due to an increase in computational power.

The natural discovery approach is based on the initial identification of AAV2. Second generation viruses, e.g. AAV5, and the newest, i.e. AAV9, were discovered through isolation from human or NHP tissues. For instance, AAV9 has been shown to cross the BBB when administered *intravenously* [19] making it a promising candidate for CNS transduction. A major caveat to these human-isolated capsid structures, however, is the notion that ~40-80% of humans (depending on serotype) have been infected with AAV1-9 throughout their lives and consequently possess an immune system response to clear these infections. Thus, such individuals would not benefit from therapeutic transgene expression. Alternatively, isolation of AAV capsid structures from rhesus macaques, e.g. AAVrh.8, AAVrh.10, and AAVrh.43, show less immunogenicity in humans. Another interesting, and evolutionary informative, approach to produce potent viral capsids is the 'ancestry by design' approach devised by researchers at Harvard University[9]. Viral lineage reconstruction yielded the identification of a common ancestor of AAV serotypes 1, 2, 8, and 9, termed Anc80, and which is also closely related to AAVrh.10 (with only 7.8% amino acid sequence divergence). Most importantly, this ancestor was tested in murine and macaque animal models

[9] https://hms.harvard.edu/news/ancestry-design (accessed 30 January 2020)

in its transduction efficiency and was found to be equally potent, or even superior, to AAV 2 and 8 in transducing liver, muscle and retinal cells. Concomitantly, Anc80 produced no overt toxicity and retained some transduction capacity in AAV8 pre-immunized animals [20].

Another, even less immunogenic approach, encompasses capsid isolation from other species, e.g. pig. Indeed, it has been shown that a porcine capsid transduces mouse organs equally efficient as, e.g. AAV5 or AAV8 [18]. It remains to be seen which further improvements can be achieved through natural discovery.

The rational design approach encompasses active engineering of the capsid protein(s). For instance, displaying so-called designed ankyrin repeat proteins (DARPins) on the capsid allowed efficient antibody-like recognition of surface markers on individual cell types [21]. As alluded to in Figure 4, the viral capsid can, upon escape from the endosome, also be degraded by the cell's proteasome. To reduce this, mutations were introduced into capsid surface-exposed tyrosine residues effectively evading ubiquitination, i.e. a signal for proteasome-mediated degradation, and leading to a 30-fold increase in cellular transduction in a mouse model, and a 8-11-fold increased transduction in HeLa[10] cells *in vitro* [22]. A more recent approach in rational design took aim at the issue of pre-existing immune responses, most notably the presence of neutralizing antibodies (NAbs). Evolution of so-called capsid antigenic motifs (CAMs) yielded a synthetic CAM, i.e. CAM130, which demonstrated reduced NAb recognition *in vitro* and *in vivo* [23].

Insufficient knowledge regarding AAV binding, internalization, endosomal/lysosomal escape, uncoating and gene expression yielded only few successful rational design approaches. Directed evolution, on the other hand, utilizes selective pressure, effectively mimicking natural evolution, to yield superior capsid variants. For instance, error-prone PCR spanning the *cap* gene can aid in creating a library for screening of advantageous capsid properties. Recently, Cre recombination-based AAV targeted evolution (CREATE) led to the identification of AAV.PHP.B, a variant that leads to superior transduction of brain tissue in a mouse model after intravenous administration [15]. However, it was shown that this superiority is only achieved in a specific mouse species and could not be replicated in NHPs [24]. Further employment of the CREATE approach yielded newer variants that enhanced neuronal transduction after *i.v.* administration [25]. It remains to be seen,

[10] HeLa is an immortalized cell line derived from cervical cancer cells from Henrietta Lacks, a patient who died of cancer in 1951. It is widely used in basic and biomedical research until today and led to many scientific discoveries, including the development of the first vaccine against poliomyelitis.

however, whether any of these newly developed and highly specialized capsid variants finds its way into a pre-clinical development program.

One last approach for capsid design is still in its infancy but involves the ever-growing field of bioinformatics. An *in silico* approach yielded a capsid variant library that produced an AAV2-based vector with a transduction efficiency in mouse liver equal to AAV8, which represents a ~20-fold increase to the normal AAV2 serotype [26].

3.1.3.2. rAAV Genome Design

Due to the size constraint of the viral genome, as outlined in 2.1.2., only a limited number of possibilities for genomic design exist. These include gene replacement, gene editing, or gene silencing and depend on the therapeutic target.

By far the most common approach is gene replacement, which encompasses the delivery of a therapeutic gene to target a specific (CNS) disease. For instance, gene therapy of Canavan disease[11], aimed at replacing a defective gene encoding an important neuronal enzyme, turned out to be well-tolerated by patients [27] and in fact, long-term follow-up indicated improvement of clinical status [28].

Since the discovery of gene editing tools, e.g. zinc-finger proteins (ZFPs) or the more recent CRISPR-Cas system, the vision of treating genetic disorders by correcting a specific mutation *in vivo* became fashionable, albeit quite challenging. So far, only two clinical trials employing zinc-finger nucleases (ZFNs) to treat different forms of mucopolysaccharidosis[12] are ongoing. In both cases, insertion of a correct copy of an otherwise defective gene into the albumin locus of

[11] Canavan disease, a so-called leukodystrophy, is characterized by a defective *ASPA* gene that encodes for the enzyme aspartoacylse which is required for the breakdown of N-Acetylaspartate (NAA) in the brain. Increasing intracellular accumulation of NAA is neurotoxic and leads to edema and loss of myelin, i.e. a protective sheath covering neuronal processes and acts as an insulator to regulate nerve impulses. In severe cases, patients develop life-threatening conditions within the first 2-3 decades of their lives. So far, no cure is possible.

[12] Mucopolysaccharidosis (MPS), a lysosomal storage disorder, is characterized by a lack or defective function of lysosomal enzymes to break down glycosaminoglycans (GAGs; sugar carbohydrates). In MPS I a defective *IDUA* gene leads to the expression of deficient iduronidase, whereas in MPS II a deficient iduronate sulfatase if produced by a defective *IDS* gene. In both cases, heparan and dermatan sulfates accumulate in cells and lead to intellectual disability, amongst other features.

hepatocytes (via an rAAV2/6 vector) is devised to yield life-long therapeutic enzyme production (NCT02702115, NCT03041324).

The 2006 Nobel Prize in Physiology or Medicine was awarded for the discovery of RNA interference (RNAi)[13] and has since been envisaged and developed to silence genes *in vivo* for disease treatment. AAV delivery of a microRNA (miRNA) in a mouse model of Huntington's Disease (HD)[14] indicated reduction of the mutated huntingtin mRNA [29].

In summary, gene replacement is by far the most developed approach. It remains to be seen whether gene editing can prove itself valuable, also from an ethical point of view. Utilization of the CRISPR-Cas system, which is derived from bacteria, might lead to an immunological response and render the therapeutic approach ineffective. Gene silencing, on the other hand, might be a worthwhile approach for certain diseases, as it employs a system that is encoded in the human genome. The CRISPR-Cas-associated/induced DNA double strand breaks are repaired, amongst other repair processes such as homologous recombination, via non-homologous end joining (NHEJ), which usually leads to insertion or deletion mutations. Consequently, frameshifts in coding regions lead to protein truncation and inactivity upon translation [30]. This error-prone NHEJ repair, therefore, bears the risk of inducing unwanted off-target effects that cannot be repaired *in vivo*.

3.1.3.3. Regulation of rAAV Genome Expression

Regulating the expression of a therapeutic gene can be influenced by three main factors, (1) controlling transcription itself, (2) utilizing post-transcriptional regulatory elements, and (3) translational control.

Controlling the level of transcription is the most advanced and employs the incorporation of specific promoters to drive therapeutic gene expression in the nucleus. For instance, strong ubiquitous promoters, such as those from cytomegalovirus (CMV), the synthetic CAG promoter or the chicken β-actin (CBA) promoter have found their way into many pre-clinical rAAV gene

[13] https://www.nobelprize.org/prizes/medicine/2006/summary/ (accessed 13 December 2019)

[14] Huntington Disease is a trinucleotide repeat disorder in which the CAG trinucleotide is excessively repeated in the *HTT* gene. The mutant protein product, mHTT, accumulates and increases the death rate of neurons. So far, no cure is available, but some forms of treatment exist.

therapies [31]. An alternative approach would be the incorporation of cell type-specific promoters (if a specific one can be identified) or the utilization of mini promoters to restrict expression to the CNS. *Intravenous* injection of rAAVs carrying specific mini promoters into neonatal mice resulted in cell type-specific transgene expression, as evidenced by histological X-gal staining of a Cre/lacZ reporter or GFP immunofluorescence – this study was a proof of principle, so no therapeutic genes were delivered. For instance, Ple264, covering the regulatory region of the *NR2E1* gene, showed specific targeting of Müller glia cells, a specialized cell type providing both functional and structural support to retinal cells. In the context of brain-targeted gene therapy, mini promoters spanning the regulatory region of the *CLDN5* gene allowed specific transgene expression in endothelial cells of the BBB [32]. Consequently, designing cell type-specific mini promoters might bring about a crucial advancement in regulating and targeting transgene expression in (brain) regions of interest.

Novel rAAVs allow *i.v.* administration and therefore systemic exposure. This, however, might lead to off-target effects in other tissues, e.g. liver or heart or lead to induction of an immune system response. The possible effects of *intravenous* dosing regarding liver toxicity and immune responses will be discussed in sections 3.2. & 3.4. and 3.3., respectively. Cardiac toxicity is another concern for regulatory agencies and will also be addressed in section 3.2. It has been shown that incorporating cell type-specific microRNA binding sites into the sequence of an rAAV vector effectively led to a repression of viral expression in off-target tissues (where these miRNAs are present) but maintained therapeutic gene expression in CNS tissue (where these miRNAs are less abundantly expressed) [33].

The last aspect, which is mostly unexplored for gene therapy to date, incorporates translational control. Optimal codon usage or their modification could bear potential to enhance therapeutic gene expression. Fine-tuning translation of the open reading frame (ORF) might be another aspect to consider.

3.2. Disorders of the Central Nervous System

3.2.1. Alzheimer Disease (AD)

Alzheimer Disease (AD) is a slowly progressing neurodegenerative disease that is attributable to the majority of dementia cases worldwide. The exact cause of brain atrophy is not known, however, two leading hypotheses prevail that are mirrored by microscopic findings of cellular deposits in brains of deceased AD patients.

The amyloid hypothesis posits that the transmembrane-bound amyloid precursor protein (APP) is processed in the wrong way which leads to misfolding and extracellular aggregation of amyloid beta (A_β) peptides. These aggregates further clump together to form clumps or deposits that are well-known as A_β plaques, a specific feature of AD.

The tau hypothesis, on the other hand, posits an intracellular disease mechanism. Tau is a microtubule-associated protein, a network of cytoskeletal proteins to transport cargos within cells, that is normally regulated by phosphorylation. In AD, tau becomes hyperphosphorylated leading to the formation of so-called neurofibrillary tangles, i.e. aggregated, hyperphosphorylated tau protein, which disintegrates the neuron's cytoskeleton.

AD appears to be a very complex disease and many genetic causes have been identified. Subsequently, some of these targets are currently under clinical investigation [34], [35], however, no single target could be identified so far. Nonetheless, some pre-clinical development programs evaluate the efficacy of AAV-based gene therapy [31], mostly in mice as other species turned out to be inferior model organisms for AD pathology.

The majority of AD pre-clinical trials involves *intraparenchymal* vector administration (refer to 3.1.1. and Table 3 in section 3.1.4.). About fifty percent of all studies presented here employ means to induce an immune system response. For instance, virally-induced expression of interleukin 4 or 10 yields reduced astro- and microgliosis[15] and improves spatial learning [36], [37]. Other studies introduce viruses encoding single-chain antibodies directed against A_β and report a reduction in A_β deposits and increased cognitive function [38], [39]. Brain cholesterol

[15] Astrocytes and microglia are two important types of glia cells that perform a plethora of supportive functions within the CNS. For instance, astrocytes form, together with other cell types, the BBB, maintain chemical homeostasis to support neurons and re-cycle neurotransmitters. Microglia are CNS-specific immune cells capable of phagocytosis.

metabolism is another pre-clinically pursued target as gene variations in CYP46A1 have been identified as risk factors [40]. Studies have shown that AD patients (and AD mice) possess less CYP46A1 as compared to healthy controls. Subsequent viral delivery of CYP46A1 in AD mice yielded an increased cholesterol metabolism and reduced A_β and Tau pathology [41], [42]. As mentioned earlier, the amyloid hypothesis posits wrong (pathologic) processing of APP. Consequently, shifting APP processing towards the non-amyloidogenic pathway is also under investigation. For instance, virally-delivered α-secretase enhanced A_β plaque clearance and restored spatial reference memory in AD mice [43].

Taken together, the exact cause for AD is not known. Research has identified several mechanisms that are implicated in disease onset and progression. Consequently, different avenues are currently pursued with the aim of decreasing A_β plaque burden, Tau neurofibrillary tangles and ameliorating brain atrophy and memory deficits/loss.

3.2.2. Parkinson Disease (PD)

Parkinson Disease (PD) is a neurodegenerative disease that mostly affects the motor system. Environmental as well as genetic factors have been identified that lead to progressive cell death spreading throughout the brain. Most prominent is the death of neurons in the substantia nigra which leads to loss of dopaminergic signaling. Mutations in the *SNCA* gene, which gives rise to the α-synuclein protein, lead to the aggregation of insoluble fibrils that bring about various pathological states, e.g. impairment of microtubules, synaptic dysfunction, dysregulated calcium signaling. Other genetic mutations have also been identified, e.g. *PINK1* (mitochondrial), *DJ-1* (oxidative stress response), *LRRK2* (kinase). Several lines of treatment exist, albeit only symptomatic, e.g. Levodopa (L-DOPA), which is a precursor of dopamine (DA), capable of crossing the BBB where it is converted to functional DA and brings about an amelioration of motor impairment [44]. Also, gene therapies aimed at protecting dopaminergic neurons by overexpressing certain proteins are currently developed [31].

Pre-clinical studies specifically targeting α-synuclein were conducted and showed reduction of aggregates and neuronal atrophy [45], [46]. Providing neurotrophic factors, most prominently GDNF (glial cell-derived neurotrophic factor), increased the number of tyrosine hydroxylase (TH,

which catalyzes the conversion of L-DOPA) expressing neurons and led to enhanced levels of dopamine, reduced neuronal degeneration and improved motor performance [47], [48], [49], [50], [51], [52]. AADC (aromatic L-amino acid decarboxylase) is another L-DOPA converting enzyme which was tested in pre-clinical animal models (usually with co-administration of L-DOPA itself). These studies showed robust and year-long expression of AADC (6-8 years in NHPs, however, low levels of neuronal transduction were reported) which reported an increased neuronal ability to produce dopamine [53], [54]. Genetic ablation studies employing shRNAs were also tested with some success. For instance, ablating p11, a scaffold protein implicated in neurotransmitter receptor function and DA replacement therapy-induced dyskinesia (involuntary movement) was found to reduce motor behavior impairment [55]. Genetic targeting of the Rho-associated kinase 2 (ROCK2), an enzyme involved in inhibitory signaling throughout the CNS, led to protection of dopaminergic neurons, increased TH positive cells and improved motor behavior [56].

Taken together, about a third of recent pre-clinical studies assessed the potential of neurotrophic factors to ameliorate PD symptoms. The second third provided key enzymes (mostly AADC) to increase DA levels in affected brain regions while the last third pursued different treatment avenues such as genetic ablation or providing transcription factors to induce neuronal protection.

3.2.3. Huntington Disease (HD)

As alluded to in footnote 12, HD is a neurodegenerative disease that is characterized by the CAG trinucleotide repeat expansion in exon 1 of the human huntingtin (*HTT*) gene, which is primarily expressed in the brain and involved in several important functions, e.g. transcription, DNA maintenance, protein transport, energy metabolism, cell signaling, etc. The three basepairs CAG encode the amino acid glutamine (Q, according to the international amino acid letter code), which leads to the formation of so-called polyglutamine (polyQ) tracts at the N-terminal end of the resulting, mutant huntingtin protein (mHTT). mHTT is an aggregation prone protein and leads to cytoplasmic aggregates and nuclear inclusions which accumulate over time and eventually lead to impairment of neuronal functions and degeneration. To date, only symptomatic treatment paradigms could be devised. Consequently, gene therapy could be a promising approach to silence this defective gene [57].

Due to the size of the human *HTT* gene, i.e. ~180 kb, engineering of adequately sized viral vectors is problematic. An alternative approach encompasses incorporation of a mHTT-targeting miRNA delivered via an AAV5 vector that was recently evaluated in pre-clinical models. A single *intraparenchymal* injection of AAV5-miHTT in both mice and minipigs yielded widespread neuronal transduction and reduced mHTT mRNA levels [58], [59]. These results culminated in the initiation of a phase I/II clinical trial investigating rAAV5-miHTT (also known as AMT-130 by the sponsoring company) which is currently recruiting and expected to run until 2026 (NCT04120493).

Almost all pre-clinical studies conducted so far employ *intraparenchymal* injections and a 50:50 split between treatment approaches exist. Some studies evaluated the potential of introducing virally-expressed neurotrophic factors such as GDNF or BDNF (brain-derived neurotrophic factor) and found reductions in neuronal atrophy [60], [61], [62], [63]. Interestingly, similar to AD pathology, reduced cholesterol metabolism has been implicated in HD. Consequently, one study showed that virally-expressed CYP46A1 reduced neuronal atrophy with a concomitant increase in motor performance in mice [64]. The remaining 50 percent of pre-clinical studies assessed the potential of mHTT protein and/or RNA reduction by means of RNAi. Reductions ranging from 40-60% in mHTT mRNA levels led to reduced brain atrophy and improvements in motor performance [65], [66], [67], [68], [69], [70]. Another study indicated the potential of mHTT repression through zinc-finger proteins which yielded ~60% reduction in mRNA levels and improved motor performance [71].

Taken together, pre-clinical research currently pursues two different treatment approaches. One is rather symptomatic by providing neurotrophic factors to reduce brain atrophy. The other approach aims at regulating mHTT levels by RNAi with some success, as evidenced by the recently initiated clinical trial. However, no reparative approach currently exists, mostly due to the size of the mutated gene itself.

3.2.4. Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), or colloquially known as Lou Gehrig's Disease (named after US baseball player Lou Gehrig who developed ALS in 1938 and died of it 3 years later), is a motor neuron disease that damages both upper and lower motor neurons, i.e. neurons in the motor

cortex of the brain and neurons in the spinal cord, respectively. Despite ongoing research, the exact genetic cause is not known and patients are left with symptomatic treatment only. Several possible causative genes have been identified, e.g. *SOD1*, *PFN1*, *TARDBP*, *FUS*, *C9ORF72*, which renders development of a targeted gene therapy difficult [72]. Nevertheless, initial treatment paradigms employing rAAVs haven been devised [31] and it remains to be seen whether they prove efficacious in ameliorating the disease phenotype.

Most pre-clinical studies assessed reducing mRNAs from mutated *SOD1* through RNAi which was delivered via the *intrathecal* route. Virally-delivered knockdown resulted in improved neuromuscular functions, attenuated motor neuron decline, increased muscle strength and overall increased survival [73], [74], [75], [76]. Other studies assessed the effects of providing neurotrophic factors such as IGF-1 (insulin-like growth factor-1) or VEGF (vascular endothelial growth factor) and found increased muscle strength, decreased motor neuron decline and increases in lifespan [77], [78]. Another interesting study provided an RNA editing enzyme as it was shown that ALS patients suffering from the sporadic form have reduced levels of ADAR2 (adenosine deaminase acting on RNA 2) which leads to motor neuron death. A single *intravenous* delivery of AAV9-ADAR2 rescued motor neurons and prevented motor behavior dysfunction [79].

Taken together, the majority of gene therapy approaches for ALS comprise (mutated) gene regulation through RNAi, while neurotrophic factors provide a symptomatic treatment without addressing the root cause of the disease. Another interesting avenue that was recently pursued addressed excitotoxicity due to excess levels of intracellular calcium [80].

3.2.5. Spinal Muscular Atrophy (SMA)

Another CNS disease affecting motor neurons is spinal muscular atrophy (SMA) which leads to loss of these cells and progressive muscle wasting. The disease is categorized into four types, depending on age of onset and the genetic culprit has been identified. Humans carry two *SMN* (survival of motor neuron) genes, i.e. *SMN1* and *SMN2*, which are both constitutively transcribed. A single basepair difference alters the *SMN2* pre-mRNA processing (skipping of exon 7) which leads to a truncated protein that is rapidly degraded. This, however, has no effect in healthy people as enough functional SMN1 protein is available. Unfortunately, SMA patients harbor

mutations in the *SMN1* gene so that a complete loss of SMN protein will lead to motor neuron death. Recently, different treatment approaches have been developed and approved. Nusinersen, an anti-sense oligonucleotide, acts as a splicing modifier so that the *SMN2* gene is functionally converted into *SMN1* (incorporation of exon 7), thereby increasing the level of available SMN protein in the CNS. Furthermore, Onasemnogene abeparvovec-xioi, is an scAAV9-based gene therapy delivering the *SMN1* gene into patients' nuclei. In addition, small molecules are currently under development, i.e. branaplam and risdiplam, that follow the same approach as nusinersen [81]. Especially risdiplam could be advantageous as it targets all SMA types and is administered orally (as compared to nusinersen which has to be administered via *intrathecal* injection). Onasemnogene abeparvovec-xioi is only approved for type I SMA (so far only in the United States) and has to be given *i.v.* The reasoning for *i.v.* delivery was explained by the manufacturer's chief scientific officer, highlighting that SMN protein is also required in peripheral tissues early in development (based on animal models) and only compartmentalized later in life, i.e. in motor neurons of the spinal cord[16]. For this targeted therapy, both *intrathecal* and *intravenous* delivery are explored. Issues arising from these approaches, i.e. liver and cardiac toxicity, required immune suppression, dose limitations and recent clinical findings are discussed in the respective route of administration sections (3.1.2. and 3.1.3.).

3.2.6. Lysosomal Storage Diseases (LSDs)

Lysosomal storage diseases (LSDs) are metabolic disorders and encompass a group of ~70 genetically distinct diseases. Lysosomes contain a plethora of distinct hydrolytic enzymes essential for macromolecule degradation. Deficiency of such enzymes leads to intracellular accumulation of waste products resulting in cellular dysfunction, (neuro)inflammatory responses and overall organ function impairment. Classification of LSDs is based on the type of stored material, i.e. mucopolysaccharidoses, mucolipidoses, glycoproteinoses, lipid storage disorders and glycogen storage diseases. Further subclassification is based on the molecular defect, e.g. enzymatic protein defects, post-translational processing defects, trafficking defects of lysosomal enzymes. To date, only symptomatic treatment is available, e.g. enzyme replacement therapy (ERT). While this form of treatment represents no cure (and patients depend on ERT for the

[16] https://smanewstoday.com/2019/05/30/intravenous-vs-intrathecal-delivery-zolgensma-particular-routes-different-sma-types/ (accessed 30 January 2020)

remainder of their lives) it showed some success in certain diseases, e.g. Gaucher Disease or Fabry Disease. *In vivo* gene therapy employing AAV vectors could provide a sustainable efficient protein source with an ideally single treatment [31], [82].

3.2.7. Epilepsy

Epilepsy is characterized by recurrent epileptic seizures of mostly unknown origin. Research has identified many genetic culprits, such as mutations in ion channels that cause ionic imbalance(s) and lead to repetitive, excessive firing of excitatory neurons. Other factors may also play a role in inducing an epileptic seizure, e.g. stress, chronic inflammation, flickering light. Currently, anticonvulsant medication is the only, life-long, treatment available. Recent progress in disease etiology has opened new avenues for gene therapy, e.g. gene overexpression or gene silencing via RNAi. To date, most AAV-based gene therapies focus on delivery of neuropeptide Y (NPY) which has been shown to suppress epileptic activity [31].

For instance, about fifty percent of all pre-clinical studies assessing gene therapy approaches focus on viral overexpression of NPY via *intraparenchymal* injection. The most significant findings are reduced seizures in general and reduced seizure sensitivity [83], [84], [85], [86]. Another neuropeptide, galanin, was also found to reduce seizure sensitivity [87]. Viral (over)expression of GDNF increased the seizure induction threshold which was correlated with a reduction in seizures overall [88]. Silencing of certain genes was also assessed pre-clinically. NMDA receptor 1 (NMDAR1), which can be manipulated to control over-excitation, was targeted and diminished via RNAi and found to significantly decrease seizure sensitivity [89]. Viral downregulation of ADK (adenosine kinase), a negative regulator of the brain's endogenous anticonvulsant adenosine, almost completely abolished recurrent seizures [90].

Taken together, most pre-clinical studies to date analyzed overexpression of neuropeptide Y to reduce seizures and/or seizure sensitivity. However, knockdown approaches to regulate overexcitation prove a worthwhile endeavor.

3.2.8. Neuropathic Pain

Neuropathic pain has a complex and diverse etiology. Central neuropathic pain is a result of diseases affecting the CNS, e.g. PD, stroke, lesions of the spinal cord (for instance multiple sclerosis, MS). Peripheral neuropathic pain mostly involves myelinated and unmyelinated nerve fibers in the PNS. Several lines of treatment exist that ameliorate pain sensation [91]. Recent progress in genetic screening identified several ion channel types, e.g. sodium ($Na_v1.3$, $Na_v1.7$), transient receptor potential cation channel subfamily V member 1 (TrpV1), which might be modified to ameliorate the pain sensation even further [31].

Genetic ablation with shRNAs targeting $Na_v1.3$ or TrpV1 reduced allodynia[17] in pre-clinical animal models of pain [92], [93]. Another approach encompassed *intrathecal* delivery of a viral vector leading to expression of an analgesic, i.e. pain relieving, gene (ppβEP). This approach resulted in a reversal of allodynia for up to 3 months [94].

[17] Allodynia is characterized as pain sensitization following non-painful stimuli.

4. Challenges and Development of AAV-Based Gene Therapies for CNS Disorders

4.1. Routes of Administration

Depending on the localization of a specific CNS disease modality, i.e. region-specific (e.g. PD, SMA, ALS) or systemic (e.g. LSDs, AD), different routes of administration have been devised that carry pros and cons in their utilization. Figure 5 provides an overview of current administration routes.

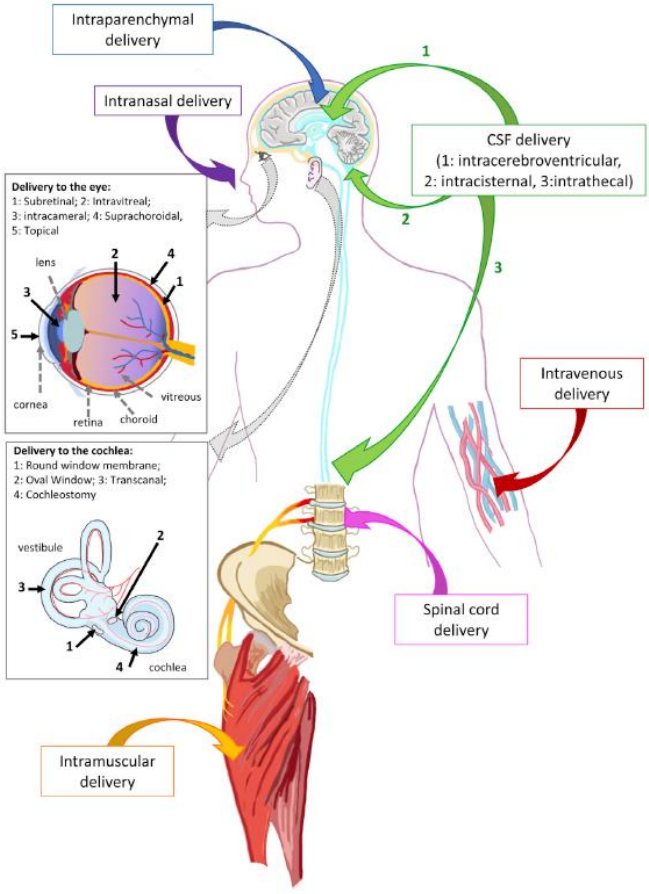

Figure 5 In vivo Routes of Administration to Treat CNS Disorders
Intraparenchymal, intra-CSF, *and* intravenous *are the most important routes of administration (see main text). Intramuscular* or delivery to the spinal cord might be employed to treat very compartmentalized disease states, e.g. SMA. Other, tissue-restricted diseases might be treated by injection into the cochlea (inner ear) or the retina[18]. *Intranasal* delivery might be employed to treat LSDs [96].
Taken from: Hudry and Vandenberghe, "Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality.," *Neuron*, vol. 101, p. 842, 2019

[18] An EMA-approved AAV-based gene therapy, voretigene neparvovec, *treats a genetic defect (in the RPE65 gene) in retinal pigment epithelium cells (RPE cells). Cellular uptake of the virally delivered cDNA leads to production of functional enzymes, which prevents vision impairment/loss.*

The following sections will introduce the currently most-applied administration techniques and highlight issues regarding optimal dosing.

4.1.1. Intraparenchymal

Intraparenchymal administration is a direct injection of AAV-containing solution into brain tissue. Upon administration, passive diffusion of the therapeutic solution allows targeted delivery to a specific region with minimal off-target biodistribution. Furthermore, since it is a compartmentalized therapy much less vector solution can be injected, e.g. as compared to *i.v.* administration. Convection-enhanced delivery (CED) [97], [98], which employs positive pressure to increase parenchymal infusion by temporally expanding the extracellular space yielded a significant increase in infusion volumes. A recent advancement of this technique was the real-time coupling with magnetic resonance imaging (MRI) to visualize tissue distribution of the infusion (by co-injection with an MR visible tracer) [99]. However, most pre-clinical studies employing *intraparenchymal* administration still relied on passive diffusion of AAV solution into target tissues.

One prominent example of limited efficacy of *intraparenchymal* injection (into the striatum of PD patients in a phase I/II first-in-man clinical trial) was ProSavin, a lentiviral-based vector aimed at expressing three key enzymes involved in dopamine biosynthesis [100]. While this study demonstrated a satisfying safety profile, no benefit over placebo could be determined. A recent long-term follow-up study corroborated the initial findings and concluded that higher viral titers might be required for maximal benefit [101]. This represents a scenario in which *intraparenchymal* administration of higher volumes might not be feasible. Therefore, a new lentiviral vector has been developed and pre-clinically evaluated with promising results such as improved motor outcome and high tolerability [102].

4.1.2. Intrathecal

Administration of an AAV-containing solution can also be done by injection into the cerebrospinal fluid (CSF). Three different routes, based on anatomical location, have been tested, i.e. *intracerebroventricular* (into either of the four cerebral ventricles), *intracisternal* (cisterna

magna), and *intrathecal* (lumbar puncture). These approaches are more suited to target site-specific disease modalities, e.g. ALS, SMA, neuropathic pain. In contrast to *intraparenchymal* administration, these methods require slightly larger injection volumes but still less than the *i.v.* route. A recent evaluation of *intrathecal* administration routes in large animals found that *intracisternal* delivery appears to result in more efficient AAV distribution [103]. Another study, employing MR-based tracing in NHPs, found different vector clearance kinetics [104]. Therefore, careful pre-clinical evaluation should be applied to determine the most appropriate delivery route for a given disease. Alarmingly, a phase I clinical trial (NCT03381729) assessing *intrathecal* delivery of Onasemnogene abeparvovec-xioi to treat SMA types II and III was put on clinical hold due to earlier pre-clinical dorsal root ganglion (DRG) inflammation and damage in monkeys[19]. The cause of this reported damage is not known. Luckily, clinical trial subjects that received the *intrathecal* injection did not report any negative issues attributable to DRG inflammation and/or damage.

4.1.3. Intravenous

Since the discovery that certain AAV serotypes can cross the BBB [19], *intravascular* administration has gained more attention as it allows uniform distribution and non-invasive targeting of CNS tissue. However, due to its systemic application, several problems arise. First, *i.v.* administration requires (much) larger doses/volumes as compared to *intraparenchymal* or *intra-CSF* applications. Sufficient quantities of rAAV vectors need to be manufactured, as a consequence. Second, systemic administration leads to exposure of peripheral organ systems, which might cause (severe) immune responses against the exposed rAAV, e.g. hepatoxicity. Third, as alluded to in 2.1.3.1., a certain percentage of the population might possess NAbs due to previous infections with AAVs. This could have a profound negative impact on the ability of an rAAV vector to transduce target cells, essentially rendering the gene therapy ineffective.

Interestingly, Onasemnogene abeparvovec-xioi was recently approved in the United States and is used via a single *i.v.* administration[20] to target a specifically located disease, i.e. SMA type I, in children up to 2 years of age. As alluded to earlier, *i.v.* administered treatments require large

[19] https://smanewstoday.com/2019/11/11/cause-of-inflammation-that-led-to-avxs-101-trial-hold-unknown-novartis-says/ (accessed 30 January 2020)
https://www.curesma.org/avexis-community-statement-clinical-hold2019/ (accessed 30 January 2020)
[20] FDA Package Leaflet Zolgensma (accessed 12 December 2019)

volumes. This is also the case in this young treatment population where patients weighing from 2.6-13.5 kg should receive a prescribed volume ranging from 16.5-74.3 ml (to establish the recommended dose of 1.1 x 10^{14} viral genomes per kg bodyweight). Also, clinical studies highlighted systemic effects that must be monitored in the days and weeks after *i.v.* infusion. For instance, decreased platelet counts, elevated troponin-I levels (sign for cardiac toxicty) and elevated aminotransferases (sign for liver toxicity) were observed. In fact, ~30% of clinical trial subjects showed elevated aminotransferase levels, i.e. above the upper limit of normal. Furthermore, baseline NAb titers directed against AAV9 should be lower than 1:50 (higher baseline titers have not been evaluated). After dosing, titers greater than 1:800,000 have been observed in most patients. Re-administration under such high titers has not been tested but it can be assumed that the therapy would not be effective at all or require excessive immunosuppression. Consequently, all these parameters should be evaluated at baseline, i.e. before dosing, and as indicated in the prescribing information[14].

4.1.4. AAV Injection Dose & Volume Comparison Depending of Route of Administration

Tables 3-5 provide a systematic overview of pre-clinical studies utilizing transgene delivery via *intraparenchymal*, *intrathecal*, and *intravenous* administration.

Table 3 Pre-clinical Studies Utilizing Intraparenchymal Injection

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Mouse	AD	Hippocampus	9	SYN	APPsα	2 x 10^{10}	4	[43]
		Hippocampus	5	PGK	*CYP46A1*	2.4 x 10^{9}	4	[41]
		Cortex, hippocampus	5	CBA	*ECE1*	4 x 10^{9}	4	[105]
		Hippocampus	1	CAG	*IL4*	1 x 10^{10}	2	[106]
		Hippocampus	2/1	N.A.	*TLR5*	1-2 x 10^{10}	2	[107]
		Cortex, hippocampus	N.A.	CAG	*scFv59*	2 x 10^{9}	8	[38]
		Hippocampus	9	SYN	Mecp2-Cas9, U6sgRNA	2.3 x 10^{9}	3	[108]
		Cortex, hippocampus	5	PGK	*CYP46A1*	12 x 10^{8}	4	[42]
		Hippocampus	2/1	N.A.	*IL10*	6 x 10^{9}	4	[109]

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Mouse	AD	Hippocampus	1/2	CAG	*7ND*[21]	2×10^{10}	2	[37]
		Hippocampus	2/1	CAG	*CD74*	1×10^{9}	2	[110]
		Hippocampus	1/2	N.A.	*IL4*	2×10^{9}	2	[36]
		Hippocampus	1/2	N.A.	*ACAT1*	N.A.	2	[111]
		Hippocampus	8	EF1α	*IGF1/2*	N.A.	1	[112]
		Hippocampus	1	N.A.	*A_{β} scFv*	2×10^{9}	10^{22}	[39]
		Hippocampus, thalamus	rh.10	CAG	*APOE2*	1×10^{10}	8	[113]
Rat		Medial septum	N.A.	N.A.	*NGF*	6×10^{9}	2	[114]
NHP		Hippocampus	rh.10	CAG	*APOE2*	5×10^{12}	90	[115]
Mouse	PD	Substantia nigra	1	CBA	*RHEB*	$2\text{-}6 \times 10^{9}$	2	[116]
		Striatum	2	CAG	*P11*	2×10^{9}	2	[55]
		Midbrain	2	CMV	*NURR1, FOXA2*	1×10^{9}	1	[117]
		Hippocampus, striatum, substantia nigra	2/2	SYN	*GBA1*	2×10^{10}	10	[45]
		Substantia nigra	1/2	SYN	*ROCK2, LIMK1*	$0.25\text{-}1 \times 10^{8}$	1	[56]
Rat		Striatum	5	SYN	*TH1, GCH1*	1.3×10^{10} – 1.3×10^{11}	5	[118]
		Striatum	5	CAG	*TH1, GCH1*	9.5×10^{11}	5	[119]
		Substantia nigra	2/5	CMV	*HSP70*	N.A.	2	[46]
		Striatum	2	CMV	*CDNF*	$4\text{-}5 \times 10^{8}$	3	[47]
		Striatum	5	E1A	*GDNF*	3×10^{9}	N.A.	[48]
		Striatum	9	CAG	*EPO*	1×10^{10}	2	[120]
NHP		Caudate nucleus, putamen	5	SYN	*TH1, GCH1*	2.6×10^{12}	80	[118]
		Cortex, striatum	2	CMV	*AADC*	N.A.	180[14]	[53]
		Subthalamic nucleus	N.A.	N.A.	*GAD*	$3\text{-}6 \times 10^{10}$	20	[121]
		Putamen	2	CMV	*AADC*	6×10^{9} – 5×10^{11}	200[14]	[122]
		Striatum	2	N.A.	*AADC*	3.6×10^{11}	180[14]	[54]
		Caudate putamen	2	CAG	*NTN*	3×10^{11}	150	[49]

[21] CCL2 mutant protein

[22] Delivered with CED

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
NHP	PD	Substantia nigra, putamen	2	CMV	*GDNF*	8.3×10^{10} – 8.3×10^{11}	50-75[14]	[50]
		Putamen	2	CMV	*GDNF*	9.9×10^{11}	150[14]	[51]
		Caudate putamen	5	N.A.	*GDNF*	4×10^{11}	40	[52]
		Putamen	2	CMV	*AADC*	3×10^{11}	200	[123]
Mouse	HD	Striatum	1	U6	anti-Htt mRNA	4×10^{10}	10	[65]
		Striatum	5, rh.10	PGK1, CAG	*CYP46A1*	6×10^{9}	4	[64]
		Striatum	2	CBA	*CNTF*	2.7×10^{9}	1	[71]
		Striatum	2/1	CAG	*ZF11xHunt*	4.4×10^{9}	6	[124]
		Striatum, cerebellum	1	CMV	*shHD2.1*	6×10^{10}	12	[66]
		Striatum	2	CAG	*GDNF*	4×10^{9}	4	[60]
		Striatum	2	CAG	*NTN*	4×10^{9}	4	[61]
		Striatum	2/1	CAG	anti-Htt mRNA	2×10^{9}	2	[67]
Rat		Striatum	1/2	CAG	*BDNF*	4×10^{9}	4	[62]
		Striatum	1/2	NSE	*HD70, HD20, Hdh8, shHD2*	3×10^{9}	3	[68]
		Striatum	1/2	CAG	*BDNF*	4×10^{9}	6	[63]
NHP		Putamen	1	U6	*miHDS1*	6.8×10^{10}	68	[69]
Mouse	ALS	Cerebellum	1, 2	N.A.	*IGF1*	4×10^{10}	6	[77]
Mouse	LSD	Thalamus	2/1	CBA	*GLB1*	$4.8\text{-}7.2 \times 10^{10}$	1-4	[125]
		Hippocampus	2/1	CBA	*GLB1*	4.1×10^{10}	1	[126]
		VTA, Hippocampus, Striatum	1, 9, rh.10	GUSB	*GUSB*	$1.2\text{-}1.3 \times 10^{10}$	1	[127]
		Striatum	5	PGK1	*NAGLU*	1×10^{9}	5	[128]
		Striatum	2, 5	PGK1	*IDUA*	1×10^{9}	5	[129]
		Cortex, cerebellum	5	CBA	*NAGLU*	1.5×10^{10}	12	[130], [131]
		Cortex, hippocampus, thalamus	2, 2/5	CBA	*GALC*	4.4×10^{7} – 2.4×10^{8}	12	[132]
		Striatum	5	RSV	*GUSB*	3×10^{9}	5	[133]
		Cortex, hippocampus, thalamus, cerebellum	2/5	CBA	*PPT1*	1.2×10^{10}	12	[134]

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Mouse	LSD	Cortex, hippocampus, hypothalamus, striatum, cerebellum	8	CBA	*ASM*	1.2×10^{11}	N.A.	[135]
		Cortex, thalamus, cerebellum	2, 5	CBA	*CLN2*	3.6×10^{9}	18	[136]
		VTA, striatum	rh.10	CAG	*ARSA*	2.3×10^{9}	1-2	[137]
		Striatum	2/1	CAG	*HEXA/B*	9.9×10^{9} – 1.4×10^{10}	3	[138]
		Striatum, hippocampus, cerebellum	rh.10	CAG	*CLN3*	3×10^{10}	0.5	[139]
		Striatum, midbrain	TT	CAG	*HGSNAT*	$2.6\text{-}3.5 \times 10^{9}$	6	[140]
		Striatum	rh.10	PGK	*SGSH, SUMF1*	7.5×10^{9}	2.5	[141]
Cat		Thalamus, cerebellum	1, rh.8	CBA	*HEXA/B*	3×10^{11} – 4.2×10^{12}	90	[142]
		Thalamus, cerebellum	1, rh.8	CBA	*GLB1*	3×10^{12} – 1.2×10^{13}	94	[143]
Dog		Putamen, white matter	5	PGK	*IDUA, NAGLU*	5×10^{11} – 6.5×10^{12}	320	[144]
NHP		Putamen, thalamus, white matter	5	PGK	*ARSA*	3.8×10^{11} – 1.9×10^{12}	120	[145]
		White matter	rh.10	CAG	*CLN2*	1.8×10^{12}	180	[146]
Rat	Epilepsy	Piriform cortex	2	CAG	*NPY*	1×10^{10}	4	[83]
		Hippocampus	N.A.	CMV	*FIB-GAL, GAL*	1.75×10^{7} – 2.4×10^{9}	1-2	[87]
		Cortex	N.A.	CMV	*NR1A, PINA*	1×10^{9}	1	[89]
		Hippocampus	2	CAG	*GDNF*	$4\text{-}6 \times 10^{9}$	3-6	[88]
		Hippocampus	1/2	CAG	*NPY*	6×10^{10}	12	[84]
		Hippocampus	1/2, 2	NSE	*NPY*	6×10^{9} – 1.2×10^{10}	6-12	[85]
		Hippocampus	1/2	NSE	*NPY*	6×10^{10}	6	[86]
		Hippocampus	8	gfaABC1D	*ADK*	2×10^{9}	2	[90]

Table 4 Pre-clinical Studies Utilizing Intrathecal Injection

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Mouse	AD	ICV	8	N.A.	*BDNF*	2×10^9	4	[147]
		ICV	9, 2g9	N.A.	*MIR137g NRA*	3.5×10^9	2-3	[148]
NHP		ICV, IC	rh.10	CAG	*APOE2*	5×10^{13}	1,000-1,300	[115]
Mouse	PD	ICV	4	N.A.	*BDNF, ΔB2Noggin*	3.9×10^9	3	[149]
NHP						$1.7\text{-}1.9 \times 10^{12}$	40	
Mouse	ALS	ICV or IT	6, 9	CMV, gfaABC1D	*SOD1 miRNA*	N.A.	3 or 10	[73]
		ICV	4	N.A.	*IGF1, VEGF*	$4\text{-}8 \times 10^{10}$	20	[78]
		ICV	9	CAG	*SOD1 miRNA*	1×10^{11}	4	[74]
		IT	rh.10	N.A.	*SOD1 miRNA*	2.4×10^{10}	8	[75]
NHP						2.7×10^{12}	250	
		IT	rh.10	U6, CAG	*SOD1 miRNA*	6×10^{12}	300	[76]
Mouse	SMA	ICV	9	CAG	*SMN1*	$2.7 \times 10^{12} – 3.3 \times 10^{13}$	5	[150]
		ICV, IT	8	CAG	*SMN1*	$1.7\text{-}5 \times 10^{10}$	10	[151]
		ICV, IT	9	CAG	*SMN1*	$1 \times 10^9 – 5 \times 10^{10}$	6	[152]
Pig		IT				3×10^{12}	1,500	
NHP		IC, IT				2.5×10^{13}	6,000	
Mouse	LSD	ICV	2/1	CAG	*BGAL*	1×10^{10}	4	[153]
		ICV	2/5	CMV	*SGSH, SUMF1*	$1.2\text{-}6 \times 10^{10}$	2	[154]
		IC	2	CMV	*NAGLU*	$1\text{-}5 \times 10^{10}$	15	[155]
		IC	9	CAG	*SGSH*	5×10^{10}	5	[156]
		ICV	1, 9	CAG	*ASA*	N.A.	20	[157]
		ICV	4	RSV	*GUSB*	1×10^{10}	10	[158]
		ICV	1, 2, 5	GUSB	*GUSB*	1.8×10^{10}	4	[159]
		ICV	1	CMV	*GALC*	6×10^{10}	4	[160]
		IC	9	CAG	*NAGLU*	3×10^{10}	N.A.	[161]
		ICV	9	CMV	*SUMF1*	1.2×10^{10}	6	[162]
		IT	2	CMV	*IDUA*	$2 \times 10^9 – 4 \times 10^{10}$	50-100	[163]
		ICV	8	CAG	*IDUA*	2×10^{10}	5	[164]
		ICV	1	N.A.	*ASA*	2×10^{11}	20	[165]
Cat		IT	9	CMV, CB	*IDUA*	N.A.	1,000-2,000	[166]
		IC	1	GUSB	*MANB*	1×10^{13}	N.A.	[167]
Dog		IC	9	CAG	*SGSH*	2×10^{13}	1,000	[156]
		IC	9, rh.10	CBA	*GUSB*	N.A.	1,000-2,000	[168]
		IT	9	CAG	*IDUA*	1×10^{12}	1,000-2,000	[169]

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Dog	LSD	IC	9	CAG	*NAGLU*	6.5×10^{12}	N.A.	[161]
NHP		IT	9	CAG	*IDUA*	3×10^{12}	1,000	[169]
Mouse	Pain	IT	9	U6	*TRPV1*	2×10^{13}	10	[93]
Rat		IT	8	CMV	*IL10, ppβEP*	3×10^{10}	15	[94]
		IT	2/5	U6, CMV	*SCN3A*	3.5×10^{11}	5	[92]

Table 5 Pre-clinical Studies Utilizing Intravenous Injection

Pre-clinical Model	Disease	Injection Site	AAV Serotype	Promoter	Transgene	Dose [viral genomes]	Volume [µl]	Ref.
Mouse	AD	IV	9	SYN	*MME*	$0.5\text{-}15 \times 10^{11}$	100	[170]
Mouse	HD	IV	9	U6, CMV	anti-Htt mRNA	6.25×10^{11} – 3×10^{12}	110-300	[70]
Mouse	ALS	IV	9	SYN	*ADAR2*	2.14×10^{12}	N.A.	[79]
		IV	rh.10	U6, CAG	*SOD1*	6×10^{12}	200	[76]
Mouse	SMA	IV	9	PGK	*SMN1*	4.5×10^{10}	70	[171]
		IV	9	CAG	*SMN1*	3×10^{11}	60	[172]
		IV	9	CBA	*IGHMBP2*	5×10^{11}	100	[173]
		IV	9	CMV	*SMN1*	1×10^{11}	10	[174]
NHP		IV	9	CAG	*SMN1*	$1\text{-}5 \times 10^{12}$	10,000	[172]
Mouse	LSD	IV	9	N.A.	*GUSB*	1×10^{12}	N.A.	[175]
		IV	rh.74	U1a	*SGSH*	5×10^{12}	150-200	[176]
		IV	8	EF1α	*IDS*	1×10^{11}	200	[177]
		IV	2	CMV	*NAGLU*	4×10^{11}	100-150	[178]
		IV	9	CAG	*ASA*	2×10^{12}	100	[179]
		IV	9	CMV	*NAGLU*	1×10^{13}	150-200	[180]
		IV	rh.10	CAG	*GALC*	7.6×10^{9}	10	[181]
		IV	9	CAG	*SGSH*	1×10^{12}	200	[182]
		IV	9	CMV	*HEXB*	3.5×10^{13}	100	[183]
		IV	9	CAG	*BGAL*	$1\text{-}3 \times 10^{11}$	200	[184]
Cat		IV	8	TBG	*IDUA*	5×10^{12}	1,000	[185]
Dog		IV	9, rh.10	CBA	*GUSB*	N.A.	1,000-2,000	[168]
NHP		IV	9	CMV	*NAGLU*	$1\text{-}2 \times 10^{13}$	5,000	[186]

As shown in Table 3, direct delivery to the CNS, i.e. *intraparenchymal*, requires the least amount of viral vector-containing solution to be injected. This amounts to ~1-15 µl for rodents and ~50-300 µl for larger animals. A similar relationship, i.e. 2 orders of magnitude, is evident for the

required dose in viral genomes (10^{7-10} for rodents and 10^{10-13} for larger animals). These low levels of volume might be especially suited for pre-clinical (academia-sponsored) studies as vector production is typically not done in large quantities. Furthermore, *intraparenchymal* injections are fairly easy to perform, the only risks being (1) mis-targeting of the vector due to wrong needle placement; (2) glia cell activation due to insertion of the needle into brain tissue; (3) tissue edema due to increased pressure exerted by the 'additional' liquid volume present; (4) post-operative surgical site infections.

Intrathecal injections require intermediate volumes (Table 4). About 5-100 µl for rodents and ~0.1-6 ml for larger animals, respectively. A similar relationship, i.e. 2-3 orders of magnitude, is evident for the required dose in viral genomes (10^{9-11} for rodents and 10^{12-13} for larger animals). These routes of administration also require an invasive procedure and might therefore bear similar risks as described above.

Peripheral delivery, i.e. *intravenous*, is technically easy and non-invasive. However, it requires the largest volumes to be administered (Table 5) which is especially challenging in terms of (large-scale) continuous production. Injection volumes are approximately one order of magnitude larger than for *intrathecal* delivery, i.e. ~100-200 µl for rodents and ~1-10 ml for larger animals. Interestingly, the dose remains relatively similar. This might be due to the already high AAV titers administered. Another potential culprit in this administration setting is the systemic exposure. AAVs with broad tissue tropism might transduce cells in off-target compartments or induce unwanted or unexpected toxicities, e.g. in visceral organs like kidney and liver (see below).

4.2. AAV Leakage Into Off-Target Tissues

AAV vector leakage and subsequent biodistribution in off-target tissues is mostly a concern in *intraparenchymal* and *intrathecal* routes of administration. Unfortunately, only a good dozen of pre-clinical studies evaluated and reported direct leakage into off-target tissues. Of those, only two (both employing *intraparenchymal* injection) reported no apparent leakage of AAV vectors into the blood stream across the BBB [37], [109]. Another study hypothesized that capsid properties of AAV5 effectively limited extravasation into the circulation [18]. As outlined in Table 5, AAV9 is the most common of *intravenous* vector delivery vehicles. Due to this specific property,

extravasation into the circulation (after *intraparenchymal* or *intrathecal* administration) represents a potential problem for off-target tissue transduction. As a result, one study showed that an engineered capsid reduced leakage 100-fold [148], thereby maintaining utilization of novel AAV9 capsids with reduced potential for systemic leakage.

Preservation of dura[23] integrity, as visualized by real-time MRI during *intrathecal* injections, revealed the importance of exact needle placement for AAV infusions [104]. Indeed, two studies reported prior implantation of an *intrathecal* infusion catheter and applying direct pressure on the spinal injection site to avoid excessive leakage [76], [115]. The exact cause for Onasemnogene abeparvovec-xioi's mononuclear cell inflammation and DRG damage or loss upon *intrathecal* administration and the resulting partial clinical hold remain to be determined[15]. However, it might be worth hypothesizing that dura integrity was compromised or AAV leakage resulted in the inflammatory response seen (at least in this small sponsor-initiated pre-clinical monkey trial).

Other studies that mentioned leakage either assessed it qualitatively or quantitatively or hypothesized it due to insufficient target cell transduction. Those experimentally assessing leakage found, e.g. AAV2 leakage into adjacent brain regions and CSF [51]; AAV9 leakage into the bloodstream and subsequent transduction of 3-5% of hepatocytes [156]; AAV9 leakage into lung and liver [96]. Insufficient/off-target transgene expression was hypothesized to be caused by leakage along the needle tract in several studies, e.g. non-specific transduction of cells in the hippocampus [85]; various degrees of cell transduction (low, medium, high) [75]; reduced enzyme activity due to wrong needle placement and subsequent extravasation into CSF [143]; and off-target cell transduction [125], [145].

One study that specifically assessed the biodistribution after *intraparenchymal* injection (into the subthalamic nucleus) found minor levels of viral DNA in kidney and lung, while most other non-brain tissues were negative for AAV genomes [187]. Another study evaluating biodistribution after *intraparenchymal* injection found trace amounts of viral DNA in distant brain regions and spleen [188].

The two most important visceral organs prone to off-target effects due to leakage or enhanced biodistribution are kidney and liver. Only a very limited number of *intraparenchymal* injection

[23] Dura mater; the outermost of three layers of connective tissue protecting the CNS

studies evaluated transgene expression in these organs (off all disease modalities affecting the CNS). Most *intravenous* and some *intra-CSF* injection studies targeting LSDs assessed transgene expression and/or protein/enzyme function in these compartments. Concomitantly, some studies also evaluated toxicity. For instance, no pathologies in liver tissue were found in several studies, [146], [147], [161]. Overt toxicity, e.g. loss of hepatocytes or liver tumors, however, were found in a few other studies [144], [183], [184], [186]. The effect of gene therapy on kidney tissue was assessed in even fewer studies (n=3). Of those, two found no signs of pathology [22], [147], while the third found some, not further characterized, abnormalities [146].

Another major site of concern of regulatory agencies regarding leakage is the germline. As outlined in Table 1 the European guideline for 'Non-clinical testing for inadvertent germline transmission of gene transfer vectors' calls for testing of germline transmission in gonads, gametes and semen through pre-clinical biodistribution studies. Especially parenteral administration poses the risk of germline exposure depending on dose levels, route of administration and tissue tropism of the vector. Of all the pre-clinical studies assessing AAV vector approaches for gene therapy presented in this manuscript only two clearly stated that this possibility was assessed [143], [188]. Both studies employed *intraparenchymal* delivery.

Cardiac toxicity might also be of concern, especially regarding systemic, i.e. *intravenous*, administration. Only a handful of studies also assessed cardiac tissue but found negligible levels of transduction, i.e. ~1-10% of transgene-expressing cardiomyocytes and/or cardiac ganglia [13], [25], [170], [178], [185]. Especially Zincarelli et al. looked at different AAV serotypes and their effects on cardiac function after *i.v.* administration (via the tail vein in mice) [13]. In functional analyses employing echocardiographic parameters no negative impact was found.

4.3. Induction of Immune Response

The immune system can be classified into two parts, i.e. innate and adaptive. For AAV-based gene therapy, mostly humoral responses by the adaptive immune system play a central role in their therapeutic efficacy. While AAV-elicited immunological responses mostly prevent re-dosing new research and development paradigms have been developed to circumvent this roadblock in (possibly) life-long treatment settings.

Innate immune responses are elicited through the recognition of so-called pathogen-associated molecular patterns (PAMPs). These can be, e.g. viral nucleic acids or membrane (capsid) proteins. Interaction with pattern recognition receptors (PRRs) on immune cells leads to rapid and non-specific elevation of pro-inflammatory cytokines or interferons. The innate immune system always elicits the same response without any so-called immunological memory.

Responses of the adaptive immune system take place after innate immunity and lead to the formation of a humoral (blood-borne) response, i.e. antibodies, and a cellular response (cytotoxic T cells). In short, pathogenic molecules (antigens) are presented by so-called antigen-presenting cells (APCs). This leads to the activation and expansion of lymphocytes (B and T lymphocytes) which eventually eliminate the pathogen(s) through humoral and/or cellular responses. After elimination, cells of the adaptive immune system become quiescent, i.e. non-reactive, and now possess a so-called immunological memory which can rapidly re-activate upon re-exposure to the same pathogen. As mentioned in 2.1.3.1. it is believed that about two thirds of the worldwide population have been exposed to AAVs in their lifetimes (this varies by region, however) and subsequently possess at least a humoral response, i.e. circulating neutralizing antibodies (NAbs). Consequently, this might be a crucial factor for developing an efficacious AAV-based gene therapy.

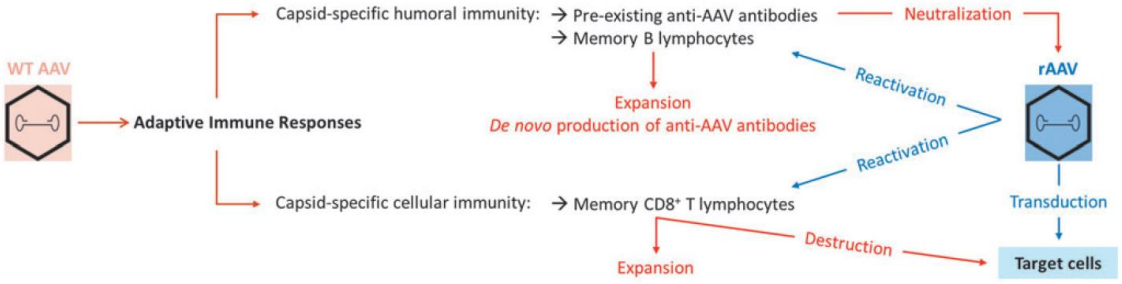

Figure 6 Adaptive Immunity in the Context of AAV-Based Gene Therapy

Wild-type AAV capsid proteins are recognized and processed by the adaptive immune system which leads to humoral (circulating antibodies and memory B cells) and cellular (memory T cells) responses. *In vivo* administration of a therapeutic AAV vector of the same serotype (or bearing similar capsid structures) leads to neutralization of vector particles by circulating neutralizing antibodies (NAbs). Furthermore, memory B cells trigger the production of new NAbs while memory T cells can elicit a cellular response, ultimately destroying transduced cells. These types of responses greatly influence therapeutic efficacy.

Taken from Vandamme et al., "Unraveling the Complex Story of Immune Responses to AAV Vectors Trial After Trial.," *Hum. Gene Ther.*, vol. 28, p. 1062, 2017.

Only a limited number of studies assessed the presence and effects of NAbs (Table 6). Some studies evaluated the effect of pre-existing NAbs and came to the conclusion that transgene expression is not affected. Interestingly, one study reported a significant rise in NAb titer six months after administration [188], however, *intraparenchymal* treatment still seemed efficacious. Another study, employing *intrathecal* administration, reported efficient transduction of and continuous expression in CNS cells while transduction of PNS cells was blocked [156].

Table 6 Pre-clinical Studies Assessing Neutralizing Antibodies (NAbs)

Route of Administration	Outcome	Ref.
Intraparenchymal	Pre-existing NAb titers did not affect transgene expression	[123]
	Detection of NAbs in previously naïve animals	[69] [145] [146]
	Significant rise in NAb titer after administration (100-fold)	[188]
	90% of phase I trial participants had low/moderate NAb elevations	[27]
Intrathecal	Ventricular injection produced higher NAb titer as compared to cisternal injection	[115]
	NAbs detectable but not affecting transgene expression	[161]
	Very limited detection of NAbs in previously naïve animals	[103]
	Efficient transduction of CNS but blockage of peripheral organ transgene expression due to NAbs	[156]
	No NAbs detected	[104]
Intravenous	Detection of NAbs in previously naïve animals	[24]
	NAbs detectable but not affecting transgene expression	[169]
	No effect of NAbs	[76] [168]
	No NAbs detected	[179]
In vitro	Development of novel capsid variants enables NAb evasion	[23]

It might be worthwhile to consider that circulating NAbs do not necessarily play a role in *intraparenchymal* and *intrathecal* delivery, as NAb levels should be relatively low in tissue or CSF. Conversely, *intravenous* administration is at much greater risk of being rendered ineffective, as AAV vector particles will almost instantaneously be detected by either circulating NAbs or memory B cells. In the limited set of studies that assessed NAbs after *intravenous* administration, however, no negative effect in terms of efficacy was observed.

Several strategies have been devised to control immune responses in the clinical setting [190]. These can either be directed against capsid- or transgene-mediated immunity. To circumvent viral vector depletion by NAbs study participants should (1) be screened for pre-existing NAbs; (2) receive immunosuppressive drugs[24]; or (3) receive vectors bearing less prevalent serotype structures. Plasmapheresis was recently demonstrated to effectively reduce pre-existing NAbs directed against AAV8 [191]. This approach might open new avenues not just for treating patients with pre-existing NAb titers but would also allow AAV vector re-administration. Another interesting approach encompasses AAV treatment with co-administration of nanoparticles containing rapamycin. *Intravenous* vector and nanoparticle co-administration into initially seronegative NHPs successfully prevented the formation of NAbs directed against the AAV serotype [192].

Re-dosing of AAV gene therapies is also a special concern for another immune-privileged part of the CNS, the eye. It was feared that ocular administration to one eye could elicit an immune response that dampens or even prevents successful treatment of the second one (or even leads to damage). Studies of the different ocular compartments and subsequent AAV administration revealed an immune-deviant response for *subretinal* injections, while the *intravitreal* route elicited NAb formation that could potentially damage the contralateral eye [193]. Indeed, the only approved AAV gene therapy for a retinal disease, i.e. voretigene neparvovec, must be administered *subretinally* with a concomitant immunosuppressive regimen.

Regarding immunity directed against the transgene itself, (1) giving immunosuppressive drugs; (2) inducing immune tolerance; or (3) avoiding APC presentation, might be worthwhile avenues to pursue successful clinical trials.

4.4. AAV Host Genome Integration & Genotoxicity

As mentioned in 2.1.2. viral nucleic acid sequences are episomal and do not integrate into the host cell's genome (unlike retro- or lentiviruses which readily do so). However, in a mouse model of *intravenous* rAAV2 injection a low number of random integration into liver cell genomes was

[24] Immunosuppression/immunomodulation is the standard of care for patients receiving *in vivo* gene therapies, as evidenced by voretigene neparvovec (requires immunomodulation by prednisone) and onasemnogene abeparvovec-xioi (requires immunosuppression by corticosteroids).

observed [194]. In fact, several more studies that assessed *i.v.* gene delivery found genomic integrations that were furthermore associated with hepatocellular carcinoma (HCC) [195]. One striking observation in some of these studies was transgene integration into the murine-specific *Rian* locus which led to upregulation of tumor-driving microRNAs [196]. In humans, it was reported that at least AAV2 genomic sequences (wild-type, i.e. not used as gene therapy) were found in HCC patient samples, indicating a low possibility (~7%) of viral-driven formation of HCC [197]. However, follow-up studies of AAV2 *i.v.* injected NHPs and humans (1 month and 1 year after administration, respectively) found an overall low rate of integration and did not observe integration events associated with the formation of HCC [198]. The number of subjects analyzed, i.e. 6 NHPs and 3 humans, however, is obviously not enough to draw any (statistically meaningful) conclusions. Also, the most recent long-term data in a dog hemophilia trial revealed genomic integrations across the canine genome, some closely localized to regions associated with growth and cellular transformation. However, this study did not establish a direct link between elevated levels of FVIII (which serves as a marker for liver tumors) and HCC. Consequently, more such analyses are warranted and definitely welcome by regulatory authorities but sponsors and research institutes alike.

Pre-clinical development programs should incorporate points discussed in EMA's reflection paper regarding rAAVs[25], such as animal models, vector persistence, tissue tropism and reactivation. In terms of clinical development, patients exposed to therapeutic AAVs with the (hypothetical) potential of integration or latency should have a monitoring plan in place for 3, 6, and 12 months and then yearly after administration for the following 5 years[26].

None of the pre-clinical studies presented in Tables 3-5 evaluated transgene integration. While this might be negligible for *intraparenchymal* and *intrathecal* routes of administration (where little or no leakage and exposure to off-target tissues occurs) this might be warranted for *intravenous* applications with systemic AAV exposure. At least those studies assessing *i.v.* delivery in larger animals could have integrated tissue (liver) biopsies to screen for genomic integration events. As will be discussed in 5.2. developers are referred to the ICH S6 guideline (carcinogenicity section) and evaluate the potential on a weight of evidence basis.

[25] Quality, non-clinical and clinical issues relating specifically to recombinant adeno-associated viral vectors (CHMP/GTWP/587488/2007)

[26] Guideline on follow-up of patients administered with gene therapy medicinal products (EMEA/CHMP/GTWP/60436/2007)

4.5. Presence of Endotoxins

Endotoxins are outer cell membrane components of Gram-negative bacteria. More specifically, the term refers to lipopolysaccharide (LPS) of various bacterial pathogens, e.g. *Escherichia coli*, *Salmonella, Shigella*, etc. Already low quantities of endotoxins can cause severe immune reactions in humans, e.g. inflammation, fever, apoptosis (i.e. programmed cell death). Therefore, quantification is a requirement for pharmaceutical manufacturing (according to Good Manufacturing Practice, GMP) and of paramount importance to patients subjected to treatment.

Annex 14 of the ICH Q4B guideline[27] describes the interchangeable use of test methods established in the respective member state pharmacopoeias. The European Pharmacopoeia (Ph.Eur. 2.6.14. Bacterial Endotoxins) warrants the test method using *Limulus polyphemus* amoebocyte lysate (LAL) which is based on the coagulation of horseshoe crab blood exposed to endotoxins.

Nearly all pre-clinical studies listed in Tables 3-5 did not mention whether endotoxins were determined. This might be understandable for research laboratories that obtained rAAV vectors from commercial production facilities that readily perform endotoxin tests in their service packages. However, some institutions that produced viral vectors on their own performed purification steps (see table below) and a fraction of those even determined endotoxin levels.

Table 7 Pre-clinical Studies Describing Purification & Endotoxin Quantification

Purification Method	References
Cesium chloride (CsCl) density gradient	[13], [18], [19], [77], [80], [104], [141], [162], [163], [178]
+ endotoxin determination	[113]
Iodixanol density gradient	[15], [21], [25], [87], [112], [144]
+ endotoxin determination	[24], [139], [146], [148]
Heparin chromatography	[63]
+ endotoxin determination	[27]

[27] ICH Q4B Annex 14 Bacterial endotoxins tests

Endotoxins are known to also cause adverse effects in pre-clinical research animals. Therefore, a more thorough description, of at least purification methods, is warranted, especially when progression into clinical development is envisaged. Recent advancements in endotoxin removal based on detergents might simplify this procedure [199] and increase uptake in the pre-clinical setting.

5. AAV Gene Therapies in the European Guideline on Quality, Non-Clinical and Clinical Aspects

5.1. AAV Manufacture

The quality section of the European GTMP guideline describes specific requirements towards development and manufacture. For instance, it provides guidance on design & development genetics, drug substance (DS) and drug product (DP) manufacture, controls, characterization, specifications, and stability.

Regarding GTMP vector design, special emphasis should be given to pathogenicity in humans, replication deficiency, tissue tropism, transduction efficiency and the potential for genome integration and/or germline transmission. For this purpose, the viral backbone, the inserted transgene and any regulatory sequence(s) should be sequenced. Furthermore, it should be established that the therapeutic sequence remains stable and unaltered.

Manufacture of the DS and DP should be clearly described and visualized by a flow diagram. Pre-determined process parameters aid in ensuring an acceptable level of consistency throughout the production process. Critical steps, as well as critical intermediates, must be identified and controlled within acceptance limits.

Characterization of the DS addresses tests to confirm the sequence of the therapeutic gene and any other element(s) incorporated into the sequence to control or regulate its activity. Genomic integrity and stability as well as vector characteristics, e.g. tissue tropism, infectivity, replication capacity, immunological profile and potential for insertional mutagenesis, should be evaluated. Characterization of the DP is not required by the guideline.

Reference to the ICH Q6B guideline is provided to specify the DS and DP. It is noted that the required tests are product and production process-specific. Data that are expected to be included in a dossier are, e.g. identity of the therapeutic sequence and the vector, infectious titer as well as number of particles (and a ratio thereof), and biological potency assay(s).

Regarding stability of both the DS and the DP reference is made to the ICH Q5C guideline. Any change during the manufacturing process warrants comparability studies according to ICH Q5E.

In summary, every GTMP is unique in its development and production. Consequently, only a limited set of quality-related studies are applicable to all drug substances/products and specific tests have to be carried out for each newly developed treatment.

5.2. AAV Pre-Clinical Development

Studies in the pre-clinical setting should provide a thorough base of evidence to allow appropriate benefit risk assessments and eventual progression into clinical trials. In general, it is encouraged to use the most pharmacologically relevant *in vivo* model. As such, the animal model should (1) be sensitive to viral infection; (2) show comparability to humans in receptor and target distribution to assess tissue tropism; (3) show a meaningful biological response to the transgene; and (4) possess an immune system response comparable to the human one. Use of transgenic disease models should be properly discussed and justified. Furthermore, administration of the GTMP should be the same as the one intended for clinical study participants. All the aforementioned parameters that should be taken into consideration usually lead to a development program employing monkeys, i.e. NHPs. However, the guideline leaves room for incorporating small rodent data, while emphasizing that limitations due to small size and short life span must be accounted for.

Primary pharmacodynamic (PD) studies are required to support the potential clinical effect, i.e. proof of concept studies. Specific expression of the transgene(s) in the intended target organ(s) should be demonstrated. Therapeutic efficacy in the pre-clinical model should be demonstrated based on disease biomarkers. Furthermore, determination of the effective dose (without toxic effects) is warranted. Tests regarding safety pharmacology should be conducted according to the ICH S7A guideline.

Pharmacokinetic (PK) studies evaluating absorption, distribution, metabolism and excretion (ADME) might not be relevant for GTMPs. Conversely, biodistribution, tissue persistence, tissue clearance, mobilization, risk of genome integration, germline transmission and virus shedding should be addressed. Biodistribution should be assessed in all relevant organ systems. Consequently, presence, persistence and clearance of the administered GTMP must be evaluated. Risk for germline transmission should also be evaluated in biodistribution studies, primarily in

gonads, gametes and semen. Non-integrating vectors, i.e. AAVs, that lead to long-term transgene expression should be investigated for unintended genome integration.

Recombinant AAVs typically encompass a one-and-done approach, i.e. a single administration entails long-term transgene expression, without the need for re-administration. Consequently, single-dose toxicity studies with an extended post-dose observation period are required for targeting non-replicating tissues, such as the brain[28]. Some cases of *in vivo* gene therapy, however, require multiple dosing regimens. Consequently, repeated-dose toxicity studies are warranted for such treatment paradigms and should incorporate endpoints as outlined in the respective guideline[29].

Standard genotoxicity studies are generally not required, except when there is concern regarding a process-related impurity, e.g. presence of a complexing material. In such a case, the ICH S2 guideline is referenced. Greater emphasis, on the other hand, should be given to potential host genome integration (also for anticipated non-integrating vectors, such as AAVs). Type and extent of these studies are contingent upon route of administration, target tissue, target organ and state of cells within the target.

Rodent bioassays addressing carcinogenicity are generally not warranted for GTMPs. However, a weight of evidence approach (according to ICH S6) should be followed to determine carcinogenic potential. Immunogenicity and -toxicity studies, especially responses of the innate immune system (complement activation), should be investigated. Local tolerance studies, e.g. *intraocular* delivery, might be warranted but depend on already available data.

In summary, few standard pre-clinical studies, i.e. PD, safety pharmacology, are required. Most other studies should be designed according to product characteristics.

[28] In animal models neurogenesis within two distinct areas of the brain, i.e. the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, has been described. There is an ongoing debate as to whether this also holds true for humans, and indeed, a recent study showed that neurogenesis takes place in the human hippocampus but drops sharply and below detectable levels in adults [200].

[29] ICH M3 (R2) Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals and marketing authorisation for pharmaceuticals (EMA/CPMP/ICH/286/1995)

5.3. Clinical Candidates

Since the first approval of *in vivo* gene therapies by regulatory authorities a lot of effort has been put into devising such approaches for a plethora of different disease modalities. Progression of pre-clinically validated candidates into clinical trials is expected to increase in the years ahead. Table 8 provides an excerpt of currently active or enrolling clinical trials through various developmental stages in the field of CNS disorders.

Table 8 Selected AAV-Based In Vivo Gene Therapy Clinical Candidates

Sponsor	Disease	AAV Serotype	Transgene	Route of Admin.	Clin. Dev. Stage	Source
Adverum Biotechnologies	Wet AMD	AAV7m8	Anti-VEGF	*Intravitreal*	I	https://adverum.com/science/#section-03
Asklepios Bipharmaceutical	Pompe Disease (LSD)	AAV2/8	*GAA*	*Intravenous*	I	https://www.askbio.com/clinical-pipeline/
Libella Gene Therapeutics	AD	N.A.	*hTERT*	*Intravenous, intrathecal*	I	https://www.libellagenetherapeutics.com/
NINDS	Giant Axonal Neuropathy	scAAV9	*GAN*	*Intrathecal*	I	https://clinicaltrials.gov/ct2/show/NCT02362438
Rocket Pharma	Danon Disease (LSD)	AAV9	*LAMP2*	*Intravenous*	I	https://www.rocketpharma.com/danon-disease-patients/
Abeona Therapeutics	MPSIII (LSD)	AAV9	*NAGLU*	*Intravenous*	I/II	https://www.abeonatherapeutics.com/clinical-trials
	Batten Disease (LSD)	AAV9	*PPT1*	*Intravenous, intrathecal*	I/II	
Amicus Therapeutics	Batten Disease (LSD)	scAAV9	*CLN3*, *CLN6*	*Intrathecal*	I/II	https://www.amicusrx.com/programs-pipeline/batten-disease/
Axovant Gene Therapies	GM1 (LSD)	AAV9	*GLB*	*Intravenous*	I/II	https://www.axovant.com/indications
	GM2 (LSD)	AAVrh.8	*HEXA*, *HEXB*	*Intraparenchymal, intrathecal*	I/II	
Prevail Therapeutics	PD	AAV9	*GBA1*	*Intrathecal*	I/II	https://www.prevailtherapeutics.com/programs/#our-pipeline
RegenXBio	Wet AMD	AAV8	Anti-VEGF	*Subretinal*	I/II	https://www.regenxbio.com/rgx-314/
	MPSI/II	AAV9	*IDUA*, IDS	*Intrathecal*	I/II	https://www.regenxbio.com/rgx-111/ https://www.regenxbio.com/rgx-121/
Spark Therapeutics (Roche Group)	LCA (Leber Congenital Amaurosis)	AAV2	hRPE65v2	*Subretinal*	I/II	https://sparktx.com/scientific-platform-programs/
	Pompe Disease (LSC)	N.A.	*GAA*	*Intravenous*	I/II	
Uniqure	HD	AAV5	miHTT	*Intraparenchymal*	I/II	http://www.uniqure.com/patients/phase-1-2-clinical-trial-of-amt-130.php
Voyager Therapeutics	PD	AAV2	AADC	*Intraparenchymal*	II	https://www.voyagertherapeutics.com/our-approach-programs/pipeline/
Lysogene	MPSIIIA (LSD)	AAV.rh10	*SGSH*	*Intraparenchymal*	II/III	http://www.lysogene.com/clinical-programs/mps-iiia-phase-i/

5.4. Conclusion & Regulatory Outlook

The purpose of this thesis was to highlight developmental challenges regarding *in vivo* AAV-based gene therapy approaches for CNS disorders. While etiology of the most debilitating diseases, e.g. AD, remains elusive and did not yet yield effective remedies, molecular mechanisms for some diseases have been characterized and led to development of treatment approaches, e.g. SMA. An overview of recent scientific literature (~30 years of AAV development) has highlighted aspects and advancements in vector design that have the potential to deliver efficacious gene therapies through several routes of delivery. Indeed, the recently US FDA approved GTMP Zolgensma[12] could pave the way for further *intravenous* delivery of therapeutic transgenes.

In Europe, approval of this type of medicine is expected in the first half of 2020. Currently, only one *in vivo* AAV gene therapy is approved by the European Medicines Agency, i.e. Luxturna[11]. In the European Public Assessment Report (EPAR) the extreme complexity of this type of medicine is highlighted. Consequently, scientific advice from regulatory authorities will aid in devising the most appropriate development program with subsequent pre-clinical testing.

Since the advent of CAR-T cell therapies[30], authorities and companies alike have a renewed interest in gene therapy as a whole. Indeed, in its strategic reflection paper[31], EMA aims to increase support for translation of ATMPs into patient treatments. It remains to be seen, however, what advancements can be achieved, especially regarding CNS complexity.

[30] Genetically engineered T cells used in immunotherapy
[31] EMA Regulatory Science to 2025

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, die Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

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