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Review article

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AAV-based in vivo gene therapy for neurological disorders

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Abstract

Recent advancements in gene supplementation therapy are expanding the options for the treatment of neurological disorders. Among the available delivery vehicles, adeno-associated virus (AAV) is often the favoured vector. However, the results have been variable, with some trials dramatically altering the course of disease whereas others have shown negligible efficacy or even unforeseen toxicity. Unlike traditional drug development with small molecules, therapeutic profiles of AAV gene therapies are dependent on both the AAV capsid and the therapeutic transgene. In this rapidly evolving field, numerous clinical trials of gene supplementation for neurological disorders are ongoing. Knowledge is growing about factors that impact the translation of preclinical studies to humans, including the administration route, timing of treatment, immune responses and limitations of available model systems. The field is also developing potential solutions to mitigate adverse effects, including AAV capsid engineering and designs to regulate transgene expression. At the same time, preclinical research is addressing new frontiers of gene supplementation for neurological disorders, with a focus on mitochondrial and neurodevelopmental disorders. In this Review, we describe the current state of AAV-mediated neurological gene supplementation therapy, including critical factors for optimizing the safety and efficacy of treatments, as well as unmet needs in this field.

Sections

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Introduction

The concept of gene therapy is disarmingly straightforward — using genetic material as therapeutic tools to treat human disease — yet the development and translation of such therapy is complicated and challenging. The first adeno-associated virus (AAV) gene therapy clinical trial for neurological disorders was conducted more than 20 years ago in Canavan disease, a rare and fatal inherited disease caused by a defective aspartoacylase (*ASPA*) gene. The gene therapy, which used a recombinant adeno-associated virus serotype 2 (rAAV2) vector to deliver a functional *ASPA* gene, was safe and associated with modest phenotypic improvement and a decrease of accumulated toxic *N*-acetyl-aspartate[1,2].

The majority of efforts to apply gene therapy to neurological disorders in the past decades have focused on such in vivo approaches. In these cases, the gene product is delivered directly into patients through routes with varying invasiveness, from minimally invasive (intravenous) to very invasive (intraparenchymal (IPa)). With an appropriate vector, gene supplementation (Fig. 1) or small RNA sequences to induce gene silencing or gene editing can lead to expression of a therapeutic, function-restorative protein. The concept of gene supplementation has now expanded to not only restoring defective protein function for monogenic diseases but also correcting signalling pathways or cellular responses for common, complex multigenic diseases through the expression of an effector gene (Box 1).

For ex vivo gene transfer, cells such as haematopoietic stem cells are isolated from the affected patient or donor, genetically modified outside the body and transferred back into the patient[3]. Ex vivo gene therapy has been clinically tested on multiple neurological conditions, such as mucopolysaccharidosis type IIIA (MPSIIIA)[4], MPSI-Hurler variant[5] and metachromatic leukodystrophy[6], and is approved for the treatment of cerebral adrenoleukodystrophy[7]. Challenges for ex vivo gene therapy include potential immune responses[8], transplantation-related complications and insertional mutagenesis caused by retroviral vectors. In vivo gene therapy may also be advantageous for time-sensitive treatment, because ex vivo cell transduction, expansion and engraftment can take several months. Additionally, in vivo gene therapy can induce therapeutic effects in broad central nervous system (CNS) regions, or specific cell types in the CNS.

This Review focuses on the use of AAV for in vivo gene supplementation. However, it is important to recognize the availability of numerous other viral and non-viral vector technologies (that is, adenovirus, lentivirus, herpesvirus and lipid nanoparticles), which each have advantages and disadvantages[9,10] (Table 1). AAV has become a prominent vector for in vivo gene transfer due to numerous features including its overall favourable safety profile. Currently, there are more than 300 clinical trials — completed and ongoing — that use AAV as the gene transfer vector. Among those, more than a third are in neurological disorders, including monogenic diseases and those with multigenic complex aetiology.

Several clinical trials have proven the overall therapeutic benefit and safety of AAV-mediated gene supplementation for neurological disorders, which led to multiple approvals in the United States and Europe. In 2017, the US Food and Drug Administration (FDA) approved voretigene neparvovec-rzyl (Luxturna), an AAV serotype 2 (AAV2) gene therapy for the treatment of Leber congenital amaurosis. Two years later, an AAV9 gene therapy product, onasemnogene abeparvovec (Zolgensma), was approved for the treatment of spinal muscular atrophy (SMA). In 2022, the European Medicines Agency (EMA) approved eladocagene exuparvovec (Upstaza), which uses AAV2 to treat aromatic

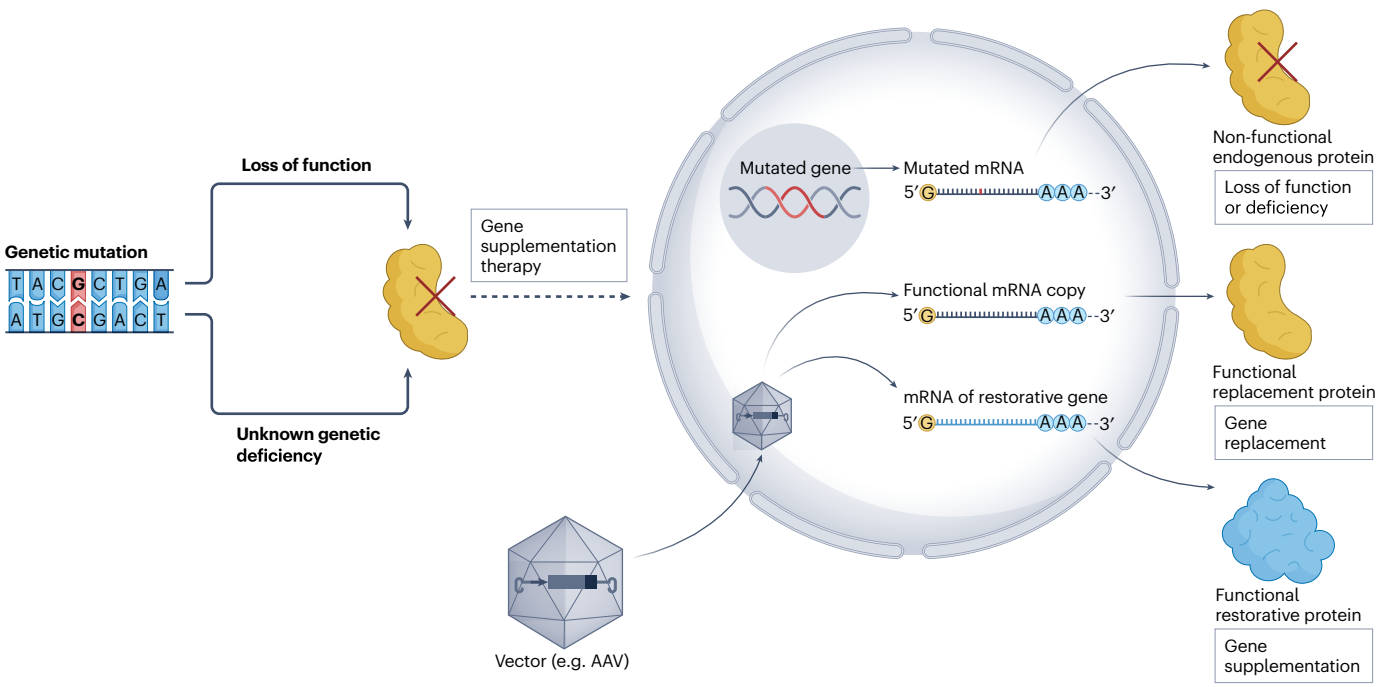

Fig. 1 | Gene replacement and supplementation therapy. Genetic mutation(s) can lead to the loss of function or deficiency of the encoded protein. A vector, such as an adeno-associated virus (AAV), can deliver a gene replacement therapy to provide a healthy copy of the mutated gene, or a gene supplementation therapy to provide a gene to functionally compensate for the mutated gene. The DNA-containing virus transduces target cells, uncoating and transcribing the healthy copy of the gene into mRNA, thus leading to the translation of the functional protein. The mutated gene still exists in the cell; however, it results in loss of function of the protein.

Box 1

Other approaches for in vivo gene therapy for neurological disorders

Gene supplementation is not the only gene therapy approach that can be used to treat neurological disorders. With the development of new technologies, gene silencing and genome editing are also being explored for precision gene therapy.

Gene silencing

For diseases caused by toxic gain-of-function mutations, gene silencing is a potential approach to eliminate the mutated gene product. Currently, RNAi is mediated by synthetic small interfering RNAs, artificial microRNAs (amiRNAs) or short hairpin RNAs (shRNAs). The design of these RNAs is based on the mutated sequence, and both amiRNAs and shRNAs can be cloned into a vector cassette to mediate RNAi in vivo. Vector-delivered shRNAs can reach high expression levels in vivo, and in some cases this may saturate endogenous RNAi machinery[176]. Therefore, amiRNAs have been developed as a safer alternative to shRNAs to produce small interfering RNAs in vivo[177].

Both small interfering RNA/shRNA and amiRNA strategies have shown efficacy in animal models of Huntington disease[178,179]. Preliminary results from a clinical trial of an amiRNA for Huntington disease suggested that the strategy was safe and effective, as the mutant huntingtin protein was reduced by a mean of 53.8% in treated patients[180]. For amyotrophic lateral sclerosis, microRNA (miRNA) targeting mutations of the superoxide dismutase 1 (*SOD1*) gene was tested in two patients and was shown to be safe[144].

A combination knockdown and addition strategy can also be designed. For example, spinocerebellar ataxia 7 is caused by a CAG-repeat expansion which could benefit from genetic silencing, but maintaining the wild-type (WT) allele is challenging due to the unique genetic variations between patients. Therefore, a broadly applicable non-allele-specific RNAi, which knocks down both mutant and WT alleles, in combination with a knockdown-resistant gene addition therapy was designed. This single construct design showed success in both knocking down and replacing the affected allele in patient fibroblast samples[181].

Genome editing

With the development of various gene editing tools, gene editing has been explored as a cutting-edge approach for personalized medicine, enabling precise changes in the human genome to correct loss-of-function or gain-of-function mutations. One of the earlier gene editing breakthroughs was the discovery of engineered nucleases, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)[182–184]. These nucleases have not been widely used in therapeutic applications as they are difficult to synthesize[185], have potential off-target effects and would have to be specifically engineered for each individual pathogenic allele.

With the discovery of CRISPR–Cas9, which uses single-guide RNAs and the Cas9 nuclease to generate double-stranded breaks in a specific DNA sequence, gene editing is becoming the next generation of gene therapy. However, similar to TALENs or ZFNs, the CRISPR–Cas9 system also requires double-stranded breaks, which may lead to undesired effects such as random mutagenesis and cell death. Base editors and prime editors were invented to directly correct gene mutations without generating double-stranded breaks[186]. Base editors utilize DNA-binding proteins to bring either adenine or cytidine deaminase to the mutation site, to create either C•G to T•A or A•T to G•C alterations in the genetic code. Alternatively, prime editors — which also use DNA-binding proteins to locate the affected loci — take a more general editing approach via the use of prime editor guide RNAs and reverse transcriptase to replace or insert any desired sequence. When compared with other editing techniques, prime editing has a relatively low rate of random insertions/deletions (fewer than 1%), but also has overall decreased editing efficiency which will require further improvement[186]. Another approach to circumvent constitutive off-target editing is RNA editing technology, which uses catalytically inactive RNA-targeting CRISPR–Cas13 with adenine deaminase acting on RNA domain (ADAR2)[187].

Currently, clinical trials using gene editing approaches have been limited to non-CNS disorders, such as for Leber congenital amaurosis, which uses adeno-associated virus (AAV)-delivered CRISPR–Cas9 editing (ClinicalTrials.gov identifier NCT03872479). For neurological disorders, studies using these emerging technologies are at the preclinical stage. According to publicly disclosed information, zinc finger targeting transcription factors delivered by AAV are in development for tauopathies, synucleinopathies, amyotrophic lateral sclerosis/frontotemporal disorders (FTD) with *C9ORF72* mutation, Huntington disease and autism. CRISPR–Cas-based gene regulatory approaches are also being developed for a few undisclosed neurological disorders[188]. To translate these studies into clinical trials, the same considerations for translational success of gene addition therapy (see the main text) can be applied. However, additional safety aspects need to be considered, such as off-target gene editing and on-target gene editing-related side effects.

L-amino acid decarboxylase (AADC) deficiency. Recently, with the advancements of small interfering RNA and gene editing tools, including the CRISPR–Cas system and base editors (Box 1), the scope and toolkit of gene therapy has expanded; all of which have utilized AAV for genetic transfer.

For neurological disorders, gene supplementation is the most advanced among gene therapy strategies, and thus is the focus of this Review. First, we provide a brief summary on the evolution of AAV as a therapeutic vector, followed by an overview of representative clinical trials that are using AAV gene supplementation therapy for neurological disorders. We discuss lessons learned from those clinical trials, and how they have informed translational considerations when designing preclinical AAV gene therapy studies. We highlight two categories of diseases that face added mechanistic challenges and represent the

Table 1 | Comparison of vector systems for gene delivery

Vector type	CNS transduction efficiency	Immunogenicity	Packaging capacity	Genome	Integration propensity	Gene expression stability
Adenovirus	High	High	25 kb	dsDNA	Low-integrating	Transient
Lentivirus	Moderate	Low	8 kb	ssRNA	Integrating	Stable
γ-Retrovirus	Moderate	Low	10 kb	ssRNA	Integrating	Stable
AAV	Moderate	Low	4.5 kb	ssDNA	Low-integrating	Stable
HSV-1	High	High	50 kb	dsDNA	Low-integrating	Transient
Non-viral vector	Low	Low	Large	–	Non-integrating	Transient

AAV, adeno-associated virus; CNS, central nervous system; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

latest frontier in preclinical and early developments. Additionally, we discuss potential safety events as a growing area of concern. Lastly, we propose some approaches to maximize efficacy while minimizing toxicity, along with examples of promising preclinical studies based on those approaches.

Basic AAV vector design

AAV was discovered in the 1960s and has become recognized as an ideal vector for therapeutic gene delivery given its low incidence of integration into host genomes, low immunogenicity, broad serotype-dependent tissue tropism and transduction efficiency[11,12]. The wild-type (WT) AAV capsid is composed of 60 subunits, encapsulating an ~4.7 kb single-stranded DNA (ssDNA) genome. The genome contains the major viral open reading frames for the *REP* (replication) and *CAP* (capsid) genes, which are flanked by two inverted terminal repeats (ITRs) (Fig. 2a).

To use AAV for transgene delivery, recombinant AAV (rAAV) is generated by replacing *REP* and *CAP* with sequences encoding a gene of interest and regulatory elements (Fig. 2b). More than 100 natural variants and several serotypes of AAV have been characterized that vary in their binding affinities to different cell surface receptors, which lead to serotype/variant-dependent tissue tropisms. Some AAV capsids have been formally tested to be serologically distinct (that is, serotypes), whereas most newly discovered or engineered capsids are better defined as variants, which have not been formally assessed to be independent serotypes.

An ideal rAAV for treating neurological disorders would have high transduction efficiency for CNS tissues and be able to cross the blood–brain barrier (BBB). AAV9 is one such capsid that meets these criteria[13] and is the most favoured for neurological applications. AAV9 was isolated from human tissue and is a distinct serotype that shows relatively high transduction efficiency in the brain[14,15]. Many new capsids have also been generated by making modifications to certain regions of the AAV capsid to drive cell tropism and mitigate toxicity[16–20]. However, further studies are needed to explore and validate the efficiency and safety of new capsid variants.

Different AAV serotypes exhibit unique specificity and transduction efficiency, determined by cell surface AAV attachment factors — glycan moieties that vary by cell type and primarily bind AAV and its co-receptors to induce AAV cellular entry via endocytosis[21]. The first AAV receptor identified was for AAV2 — which binds to the heparan sulfate proteoglycan (HSPG)[22]. Other serotypes such as AAV1, AAV4, AAV5 and AAV6 attach to sialylated glycans[23,24], whereas AAV9 uses galactose as its primary receptor[25]. Overall, the receptors and co-receptors utilized by various AAV capsids are important mediators of their tropism and transduction efficiency, and are promising targets for identifying more efficient AAVs or mitigating off-target transduction (discussed below).

Compared with serotype receptor specificity, the process of AAV intracellular trafficking is less understood, with most of our knowledge coming from studies on AAV2. However, individual AAVs can differ in their efficiencies to faithfully traffic their genomes to the nucleus. Following cellular entry, the endocytic vesicles deliver AAV to the *trans*-Golgi network through the late endosomes or recycling endosomes, or using endoplasmic reticulum-derived transport vesicles. The capsid then travels from the Golgi into the nucleus through the nuclear pore complex in an importin β-dependent manner. Within the nucleus, the genome is released from the capsid to begin the replication process[26]. The ssDNA of AAV is converted to double-stranded DNA (dsDNA) through either second strand synthesis or strand annealing, and this double-stranded genome remains as a circular episome[27] or, in rare cases, integrates into the host genome[28].

Understanding these processes of cell binding, trafficking and uncoating could enable the design of AAVs that show higher tissue tropism, increased cell specificity, enhanced intracellular trafficking and/or greater overall potency. However, it remains unknown whether all AAVs exhibit the same trafficking processes, or what determinants are required for forming a stable circular episome.

An important modification that has been made to the initial rAAV is to change its single-stranded genome to a double-stranded, or self-complementary, structure by deleting the AAV terminal resolution site and D sequence from the 5′ ITR[29,30] (Fig. 2c). By obviating the need to covert ssDNA to dsDNA within the host cell, self-complementary AAV improves transduction efficiency by tenfold or more compared with single-stranded AAV. However, as the amount of ssDNA loaded into the capsid remains limited to ~4.7 kb (4.4 kb without ITRs), the maximum size of the double-stranded transgene cassette is reduced to ~2.2 kb — half the size of the single-stranded genome.

Initially, AAV2 was the most widely studied AAV serotype, but the toolkit of additional serotypes/variants of AAV has greatly expanded over the past two decades[15], leading to greater capabilities to transfer genes to many tissues including the CNS. One of the most commonly used AAVs for CNS tissue is AAV9, which has been applied in at least 15 clinical trials[31], such as for giant axonal neuropathy[32,33] and the FDA-approved therapy for SMA[34]. As our understanding of AAV design continues to grow, there is optimism that stronger CNS-tropic AAV capsids can be identified, which would enhance the efficacy and safety of gene therapy.

CNS gene supplementation in clinical trials

The first FDA-approved AAV gene therapy for a neurological disorder was for the treatment of type I SMA with a single-dose AAV9/survival

motor neuron (*SMN*) gene therapy (Zolgensma) via intravenous injection[34–36]. During the clinical trial, the first 15 treated patients were event-free at 20 months of age compared with 8% survival in the natural history cohort[34]. Its success has led to increasing interest in the use of gene therapy for other genetic diseases affecting the CNS, and several clinical trials are ongoing (Table 2). For monogenic recessive diseases, the therapeutic transgene is straightforward — the mutated gene — whereas for more complex diseases, such as Parkinson disease and Alzheimer disease, the therapeutic gene could be the primary genetic cause, or a protein target that can improve neurological functions.

For broad CNS transduction, the favoured delivery vehicle is currently AAV9, with only a few studies using other capsids such as AAVhu.68 (refs. 15,37) and AAVrh.10 (ref. 15), which were isolated from human and non-human primate (NHP) tissues, respectively. To induce region-specific brain transduction, such as for Parkinson disease[38], AAV2 has been used partly due to the wealth of historical data on its spread and tropism following IPa administration, but also because of its lower tendency to spread to regions distal to the injection site compared with other AAVs[39]. The administration route also plays a critical role in the biodistribution of gene expression (Fig. 3). Several routes are under investigation in ongoing trials and are discussed in more detail below.

Zolgensma utilizes the least technically challenging route of administration — systemic intravenous delivery. Another in vivo AAV gene replacement that is currently in clinical testing using systemic delivery is in GM1 gangliosidosis, a lysosomal storage disorder caused by deficiency in the β-galactosidase (β-gal) enzyme that breaks down glycosphingolipids[40]. When this deficiency occurs, GM1 ganglioside accumulates in brain and spinal cord neurons, ultimately leading to cell death[40]. Gene therapy was effective for this disease in a naturally occurring feline model[41,42], as well as a murine model[43]. Currently, three trials are ongoing to assess the efficacy and safety of either systemically or CNS-specific delivery of functional copies of β-gal using the AAV9, AAVrh.10 or AAVhu.68 vector. As both administration routes are being tested in the same indication, results from those trials will provide valuable information for comparison of the pros and cons of each administration route. Preliminary data from a phase I/II trial using AAVrh.10 administered directly into cerebrospinal fluid (CSF) suggested that the treatment increased β-gal activity in the CSF, and there were no adverse events recorded up to a year post dosing[44].

Systemic gene supplementation therapy has advanced to clinical stage testing in other lysosomal storage disorders such as MPSIIIA, which is caused by mutations in the gene encoding *N*-sulfoglucosamine sulfohydrolase (SGSH), leading to impaired degradation of heparan sulfate. Patients show severe neurological symptoms such as dementia, seizures, deafness, loss of vision and inability to sleep for extended periods of time. Gene delivery with self-complementary AAV9 showed benefit in preclinical studies[45]. However, in clinical studies, one trial (NCT04088734) was terminated due to lack of efficacy and another trial (NCT03315182) was terminated due to commercial considerations and lack of drug supply (reported from ClinicalTrials.gov). Without additional details from these trials being made public, it is difficult to discern whether the gene therapy approaches failed because they were suboptimal or due to other practical factors such as patient selection, outcome measures or commercial barriers.

To induce global CNS-specific gene transfer, intrathecal lumbar puncture (IT-LP) administration has been tested in multiple diseases. Giant axonal neuropathy is a disease caused by autosomal recessive, loss-of-function mutations in the gigaxonin (*GAN*) gene encoding GAN protein, leading to the development of dysfunctional 'giant' axons throughout the nervous system. AAV9/*GAN* gene supplementation therapy was the first clinical trial to use IT-LP as the delivery route[33,44], and the trial has been ongoing since 2015. Preliminary trial data, which have not been peer-reviewed, reported a relative preservation of motor function in treated patients that significantly differed from the natural history of giant axonal neuropathy as assessed by the Motor Function Measures 32 (MFM32) scale[32].

Delivery via IT-LP has since been used in SMA clinical trials and for several lysosomal storage disorders including ceroid lipofuscinosis 3 (CLN3), CLN6 and CLN7. This route of delivery is also under investigation in GM2 gangliosidosis type 1 (Tay–Sachs disease) and type 2 (Sandhoff disease), which are caused by deficiencies in the hexosaminidase (HEXA) heterodimer enzyme, encoded by *HEXA* (hexosaminidase A, α subunit) and *HEXB* (hexosaminidase A, β subunit) genes. In an ongoing clinical trial for GM2 gangliosidosis, a single AAV9 vector delivering both human *HEXA* and *HEXB* in a bicistronic design[46] was administered through a single IT-LP administration (NCT04798235). As an example of IT-LP gene delivery for complex neurological disorders, AAVrh.10/apolipoprotein E2 (APOE2) is currently under clinical investigations to treat Alzheimer disease with expression of *APOE4*,

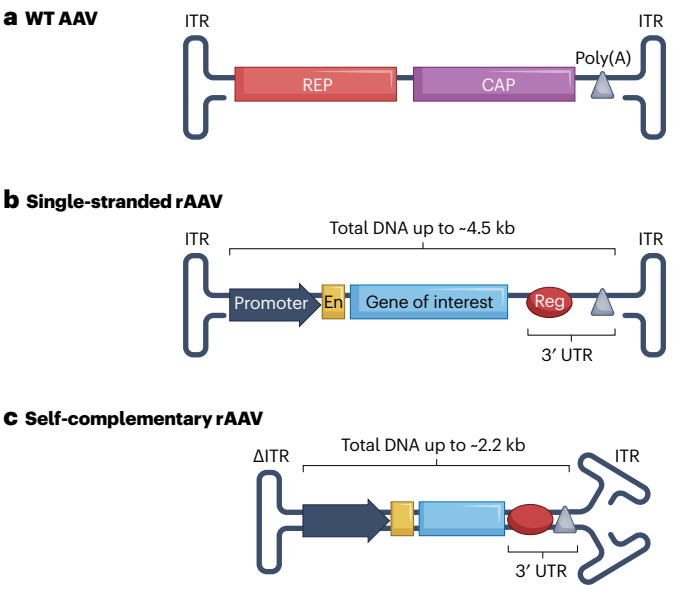

Fig. 2 | AAV vector design. a, The wild-type (WT) adeno-associated virus (AAV) genome contains the *REP* (replication) and *CAP* (capsid) genes, Poly(A) tail and inverted terminal repeats (ITRs). The *REP* gene is responsible for AAV genome replication, whereas the *CAP* gene encodes the capsid proteins, which are responsible for the viral genome packaging. The ITRs are critical for both genome replication and packaging, as they are recognized by the REP and CAP proteins. **b**, A single-stranded recombinant AAV (rAAV) in which the *REP* and *CAP* genes are replaced with a cell-specific or ubiquitous promoter, enhancer (En), gene of interest and 3′ untranslated region (UTR), while also including regulatory elements (Reg) and a Poly(A) tail. These elements drive the expression of the gene of interest in a time and site-specific manner. The foreign DNA packaging capacity is 4.5 kb. **c**, In a self-complementary rAAV cassette in which the 5′ ITR is mutated, the single-stranded genome folds over to a double-stranded, or self-complementary, structure, and thereby the foreign DNA packaging capacity is decreased to 2.2 kb. This structural change leads to higher transduction efficiency and more stable transgene expression. All elements contained in the cassette are similar to those of the single-stranded rAAV.

Table 2 | Selected ongoing AAV-based gene supplementation clinical trials for neurological disorders

Clinical trial identifier	Disease	Sponsor	AAV serotype	Transgene	Route of administration	Dose	Phase
NCT05040217	Alzheimer disease	UCSD	2	*BDNF*	IPa (entorhinal cortex)	Single dose	I
NCT03634007	Alzheimer disease	Lexeo Therapeutics	rh.10	*APOE2*	IT-LP	5E10, 1.6E11, 5E11 vg ml^{-1}	I/II
NCT04408625	FTD-GRN	Prevail Therapeutics	9	*PGRN*	ICM	3 ascending doses	I/II
NCT04747431	FTD-GRN	Passage Bio	1	*PGRN*	ICM	3.3E10, 1.1E11, 2.2E11 vg g^{-1} brain mass	I/II
NCT04167540	Parkinson disease	Brain Neurotherapy Bio	2	*GDNF*	IPa (putamen)	Single dose	I
NCT04127578	Parkinson disease	Prevail Therapeutics	2	*GBA1*	ICM	2 ascending doses	I/II
NCT05603312	Parkinson disease	MeiraGTx	2	*GAD*	IPa (subthalamic nuclei)	Single dose	I/II
NCT03562494	Parkinson disease with motor fluctuations	Neurocrine Biosciences	2	*AADC*	IPa (putamen)	Single dose of up to 3.6E12 vg	II
NCT04411654	Type 2 Gaucher disease	Prevail Therapeutics	2	*GBA1*	ICM	2 ascending doses	I/II
NCT04680065	Multiple system atrophy	Brain Neurotherapy Bio	2	*GDNF*	IPa (putamen)	Single dose	I/II
NCT05541627	Early manifest Huntington disease	AskBio	rh.10	*CYP46A1*	IPa (striatum)	2 ascending doses	I/II
NCT05606614	Rett syndrome	Taysha Gene Therapies	9	mini*MECP2*	IT-LP	2 ascending doses	I/II
NCT04737460	CLN7	UTSW	9	*MFSD8*	IT-LP	5E14, 1E15 vg	I/II
NCT03770572	CLN3	Amicus Therapeutics	9	*CLN3*	IT-LP	2 ascending doses	I/II
NCT05228145	CLN5	Neurogene	9	*CLN5*	ICV and intravitreal	2 ascending doses	I/II
NCT05089656	SMA type 1	Novartis Pharmaceuticals	9	*SMN1*	IT-LP	1.2E14 vg	III
NCT05747261	SMA type 1	Biocad	9	*SMN1*	Intravenous	3 ascending doses	I/II
NCT05614531	SMA type 1	Hangzhou Jiayin Biotech	9	*SMN1*	Intravenous	3 ascending doses	I/II
NCT04998396	Canavan disease	Aspa Therapeutics	9	*ASPA*	Intravenous	2 ascending doses	I/II
NCT04833907	Canavan disease	Myrtelle	Olig001	*ASPA*	ICV	3.7E13 vg	I/II
NCT05518188	SPG50	UTSW	9	*AP4M1*	IT-LP	Single dose	I/II
NCT04669535	Tay–Sachs disease and Sandhoff disease	Sio Gene Therapies	rh.8	*HEXA* and *HEXB*	IPa (bilateral thalamic)/ICM/ IT-LP	4 ascending doses	I
NCT03952637	GM1 gangliosidosis	NHGRI	9	*GLB1*	Intravenous	1.5E13, 4.5E13 vg kg^{-1}	I/II
NCT04798235	GM2 gangliosidosis	Queen's University	9	*HEXA* and *HEXB*	IT-LP	Single dose	I/II
NCT04519749	Fabry disease	4D Molecular Therapeutics	9	*GLA*	Intravenous	3 ascending doses	I/II
NCT04046224	Fabry disease	Sangamo Therapeutics	2/6	*GLA*	Intravenous	0.5E13, 1E13, 3E13, 5E13 vg kg^{-1}	I/II
NCT04040049; NCT04455230	Fabry disease	Freeline Therapeutics	9	*GLA*	Intravenous	2 ascending doses	I/II
NCT02852213	AADC deficiency	Krzysztof Bankiewicz, Ohio State University	2	*AADC*	IPa (substantia nigra pars compacta and the ventral tegmental area)	1.3E11 vg	I
NCT02362438	Giant axonal neuropathy	Taysha Gene Therapies	9	*GAN*	IT-LP	4 ascending doses	I
NCT04771416	Early infantile Krabbe disease	Passage Bio	hu.68	*GALC*	ICM	1.5E11, 5E11 vg g^{-1} brain mass	I/II
NCT03580083	MPSI (Hurler syndrome)	Regenxbio	9	*IDUA*	ICM	1E10, 5E10 vg g^{-1} brain mass	I/II

Table 2 (continued) | Selected ongoing AAV-based gene supplementation clinical trials for neurological disorders

Clinical trial identifier	Disease	Sponsor	AAV serotype	Transgene	Route of administration	Dose	Phase
NCT04571970	MPSII (Hunter syndrome)	Regenxbio	9	*IDS*	ICM	6.5E10 vg g^{-1} brain mass	I/II
NCT03566043	MPSII (Hunter syndrome)	Regenxbio	9	*IDS*	ICM	1.3E10, 6.5E10, 2E11 vg g^{-1} brain mass	I/II
NCT02716246	MPSIIIA	Abeona Therapeutics	9	*SGSH*	Intravenous	0.5E13, 1E13, 3E13 vg kg^{-1}	I/II
NCT05152823	IGHMBP2-related diseases	Nationwide Children's Hospital	9	*IGHMBP2*	IT-LP	Single dose	I/II

AADC, aromatic L-amino acid decarboxylase; AAV, adeno-associated virus; *APOE2*, apolipoprotein E2; *ASPA*, aspartoacylase; *BDNF*, brain-derived neurotrophic factor; CLN, ceroid lipofuscinosis; *CYP46A1*, cytochrome P450 family 46 subfamily A member 1; FTD-GRN, progranulin-related frontotemporal dementia; *GAD*, glutamic acid decarboxylase; *GALC*, galactosylceramidase; *GAN*, gigaxonin; *GBA1*, glucocerebrosidase β1; *GDNF*, glial cell line-derived neurotrophic factor; *GLA*, α-galactosidase; *GLB1*, β-galactosidase; *HEXA*, hexosaminidase A, α-subunit; *HEXB*, hexosaminidase A, β-subunit; ICM, intracisterna magna; ICV, intracerebroventricular; *IDS*, iduronate 2-sulfatase; *IDUA*, α-L-iduronidase; IPa, intraparenchymal; IT-LP, intrathoracic lumbar puncture; *MECP2*, methyl cytosine-phosphate-guanine (CpG) binding protein 2; MPS, mucopolysaccharidosis; *PGRN*, progranulin; SMA, spinal muscular atrophy; *SMN1*, survival motor neuron 1; *SGSH*, *N*-sulfoglucosamine sulfohydrolase; SPG50, spastic paraplegia 50; vg, viral genomes.

the primary genetic risk factor for sporadic Alzheimer disease. The strategy is based on the rationale that APOE2 is neuroprotective, which can offset the adverse effects of APOE4 (ref. 47).

Other intra-CSF administration routes, including intracisterna magna (ICM) and intracerebroventricular (ICV), are also being evaluated in clinical trials, including in combination with other routes. For example, a single patient with Canavan disease, a neurodegenerative disease caused by mutations in the *ASPA* gene and characterized by spongy degeneration of the white matter, was treated with a combination of intravenous and ICV administration (NCT05317780). The vector is driven by a ubiquitous chicken β-actin (CB6) promoter with a cytomegalovirus (CMV) enhancer. So far, no adverse events have been reported. By combining systemic and CNS-specific gene delivery routes, both the CNS and peripheral organs will be transduced, thereby allowing for maximal gene expression and therapeutic efficacy.

Another trial uses combinational administration in Tay–Sachs disease, a fatal neurological condition caused by lack of the HEXA enzyme and subsequent toxic build-up of gangliosides in neuronal cells. Two children were administered an equimolar combination of AAVrh8-*HEXA* and AAVrh8-*HEXB* gene therapies[48]. One child received a combination of IT-LP (25% of total dose) and ICM (75% of total dose) administration at 30 months of age, and remained seizure-free at 5 years of age while maintaining the same anticonvulsant therapy as before the gene therapy intervention. At 7 months of age, the other child received a combination of IPa (bilateral thalamic) administration and IT-LP infusion. Disease in this patient stabilized by 3 months post treatment, but resumed progression at 6 months post treatment with anticonvulsant-responsive seizure developing at 2 years old. Both patients showed increased CSF HEXA activity.

This study demonstrated the overall safety of using a combination of administration routes[48]. It is also worth noting that, in this study, the ICM infusion was done through adaptation of an intravascular microcatheter, which was safely navigated intrathecally under fluoroscopic guidance to the cisterna magna for delivery. This adaptation avoided the risk of medullary injury that can be associated with ICM administration[49].

Another trial in GM gangliosidosis evaluated the safety and efficacy of delivering the gene therapy through combining bilateral thalamic injection with dual ICM and IT-LP administration into CSF using the microcatheter approach. Preliminary data from this trial showed that treatment was associated with movement disorders in juvenile patients (who experience disease onset after age 2 years), but not in infant patients (who die within the first several years of life if untreated). Therefore, future studies have planned dose-escalation evaluations only in the infant patient population[50]. Comparing clinical data of this trial with the trial discussed above that used single IT-LP to deliver a bicistronic human *HEXA* and *HEXB* will provide valuable information for selecting vector designs and administration routes for GM2 gangliosidosis.

A study to deliver the β-glucocerebrosidase (*GBA*) gene for patients with Parkinson disease has also used the ICM route. This is a specific case where gene supplementation therapy is being used for a complex neurodegenerative disease. The rationale for this treatment is that mutations in *GBA* are the most common genetic risk factor for Parkinson disease[51], which led to the development of gene therapies for the subpopulation of patients with Parkinson disease who carry *GBA* mutations. The *GBA* gene encodes glucocerebrosidase (GCase), a lysosomal enzyme that hydrolyses glucosylceramide to glucose and ceramide. The loss of GCase function results in disturbed cholesterol metabolism, which is critical for establishing synaptic integrity[52]. As the protein of GBA is ubiquitously expressed throughout the neuroaxis, using gene therapy to broadly induce its expression in the CNS could potentially restore synaptic function and mitigate the progression of Parkinson disease[53,54].

The IPa administration route can also achieve diffuse expression through multiple sites of stereotactic injection of AAV2 vector, such as in the studies of MPSIIIA[55], MPSIIIB[56,57], Canavan disease[2], CLN2 disease[58] and Alzheimer disease[59]. According to the results from these studies, although the treatment is safe, efficacy is minimal, likely due to insufficient gene transfer across all brain regions. For example, in the trial in Alzheimer disease using AAV2 to deliver nerve growth factor (*NGF*) into the nucleus basalis of Meynert, post-mortem tissue analysis indicated that NGF did not reach cholinergic neurons due to limited distribution and inaccuracy of the stereotactic targeting, even with 15 injection sites[59].

Parkinson disease is also an example that can benefit from a localized therapeutic approach, as the primary pathological insult is well defined – the degeneration of dopamine-containing neurons in the substantia nigra[60]. Several gene supplementation therapies are in phase I or II clinical trials for Parkinson disease (Table 2). The therapeutic transgenes include *AADC*, glutamic acid decarboxylase (*GAD*), glial cell line-derived neurotrophic factor (*GDNF*) and neurturin (*NTN*). A similar approach for these Parkinson disease trials was successfully applied to AADC deficiency, which is a neurodevelopmental

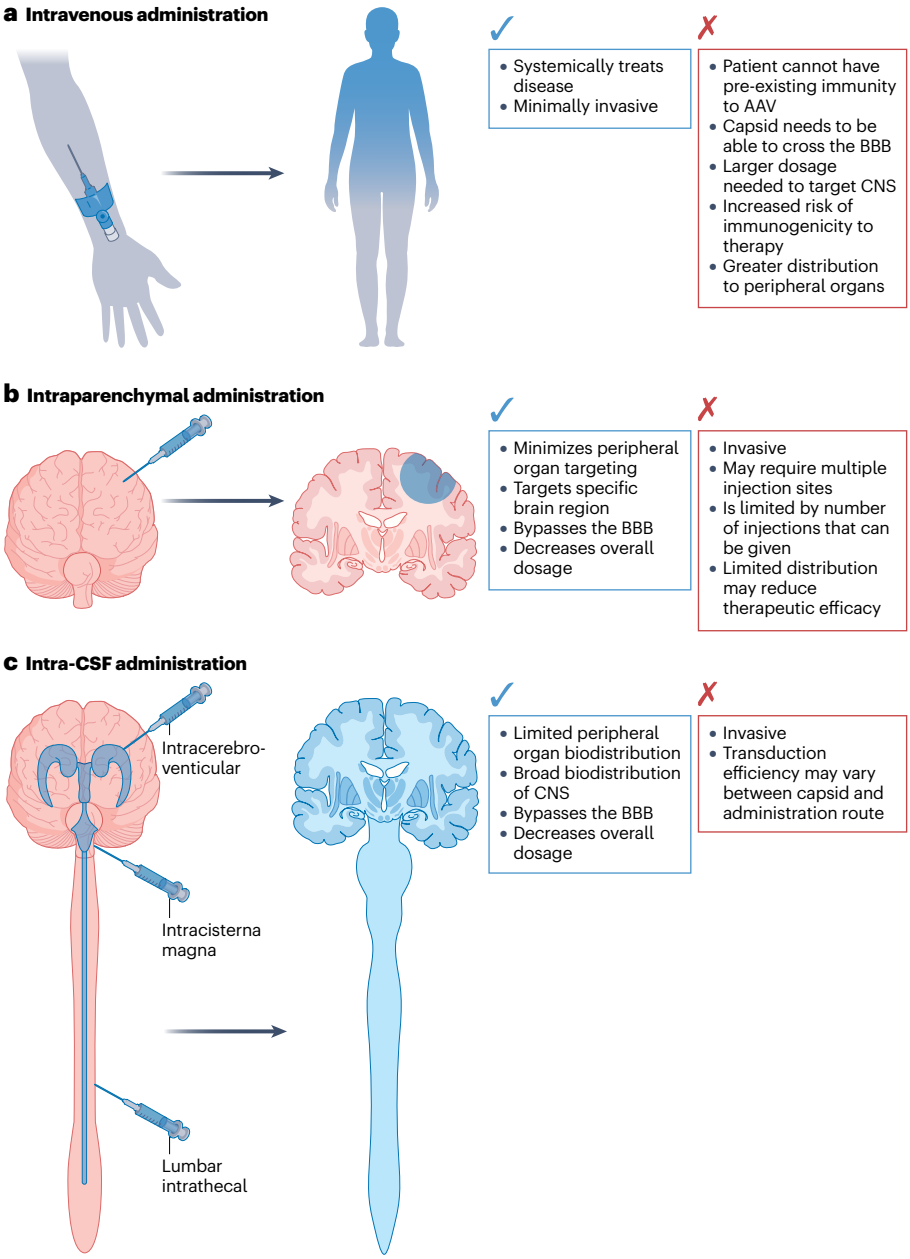

Fig. 3 | Routes of administration for brain-targeted gene therapy. Different routes of administration are associated with different distribution of the gene therapy (indicated by blue shading), and have different pros and cons. **a**, Gene therapy can be administered intravenously, which circulates the gene therapy throughout the body. **b**, Intraparenchymal (IPa) administration directly delivers the gene therapy into specific regions of the brain. **c**, Intra-cerebrospinal fluid (intra-CSF) administration delivers the gene therapy through either intracerebroventricular (ICV), intracisterna magna (ICM) or lumbar intrathecal routes. AAV, adeno-associated virus; BBB, blood–brain barrier; CNS, central nervous system.

disorder characterized by the deficiency of catecholamines and serotonin[61]. Children with AADC deficiency show various motor, autonomic and behavioural dysfunctions. Using AAV2 as the delivering vector, the gene therapy eladocagene exuparvovec (Upstaza) received market approval for the treatment of AADC in Europe in 2022. According to data from 26 patients who received bilateral injections in the putamen, improvements were seen in motor and cognitive function, as well as an increase in dopamine production within 12 months of treatment, with improvements lasting at least 5 years. Other symptoms, such as mood, seating, temperature and oculogyric crises, also improved[62].

To enhance vector distribution to target regions, vectors can be delivered into connecting regions through IPa administration and take advantage of axonal transport mechanisms[63,64]. An ongoing clinical trial using the axonal transport approach is for AADC deficiency. AAV2/AADC was administered directly to the midbrain, specifically to the substantia nigra pars compacta and the ventral tegmental area, and this was shown to be safe and effective in improving dopamine levels and motor function in children. Administering the treatment to the substantia nigra pars compacta and ventral tegmental area, instead of the putamen, can increase AADC enzyme activity in midbrain dopaminergic neurons that are involved in the pathological pathways, including nigrostriatal, mesolimbic and mesocortical pathways. Additionally, this administration takes advantage of neuronal projections from the substantia nigra pars compacta and ventral tegmental area to the striatum, through which AAV is distributed as

well[65]. To address the limitation of incomplete vector distribution, an image-guided convection-enhanced delivery technique was used to enhance the AAV biodistribution, beyond traditional stereotactic surgical methods[65–67]. With convection-enhanced delivery alone, no more than 10% of the putamen can be transduced with AAV. However, in conjunction with an imaging technique, such as magnetic resonance imaging of a tracer, larger volumes of AAV can be infused, leading to greater distribution in the desired brain region[68,69].

All these trials use AAV2 as the delivery vehicle, thereby efficiently transducing neurons, which is beneficial for Parkinson disease as it is caused by neuronal dysfunction in the basal ganglia[70]. Specifically, the treatment delivering the *AADC* transgene is meant to increase dopamine by supplying the enzyme that converts the precursor of dopamine (L-dopa; which is the active ingredient in the oral drug levodopa used to treat Parkinson disease) to dopamine. Combined oral levodopa and direct infusion of the AAV2/*AADC* therapy into the striatum was not associated with serious adverse events (based on data from NCT01973543). Further, the two high-dose gene therapy cohorts showed therapeutic benefits of reduced requirements for Parkinson disease medications, and improved motor function and quality of life[71]. AAV2/*GAD* gene supplementation, administered into the subthalamic nucleus, was intended to resolve striatal neurochemical imbalances by increasing GABAergic neuronal signalling[72]. Clinical data have shown motor benefits from the treatment over sham controls[73]. Both AAV2/*GDNF* and AAV2/*NTN* therapies were delivered directly into the basal ganglia to restore function of the degenerating nigrostriatal neurons. Treatment with AAV2/*GDNF* has recently been shown to be safe; further evaluations are needed for efficacy[74]. AAV2/*NTN* treatment led to modest symptomatic improvement, especially for patients treated at an earlier disease stage[75,76].

Considerations for translational success

To lead to a successful clinical trial of a candidate gene therapy, a well-designed preclinical study is critical. Dose, administration routes and timing are all important factors to consider for each disease[77]. For diseases that are cell autonomous, it is important to transduce as many affected cells as possible. For diseases that are non-cell-autonomous — meaning that the mutated allele-encoded protein can be secreted and affect the phenotype of neighbouring cells — the number of affected cells transduced is less crucial, as each transduced cell can subsequently secrete the functional protein to correct other affected cells. For example, GM2 gangliosidosis and CLN7 disease are both lysosomal storage diseases, but GM2 gangliosidosis is non-cell autonomous whereas CLN7 is cell autonomous. CLN7 disease is caused by dysfunction of the membrane-bound MFSD8 protein, making broad, functional distribution of the protein therapeutically challenging.

Another factor that must be considered is the type of disease that needs to be treated. Neurodegenerative diseases typically have a window wherein a treatment might slow or halt further degeneration, but also a point after which the damage may be irreversible, especially when the targeted cell population is lost through the disease process.

For neurodevelopmental diseases, all of the neural cells may be present and targetable. However, if a gene plays a crucial role in early development, it remains an open question whether gene restoration after that neurodevelopmental window will provide a substantial benefit; this will likely differ considerably from one disorder to another. Lastly, treatment durability has often been raised as a potential issue for gene therapy, as it is currently performed as a one-shot treatment. For diseases caused by postmitotic cells, this issue is, in general, less of a concern. However, for other diseases in which the pathology involves mitotic cells, such as in Krabbe disease, therapeutic efficacy can be diminished due to proliferation of other brain cell types, including oligodendrocyte progenitor cells, astrocytes and microglia, around the inflammatory sites[78].

To successfully translate a study from bench to bedside, it is critical to assess the efficacy and safety of the treatment, both of which can be affected not only by the state of the disease but also by the choice of administration route, dose and timing of treatment. A recent preclinical study on gene therapy for CLN7 disease (which has translated into a clinical trial: NCT04737460) provided a good example of how those factors impact treatment efficacy[79]. In this section, we also use other examples to discuss each of these factors, along with potential issues associated with different targets and disease types.

Administration route

Unlike traditional drug treatment, the administration route can significantly affect therapeutic efficacy and potential adverse events in gene therapy. The same vector can be tested with different routes and exert different effects. The choice of administration route largely depends on the characteristics of the disease and the transduction efficiency of the AAV capsid (Fig. 3).

For diseases that are cell autonomous and associated with dysfunction in specific brain regions, IPa administration via a stereotaxic injection to specific brain regions can be used, such as for AADC deficiency and Parkinson disease (discussed above). An advantage of IPa administration is to minimize biodistribution to the peripheral organs, which may limit the overall immunogenicity of the vectors, as well as significantly lower the vector dose required. However, the minimized biodistribution may also mean limited transduction of the target region. To address this, the current approach is to use the image-guided convection-enhanced delivery technique discussed above.

For diseases that require diffuse treatment (including non-cell autonomous and cell autonomous) of both CNS and peripheral tissues, a systemic administration route may be used, such as intravenous administration. However, this approach requires an AAV serotype that can cross the BBB efficiently, and a higher dose that may lead to toxicities, as shown in recent clinical studies[80]. Thus, compared with other routes, systemic administration is less often used as a single route of delivery for CNS indications.

To achieve broad CNS transduction, there has been a recent shift towards intra-CSF administration routes, including IT-LP, ICM and ICV. Administering directly into CSF enables bypassing of the BBB, allowing higher transgene copy number delivery to the brain and lessening off-target gene transfer to peripheral organs such as the liver. Another benefit of intra-CSF administration is that it tolerates a certain degree of pre-existing circulating anti-AAV antibodies[81,82].

A recent study compared the three intra-CSF routes, as well as the IPa route, using 29 different AAV capsids in NHPs[83]. Transduction efficiency varied based on the capsid–route combination, indicating that each administration route and capsid need to be individually evaluated prior to implementation in the clinic[83]. A recent literature review on preclinical studies using intra-CSF routes over the last decade concluded that the relative advantages and disadvantages of different intra-CSF routes of administration are still not well established, and suggested there is a need for more standardized approaches to facilitate a more reliable comparison[31]. Further, it could be necessary to understand individual cases in order to design tailored therapies for safe and maximally effective gene transfer to the brain.

Timing of treatment

The timing of therapeutic intervention can greatly impact the efficacy of gene therapies[84]. Preclinical gene therapy studies on SMA, MPSIIIA and CLN7 disease showed that earlier treatment (including pre-symptomatic) led to substantially better therapeutic efficacy[45,79,84,85]. It has also been clearly demonstrated clinically that earlier intervention leads to better rescue, as shown in the clinical trial for AADC deficiency[62] and the SMA phase I/IIa clinical trial. In the latter trial, early therapeutic intervention (1–2 months of age) in a group of infants who had high motor function led to maintenance of those functions as well as gain of other normal motor function skills such as sitting unassisted, talking and even standing independently[86]. Patients with more progressive disease (5–6 months of age) also gained improvement in motor function, but to a lesser extent[86].

From a translational standpoint, it is often difficult to precisely predict effective treatment ages in humans from studies in preclinical models such as mice, due to the drastic differences in the pace of their ageing and development. For CLN7 Batten disease, for example, mice develop a severe and lethal phenotype by 8 months of age (well into adulthood), whereas human patients typically die in early childhood. However, it may be reasonable to deduce the optimal time for treatment, in terms of intervening either pre-disease or post-disease onset, or both, from preclinical studies. Especially for early-onset and/or rapidly progressive diseases, transformative treatment benefits may often require treatment before or soon after the onset of symptoms. In the meantime, preclinical studies should be designed to suggest an intervention window in which gene therapy can mitigate disease phenotypes, with an understanding that clinical studies may be needed to fully answer the question of when the timing of the treatment can be effective. Further, the extent of disease progression at the time of intervention should be taken into consideration when interpreting the efficacy of either preclinical or clinical studies.

Assessment of potential immune responses

A primary concern of gene therapy is the triggering of a host immune response which can be elicited by the AAV capsid, transgene cassette and/or therapeutic protein. Immune responses are an underappreciated confounding variable when interpreting the safety and efficacy of gene therapy approaches, both preclinically and clinically. In either case, an otherwise 'safe' gene therapy approach could cause unacceptable toxicity due to immune responses that could potentially be mitigated if properly recognized. There have been many excellent reviews discussing different types of AAV-induced immune responses and their possible mechanisms[87,88]. In clinical trials, immunosuppression such as corticosteroid and rapamycin treatments have been widely incorporated before, during and after AAV gene therapy infusion, although the regimen still needs to be defined for individual cases, according to criteria such as the absence or presence of the neutralizing antibodies and the patient's cross-reactive immunological material (CRIM)-negative or CRIM-positive status.

Other novel approaches to evade immunity against AAV are being tested preclinically and clinically. For example, unmethylated cytosine-phosphate-guanine (CpG) depletion can be performed as a genome modification to facilitate immune evasion. Bacterial and viral genomes commonly have unmethylated CpG motifs which the innate immune system can recognize through Toll-like receptor 9 (TLR9)[89]. Depletion of CpG motifs in the vector cassette and ITRs can help maintain long-term vector expression and decrease cytotoxic T lymphocyte responses[89–91]. CpG depletion can only address a portion of the immune responses, but in concert with immunosuppression regimens that are currently used in clinical trials, it could result in more comprehensive immune evasion. Additionally, the AAV manufacturing process should be standardized and optimized to minimize the vector-associated immunotoxicities. A comprehensive summary of the pharmacological approaches to minimize immune responses against AAV can be found in a recent review[92].

Furthermore, the CNS — although considered immune privileged — also harbours efferent and afferent connections to the peripheral immune system. Immune responses in the brain are elicited not only by gene therapies administered systemically but also by therapies administered through IPa and intra-CSF routes[93]. Notably, studies have shown that certain serotypes, such as AAV9, that transduce a wide range of cell types exert stronger immune responses than serotypes that only transduce neurons, such as AAV2 (refs. 94,95). A preclinical study in NHPs treated with AAV9 via IPa or ICM administration showed that immune responses were induced due to transduction and activation of glial cells, such as astrocytes and microglia, leading to upregulated major histocompatibility complex (MHC) class II expression and T cell infiltration. Pathological analysis of injected NHPs showed extensive necrosis and vessel infiltration in the striatum[96]. Additionally, multiple studies have shown that AAV9–GFP administered through IT-LP induced immune responses, such as MHC class II expression[96] and T cell infiltrations[97] in the brain. The T cell reactivity can be partially mitigated by rapamycin treatment to dampen T cell responses[97].

Animal models

A major obstacle for conducting preclinical studies can be the availability of animal models of disease. A good animal disease model is one that mimics patient biochemical and physiological phenotypes. It not only provides indications of safety and efficacy of the gene therapy but also facilitates proper design of the gene therapy through similar mechanisms of action.

Genetically modified animal models, especially mice and rats, are often used in preclinical studies to assess the efficacy and safety of gene therapies. However, due to genetic differences between mice and humans, creating a model that mimics human disease can sometimes be challenging, especially for rare diseases, which are generally less studied. For example, this has been a challenge in the development of a gene therapy for an early-onset neurodegenerative disease called *SURF1* (Surfeit locus protein 1)-related Leigh syndrome, which has only one viable animal model available[98]. Even with complete knockout of *Surf1* in mice, disease-related phenotypes are not recapitulated, including a lack of neurological dysfunction and even longer life spans than WT mice[99]. *Surf1* knockout pigs have also been generated in the past, but their preweaning lethality (life span <3 days) limited their utility in *SURF1* AAV gene therapy development[100]. On the other hand, Krabbe disease is recapitulated in naturally occurring murine and canine models, thereby providing useful measurable read-outs for preclinical evaluations[101,102].

Due to the general differences between species, NHPs have become a favoured model for evaluating AAV immunogenicity and vector biodistribution. However, there are ethical debates surrounding the use of NHPs in scientific research, and outbred large animal models inherently show greater variability in studies than smaller inbred rodent models. Therefore, it is pertinent to develop better in vivo and in vitro disease models, such as organoids[103], as well as to expand our understandings of the differences between animal models and human disease. Through this, one would be able to translate gene therapy from the bench to the bedside more effectively.

New frontiers in CNS gene supplementation therapy

Among the diseases we have discussed, two major disease categories with CNS indications — primary mitochondrial diseases and neurodevelopmental diseases — present additional mechanistic challenges for therapeutic targeting, and such efforts thus largely remain in preclinical development.

Primary mitochondrial diseases

Primary mitochondrial diseases represent a group of rare diseases caused by heterogeneous genetic mutations in mitochondrial proteins encoded from either the nuclear genome or the mitochondrial genome (which encodes 13 proteins in total), leading to mitochondrial defects. Mitochondrial dysfunction can cause defects in one or multiple organs, and can often result in neurological manifestations[104]. The challenges for developing gene therapies for primary mitochondrial diseases include developing ideal animal models, achieving optimal expression in multiple organ systems, as many of the diseases show multisystemic symptoms, and delivering the therapeutic gene into mitochondria for those caused by mitochondrial DNA mutations.

Our group has recently published a preclinical proof-of-concept study on using AAV gene therapy for *SURF1*-related Leigh syndrome, which is a severe early-onset neurodegenerative mitochondrial disease[98]. Other studies have demonstrated that AAV gene therapy can rescue phenotypes in *Ndufs3* and *Ndufs4* knockout mouse models, which represent other common causes for Leigh syndrome[105–108].

SLC25A46-related mitochondrial disorders can lead to Charcot–Marie–Tooth type 2A neuropathy[109], Leigh syndrome[110], optic atrophy, progressive myoclonic ataxia[111] and lethal congenital pontocerebellar hypoplasia[112]. A recent study has demonstrated that early systemic treatment with AAV can mitigate the neurological phenotypes in an *Slc25a46* knockout mouse model[113]. Other mitochondrial diseases with neurological manifestations that have shown benefit from gene therapy intervention include other forms of Leigh syndrome, ethylmalonic encephalopathy and mitochondrial neurogastrointestinal encephalomyopathy[108,114–118] (see Supplementary Table 1).

Neurodevelopmental disorders

Neurodevelopmental disorders are a broad class of disorders that are starting to be evaluated for gene supplementation therapy. The therapeutic potential of this approach has been questioned for many neurodevelopmental disorders, in part because they typically manifest during early developmental stages with an unclear potential for disease reversibility. Nonetheless, the potential of gene supplementation therapy is being investigated preclinically in several neurodevelopmental disorders (see Supplementary Table 2). For example, Prader–Willi syndrome is a rare multigenic, neurodevelopmental disorder that results in developmental delays, intellectual disability, hypothalamic hypogonadism and comorbid obesity[119]. It has been shown that the expression of brain-derived neurotrophic factor (BDNF) is reduced in Prader–Willi syndrome due to it being downstream of one of the affected genes, *MAGEL2*. Recently, a study demonstrated that delivering a self-regulating AAV1–BDNF gene therapy via hypothalamic injections decreased body weight and improved cognitive and behavioural function in *Magel2*-null mice[120].

Another neurodevelopmental disorder, fragile X syndrome, is under preclinical investigation for AAV gene supplementation[121]. Studies have evaluated the administration route, the vector serotype, cell-specific and ubiquitously driven transgene expression, and overexpression of the fragile X mental retardation protein (FMRP)[121–123]. Overall, these studies have indicated that gene supplementation for fragile X syndrome has the potential to ameliorate some disease phenotypes. Other neurodevelopmental disorders that are under preclinical investigation for gene transfer include Angelman syndrome, SLC13A5 deficiency disorder, *SLC6A1*-related disorder, tuberous sclerosis and CDKL5-deficiency disorder[124–127].

In addition to the challenge of early manifestation of neurodevelopmental disorders, many genes involved are dose-sensitive and highly regulated (for a review, see ref. 125), and some undergo alternative start codons or alternative splicing to produce multiple isoforms with different functions. One potential solution to address the issue of dose-sensitivity is a self-regulated gene supplementation therapy approach that has recently been authorized for a clinical trial in Rett syndrome[128]. Rett syndrome is an X-linked neurodevelopmental disorder caused by inactivating mutations in the gene methyl-CpG binding protein 2 (*MECP2*). The expression level of *MECP2* needs to be precisely regulated as either underexpression or overexpression may lead to developmental delays. The gene supplementation therapy incorporates a panel of microRNA (miRNA)-targeting sites that can bind to specific endogenous miRNAs that are induced by overexpression of *MECP2*. With this regulated system, the therapeutic vector was able to extend the life span of treated *Mecp2* knockout mice without deleterious *MECP2* overexpression[129]. An instability-prone *Mecp2*, generated by incorporating a shorter 3′ UTR sequence, has also been tested preclinically as a gene therapy approach to address this challenge[130].

Epilepsy, characterized by intermittent episodes of pathological neuronal activity, can often coincide with neurodevelopmental disorders and is a general class of disorders with emerging gene therapy development. However, many genes involved in epilepsy are also dose-sensitive. A recent preclinical study has developed a gene therapy approach to treat brain circuit disorders, such as epilepsy, by modulating neuronal activity. The therapeutic transgene is potassium voltage-gated channel subfamily A member 1 (*KCNA1*), overexpression of which can reduce neuronal excitability and, thus, epileptic activity[131]. By using an immediate early gene promoter, such as the *cfos* promoter, which is responsive to neuronal activity, the expression of *KCNA1* is autonomously regulated at the cellular level by the activity of the transduced neurons. The study showed proof-of-concept evidence in both mouse epileptic models and human cortical spheroid models[132].

The issue of multiple protein isoforms is challenging to address, due to the limited packaging capacity of AAV vectors. An innovative design addressed this issue for Angelman syndrome[133]. Angelman syndrome is caused by loss of the maternal *UBE3A* allele, leading to lack of UBE3A protein expression. Within normal mature neurons, UBE3A protein is translated as two isoforms that differ in their extreme amino termini through alternative splicing. These two isoforms are expressed at a regulated ratio, with the shorter isoform in excess. It is critical for a gene therapy approach to mimic the endogenous expression pattern. By manipulating the Kozak sequence for selective strength of alternative start codons, two isoforms of UBE3A protein were produced from a single engineered open reading frame at a close to endogenous ratio. Angelman syndrome mice treated with this vector showed improved motor learning, and decreased seizure phenotypes[133].

A gene therapy treatment for Dravet syndrome, also a developmental and epileptic encephalopathy, has recently been authorized for a clinical trial (NCT05419492). It potentially addresses the challenges of cell type specificity and limited packaging capacity of AAV vectors. Dravet syndrome is caused by haploinsufficiency of the *SCN1A* gene,

Glossary

Capsid
The structural protein surrounding the genome of an encapsulated virus, including adeno-associated viruses (AAVs).

Convection-enhanced delivery
An experimental gene therapy delivery technique that uses a catheter to insert a thin tube into the brain and applies pressure to deliver the vector.

Cross-reactive immunological material
(CRIM). Typically refers to the presence of 'self' antigens by an individual, such that their immune system is tolerant to those antigens.

Episome
A closed circular extrachromosomal DNA molecule formed from a viral genome that serves as a transcription template.

Haploinsufficiency
When one copy of a gene is mutated, which leads to loss of function of the protein, only half the amount of functional protein is produced, and that is not enough to support normal cellular functions.

Hepatotoxicity
Liver-related adverse effects usually indicated by increased aspartate aminotransferase and alanine aminotransferase levels, sometimes accompanied by thrombocytopenia and coagulopathy.

Immediate early gene
A gene that is activated rapidly and transiently in response to a wide variety of cellular stimuli, such as neuronal activity.

Intraparenchymal
Within the functional tissue of an organ, which in this Review refers to the brain.

Kozak sequence
A nucleic acid motif for initiation of translation in vertebrates. The consensus sequence is GCCRCCAUGG, where R is a purine (A or G) and AUG is the initiation codon.

Open reading frame
A start codon followed by a portion of in-frame DNA sequence that does not include a stop codon.

Promoter
The upstream element to a gene that can control the timing and cell specificity of expression through the recruitment of transcriptional machinery.

Serotype
A virus classification based on surface antigen expression and determined by immunological responses in host serum.

Variant
Similar to serotype, a viral variant is classified according to surface antigen expression or other characteristics, but is not determined by immunological responses in host serum.

which encodes the α-subunit of the voltage-gated type I sodium channel ($Na_v1.1$). The reduction of $Na_v1.1$ through loss-of-function mutations primarily leads to dysregulated action potentials of GABAergic inhibitory neurons[134]. Thus, a GABAergic neuron-specific gene therapy would be ideal. Another challenge for a gene supplementary therapy for this disease is that the *SCN1A* coding sequence is 6,027 bp, which exceeds the packaging capacity of AAV. To address these two challenges, a gene therapy approach with a GABAergic neuron-specific expression element — comprising enhancer, promoter, 5′ UTR and intronic sequences — was engineered to drive the expression of a transcriptional factor domain to increase the expression of the normal copy of the *SCN1A* gene[135]. In preclinical evaluations, Dravet mice showed elevated *SCN1A* transcription in transduced GABAergic neurons, no elevated *SCN1A* transcription in excitatory neurons, diminished spontaneous seizures and prolonged life span[135].

Potential neurological safety concerns

The most prominent advantage of AAV over other viral vectors is its in vivo safety, as it has lower immunogenicity and minimal host genome integration. However, AAV has lower transduction efficiency, especially compared with adenovirus. This necessitates a higher therapeutic dose to achieve the desired effects, which in some cases has induced toxic events.

The most common adverse event observed is hepatotoxicity, which has been seen in multiple preclinical animal studies as well as clinical trials. Available evidence[80] suggests that high-dose AAV treatment and pre-existing liver conditions contribute to severe hepatotoxicity. The possibility of AAV integration into the genome has also been identified as a risk of gene therapy, but has only been seen in animal models[136–139]. In this section, we focus on potential neurological toxicities identified in preclinical and clinical AAV gene therapy studies (Table 3).

One complication that has been observed in AAV-treated patients is thrombotic microangiopathy (TMA), which is characterized by arteriole and capillary endothelial pathology and microvascular thrombosis. Thus, TMA may lead to ischaemia in multiple major organs including the brain. Nine out of 1,400 patients with SMA who received AAV9/*SMN* through intravenous administration developed TMA with immune complement activation 1 week post treatment[140]. A fatal case was reported 3 months post treatment[141]. Although the exact mechanism linking TMA to high-dose systemic delivery of AAV is unknown, it has been associated with complement activation, which could potentially be prevented or treated with complement inhibitors.

One challenge for preclinical studies of TMA is that acute thrombocytopenia and transaminase elevation have only been reported in NHPs[37]. Thus, in small animals, biomarker studies of acute complement activation need to be performed as an indicator of TMA for further investigation. Additionally, all the reported cases of TMA resulted from intravenous administration of high doses of AAV. Conceptually, an intra-CSF route of administration could reduce circulating levels of AAV and minimize the risk of TMA. Therefore, it could be valuable to assess TMA in patients with SMA participating in a clinical trial (NCT03381729) involving a lower overall dose of AAV administered by IT-LP.

Another adverse finding is pathologies in the dorsal root ganglion (DRG) — a collection of primary sensory neuron cell bodies located bilaterally along the spine. DRG pathology has been extensively described as primarily a histopathological finding in intra-CSF or intravenous AAV-treated NHPs, which includes minimal to moderate infiltration of mononuclear inflammatory cells, proliferating resident satellite cells and degeneration of primary sensory neurons within the cervical, thoracic and lumbar DRG[142,143]. In clinical trials, only one case of putative DRG toxicity has been reported. This was for a familial patient with amyotrophic lateral sclerosis, who received an AAV gene therapy dose of 4.2×10^{14} viral genomes (vg) through IT-LP administration. Sensory neuropathy developed in the patient and correlated with neuronal loss in the DRG that was observed at 15.6 months post treatment[144].

The observed DRG pathology in preclinical studies may be linked to overexpression of a transgene, as inclusion of DRG-specific miRNA binding sites in the expressed transgene mRNA (to specifically silence expression in DRG) prevented DRG toxicity in NHPs[145]. Supporting the hypothesis that the DRG pathology is driven by the transgene expression rather than the AAV capsid, injection of similar doses of AAV9

Table 3 | Adverse effects of CNS-targeted AAV gene therapy

Adverse event	Transgene	Species	Disease	Age	Route of administration	Dose	Duration (post dosing)	Potential cause	Refs.
Acute liver damage	Human *SMN1*	Human	SMA	<8 months	Intravenous	1.1E14 vg kg^{-1}	1 month	Immune response to AAV9	172
Subacute liver failure	Human *SMN1*	Human	SMA	6 months	Intravenous	1.1E14 vg kg^{-1}	7 weeks	Unknown	172,173
				20 months			8 weeks	Unknown	
TMA	Human *SMN1*	Human	SMA	<8 months	Intravenous	1.1E14 vg kg^{-1}	1 week	Complement activation	141
Acoustic neuroma; frontal/intracranial haemorrhage; acute ischaemic stroke	*NTN*	Human	Parkinson disease	18–65 years (20 patients); >65 years (4 patients)	IPa (subthalamic nuclei and putamen)	2.4E12 vg	5 years	Unknown	NCT00985517
Inflammation and degeneration of the DRG	Human *IDS*	Rhesus macaques	MPSII	NA	ICM	5E13 vg	90 days	Transgene mRNA/protein overexpression	174
Inflammation and degeneration of the DRG	*IDUA*	Rhesus macaques	MPSI	NA	ICM	1E13 vg	180 days	Transgene mRNA/protein overexpression	174
Cell infiltration	Arylsulfatase A	African green monkey	Metachromatic leukodystrophy	2–4 years	IPa	1.5E12 vg	13,26 and 52 weeks	Transgene mRNA/protein overexpression	147
Acute liver failure	Human *SMN1*	NHPs	SMA	14 months	Intravenous	2E14 vg kg^{-1}	4 days	DNA damage/ endoplasmic reticulum stress, constitutive transgene overexpression, innate immune response to AAV vector	37
Thrombocytopenia							5 days		
Proprioceptive deficits and ataxia		Piglets		7–30 days			14 days		
Hepatocellular carcinoma	Human *GUS*	Mouse	MPSVII and WT	Neonatal	Intravenous	1.5E11 vg per mouse	>1 year	AAV integration in the *Rian* locus	138
Hepatocellular carcinoma	*GALC*	Mouse	Krabbe disease	Neonatal	ICM and IT-LP	2.3E10 vg per mouse	>1 year	AAV integration in the *Rian* locus	175

AAV, adeno-associated virus; *ARSA*, arylsulfatase A; CNS, central nervous system; DRG, dorsal root ganglion; *GALC*, galactocerebrosidase; *GUS*, β-glucuronidase; ICM, intracisterna magna; *IDS*, iduronate 2-sulfatase; *IDUA*, α-L-iduronidase; IPa, intraparenchymal; IT-LP, intrathecal lumbar puncture; MPS, mucopolysaccharidosis; NA, not available; NHP, non-human primate; *NTN*, neurturin; SMA, spinal muscular atrophy; *SMN1*, survival motor neuron 1; TMA, thrombotic microangiopathy; vg, viral genomes; WT, wild type.

vectors containing intact or disrupted transgene expression cassettes showed DRG pathology only when the AAV9 capsids contained an intact transgene expression cassette[143]. In accordance with the notion that lower DRG transgene expression might minimize DRG pathology, preliminary safety data from a clinical trial for CLN7 demonstrated stable or improved sensory nerve function[146] (S. Gray, unpublished observations). The CLN7 example is notable, as the intrathecal doses were 5×10^{14} or 1×10^{15} vg per patient, which is the highest dose used in humans so far, and the minimal JeT promoter was used to drive expression[79]. DRG pathology may be more specific to neurological targeted gene therapies as studies have suggested that, apart from a higher dose, using intra-CSF administration routes may promote the occurrence of DRG pathology[142].

Delivering AAV vectors directly to specific brain regions via IPa administration is an efficient approach to focally target CNS cells. However, this administration route may lead to inflammation due to local transgene overexpression at the injection site and needle track, according to brain MRI findings. For example, in a gene therapy trial for late infantile Batten disease, CLN2 disease, MRI images indicated T2 hyperintensities, diffusion hyperintensity and restriction of diffusion at the site of IPa injection, indicating brain ischaemia and demyelination within 48 h post treatment. This persisted in 7 out of 13 subjects at 18 months post treatment, although MRI findings did not translate clearly to adverse clinical effects. Preclinical animal studies have also reported neuroinflammation signs from IPa administration at the injection tracks starting between 7 and 90 days post dosing[58,147].

Improving efficacy and safety

To overcome the limitations discussed above, numerous modifications have been made to the AAV vector design to increase cell specificity and transduction efficiency, and to limit host immunogenicity. These approaches encompass capsid engineering to target cell-specific transduction, inclusion of cell-specific regulatory elements to target transgene expression, gene detargeting through the use of endogenous miRNA binding sites and codon optimization in the transgene itself to improve protein translation efficiency (Fig. 4).

Capsid engineering

AAV capsids play an important role in viral biology, as they mediate viral entry and intracellular trafficking, and assist transcription and genome

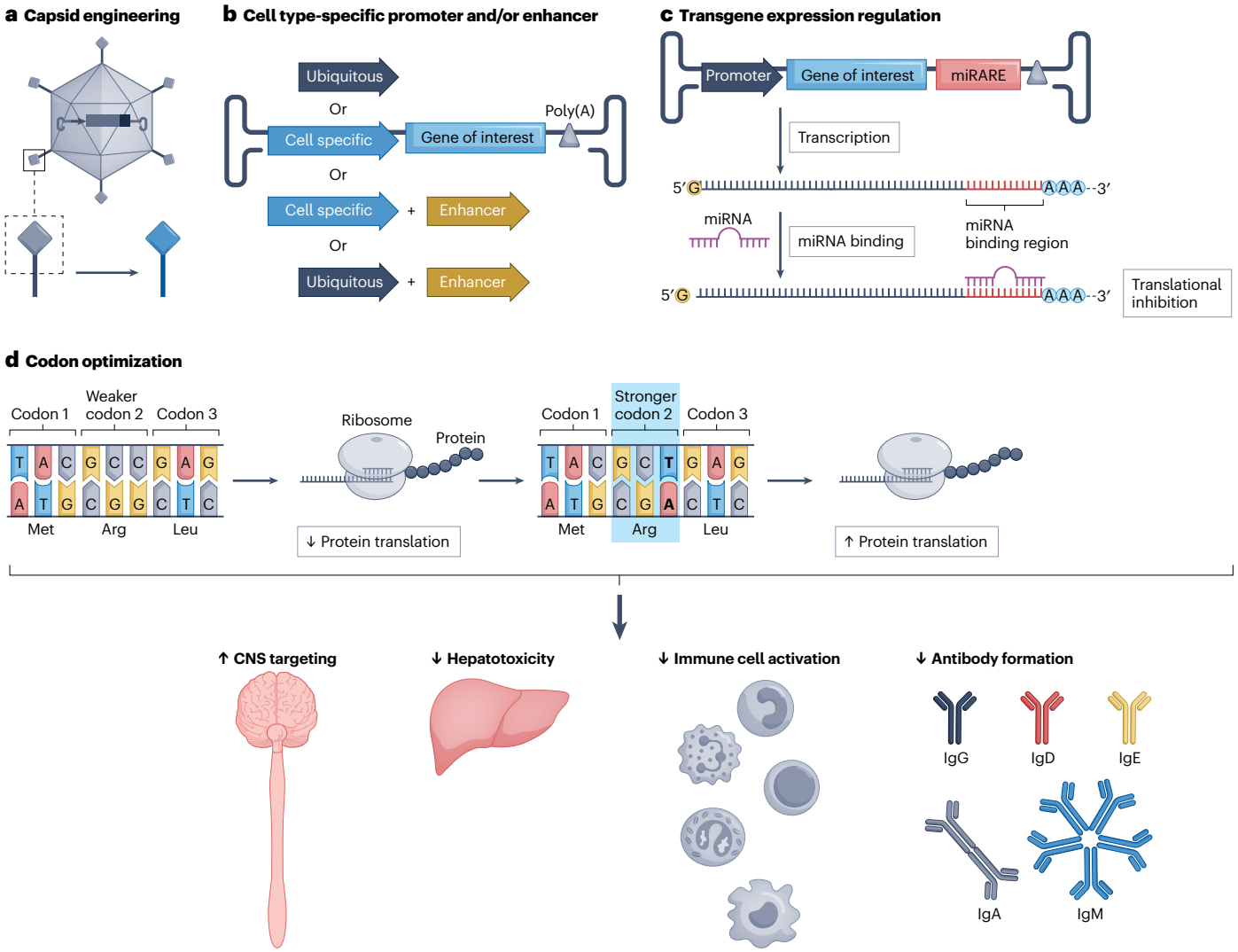

Fig. 4 | Mitigating AAV off-targeting and toxicity. a–d, Potential strategies to reduce the risk of adeno-associated virus (AAV) off-targeting and toxicity include capsid engineering (panel **a**), cassette modifications to target specific cell types (panel **b**), inclusion of regulatory elements in the cassette to detarget transgene expression in unwanted cell types (panel **c**) and codon optimization to promote protein translation (panel **d**). All of these methods increase central nervous system (CNS) targeting, decrease hepatoxicity, decrease immune cell activation and mitigate antibody formation against AAVs. miRARE, microRNA-responsive autoregulatory element; miRNA, microRNA.

synthesis[148,149]. AAV serotypes vary in capsid composition and structure, which determines cell surface receptor binding[148]. This composition can then be manipulated by introducing specific mutations to achieve greater transduction levels, decreased immunogenicity and increased cell specificity (Fig. 4a).

One example of a cell type-specific AAV capsid is AAV.Olig001. This is an engineered oligodendrocyte-specific AAV capsid, and is in clinical testing for in vivo gene supplementation therapy for Canavan disease (Table 2), a disease which primarily affects oligodendrocytes. Capsid engineering may also alter binding and transduction. Following systemic administration, CNS transduction improved when location-specific point mutations were introduced in the HSPG-binding region of AAV2, but not in AAV5 or AAV8, pointing to contextual differences specific to each capsid[150].

Cre recombination-based AAV targeted evolution (CREATE) is an approach to engineer new AAV capsids that involves generating and screening a library of AAV variants engineered to include a random seven amino acid peptide in the capsid[151,152]. Early iterations of AAV9-derived capsids discovered through this method showed significantly improved efficiency of crossing the BBB and transducing neurons in mice, but did not translate to NHPs or humans[153–155]. More recently created variants targeted the CNS more efficiently than naturally occurring AAVs while reducing targeting of the liver, and crossed the BBB of both mice and marmosets[18]. Additionally, capsid engineering created two AAV variants with peripheral nervous system preference in rodents and NHPs[20]. It is worth noting that the affinity and transduction efficiency in different cell types of a given AAV variant may vary among species. Thus, for translational purposes, any novel capsid

should be tested in both NHPs and mice[18,156–158] before determining its suitability for human translation. It should also be validated by multiple independent groups prior to broad implementation in the field. Additionally, capsids that are found to work in NHPs but not in mice set a complicated translational path to establish an effective dose, as most gene therapy pharmacology studies are typically done in genetic mouse models.

Modifying the transgene cassette

Modifications to the transgene cassette can be made using cell-specific promoters or regulatory elements, such as enhancers[159] (Fig. 4b), to ensure the construct is being expressed in the intended cell type[160].

Promoters are the most common choices for cell targeting. Cell-specific promoters are used to drive the expression of gene vector cassettes in specific cell types while concurrently limiting expression in off-target cells and/or organs. For example, when designing a gene therapy for amyotrophic lateral sclerosis, different promoters were tested within the context of the engineered CNS-enhanced AAV–PHP.B capsid, for expression of transactive response DNA-binding protein 43 kDa (TDP-43) in neurons with limited off-target transgene expression[161]. Through the use of the promoter for synapsin — a gene that is endogenously expressed in neurons — the investigators were able to enhance neuronal targeting in rats more effectively than the more traditional ubiquitous promoter, chicken β-actin with a CMV enhancer (CBA)[161]. Use of the synapsin promoter-driven construct also generated less liver expression than the CBA construct and completely avoided other off-target cell types, such as cardiomyocytes[161].

Transgene expression detargeting

Transgene expression detargeting can be performed by using RNAi to regulate transgene expression in on-target cells, as well as restrict expression in off-target areas of high transduction efficiency, such as the liver and DRG (Fig. 4c). Understanding which cell type(s) expresses a certain miRNA can enable a more targeted gene therapy approach. When endogenous miRNAs are only expressed in off-target cell types, or show inducible expression associated with transgene overexpression, the gene therapy can be designed to incorporate specific binding sites for those miRNAs, leading to silencing of the transgene in off-target cells. This has advantages due to the small size of miRNA binding sites, which are easier to fit within the limited space of a rAAV genome.

One example is miRNA detargeting in the context of AAV9 to minimize expression in peripheral tissues when the CNS-directed therapy was systemically delivered. By using miRNA-122 and miRNA-1 target binding sites in the 3′ UTR of the rAAV9 β-gal vector, the expression in skeletal muscle, heart and liver was decreased[162]. miRNA-mediated transgene regulation has also been used to avoid transgene overexpression in a gene therapy approach for Rett syndrome (discussed above)[128,129].

Codon optimization

The transgene cassette can also be modified to alter transgene expression, through techniques such as codon optimization in the transcript itself. For codon optimization (Fig. 4d), inefficient codons are replaced with more efficient and better translated codons to increase protein expression, and this process can also remove cryptic splice sites or alternative start codons in the transgene open reading frame[163,164]. Codon optimization assumes that uncommon codons lead to decreased protein expression due to inefficiency in translation, that all synonymous codons can be interchanged without any adverse effects on the protein and that replacing inefficient codons with more frequently used codons will lead to higher protein expression[165]. Although there is some debate on whether changing codons affects protein structure due to the rate of translation[166–169], gene therapy programmes have implemented this strategy — such as in the vector development for SMA[170] and for Krabbe disease[171].

Summary and future prospects

The development of gene therapy for neurological indications is continuously evolving. Several avenues are being actively pursued to achieve the ultimate goal of developing safe and effective gene therapies. Firstly, to achieve a better gene transfer technology, an improved AAV capsid would be enormously beneficial for more specific cell targeting capabilities and/or to increase transduction efficiency across the BBB, which could, in turn, increase therapeutic efficacy while reducing off-target toxicity. Secondly, with the development of CRISPR and other gene editing technologies, personalized and individualized medicines are on the horizon, even with the necessary progress that needs to be made to increase editing efficiency and mitigate off-target effects. Thirdly, approaches to regulate transgene expression, such as using endogenous miRNA targeting sites and cell-specific gene expression regulatory elements, should be explored in cases where transgene expression levels need to be tightly controlled. Finally, more studies are needed to explore how immune responses against the vector and transgene can be managed, which could potentially improve the therapeutic efficacy or provide the possibility of gene therapy redosing.

Additionally, as an approach to treat the disease aetiology, early intervention is critical[84]. For neurodevelopmental diseases, later treatment may miss the critical therapeutic window to keep the patient on the appropriate developmental trajectory. For neurodegenerative diseases, the disease may have progressed to a point with limited possibilities of intervention. Further, whether gene therapy can reverse disease or prevent progression will depend on the indication and the condition of the patient, and on the gene target itself. Thus, similar to many other therapies, early diagnosis will be crucial for attaining a better outcome. Recent developments and investments in newborn screening will facilitate achieving this goal.

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Author contributions

All authors researched data for the article. S.J.G. and Q.L. contributed substantially to discussion of the content. Q.L. and J.H. wrote the article. All authors reviewed and/or edited the manuscript before submission.

Competing interests

S.J.G. has received royalty income from inventions discussed in the article, through licensing agreements with Neurogene, Asklepios Biopharmaceuticals, Taysha Gene Therapies and Abeona Therapeutics. A.B. has received royalty income from inventions discussed in the article from Axovant Gene Therapies and Neurogene. Q.L. has received royalty income from inventions discussed in the article from Taysha Gene Therapies.

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