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REVIEW

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Designing and optimizing AAV-mediated gene therapy for neurodegenerative diseases: from bench to bedside

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Abstract

Recombinant adeno-associated viruses (rAAVs) have emerged as an attractive tool for gene delivery, and demonstrated tremendous promise in gene therapy and gene editing—therapeutic modalities with potential "one-and-done" treatment benefits compared to conventional drugs. Given their tropisms for the central nervous system (CNS) across various species including humans, rAAVs have been extensively investigated in both pre-clinical and clinical studies targeting neurodegenerative disease. However, major challenges remain in the application of rAAVs for CNS gene therapy, such as suboptimal vector design, low CNS transduction efficiency and specificity, and therapy-induced immunotoxicity. Therefore, continuing efforts are being made to optimize the rAAV vectors from their "core" genetic payloads to their "coat" or capsid structure. In this review, we describe current approaches for rAAV vector design tailored for transgene expression in the CNS, summarize the development of CNS-targeting AAV serotypes, and highlight recent advancements in AAV capsid engineering, aimed at generating a new generation of rAAVs with improved CNS tropism. Additionally, we discuss various administration routes for delivering rAAVs to the CNS and provide an overview of AAV-mediated gene therapies currently under investigation in clinical trials for the treatment of neurodegenerative diseases.

Keywords AAV, Central nervous system, AAV delivery, Blood–brain barrier, Neurodegenerative diseases, Clinical trials

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Introduction

Adeno-associated virus (AAV) was first identified as a contaminant of rhesus monkey kidney cell cultures that were infected with adenovirus stocks in 1965 [1]. AAV was then characterized as an autonomously replication-defective virus unless aided by co-infection of a helper virus, such as adenovirus, herpesvirus, or vaccinia virus [2, 3]. Subsequent studies classified AAV as a member of the Dependovirus genus within the Parvoviridae family. AAV comprises of a nonenveloped icosahedral viral capsid of ~ 26 nm in diameter, which encapsulates a linear single-stranded DNA genome of ~ 4.7 kb [4]. AAV can transduce both dividing and non-dividing cell types for long-term expression of gene of interest (GOI), and has relatively low immunogenicity and high affinity to neuronal or glial cells as compared with other viral vectors. Thus, AAV viral vectors provide valuable opportunities for clinical applications in the treatment of neurodegenerative diseases [5, 6].

Gene therapy holds huge potential for addressing many genetic and debilitating diseases through a one-time treatment. For central nervous system (CNS) diseases, a lack of efficient gene delivery vectors was a major challenge before the introduction of AAV [7]. Recently, AAV-mediated gene therapy has been galvanized by the marketing approval of Luxturna for RPE65-associated retinal dystrophy and Zolgensma for SMN1-linked spinal muscular atrophy. In addition, AAV-based therapies are actively being investigated in preclinical studies and clinical trials for other neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [8]. Despite these notable successes, more efforts are required in designing AAV vectors and optimizing the therapeutic strategy in areas including: (1) Viral genome accommodation and optimization; (2) AAV capsid selection for ideal efficiency and specificity; (3) Need-driven proper selection of administration routes; and (4) Understanding mechanisms of potential toxicities.

In this review, we first delve into AAV fundamentals, exploring AAV vector components, as well as the optimization approaches for each regulatory element within the genome. Then, we discuss both naturally occurring AAV serotypes and novel engineered capsids for their CNS application. Lastly, we discuss all viable routes for AAV administration in the CNS and highlight current efforts being made in clinical trials for the treatment of neurodegenerative diseases.

AAV optimizations for CNS gene therapy

The rAAV (recombinant adeno-associated virus) genome controls the transgene expression level, cell-type specificity, duration, and other important aspects of AAV therapeutics. Therefore, considerable efforts have been made to optimize components in the genome, including the inverted terminal repeat (ITR), promoter, and other regulatory elements (Fig. 1).

Inverted terminal repeats (ITRs)

The AAV genome contains symmetrical ITRs at each end, serving as the origin of DNA replication and packing signals [9]. The single-stranded AAV (ssAAV) requires the host-cell DNA polymerase machinery to produce the complementary strand for transduction which represents a major limitation that slows down the transgene expression. However, this obstacle has been circumvented by the development of self-complementary AAV (scAAV) [10, 11]. scAAV is capable of five- to 140-fold increase in transduction over ssAAV. The increased efficiency is compensated for the loss of half the packaging capacity (2.4 kb of scAAV vs. 4.8 kb of ssAAV) [12]. Notably, scAAV heightens innate immune response via enhanced activation of the TLR9/MyD88 signaling in a dose-dependent manner with induction of IL-6, TNF-α, and other proinflammatory cytokines [13, 14], suggesting increased immunogenicity associated with scAAV. The TLR9 receptor is essential for the recognition of unmethylated CpG motifs, which are highly present in ITR region [15]. Thus, Pan and colleagues adopted rational engineering method to generate CpG-free ITR with equivalent biological potency of transgene expression, but with an approximately threefold reduction in yield [16].

Promoters

The promoter serves as a critical regulatory element for protein expression in the rAAV vector genome, and determines the expression level, cell- or tissue-specificity, and spatial and temporal characteristics [17]. Ubiquitous promoters are frequently chosen to reach potent expression, with over 50% usage reported in 106 clinical trials and 76% in CNS disorders. Among the most commonly used ubiquitous promoters are cytomegalovirus immediate early enhancer/chicken β actin/rabbit β-globin (CAG) promoter, the chicken beta-acting (CBA) promoter and the cytomegalovirus (CMV) promoter [18]. On the contrary, the use of cell- or tissue-specific promoter can regulate precisely localized gene expression to minimize off-target effects and related immune-mediated toxicity. For clinical applications in the nervous system, specific promoters are also selected for manipulating gene expression in certain cell types, such as NSE (identifier: NCT00643890) and syn1 promoters with neuron-specificity (identifier: NCT04127578), and hRPE65p promoter with retinal pigment epithelium-specificity (identifier: NCT04127578).

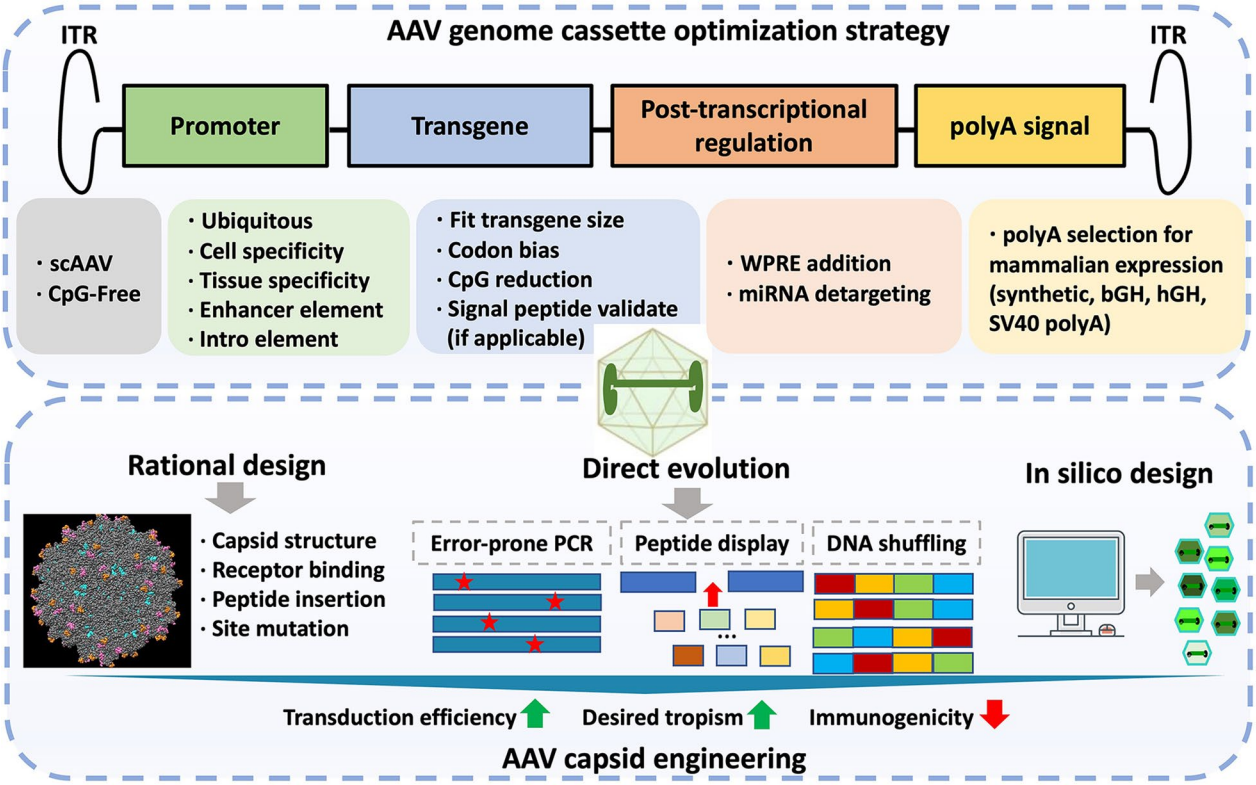

Fig. 1 Strategies for AAV genome design and capsid engineering. The top part displays each element accommodated in the viral genome cassette, as well as corresponding optimization approaches. The bottom part illustrates three major methods to engineer AAV capsids for desired properties

Regulatory elements

Enhancer elements

Enhancer element functions by amplifying transcriptional signals through physical contact with its target promoters in the presence of RNA polymerase II, while located either in the upstream or downstream of the promoter [19]. CMV enhancer is the most commonly used strong enhancer. It is usually conjugated to CBA promoter and CMV early promoter for non-specific enhancement in the CNS. However, it may collaborate with tissue-specific promoters to drive strong transgene expression in cultured neuronal cells [20, 21]. Additionally, Dimidschstein demonstrated that the mouse DLX5/6 enhancer alone can induce specific gene expression in GABAergic interneurons in the forebrain [22].

Intron elements

Intron element is generally positioned downstream of a promoter and enhances gene expression by improving mRNA stability and facilitating mRNA export to the cytoplasm [23]. Strikingly, introducing the Minute virus of mice (MVM) intron between promoter and transgene has been reported to increase 80-fold of transgene expression in liver cells compared to intron-null AAV [24]. Moreover, addition of SV40 intron confers about twofold enhancement of AAV transgene expression in lung carcinoma cells [25].

WPRE element

The woodchuck hepatitis post-transcriptional regulatory element (WPRE) is often included in AAV cassettes to improve viral titer and transduction efficiency via stabilizing mRNA and regulating transcriptional termination [26, 27]. Controversially, WPRE showed no effects on transgene expression in both in vitro and in *vivo* when combined with either EF-1α or CAG promoter in the presence of an intron element [28, 29]. Notably, WPRE was previously reported to harbor potential oncogenic activity that may hinder its translation into clinical practices [30]. However, this concern has been addressed through WPRE optimization to ensure a safe profile for clinical use.

miRNA target sequences

Endogenous tissue-specific microRNA (miRNA) expression is an effective tool to restrict foreign DNA expression in undesired tissues. It is achieved by fusing exact

complementary miRNA target sequences into the 3′ untranslated region (UTR) following gene expression cassette [31, 32]. For example, four tandem complimentary copies of the hematopoietic-specific miR-142-3p sequence were inserted into the downstream of a green fluorescent protein (GFP) reporter. It significantly prevented transgene expression in hematopoietic cells, even after the cells were superinfected with 30 copies of viruses [31]. AAVs exhibit high tropism towards liver tissue. With the development of AAV-mediated systemic gene therapy for the nervous system, efforts have been made to reduce the off-target transduction in the liver post-intravenous administration by adding miR-122 target sequences in the AAV vector [33]. Incorporation of 5 copies of miR-122 target sequences has shown enhanced inhibitory effects over 3 copies in the liver of ICR mice, while without any significant change in transduction efficiency in 293 T cells [33]. In the CNS, the endogenous miR-124 can specifically de-target transduction of viral vectors in astrocytes in mouse brain, conferring the possibility to cell type-specific engineering [34].

AAV serotypes and capsid bioengineering

Since the discovery of AAV as contaminants from adenovirus stocks, 13 AAV serotypes and more than 150 variants have been naturally isolated from human, non-human primates, and other animal species. The variants exhibit variable patterns of transduction and cell tropisms owing to the binding activity between surface topology of capsid and host cell receptor [24, 35–37]. In addition to naturally occurring AAV variants, bioengineering-based synthetic capsid development is rapidly evolving, owing to the advancements of high-throughput NGS sequencing technology and in silico biomining (Fig. 1). In this section, we discuss both naturally occurring and synthetic AAVs and their implications in neurological gene therapy.

Naturally occurring AAV serotypes

The most extensively investigated serotype by far is AAV2, known for its wide tissue tropism, especially in efficiently transducing neurons, which expands its clinical applications to neurological disorders. For instance, AAV2-based LUXTURNA® has been approved by FDA in 2017 for use in patients with Leber's congenital amaurosis [38]. The viral tropism of AAV2 results from universal distribution of its primary receptor heparan sulfate proteoglycan (HSPG) [39], as well as it multiple co-receptors such as the FGF receptor, integrins, and CD9 [40, 41]. AAV3, similar in amino acid composition to AAV2, primarily binds to HSPG as well but with lower affinity, and preferentially targets hematopoietic cells [42]. HSPG also serves as a co-receptor for AAV6 which lacks R585 and R588 residues involved in heparin binding [43]. With only 6 amino acid difference, AAV6 is similar to AAV1 in composition. Both these vectors exploit N-linked sialic acid (SIA) as a primary receptor, and are reported to possess rapid and high levels of transduction in skeletal muscles [44, 45].

In 2012, the first market AAV product Glybera®, an AAV1-based vector, was approved by the European Medicines Agency (EMA) to treat lipoprotein lipase deficiency (LPLD) in skeletal muscle and adipose tissue [46]. Besides, AAV1 and AAV5 display higher infiltrative transduction efficiency than AAV2 in all regions injected within the brain, mainly targeting the neurons [47]. This extends its clinical applications in fighting Huntington's Disease (Identifier: NCT04120493), Leber Congenital Amaurosis (Identifier: NCT00999609) and Retinitis Pigmentosa (Identifier: NCT03328130).

Apart from the discovery of AAV1-AAV6 as live viruses, PCR-based high throughput screening mechanisms have been developed to isolate new serotypes [48]. Both, AAV7 and AAV8, represent hepatocyte tropism, though their primary receptors are still unknown. Notably, AAV7 induces stronger anti-AAV immune response in the hemophilia B population than AAV8 [49], which makes AAV8 suitable for hemophilia B patients in clinical trials (Identifier: NCT01620801 and NCT00979238). On the other hand, AAV8 shows a broad range of tropism and has already been applied in clinical use (Identifier: NCT03001310 and NCT04611503 for eye disorders, NCT04174105 and NCT03173521 for Lysosomal Storage Disorders). Another validated vector with broad tropism is AAV9, binding to terminal galactose and showing efficient transduction in CNS, eye, cardiac and skeletal muscle, and liver [50–53]. Moreover, AAV9 holds the natural ability to cross the BBB and transduces the CNS after systemic administration that will be further discussed in the sections below [54].

Capsid bioengineering

The applications of several naturally occurring AAV serotypes have provided a solid foundation for clinical trials in CNS diseases. However, either limited efficiency or specificity of naturally discovered AAV, or high prevalence of neutralizing antibodies (NAbs) in human population has greatly inspired the generation of novel AAV capsid to better fit clinical demands through laboratory-driven approaches, such as rational design, direct evolution and in silico design.

Rational design

Rational design takes in-depth biological knowledge of existing AAV serotypes to predict and refine virus function with increased capsid tropism, enhanced

transduction efficiency, and reduced immune response [55, 56]. Recently, we reported two novel BBB penetrant AAVs, namely AAV.CPP.16 and AAV.CPP.21, by adopting a rational design approach of displaying cell-penetrating peptides (CPPs) into VR-VIII loop of the AAV9 capsid. Strikingly, the superiority of AAV.CPP.16 over AAV9 can translate from mice to non-human primates, showing 6- to 249-fold enhancement across 4 mouse strains and fivefold enhancement in non-human primates (NHPs) in CNS transduction after intravenous delivery [57]. Moreover, Albright and colleagues identified the essential 8 amino acid residues on the AAVrh.10 capsid that mediated transport across the BBB and grafted onto AAV1 capsid, enabling the new chimeric capsid to cross the BBB and preferentially transduced neurons in C57/Bl6 mice [58]. With extensive understanding of capsid structure, AAV receptor binding and trafficking mechanistic basis, rational design strategy yields many engineered vectors with enhanced functionalities. Thus, these new variants also promote translational study of AAV-mediated gene therapy in nervous system, such as Huntington's disease [59], Mucopolysaccharidosis IIIB (MPS IIIB) [60] and Mucopolysaccharidosis IIIC (MPS IIIC) [61].

Direct evolution

This approach has been widely used for AAV engineering, as much larger capsid library containing up to millions of variants is examined using high-throughput sequencing technology after iterative screening. The direct evolution usually generates a highly diverse capsid library by error-prone PCR, peptide display, DNA family shuffling or a combination of them followed by multiple rounds of selection for a desired phenotype [62, 63]. Of these, the peptide display stretches amino acid segments with random sequences into a capsid site that can provide affinity for specific receptors, for example, inserting between AA588 and AA589 of AAV9 capsid. Based on such strategy, Gradinaru's lab developed Cre recombination-based AAV targeted evolution (CREATE) platform and successfully identified PhP.B and PhP.eB [64, 65], showing overwhelming superiority of CNS transduction after systemic delivery in some mouse strains but unfortunately failed to translate into NHPs [66, 67]. Later, they optimized the CREATE platform by introducing peptide library into the surface-exposed AA455 loop of PhP.eB capsid and obtained a variant AAV.CAP-B10 with enhanced CNS tropism and simultaneously de-targeted from liver expression in both mice and marmosets [68]. Even the direct evolution via peptide display approach to engineering AAV capsid has gained some successes to achieve desired cell tropism and tissue specificity, further explorations are needed to understand cellular interactions of novel capsid as well as to validate its safety and efficiency prior to clinical trials.

In silico design

With technological advancements of computational modeling and machine learning, an in silico design approach to reconstruct and discover new capsid was first reported in 2015 [69, 70]. Carvalho applied in silico ancestral sequence reconstruction (ASR) method to predict the amino acid sequence of putative ancestral AAV capsid monomers using maximum likelihood (ML) methods from 75 isolates of AAV. This method identified the new AAV, namely Anc80L65, from generated capsid pool with 2048 variants. Later, more studies further characterized the vector Anc80L65, demonstrating great transduction efficiency for retinas [71], inner ear [72], and even notable expression in the CNS after systemic gene transfer [73]. Besides, machine learning-based approaches have also encouraged more efforts to de-immunize the capsid [74] and decipher AAV capsid landscape [75], and predict viral assembly [76]. Nonetheless, this next-generation design strategy requires more advanced computational resources and higher quality data for machine learning prior to widespread recognition and adoption.

AAV delivery routes

Successful gene therapies for neurological disorders not only rely on the careful design of AAV vector, including optimization of genome cassette and selection of capsid, but also require the most appropriate route for AAV administration (Fig. 2). The commonly used routes for CNS gene transfer are intraparenchymal delivery (IP), intracerebrospinal fluid (intra-CSF) delivery, intravenous (IV) delivery, and intramuscular (IM) delivery [8]. While, the best selection of administration route is associated with viral safety, transduction efficiency and pathophysiological characteristics of diseases, for example coverage of targeted region and cell- or tissue type of interest.

Intraparenchymal delivery

The direct CNS parenchymal injection is the most selected route for CNS diseases and can precisely transfer high concentrations of vectors to the target region via surgical approach, while bypassing the blockade of physiological barriers. Currently, naturally occurring serotypes AAV1, AAV2, AAV5, AAV8, AAV9 and the engineered variant AAV-DJ are the most frequently used for lP delivery [47, 63, 77]. AAV2 shows minimal diffusion and primarily transduces neurons, which has the most extensively used vector to IP delivery of therapeutic genes in clinical trials for neurodegenerative diseases, such as Alzheimer's disease (Identifier: NCT04133454 and NCT00876863) and Parkinson's disease (Identifier:

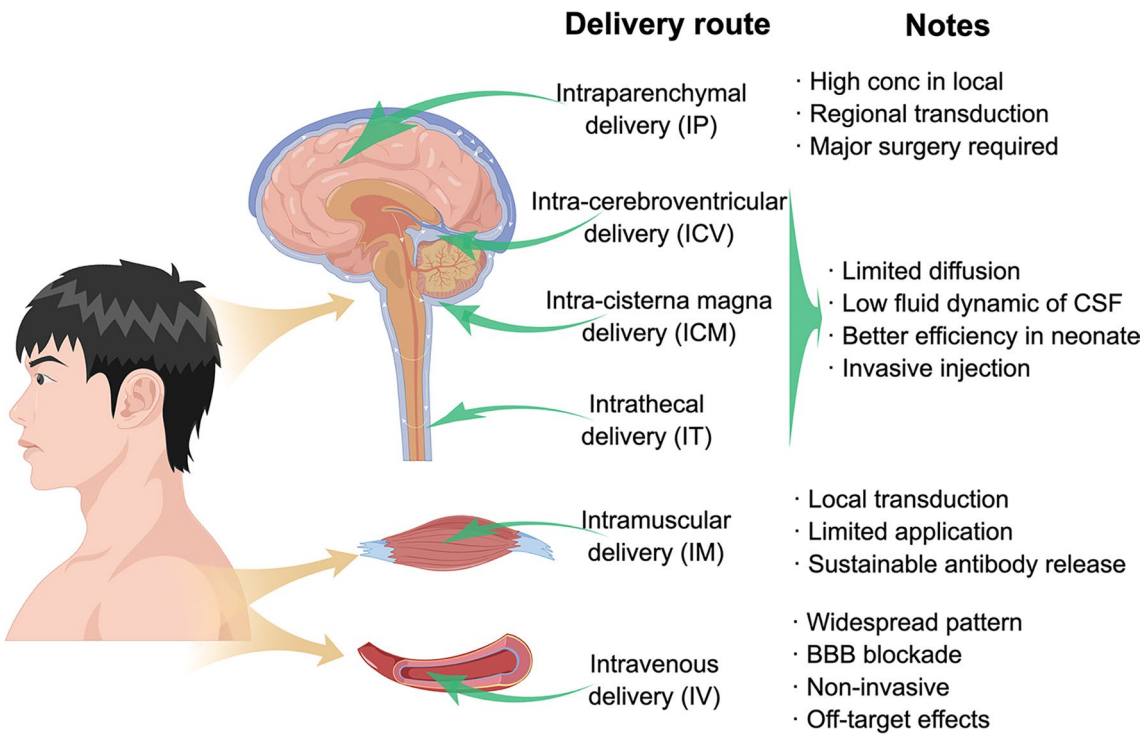

Fig. 2 AAV delivery routes for neurodegenerative diseases. The diagram shows AAV-mediated gene therapy administration routes being used in clinical trials for neurological disorders. Moreover, key pros and/or cons for corresponding approach are listed highlighting the appropriate selection of AAV-based therapy. Created with http://www.figdraw.com/

NCT04167540, NCT00643890 and NCT02418598). Similarly, AAV-DJ can only transduce a confined region but with higher efficiency than AAV2, suggesting an ideal vector for desired expression within a small area [63]. On the contrary, AAV9 and AAV.rh10 provided widespread expression patterns beyond the injection site with glia- and neuron-tropism respectively [78, 79]. Other natural discovery serotypes have also been investigated for IP delivery, displaying comparable expression levels but overlapping cell tropism [80–82]. It is noted a novel variant AAV-TT derived from AAV2 revealed strong neurotropism and exceeded transduction of AAV9 and AAVrh10 in mice, even correcting neurological deficits in the mouse model of MPSIIIC [61].

Intracerebrospinal fluid delivery

The cerebrospinal fluid (CSF) is produced in the brain ventricular by choroid plexus and flows within the cerebral ventricles, cisternal spaces, the subarachnoid space of the spine and brain, and the central spinal cord canal [83]. The CSF system envelops the CNS system, while separated by pia mater and ependyma in the subarachnoid space and brain ventricular zones respectively to form the CSF-brain barrier [84]. When therapeutic agent is administered into CSF, it moves across the barrier to the parenchymal side in the manner of different diffusion approach depending on the injection site (Fig. 3) [85]. The circulation of CSF delivers viral vectors to broader CNS regions when compared to IP injection [86]. While comparing to systemic delivery, intra-CSF route performs in a more invasive procedure, but on the other hand, attenuates the exposure to pre-existing neutralizing antibodies from vascular system and avoids the risk of liver toxicity after intravenous administration of high doses [87]. Direct injection of viral vectors into the compartment of CSF to access the CNS and is usually achieved via ICV [88], IT [89] and ICM (Fig. 3) [90, 91]. However, different routes of intra-CSF delivery led to unique pattern of viral distribution in the CNS.

IT route injects therapeutic agents via lumbar puncture, which is the minimal invasive and easy access procedure for CSF delivery, transporting most of molecules into the spinal cord with little transduction to the brain. Interestingly, Michael and colleagues adopted Trendelenburg position that lying head approximately 25° below the feet after intrathecal infusion of AAV9, resulting a marked improvement of cortical distribution with 15-fold increase in neuron transduction [92]. Comparing to IT route, ICV administration accesses the ventricles requiring an invasive stereotaxic surgery but has

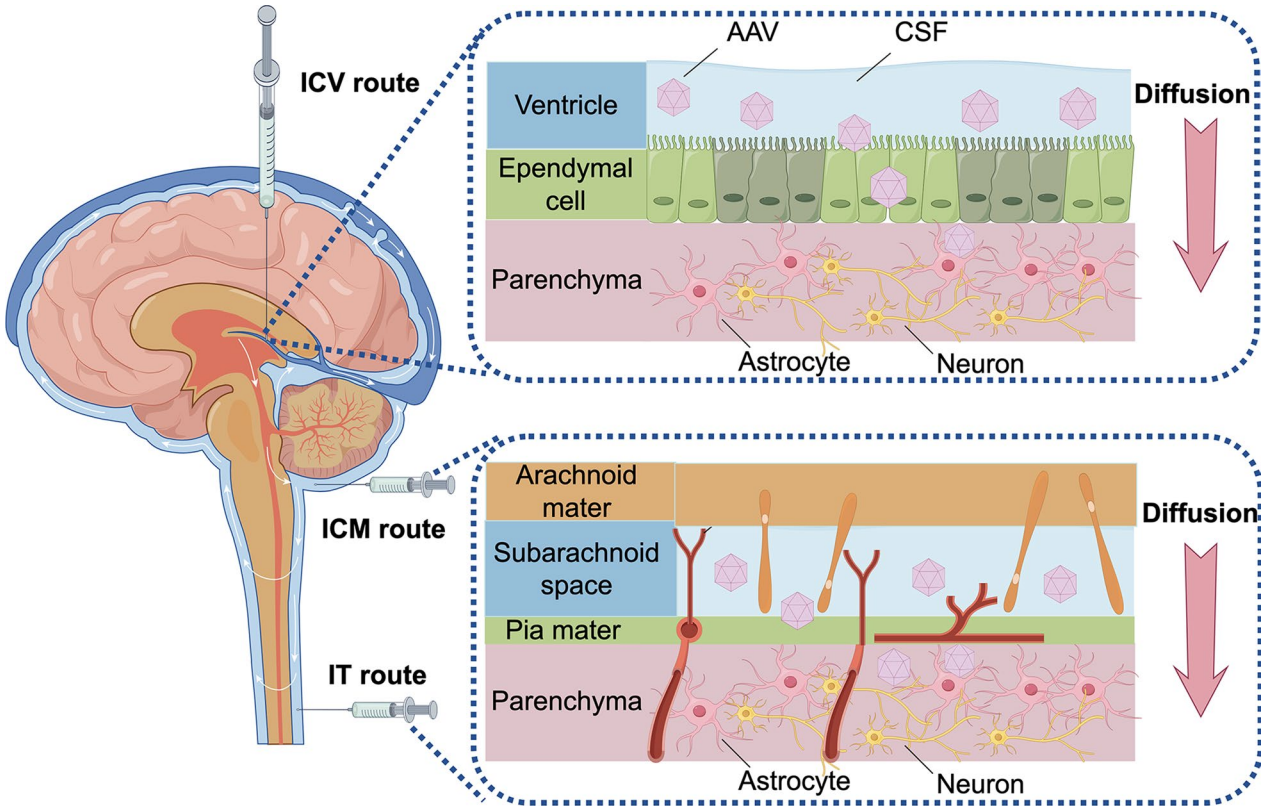

Fig. 3 Intra-CSF delivery routes. The diagram shows major routes for intra-CSF delivery and the fluid dynamics of AAV to cross the CSF-brain barrier. The upper panel demonstrates that AAV infuses with the CSF in the ventricle and cross the ependymal layer to the brain parenchyma side under diffusion effect after ICV delivery. The lower panel shows AAV enters subarachnoid space after ICM or IT injection and passes pia mater to transduce types of various brain cells in the manner of diffusion as well. Created with http://www.figdraw.com/

been demonstrated to be safe in clinical trials and achieve widespread AAV distribution in the brain [93]. Another intra-CSF delivery, ICM route, is injected into the subarachnoid space between the cerebellum and the dorsal side of the medulla oblongata, reaching a broad and balance transduction in both of brain and spinal cord than other CSF infusion [94]. However, puncture to the cisterna magna (CM) procedure avoids damage to the brain parenchymal but is invasive and high risk to induce injury to the blood vessels and brainstem [94].

The transduction efficiency of CSF delivery is not only affected by different routes, but also can be determined by the AAV serotypes. It is reported that AAV7, AAV9 and AAV.rh10 are the extensively studied gene transfer vehicles for intra-CSF delivery [95–97]. Especially, AAV.rh10 favorably transduced motor neurons in NHPs' lumbar spinal cord after IT injection rather than AAV9 to target astrocytes, which successfully mediated SOD1 silencing in adult $SOD1^{G93A}$ mice and profoundly improved both disease onset and survival outcome [97]. A clinical trial of giant axonal neuropathy (Identifier: NCT02362438), a slowly progressive neurodegenerative disorder, applied scAAV9 to carry JeT-GAN gene via IT injection, which marked the first instance of AAV-mediated gene therapy using IT delivery in humans [98]. However, the limitation of intra-CSF delivery still needs to be addressed: (1) unfavorable flow dynamics of CSF and the huge size of human CNS organs often restrict the virions to diffuse into the deep CNS regions; and (2) surgical procedure of ICM and ICV injection posing high-risk injury to the brain.

Intramuscular delivery

AAV-mediated gene therapy using IM delivery is minimally invasive in local muscle tissue and undergoes a cascade intracellular event for retrograde axonal trafficking into neuronal soma [99, 100]. This strategy is particularly suited for mitigating motor neuron diseases (MNDs), such as spinal muscular atrophy (SMA) [99] and amyotrophic lateral sclerosis (ALS) [101]. Owing to the efficiency of retrograde transportation, some AAV serotypes were utilized to achieve desired neuronal transduction in rodents, for example, AAV1 [102], AAV2 [103], AAV9 [103, 104] and AAV-DJ-8 [101]. Moreover, IM delivery of

AAV6 showed about 50% transduction of spinal motor neurons in NHPs [105]. It is noted that some studies revealed neuropathology under MNDs may impact retrograde transduction and hinder AAV-mediated gene therapy [106, 107]. Besides MNDs, another gene therapy strategy using IM delivery aims to induce sustained high-level antibody production in neurodegenerative diseases. AAV1-driven release of single-chain variable fragment (scFv) antibodies targets Aβ deposition and attenuates cognitive deficits, preventing and treating of Alzheimer's disease [108, 109]. This alternative method presents a novel therapeutic approach to attain long-term antibody production after one-dose IM infusion of AAV for neurodegenerative diseases.

Intravenous (IV) delivery

IV delivery is a non-invasive method and could take advantage of the dense vascular network in the brain and spinal cord for more uniform vector distribution but would require vectors to circumvent the obstacle of BBB [110]. AAV9 and AAV.rh10 are the leading naturally occurring serotypes that can transverse the BBB, but the transduction efficiency is superior in the CNS of neonates over that of adults, which limits AAV-mediated systemic gene therapy mostly for infants in clinical trials [8], such as for treating spinal muscular atrophy (Identifier: NCT03461289), Canavan disease (Identifier: NCT05317780) and Krabbe disease (Identifier: NCT04693598). The bioengineering of novel AAV variants in terms of BBB-crossing and CNS targeting has achieved some successes in recent years, yielding AAV.CPP.16 [57], AAV.CAP-B10 [68] and AAV.cc47 [111] with variable enhancements over AAV9 from mice to NHPs. It is also notable that some aspects should take into consideration in future work: (1) potential toxic to the dorsal root ganglia (DRG) [112]; (2) high dosage related immune response after systemic administration [113]; and (3) off-target expression in peripheral organs [113].

Mechanism and methods of AAV to cross the BBB

Intrinsic mechanisms

The BBB is an assembly of vascular endothelial cells, astrocyte, pericytes and extracellular matrix, and tightly tethered together via tight junction proteins [114]. It protects the brain by blocking entry of molecules greater than 400 Da into the CNS, concomitantly with inhibition of systemic delivery of therapeutic drugs. At present, most studies investigate the molecular interaction between AAV capsid and cellular receptors. Here, we will discuss two potential intrinsic mechanisms that mediated BBB-crossing of AAV.

Receptor-mediated transcytosis (RMT)

In a non-invasive way to deliver AAV through the BBB into the CNS, the receptor-mediated transcytosis (RMT) is the most extensively investigated, although it is still not well understood the choice of receptors responsible for AAV trafficking across the BBB. Ramirez and colleagues [115] demonstrated that AAV9 preferably crossed brain-endothelial barriers through an active, cell-mediated process, while little transduced endothelial cells. In contrary, AAV2 without BBB-crossing ability was found to be endocytosed to transduce BMVEC, hardly to diffuse across the endothelial barrier. Also, they provided evidence that the process of AAV9 transport maintained the integrity and barrier homeostasis of BBB. The transcytosis approach to transverse the endothelial barrier can be hampered by addition of two chemicals, tannic acid or filipin that interferes the transport of glycosylphosphatidylinositol-anchored proteins to the apical surface of cells and afterwards restricts its function as a receptor binding to AAV capsids [116, 117].

The receptors in brain endothelial cells, mediated AAV attachment, subsequently facilitate transcytosis through the BBB have yet been characterized with recent progress. Gradinaru lab identified AAV-PHP·B and AAV-PHP.eB with significant higher efficiencies in transporting across the BBB in C57BL/6 J mice after intravenous delivery but starkly absent in BALB/cJ mice and marmosets [64–67]. Later, it was reported that lymphocyte antigen 6 complex, locus A (LY6A), a GPI-anchored membrane protein, responsible for specific engagement to the 7-mer insertion of PHP.B and PHP.eB and transcytosis at BBB [118, 119]. However, LY6A homolog is highly and specifically presented in brain endothelial cells of C57BL/6 J mice but not in BALB/cJ mice and primates. Notably, Kay built up an in vitro transwell model for selection of human BBB-crossing AAVs and applied siRNA screen approach to identify the roles of receptors that participate in the AAV transcytosis [120]. They reported, for the first time, that scavenger receptors (MSR1, SCARB1, and SCARA3), insulin-like growth factor receptors (IGF1R and IGF2R), antibody transporting receptors (FCGRT and TMEM30A), as well as LEPR, LRP8, CNR1, EPHA2, PTAFR, and CCR2 improved AAV transcytosis, whereas INSR, AGER, AAVR, M6PR, TFRC, and LRP2 receptors participated in inhibition of AAV transcytosis.

Paracellular pathway

The tight junctions serve as a "gate" to prevent paracellular transport and maintain permeability and homeostasis of BBB. However, the TJ proteins are reported to be affected under multiple pathological conditions or by approaches causing transient disruption, compromising

the integrity of BBB and increasing influx of substances via the paracellular pathway [121, 122].

Some neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and traumatic brain injuries, may induce transiently disruption of TJs. Accumulation of the β-amyloid, a key characteristic of AD, was demonstrated to compromise BBB permeability by decreasing expression of TJ proteins, including claudin-5, occluding and ZO-1 [123]. TJ loss was also observed in the white matter of MS lesions and positively correlated with BBB breakdown [124]. Using mouse model of traumatic brain injury, our laboratory showed the breakdown of BBB permeability with increased influx of high molecular weight Dextran (155 kDa). Nonetheless, it is still unknown whethe persist TJ damage under pathological conditions could improve the access of AAV to the brain parenchyma via paracellular transport.

Approaches for increasing CNS delivery

Development of BBB-crossing AAVs

As mentioned above, the nature-discovered AAVs—for example the first BBB-crossing serotype AAV9—have provided a solid foundation for novel systemic gene therapy in CNS diseases. However, further enhancing the BBB-crossing efficiency may require development of improved capsids through new discovery and engineering (Fig. 4a). Besides AAV9, naturally isolated AAVrh.8 and AAVrh.10 from rhesus macaques were validated with enhanced CNS tropism in adult mice following IV injection [125]. AAVrh.8 was shown to better transduce neurons, astrocytes, and oligodendrocytes, while with low peripheral tropism compared to AAV9 and AAVrh.10 [126, 127]. AAVHSCs are another group of neurotropic AAVs identified from healthy CD34+human hematopoietic stem cells [128]. Like AAV9, they are classified into Clade F, supporting the claim that the ability to transverse the BBB may be a feature of Clade-F AAVs, although how they cross the BBB after systemic injection is not yet clear.

Enhanced CNS transduction by AAV.PHP.B and AAV.PHP.eB fails to translate into primates, as aforementioned. Ongoing efforts are focused on screening and validating novel AAVs with CNS tropism after systemic injection. For instance, the iTransduce capsid-engineering platform yielded a new variant, namely AAV-F, which mediated successful CNS transduction in C57BL/6 J and BALB/c mice with 65-fold and 171-fold higher

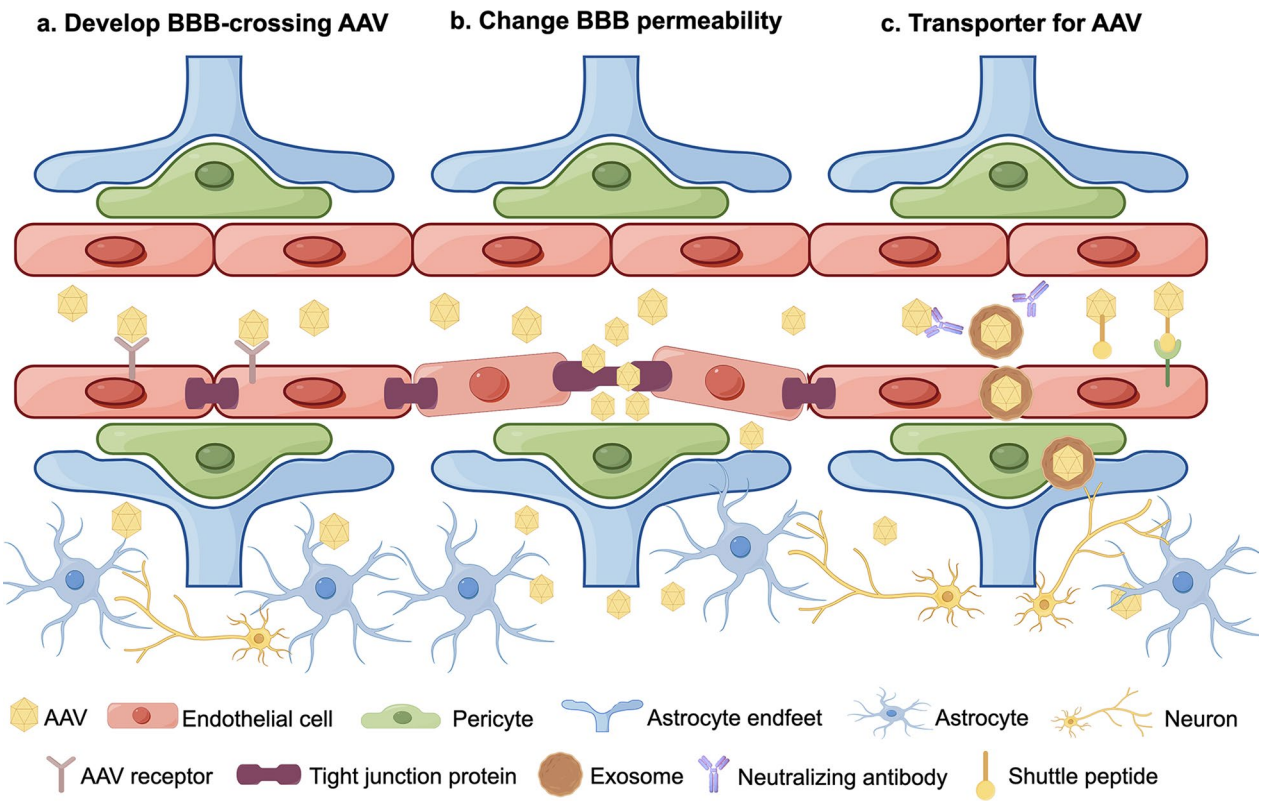

Fig. 4 Methods to increase AAV transduction in the CNS after systemic administration. The diagram shows three potential approaches for facilitating BBB-crossing and CNS transduction by AAVs. Created with http://www.figdraw.com/

transduction of astrocytes and neurons than parental AAV9 respectively [129]. Also, Voyager Therapeutics built up another platform called TRACER (Tropism Redirection of AAV by Cell-type-specific Expression of RNA) and identified ten enhanced variants in C57BL/6 mice, including 9P31 with up to 400-fold higher brain transduction [130].

Change of BBB permeability

Transient opening of the BBB can be utilized to facilitate the delivery of viral vectors to the CNS (Fig. 4b). Fu and colleagues first report that intravenous infusion of 25% mannitol solution 15–20 min before scAAV2 vector administration can increase up to tenfold of vector dispersion in mouse brain. As the mannitol-mediated BBB disruption is temporary and reversible, the BBB permeability peaks 5 min after intra-arterial (IA) infusion and lasts for 20–30 min [131, 132]. Importantly, a study showed that the optimal transduction of AAV2 vectors in the brain was achieved by IV injection of AAV 8 min after mannitol administration; delivery of the vector less than 5 min or 10 min after mannitol infusion was ten times less efficient. Considering above, mannitol-mediated BBB disruption allows broad CNS distribution of AAV vectors depending on the mannitol dose, infusion route as well as virus injection timing [133].

A more localized transient disruption of BBB is accomplished by using magnetic resonance imaging-guided focused ultrasound in combination with IV infusion of microbubbles (FUS-MB) (Fig. 4b), which induce temporary BBB opening even in NHPs [134–137]. The FUS waves induce oscillation of intravenously injected microbubbles in the desired brain region, leading to transient BBB compromise by physically disrupting tight junctions between endothelial cells [138, 139]. This method has been exploited to improve the efficiency for AAV brain penetration. It was reported that IV infusion of AAV1, AAV2 or chimeric AAV1/2 vector, which showed low BBB-crossing per se, successfully penetrated into directed brain regions after FUS-MB treatment [140–142].

Transporter for AAVs

Shuttle peptides, working as a type of transporter, have been investigated to facilitate drug delivery for several decades including in clinical trials (Fig. 4c). However, only a few peptides are showed to be capable of boosting systemic AAV delivery to the brain. For instance, a BBB shuttle peptide THR, which specifically binds to the transferrin receptor 1 (TfR1) [143], can increase the BBB-crossing efficiency of AAV9 and improve brain transduction in a dose-dependent manner in mice. Importantly, further mechanistic study revealed the THR peptide increased the AAV8 transcytosis in hCMEC/D3 endothelial cells and delivered more virion across the BBB to the brain parenchyma [143]. Another PB5-3 peptide specifically bound to AAV9 serotype and enhanced transduction efficiency in different brain cells in mice [144]. Thus, AAV9-PB5-3 complex was used to express IDUA in the mucopolysaccharidosis type I (MPS I) mouse model and achieved more than twofold higher expression with successful phenotypic correction in the brains.

During AAV production, a fraction of AAV vectors were wrapped in microvesicles/exosomes, as observed using transmission electron micrograph [145] (Fig. 4c). It is reported that exosome-enveloped AAV8 or AAV9 had enhanced CNS transduction after intravenous injection [146, 147]. Although the mechanism of exoAAV to cross BBB is still unclear, it is demonstrated that exosome endows AAV vectors with more resistance to anti-AAV neutralizing antibodies [146].

AAV therapeutics in clinical trials for neurodegenerative diseases

In the past decades, AAV-mediated "one-and-done" gene therapies have achieved promising outcomes in preclinical studies of neurodegenerative disorders and have successfully progressed to clinical trials. In this section, we review the major applications of AAV in clinical trials targeting several neurological diseases (Table 1).

Alzheimer's disease (AD)

AD is a common and progressive neurodegenerative condition that predominantly begins in people over 65 years old [148, 149]. The potential pathological mechanism of AD is attributed to the extracellular aggregates of amyloid β (Aβ) plaques and intracellular neurofibrillary tangles formed in the brain by phosphorylated tau protein [150, 151]. Currently, there are three approaches of AAV-based gene therapy strategies to treat AD, including: (1) Induction of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF),which can be retrogradely transported to the cholinergic neurons in the basal forebrain and modulate synaptic plasticity to restore impaired cognition and memory functions [152, 153]; (2) Delivering genes encoding telomerase reverse transcriptase (TERT) that can lead to accumulation of TERT in mitochondria, preventing neuronal damage and promoting neuron's longevity [154, 155]; and (3) Targeting APOE ε4, a high genetic risk factor of AD, by overexpressing APOE ε2 to reserve the balance between APOE ε4 and APOE ε2 (Identifier: NCT03634007). Despite demonstrating safeties, several clinical trials using IP-administered AAV2 gene therapies so far have unfortunately reported no positive outcomes in treating AD [156].

Table 1 Clinical trials of AAV-based gene therapy for neurodegenerative diseases

Disease	Biological name	AAV	Route	Dose	Target	Strategy	Phase	Sponsor	Identifier
Alzheimer's disease	CERE-110	AAV2	IP (nucleus basalis of Meynert)	2×10^{11} vg	NGF	Overexpress	Completed	Sangamo Therapeutics	NCT00876863
	AAV2-BDNF	AAV2	IP	N/A	BDNF	Overexpress	Phase 1	Mark Tuszynski	NCT05040217
	AAV- hTERT (LGT)	N/A	IV and IT	N/A	hTERT	Overexpress	Completed	Libella Gene Therapeutics	NCT04133454
	LX1001	AAVrh.10	ICM	1.4×10^{10} to 1.4×10^{11} vg/ml CSF, and fixed 1.4×10^{14}	APOE2	Replace	Phase 1/2	Lexeo Therapeutics	NCT03634007
Parkinson's disease	AAV2-GDNF	AAV2	IP (putamen)	9×10^{10} vg to 3×10^{12} vg	GDNF	Overexpress	Completed	National Institute of Neurological Disorders and Stroke	NCT01621581
	AAV2-GDNF	AAV2	IP (putamen)	N/A	GDNF	Overexpress	Phase 1	Brain Neurotherapy Bio, Inc	NCT04167540
	CERE-120	AAV2	IP (putamen and substantia nigra)	9.4×10^{11} to 2.4×10^{12} vg	NTN	Overexpress	Completed	Sangamo Therapeutics	NCT00985517
	VY-AADC02	AAV2	IP (putamen)	3.6×10^{12} vg	AADC	Replace	Phase 2	Neurocrine Biosciences	NCT03562494
	VY-AADC01	AAV2	IP (putamen)	9.4×10^{12} vg	AADC	Replace	Completed	Neurocrine Biosciences	NCT03065192
	AAV-GAD	AAV2	IP (subthalamic nucleus)	N/A	GAD	Overexpress	Completed	Neurologix, Inc	NCT00195143
	LY3884961	AAV9	ICM	N/A	GBA1	Replace	Phase 1/2	Prevail Therapeutics	NCT04127578
Huntington's disease	AMT-130	AAV5	IP (striatum)	6×10^{12} to 6×10^{13} vg	HTT	Silence	Phase 1/2	UniQure Biopharma B.V	NCT04120493
	VY-HTT01	AAV1	IP (putamen and thalamus)	3×10^{9} to 9.9×10^{10} vg/ml	HTT	Silence	Withdrawn	Voyager Therapeutics	NCT04885114
Canavan disease	BBP-812	AAV9	IV	N/A	ASPA	Replace	Phase 1/2	Aspa Therapeutics	NCT04998396
	rAAV9-CB6-AspA	AAV9	IV and ICV	N/A	ASPA	Replace	Available	Barry J. Byrne	NCT05317780
	rAAV-Olig001-ASPA	AAV1	ICV	3.7×10^{13} vg	ASPA	Replace	Phase 1/2	Myrtelle Inc	NCT04833907
Spinal Muscular Atrophy	AVXS-101	AAV9	IV	6.7×10^{13} to 2×10^{14} vg/kg	SMN	Replace	Completed	Novartis Gene Therapies	NCT02122952
	Onasemnogene Abeparvovecxioi	AAV9	IV	1.1×10^{14} vg/kg	SMN	Replace	Completed	Novartis Gene Therapies	NCT03505099
	EXG001-307	AAV9	IV	N/A	SMN	Replace	Phase 1/2	Hangzhou Jiayin Biotech Ltd	NCT05614531
Krabbe Disease	FBX-101	AAVrh10	IV	N/A	GALC	Replace	Phase 1/2	Forge Biologics, Inc	NCT04693598
GM1 Gangliosidosis	AAV9-GLB1	AAV9	IV	1.5e + 13vg/kg; 4.5e + 13vg/kg	β-gal		Phase 1/2	National Human Genome Research Institute (NHGRI)	NCT03952637

NGF, nerve growth factor; BDNF, brain-Derived Neurotrophic Factor; hTERT, human telomerase reverse transcriptase; GDNF, glial cell line-derived neurotrophic factor; NTN, Neurturin; AADC, aromatic l-amino acid decarboxylase; HTT, huntingtin; ASPA, aspartoacylase; SMN, survival motor neuron; GALC, galactocerebrosidase; β-gal, beta-galactosidase

Parkinson's disease (PD)

Compared to AD which has broad brain pathologies, PD is selectively associated with the death of dopaminergic neurons in a confined region of the substantia nigra and consequent impairment of gait and movement [157, 158]. AAV-mediated clinical trials for PD treatment are performed via three different target approaches: (1) Induction of protective neurotrophic factors, for example glial cell line-derived neurotrophic factor (GDNF, identifier: NCT01621581 and NCT04167540) and neurturin (identifier: NCT00985517), which can protect and regenerate damaged neural tissues [159, 160]; (2) Increasing dopamine production by AAV-mediated expression of the L-aromatic acid decarboxylase (AADC) enzyme, conferring benefits to some PD patients with moderately motor improvement [161]; and (3) Induction of glutamic acid decarboxylase enzyme as a neuromodulator that can evoke the synthesis of a neurotransmitter Gamma-aminobutyric acid (GABA) to perform inhibitory modulation of neuronal activities in the subthalamic nucleus [162, 163]. Due the localized nature of PD target regions, the most commonly tested AAV serotype so far is AAV2, which often is directly injected into the putamen. Recently, a initiated trial has utilized AAV9 instead to replace mutated GBA1 gene, a high prevalence genetic risk factor for PD. ICM administration route was selected in this trial to maximize broad distribution of AAV9 in the brain [164].

Huntington's disease (HD)

HD is a fatal inherited disorder caused by the expansion of trinucleotide (CAG) repeats in the first exon of huntingtin (HTT) gene on chromosome 4. This genetic mutation can result in neuronal degeneration or death, triggering uncontrollable dance-like movements and abnormal neuropsychiatric signs [165, 166]. A preclinical study for HD applied AAV2-mediated RNAi silencing of HTT gene and proved to be safe without induction of motor deficits, neuronal degeneration, or immune response [167]., Subsequently, this approach has been translated into a clinical trial using AAV5 to deliver the gene therapy via intrastriatal injection (Identifier: NCT04120493). Notably, another clinical trial (Identifier: d) designed to use AAV1 serotype has been withdrawn by the sponsor Voyager Therapeutics. It is believed that systemic delivery using a BBB-crossing AAV vector may be another approach of genetically silencing the HTT mutant gene [168].

Canavan disease (CD)

Canavan disease is a rare fatal neurodegeneration disease caused by deficiency of the aspartoacylase (ASPA) enzyme. An AAV9-based gene therapy approach is currently being investigated to replace the ASPA gene via single IV injection (NCT04998396). Additionally, another clinical trial for CD has similarly utilized AAV9 as delivery tool by simultaneous administration through IV and ICV routes to a single subject (NCT05317780). Notably, a preclinical study has demonstrated that targeting oligodendrocytes using a novel AAV capsid variant AAV/Olig001 is quite efficient for treating CD [169]. Subsequently, AAV/Olig001 expressing the ASPA enzyme has been translated into a clinical trial for ~ 6 years old children with CD (Identifier: NCT04833907).

Spinal muscular atrophy (SMA)

SMA is a rare and severe genetic disorder caused by the mutated SMN1 gene coding SMN protein crucial for motor neuron survival [170]. With AAV9's inherent ability to target motor neurons, AAV9-based Zolgensma® for the treatment of SMA has been established as the first FDA-approved drug for systemic gene therapy in CNS diseases [171]. Despite its approval, Zolgensma is not without limitations and controversies. For instance,, the dosage set by Zolgensma is very high (1.1×10^{14} vg/kg) given the limited BBB-crossing efficiency of AAV9, which greatly increases the manufacturing cost and price tag of the drug. Moreover, high dosage of AAV9 vectors required to reach therapeutic outcome can lead to severe immune-related toxicity and liver damage. To dampen the immune response, immunomodulation medicine such prednisolone or corticosteroid is typically co-administered with Zolgensma. Still, patient fatalities have been reported due to acute liver failure after intravenous delivery of Zolgensma [172].

Conclusions and perspectives

AAV vectors are emerging as the most advanced and promising gene delivery toolkit for basic research and clinical translation. With marketing approvals of Zolgensma and Luxturna, a variety of AAV-mediated gene therapies have progressed into clinical trials. Despite the great potential of AAV based gene therapies for neurodegenerative diseases, challenges and obstacles remain. Success of such therapy requires comprehensive consideration of key factors that could determine the final outcome such as: (1) Administration route must fit the targeting area with a minimally invasive approach; (2) Serotype or capsid selection must strike a proper balance between cell- or tissue-tropism and the chosen delivery routes; and (3) The design of viral genome must accommodate the combination of regulatory elements and increase the expression efficiency and specificity. Moreover, previous epidemiological analyses show that almost 40–80% of the human population are positive for AAV neutralization antibodies. Efforts are underway to

address such pre-existing immunity against AAV gene therapy. These endeavors are crucial for achieving the full potential of AAV-based gene therapy strategies in the treatment of neurodegenerative diseases.

Abbreviations

AADC	L-aromatic acid decarboxylase
AAV	Adeno-associated viruse
AD	Alzheimer's disease
ASPA	Aspartoacylase
ASR	Ancestral sequence reconstruction
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
CD	Canavan disease
CNS	Central nervous system
CPP	Cell-penetrating peptide
CSF	Cerebrospinal fluid
MND	Motor neuron disease
DRG	Dorsal root ganglia
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
HD	Huntington's disease
ICM	Intra-cisterna magna
ICV	Intra-cerebroventricular
IM	Intramuscular
IP	Intraparenchymal
ITR	Inverted terminal repeat
IT	Intrathecal
IV	Intravenous
Ly6a	Lymphocyte antigen 6 complex, locus A
MPS	Mucopolysaccharidosis
MS	Multiple sclerosis
NGF	Nerve growth factor
NHP	Non-human primate
PD	Parkinson's disease
RMT	Receptor-mediated transcytosis
SMA	Spinal muscular atrophy
TERT	Telomerase reverse transcriptase
TJ	Tight junction
UTR	Untranslated region
WPRE	Woodchuck hepatitis post-transcriptional regulatory

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