Gene therapy for neurological disorders: progress and prospects

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Abstract | Adeno-associated viral (AAV) vectors are a rapidly emerging gene therapy platform for the treatment of neurological diseases. In preclinical studies, transgenes encoding therapeutic proteins, microRNAs, antibodies or gene-editing machinery have been successfully delivered to the central nervous system with natural or engineered viral capsids via various routes of administration. Importantly, initial clinical studies have demonstrated encouraging safety and efficacy in diseases such as Parkinson disease and spinal muscular atrophy, as well as durability of transgene expression. Here, we discuss key considerations and challenges in the future design and development of therapeutic AAV vectors, highlighting the most promising targets and recent clinical advances.

Capsid
The protein shell of the virus that protects the adeno-associated viral genome and mediates entry into and trafficking within the host cell.

Intraparenchymal
(IPa). Direct delivery of an agent into the tissue of interest.

Intrathecal
(IT). A route of access into the spinal cord cerebrospinal fluid via the space under the arachnoid membrane.

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In the past several years, there has been much progress towards realizing the potential of AAV vectors for therapeutic gene delivery for neurological disorders. Several key advances have converged. Mutations for many, if not most, monogenic neurological disorders have now been identified and, together with an ever-increasing understanding of their mechanisms of disease pathogenesis, have revealed novel therapeutic targets. Although many of these genetic targets are not readily 'druggable' with conventional drug modalities, they are potentially druggable with adeno-associated viral (AAV) gene therapy, which enables both gene replacement to address loss-of-function mutations and gene silencing to address gain-of-function mutations (for example, replacing survival motor neuron protein (*SMN*) for spinal muscular atrophy (SMA)[1–3] or silencing superoxide dismutase 1 (*SOD1*) for amyotrophic lateral sclerosis (ALS)[4–6] or huntingtin (*HTT*) for Huntington disease[7–9]) (TABLE 1).

Transgenes that encode therapeutic proteins[2,3,10–21], microRNAs (miRNAs)[4–9], antibodies[22,23] and Cas9 – guide RNA[24,25] for gene editing have been successfully delivered to the central nervous system (CNS) with AAVs in mice and other species. In addition, multiple capsids have been used across species to successfully target a variety of tissues and cell types within the CNS, including neurons, astrocytes and oligodendrocytes[26–38].

Important advances in optimizing delivery of AAVs into the CNS have been made. In preclinical studies, various routes of AAV delivery, including intraparenchymal (IPa)[39], intrathecal (IT)[5,15,40], intracerebroventricular (ICV)[16], subpial[41] and intravenous (IV) administration[3,20,42,43], have been shown to achieve therapeutic levels of gene expression in the CNS. Intramuscular (IM) administration has been demonstrated to provide a depot for monoclonal antibody production and delivery[44,45], with the theoretical potential to provide a source of antibodies that can cross the blood–brain barrier (BBB) in sufficient quantities, albeit to a limited extent (see below), for clinical benefit. Notably, in 2016, novel, engineered AAV capsids were reported to have unprecedented ability to transfer genes to the CNS in the adult mouse after systemic administration[46], with a >40-fold enhancement over the previous standard AAV9, potentially transforming our ability to treat neurodegenerative diseases with AAV gene therapy.

A 'one-and-done' therapeutic approach that provides durable pharmacology and efficacy is attractive, particularly with neurological disorders that require more complex CNS dosing strategies, and is a key feature of AAV gene therapy. Importantly, early gene therapy clinical trials with IPa CNS administration may not have provided sufficient biodistribution to the intended brain targets, but improvements in AAV delivery have enabled more widespread therapeutic transgene expression. Recent clinical trials have demonstrated the relative safety of AAV gene therapy in >200 patients with various CNS diseases, including IPa, IT and IV administration (TABLE 2). Importantly, evidence of clinical efficacy in several CNS disorders, including Parkinson disease (PD)[47], aromatic-L-amino-acid decarboxylase (AADC) deficiency[48] and type I SMA, has recently been reported. Durable transgene expression in neurons of the putamen has also been demonstrated, with >4 years

Table 1 | **Examples of promising molecular targets for AAV gene therapy**

Anatomy	Disease type	Representative disease(s)	Target gene (protein)	Target validation	Mechanism	Refs
Cortical/global brain	Neurodegenerative	Alzheimer disease	*APOE* ε4	GRF	Silence/immunotherapy	230,231
			APOE ε2	GRF	Replace	189,232
			APP (amyloid-β)	• SG • P	Silence/immunotherapy	233,234
			MAPT (tau)	P	Silence/immunotherapy	22,127
		Frontotemporal dementia	*MAPT* (tau)	SG	Silence/immunotherapy	22
			GRN	SG	Replace	235
			C9orf72	SG	Silence	236
		Huntington disease	*HTT*	SG	Silence	7,8,204,237
	Developmental	Rett syndrome	*MECP2*	SG	Replace	17
Subcortical/ focal	Neurodegenerative	Parkinson disease	*AADC*	Ph	Replace	47,49,120,238
			GBA1 (also known as *GBA*; glucosylceramidase	GRF	Replace	43,239
			SNCA (α-synuclein)	• SG • P	Silence/immunotherapy	240–243
	Neurotransmitter deficiency	AADC deficiency	*AADC*	SG	Replace	48
Metabolic with diffuse CNS involvement	Lysosomal storage	Canavan disease	*ASPA*	SG	Replace	118,119
		Late infantile neuronal ceroid lipofuscinosis	*CLN2* (also known as *TPP1*)	SG	Replace	31
		Gangliosidosis 1	*GLB1*	SG	Replace	14
		Mucopolysaccharidosis IIIA and IIIB	*SGSH* and *NAGLU*	SG	Replace	147
		Mucopolysaccharidosis IIA	*IDS*	SG	Replace	126
		Niemann–Pick C1	*NPC1*	SG	Replace	–
Cerebellum	Neurodegenerative	Spinocerebellar ataxia 1	*SCA1* (also known as *ATXN1*)	SG	Silence	145
		Spinocerebellar ataxia 3	*SCA3* (also known as *ATXN3*)	SG	Silence	146
		Spinocerebellar ataxia 7	*SCA7* (also known as *ATXN7*)	SG	Silence	244
Spinal cord/ anterior horn	Neurodegenerative	Type I, II and III spinal muscular atrophy	*SMN1*	SG	Replace	1
		Amyotrophic lateral sclerosis	*SOD1*	SG	Silence/immunotherapy	4–6,245–247
			C9orf72	SG	Silence	236
			TARDBP (also known as *TDP-43*)	SG	Silence/immunotherapy	248
Dorsal root ganglia/ sensory pathways	Sensory ataxias	Friedreich ataxia	*FRDA* (also known as *FXN*)	SG	Replace	21,115
	Neuropathic pain	Post-herpetic or trigeminal neuralgia	*SCN9A* ($Na_v1.7$) and *SCN10A* (also known as $Na_v1.8$)	SG Ph	Silence	249
Peripheral nerves	Motor and sensory	Giant axonal neuropathy	*GAN*	SG	Replace	15

AADC, aromatic-L-amino-acid decarboxylase; AAV, adeno-associated viral; CNS, central nervous system; GRF, genetic risk factor; SG, single gene; P, pathology; Ph, pharmacology.

of sustained expression of AADC in patients with PD[49] and >15 years of sustained expression in nonhuman primates (NHPs)[50], supporting the one-and-done treatment concept. Finally, recent progress in chemistry, manufacturing and controls (CMC) using both mammalian (HEK293) and insect (Sf9) cells has enabled the production of AAV gene therapies at commercial quality and scale[51,52]. Together, these advances have resulted in a resurgence of interest in gene therapy for treating neurological disorders affecting the CNS.

In this Review, we focus on AAV gene therapy for CNS disorders, which to date represents the vast majority of CNS gene therapy programmes, although other viral vector platforms have been tested clinically (for example, lentivirus)[53,54]. We discuss recent progress in gene therapy for treating neurological disorders, focusing on the key features of AAV gene therapy vectors that need to be optimized for a successful therapy, including capsid selection, vector genome design and selection, and the delivery and dosing paradigm, using examples

Intracerebroventricular (ICV). A route of access into the CSF via the cerebral ventricles (typically the lateral ventricle).

Table 2 | **Ongoing AAV gene therapy clinical trials for neurological disorders**

Disease	Sponsor/drug name	Serotype	Transgene	Route of administration	Dose	Phase	Identifier
Parkinson disease	Voyager Therapeutics/ VY-AADC01	AAV2	*AADC*	Intraputaminal	• 7.5×10^{11} vg • 1.5×10^{12} vg • 4.5×10^{12} vg	I	NCT01973543
Parkinson disease	Voyager Therapeutics/ VY-AADC01	AAV2	*AADC*	Intraputaminal	9.4×10^{12} vg	I	NCT03065192
Parkinson disease	Jichi Medical University/ AAV-hAADC-2	AAV2	*AADC*	Intraputaminal	• 3×10^{11} vg • 9×10^{11} vg	I/II	NCT02418598
Parkinson disease	UCSF and NINDS/AAV2-GDNF	AAV2	*GDNF*	Intraputaminal	• 3×10^{11} vg • 9×10^{11} vg • 4×10^{12} vg	I	NCT01621581
AADC deficiency	National Taiwan University Hospital/AAV2-hAADC	AAV2	*AADC*	Intraputaminal	2.371×10^{11} vg	II	NCT02926066
AADC deficiency	National Taiwan University Hospital/AAV2-hAADC	AAV2	*AADC*	Intraputaminal	1.8×10^{11} vg	I/II	NCT01395641
AADC deficiency	Krystof Bankiewicz UCSF/ AAV2-hAADC	AAV2	*AADC*	Within the substantia nigra pars compacta and the ventral tegmental area	1.3×10^{11} vg	I	NCT02852213
SMA	AveXis/AVXS-101	AAV9	*SMN*	Intravenous	1.1×10^{14} vg/kg	III	NCT03505099
Type I SMA	AveXis/AVXS-101	AAV9	*SMN*	Intravenous	Undisclosed	III	NCT03306277
Type I SMA	AveXis/AVXS-101	AAV9	*SMN*	Intravenous	Undisclosed	III	NCT03461289
Type II SMA	AveXis/AVXS-101	AAV9	*SMN*	Intrathecal	• 6.0×10^{13} vg • 1.2×10^{14} vg	I	NCT03381729
MPS IIIA	Abeona/scAAV9.U1a.hSGSH	AAV9	*SGSH*	Intravenous	• 5×10^{12} vg/kg • 1×10^{13} vg/kg • 3×10^{13} vg/kg	I/II	NCT02716246
MPS IIIB	Abeona/rAAV9.CMV.hNAGLU	AAV9	*NAGLU*	Intravenous	• 2×10^{13} vg/kg • 5×10^{13} vg/kg	I/II	NCT03315182
MPS II	Sangamo/SB-913	AAV6	*IDS*	Intravenous	• 5×10^{12} vg/kg • 1×10^{13} vg/kg • 5×10^{13} vg/kg	I	NCT03041324
MPS I	Sangamo/SB-318	AAV6	*IDUA*	Intravenous	• 5×10^{12} vg/kg • 1×10^{13} vg/kg • 5×10^{13} vg/kg	I	NCT02702115
Batten disease	Weill Cornell/AAVrh.10CUhCLN2	AAVrh10	*CLN2* (also known as *TPP1*)	Intracranial	• 9×10^{11} vg • 2.85×10^{11} vg	I	NCT01161576
Batten disease	Weill Cornell/AAVrh.10CUCLN2	AAVrh10	*CLN2*	Intracranial	• 9×10^{11} vg • 2.85×10^{11} vg	I/II	NCT01414985
Batten disease	Nationwide Children's Hospital/ scAVV9.CB.CLN6	AAV9	*CLN6*	Intrathecal	1.5×10^{13} vg	I/II	NCT02725580
Giant axonal neuropathy	NINDS/scAAV9.JeT-GAN	AAV9	*GAN*	Intrathecal	3.5×10^{13} vg	I	NCT02362438
Metachromatic leukodystrophy	INSERM/AAVrh.10cuARSA	AAVrh10	*ARSA*	Intracranial	• 1×10^{12} vg • 4×10^{12} vg	I/II	NCT01801709

AADC, aromatic-L-amino-acid decarboxylase; AAV, adeno-associated viral; MPS, mucopolysaccharidosis; NINDS, National Institute of Neurological Disorders and Stroke; UCSF, University of California–San Francisco.

from preclinical and clinical studies. Manufacturing challenges and advances are discussed, particularly in the context of the different routes of administration and associated drug requirements. Finally, the most promising CNS drug targets for AAV gene therapy are described, along with recent clinical experience with several of these targets and diseases.

AAV capsid design and selection

The AAV capsid is the determinant of several key features of an effective or successful AAV gene therapy, including tissue tropism and distribution as well as susceptibility to interference by circulating neutralizing antibodies. Both natural and engineered capsids have been explored in efforts to identify novel AAV vectors with properties

Tropism
Specificity for a particular host tissue or cell type.

better suited for the treatment of a given neurological disorder. The natural diversity of AAVs isolated from a broad range of species has provided researchers with a large collection of AAV capsids with unique, but largely overlapping, characteristics. As discussed below, beyond the repertoire provided by nature, engineered capsids are expanding the arsenal of vectors for gene delivery to the CNS. Although no laboratory-modified AAV vectors have yet entered clinical trials targeting the brain or spinal cord, one modified AAV2 variant[55] is currently undergoing trials for several retinal diseases (NCT02935517, NCT03316560, NCT02599922 and NCT02416622), and a second laboratory-derived variant[56] is being used in a haemophilia trial (NCT03307980). Undoubtedly, more will follow as an increasing number of AAV capsids are customized for improved efficacy and safety in preclinical models and humans.

When delivered to the CNS by IPa injection, AAV vectors provide durable transduction of neurons, leading to their use in several clinical trials and hundreds of basic and applied neuroscience studies. However, different serotypes vary in terms of their efficiency of transduction of specific neuron populations, the extent of glial transduction, the degree of spread from the injection site and their ability to transduce various cell types through alternative cerebrospinal fluid (CSF) or intravascular routes of administration. Below, we provide an overview of the CNS distribution and tropism characteristics that exist within the naturally occurring capsids and highlight several recent efforts in which capsid engineering has been used to overcome barriers that stand in the way of applying AAV vectors to a wider range of CNS diseases, including those that would benefit from broader gene delivery throughout the CNS.

Naturally occurring capsids

The first AAV serotypes (AAV1–AAV4) were identified in the 1960s from adenovirus stocks[57–59]. Following the cloning of the AAV2 genome into a bacterial plasmid, the AAV2 serotype became the initial focus of AAV vector biology research. However, each of these serotypes was found to have unique transduction profiles and receptor binding properties[60,61], spurring the search for novel AAV serotypes with different biological properties. More than 100 natural AAV variants comprising at least 8 serotypes have been identified from humans, NHPs and other vertebrates[62–65]. For neurological applications, AAV2, which provides long-term expression in CNS neurons although with relatively restricted biodistribution, has been the most well-studied serotype[29]. Other serotypes (such as AAV1, AAV5, AAV9, AAVrh.10 and AAV-DJ8) that spread more broadly in the CNS and transduce cells with higher efficiency than AAV2 have been evaluated following IPa[26,27,30,32,34,36,66], IT[33,67,68], intracisternal[68–71], ICV[11,12,16,70] and intravascular dosing[13,33,67,72–76].

The extent of biodistribution after direct CNS administration is influenced by multiple factors that are governed by the capsid itself, including receptor interactions[77] and anterograde and retrograde axonal transport[78]. In general, the major challenge with translation of AAV delivery for application to CNS disorders is biodistribution and homogeneity of cellular transduction, particularly in adults. To address this delivery challenge and enable application to neurological disorders widely affecting the brain, capsid engineering will be critical.

Engineered capsids for enhanced CNS transduction

Adeno-associated viruses are dependoviruses that co-evolved with viruses such as adenoviruses or herpes simplex viruses (HSVs), which do not typically infect the CNS. Therefore, although adeno-associated viruses have a natural propensity for latency in the absence of a helper virus co-infection (a useful property for durable gene expression in the CNS), it is unlikely that adeno-associated viruses were subjected to selection pressures that would lead to their optimization as delivery vehicles for CNS gene transfer. Fortunately, laboratory-driven rational design, high-throughput screening and directed evolution strategies can be used to customize the properties of the AAV capsid for any of a number of routes of administration, which should open new options for treating neurological diseases.

Rational design and mutational analysis. Understanding structure–function relationships (for example, identification of key residues that affect capsid stability, receptor binding or trafficking) is critical for making targeted improvements to capsid function. Several AAV–receptor interactions have been mapped onto the AAV surface[79–81] (see BOX 1), and mutations that disrupt these interactions typically reduce transduction efficiency[82–84]. Interestingly, not all such mutations are deleterious, as a low-affinity *O*-linked α2,3-sialic-acid-binding mutant of AAV4 (AAV4.18) displays a new and unique tropism for subventricular zone neural stem cells when administered by ICV injection into neonatal mice[85]. In a tour de force alanine-scanning mutational analysis, Adachi et al.[86] used barcoding-based biodistribution assays to study the contribution of specific AAV9 amino acids to numerous capsid characteristics. Leveraging these data and prior studies, the authors mapped the galactose-binding site of AAV9 and grafted ten key amino acids into a heparin-binding-deficient AAV2 mutant, resulting in an AAV2-based capsid that shares several tropism characteristics with AAV9, including enhanced liver, heart and Lec2 cell transduction[86]. In a recent intriguing finding that may help shed light on the mechanism of BBB crossing used by the rh.10 AAV serotype, Albright et al.[87] identified a short linear region of the rh.10 capsid that is sufficient, when grafted into the AAV1 capsid, to convert AAV1 into a more BBB-penetrant variant. Whether the homologous region of AAV9 also shares this activity and whether the same region of rh.10 can enhance the BBB crossing efficiency of other AAV serotypes remains unreported. With the advancing knowledge of capsid structure–function relationships, rational design holds substantial promise for achieving improved biological profiles for therapeutic application. Nevertheless, designing capsids that more efficiently target specific cell types has proved challenging; for this purpose, directed evolution holds considerable promise.

Serotypes
Capsid variants or groups of capsids that have distinct neutralization properties.

Intracisternal
A route of access into the CSF via the cerebellomedullary cistern.

Dependoviruses
Genus of parvoviruses that are replication-incompetent in the absence of co-infection of the host cell with a second virus such as an adenovirus or HSV.

Box 1 | **The AAV capsid and its interactions**

The adeno-associated viral (AAV) capsid is a 25 nm icosahedral protein shell assembled from three proteins, VP1, VP2 and VP3, in an approximate 1:1:10 ratio. The VP proteins are encoded by a single open reading frame within the AAV cap gene through alternative splicing and codon usage. The AAV capsid protects the AAV genome, and its amino acid sequence and resulting structural features determine its tropism, spread and antigenicity through interactions with cellular receptors, glycans and blood proteins, including antibodies.

The core of the AAV capsid forms a β-barrel structure that is highly conserved across AAVs, but the surface of the capsid harbours more diversity. As primary receptors, AAV capsids utilize a range of proteoglycans, including heparin sulfate (AAV2, AAV3, AAV3b and AAV6), α2,3 and α2,6 *N*-linked sialic acid (SIA) (AAV1), α2,3 *O*-linked SIA (AAV4), α2,3 *N*-linked SIA (AAV5) and heparin sulfate and α2,3 and α2,6 SIA (AAV6) or terminal N-linked galactose (AAV9) [77]. Secondary receptors have also been identified for several AAVs (for example, fibroblast growth factor receptor (FGFR) and possibly αvβ5 integrin for AAV2 (REF.[210]); hepatocyte growth factor receptor for AAV3 (REF.[211]); platelet-derived growth factor receptor (PDGFR) for AAV5 (REF.[212]); and laminin receptor for AAV2, AAV3, AAV8, and AAV9 (REF.[213])). These receptor interactions influence the biodistribution and tropism of the AAV capsid. In addition, a possible AAV receptor (AAVR) has been identified recently as a necessary cofactor for transduction by most, but not all, AAVs vectors [214]. Interestingly, AAV2 and AAV5 interact with distinct AAVR ectodomains whereas AAV1 and AAV8 interact with multiple domains[215]. Whether the AAV–AAVR interactions are important for binding or post-attachment trafficking is still an area of active investigation[214].

Safely transporting the recombinant genome through the extracellular milieu and making contact with cell surface receptors is not the capsid's only task; it must also traffic intracellularly through endosomes and deliver the packaged genome to the host cell nucleus, feats it accomplishes through an acidic pH-dependent exposure of a buried amino-terminal phospholipase A2 domain and multiple nuclear trafficking signals[216–218]. Surprisingly, the AAV capsid may also have post-genome release effects on transcription. Several transduction-defective AAV capsid mutations have been identified that block mRNA expression from the recombinant genome without altering the efficiency of DNA uncoating in the nucleus[219,220]. The continued study of AAV vector biology is critical for informing AAV capsid engineering efforts aimed at optimizing the AAV capsid for specific gene therapy applications.

Directed evolution. Directed evolution has been the most successful approach to date for developing AAV capsids with improved biological characteristics. The power of this approach stems from it being high throughput, as millions of sequences can be tested against each other with the most potent sequences being enriched over iterative selections, and the lack of a need for prior knowledge of an enhancement mechanism. Applications include enhancing transduction efficiency, increasing resistance to pre-existing neutralizing antibodies, reducing immunogenicity, achieving specificity for particular tissues or cell types and improving axonal transport or spread.

The first step in applying directed evolution is to generate highly diverse capsid libraries (FIG. 1). This generation has been accomplished through homology-based recombination and shuffling[56,88–92], peptide insertion[46,93–96] or error-prone PCR[97,98]. Key to the success of this approach is that each capsid carries its own specific instruction manual (that is, the cap gene DNA sequence). Selective pressure is then applied in vivo or in vitro, and iterative rounds of selection enrich for the most potent variants. Sequences that pass the selection process have been recovered via one of several approaches, including PCR[46,96], adenovirus-based AAV genome replication[56,87] or fluorescence-activated cell sorting followed by PCR[95] (FIG. 1).

For CNS applications, directed evolution or high-throughput selection has resulted in the identification of AAV capsids with enhanced transduction of the CNS following IPa administration (AAV-DJ)[88], increased transduction of neural stem and progenitor cells (SCH9 and AAV4.18)[85,99], enhanced retrograde transduction (rAAV2-retro)[94], selective transduction of brain endothelia (AAV-BR1)[96] and greatly increased transduction of the adult CNS after IV administration (AAV-PHP.B and AAV-PHP.eB)[46,100]. In particular, in 2016, Deverman et al.[46] reported several AAV capsids that efficiently transduced the adult mammalian CNS via the vasculature. The most notable variant, AAV-PHP.B, delivered >40-fold more AAV genomes to the CNS than AAV9 and transduced >50% of all neuron and astrocyte populations sampled after IV dosing of adult C57BL/6 mice. The CNS tropism of AAV-PHP.B has been replicated in other mouse studies[43,101,102] and extended to the rat[103], but not the marmoset[104]. A recent study reported the mouse strain-dependence of AAV-PHP.B as well as the toxicity of AAV-PHP.B.GFP in a rhesus monkey[105]. However, the contribution of the expression of a non-self-protein such as green fluorescent protein (GFP) to the observed toxicity remains to be determined, especially because GFP expression is well known to cause severe toxicity in NHPs[106]. Notably, although the toxicity was observed within a short time (by day 5) following AAV-PHP.B.GFP administration, GFP expression in the liver was clearly evident at that time. In a recently reported study in cynomolgus monkeys, AAV-PHP.B and a related variant expressing a self-protein were well tolerated and, relative to AAV9, showed enhanced transduction of several brain and spinal cord regions[107,108]. More recently, the same group that discovered AAV-PHP.B has reported an enhanced AAV-PHP.B variant (AAV-PHP.eB) that shows further improvements in neuronal transduction[100]. These AAV capsids were identified using a novel Cre-based, cell-type-specific selection method (CREATE). CREATE enables the selective recovery of sequences that transduce a specific cell population of interest within the CNS, thereby ensuring that the capsids recovered crossed the adult mouse BBB and, within the cell type of interest, mediate conversion of packaged genomes into double-stranded DNA, a late rate-limiting step required for AAV transduction[109].

To date, only one laboratory using a mouse model of mucopolysaccharidosis type VII (MPS VII) has reported disease-specific changes to the brain that blocked gene delivery by AAV9 but not an AAV engineered for CNS transduction in this model[110]. Nonetheless, disease-specific tropism changes certainly warrant close attention to ensure successful translation to the clinic.

Tissue and cell-type selectivity

The AAV capsid and the promoter within the vector genome (below) play major roles in determining cell tropism and expression. AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9 and rh.10 capsids have been studied by multiple laboratories for transduction and tropism in the CNS in several preclinical species. Similar to AAV2, capsids from most of these serotypes impart

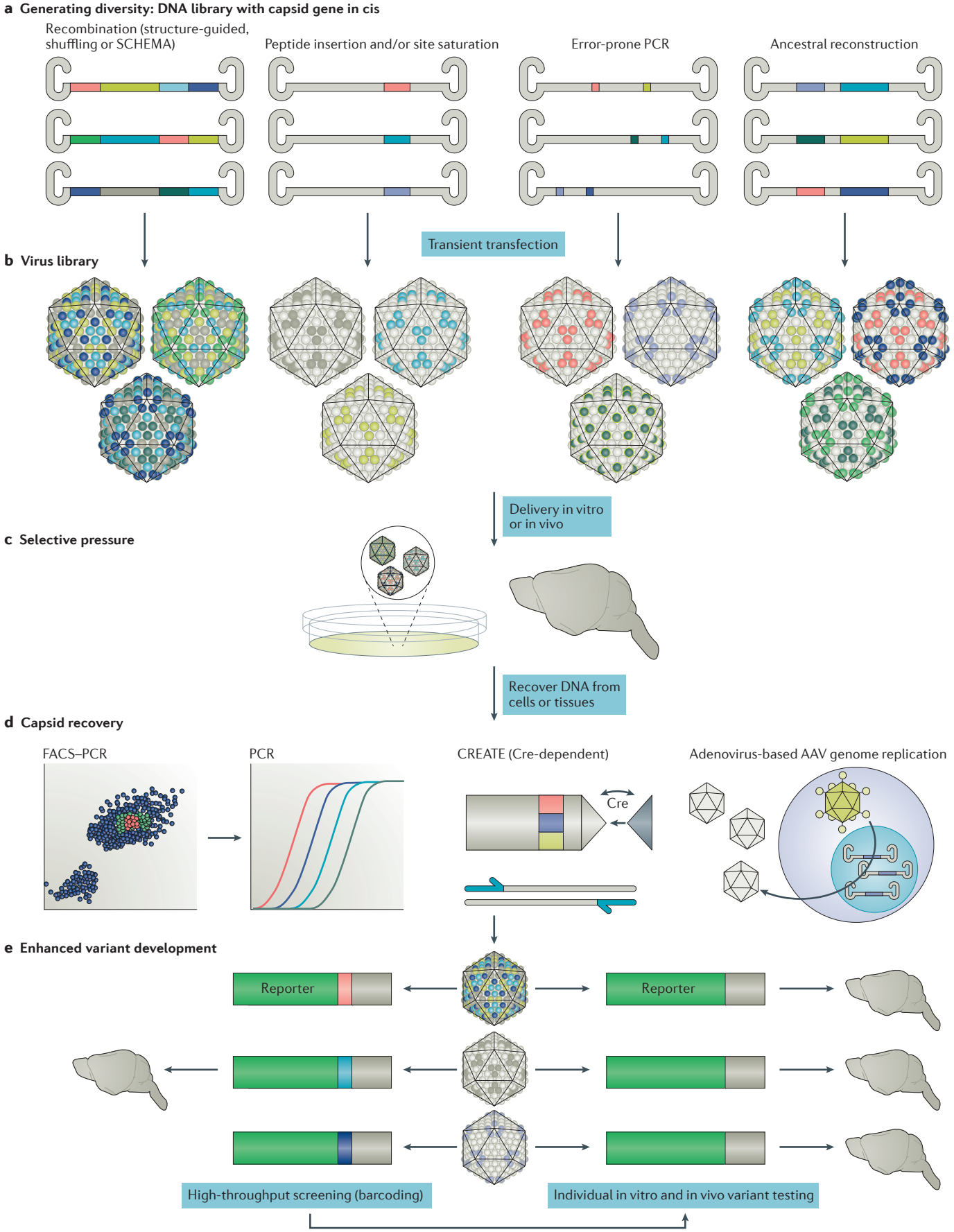

Figure 1 | **AAV engineering through high-throughput selection.** The schematic shows the major steps used to evolve adeno-associated viral (AAV) capsids through high-throughput screening and selection. A library of capsids is generated by introducing diversity within the AAV genome (part **a**). The library of capsid-containing genomes is then used to generate a virus library (part **b**), which is subsequently subjected to selection pressure (part **c**) — for example, for those that escape antibody neutralization, transduce cells in vitro or distribute to the brain or other organs. The capsid sequences are recovered by PCR, cell-type-specific Cre-based amplification or replication with adenovirus (part **d**). Multiple rounds of selection can be used to enrich for the most effective capsids. Following selection, enriched capsids are screened with barcoding-based methods and/or tested individually in animal models (part **e**). CREATE, a novel Cre-based cell-type-specific selection method; FACS, fluorescence-activated cell sorting; SCHEMA, a structure-guided approach to generating recombination libraries.

a neuronal tropism when injected directly into brain parenchyma, although species[75], region[30,36] and cell-specific differences in transgene expression have been noted[28,111]. AAV1, AAV5, AAV8, AAV9 and the less well-studied variants hu.11, hu.32, hu.48R3 and rh.8 transduce astrocytes and oligodendrocytes to a considerable extent[28,34,111]. Notably, owing to strong neuronal tropism, expression in glia may be underappreciated when ubiquitous promoters are used and only fully revealed with promoters that restrict expression to specific glial populations[111–113]. In contrast to the transduction of neural cells, microglia, pericytes and endothelial cells have proved relatively refractory to transduction following direct brain injection, although several engineered AAV vectors transduce brain endothelia when delivered into the vasculature[46,96]. Interestingly, AAV4 appears to have a propensity for transducing the ependymal cells that line the walls of the lateral ventricles after injection into or near the ventricle[26,35]. It should be noted that the virus purification method can also influence AAV tropism when administered to the brain[27,114], highlighting the importance of attention to AAV production protocols when comparing in vivo results. Nonetheless, it is clear that the AAV capsid affects cell tropism and can more or less direct the therapeutic payload to the cell type of interest for a particular CNS disease application.

Vector genome design

The interior of the AAV capsid holds the recombinant vector genome (BOX 2), which comprises the AAV inverted terminal repeat, transgene, promoter and post-transcriptional regulatory elements (FIG. 2). An AAV genome for gene replacement, for example, in the case of a monogenic disorder such as Friedreich ataxia, would contain a transgene that is a native or optimized codon sequence for the therapeutic protein[115]. For gene suppression, such as in the case of Huntington disease, the transgene may comprise a primary microRNA (pri-miRNA) or short hairpin RNA (shRNA) to inhibit the target of interest via RNA interference (RNAi)[116] or alternatively may comprise a sequence for gene editing[117] or repression. Antibodies or antibody fragments may also be expressed[22,23]. In all cases, selection of gene regulatory elements that determine where (which cells) the therapeutic transgene is expressed and to what degree (strength of expression) is critical for providing an effective and safe therapy.

The transgene

The most common therapeutic application of AAV vectors has been for gene replacement by delivery of a transgene. Gene replacement as a therapeutic strategy in the human CNS was first evaluated for Canavan disease by delivering human aspartoacylase (ASPA) cDNA to multiple brain sites with AAV2 (REFS[118,119]). The transgene was well tolerated and showed evidence of efficacy, with reduced brain atrophy and clinical stabilization over a 5-year follow-up study[119]. Transgene expression for monogenetic gene deficiencies is being evaluated for several lysosomal storage disorders, giant axonal neuropathy (GAN; NCT02362438) and type I SMA. However, transgene delivery is not limited to the replacement of gene function for monogenetic disorders. Indeed, despite its more complex aetiology, PD has been the target of numerous AAV-based clinical trials with several gene therapy strategies being tested for safety and efficacy, including delivery of AADC directly to the putamen to enhance the conversion of 3,4-dihydroxyphenylalanine (L-DOPA) to dopamine[49,120], glutamic acid decarboxylase delivery to the subthalamic nucleus to increase local GABA production[121] and neurturin[122] delivery to the striatum to provide neurotrophic support for the remaining dopaminergic neurons. Numerous additional promising AAV-based transgene delivery approaches are progressing through preclinical studies[10].

To date, CNS-targeted gene therapy trials have exclusively used human cDNAs and thus have not introduced new non-self antigens. In one recent exception, a null patient was included in the IT AAV9 GAN trial (NCT02362438). Promising preclinical results with non-self proteins include full-length humanized antibodies[44,45,123], and gene editing[124–126] and gene regulation machinery[127] may also comprise new therapeutic strategies in humans. If proved safe, and coupled to more effective capsids and routes of delivery, these non-self transgenes would greatly expand the options available for treating CNS disorders. However, caution is warranted owing to the potential severe consequences of T cell-mediated immune reactions in the CNS[106,128].

Antibodies. Antibody therapies being evaluated for neurodegenerative diseases in the clinic currently include anti-amyloid[129] and anti-tau[22,23] monoclonal antibodies for Alzheimer disease. A major challenge for systemic administration of such antibody therapies with passive immunization is the BBB, which greatly restricts entry of large proteins such as antibodies from the peripheral circulation. Only 0.1% of administered monoclonal antibody reaches the brain[130]. Direct CNS administration of an AAV vector encoding an antibody would provide considerably greater drug levels in the CNS with potentially greater therapeutic benefit, although safety would of course need to be evaluated and demonstrated. Administration of AAV rh.10 vectorized phosphorylated-tau monoclonal antibody in a mutant tau transgenic mouse has recently been reported to result in a substantial reduction of tau pathology, supporting the potential of this therapeutic approach[22]. However, further work will be needed to optimize

Box 2 | **The recombinant AAV genome**

The wild-type adeno-associated viral (AAV) genome is a single-stranded DNA (ssDNA) genome flanked by inverted terminal repeats (ITRs) with a total length of approximately 4,700 bp. The ITRs are the only AAV genome sequences required for the generation of recombinant virus. ITRs function during viral genome replication as packaging signals and during transduction by priming second-strand synthesis and episome formation in host cells[109]. Recombinant AAV vector genomes are typically constructed with AAV2 ITRs, which can be used to cross-package the genome into other AAV capsid serotypes[26,60,221], making it a fairly simple 'plug-and-play' task to generate any AAV capsid serotype or variant by co-transfection with an AAV2-based genome.

Including ITRs, the maximum recombinant genome size has a strict size limit of just under 5 kb (REFS[222,223]). Within this space constraint, a cDNA, gene or RNA interference cargo can be inserted along with regulatory elements to control expression. Following uncoating in the nucleus, conversion of the ssDNA genome to a double-stranded genome is a key rate-limiting step for transduction[109]. Second-strand synthesis requires either host cell DNA synthesis or co-transduction with complementary positive-stranded and negative-stranded AAV genomes[224]. AAV genomes less than half the wild-type genome length package as dimers that can refold as self-complementary genomes[225], and McCarty et al. applied this knowledge to design single ITR terminal resolution site mutants that force self-complementary DNA formation[226]. These self-complementary AAV genomes provide more rapid and more efficient transduction and have been used in type I spinal muscular atrophy and haemophilia trials[1,227].

transgene configurations for antibody expression in the brain. Nonetheless, AAV-mediated antibody expression in the CNS is a promising alternative to traditional passive immunization.

Gene editing. Gene editing and engineered transcriptional regulators targeted to single genes are also being explored as potential therapeutics. Zinc-finger protein (ZFP) transcription factors and nucleases and a rapidly growing CRISPR–Cas toolkit are expanding potential options for regulating endogenous transcription of individual genes and editing genes[131–137], RNA[138,139] and single bases[140–142]. Clinically, ZFPs are attractive owing to their fairly short gene length and similarity to endogenous human proteins. A ZFP-based gene-editing platform designed to insert the *IDS* gene into the active albumin locus for MPS II has entered a clinical trial and, as of the time of writing, has not been associated with any reported adverse effects[143]. This study will be an important proof of concept for in vivo gene editing. However, success in this trial will not necessarily provide a straightforward path to gene editing in the CNS. CNS gene editing will require further safety data demonstrating the tolerance of irreplaceable CNS cells to expression of a ZFP. Off-target editing remains a concern, as does the expression of a non-self-protein, which may elicit cell-mediated immune responses. Notably, pre-existing acquired immune responses against Cas9 may be common in the human population[128]. Transient editing machinery expression may mitigate these challenges. In addition, the success of gene-editing approaches that require co-transduction by multiple vectors will likely require more efficient gene delivery vectors.

Gene silencing. Another therapeutic application of AAV vectors has been gene silencing. RNAi is a natural biological process by which small interfering RNAs (siRNAs), typically 19–21 nucleotides in length, can direct sequence-specific degradation of target mRNA, leading to reduced expression of the corresponding protein. Several clinical trials in >800 patients have demonstrated that RNAi can be harnessed in humans by synthetic siRNAs for the suppression of targeted genes and is generally well tolerated (for example, NCT01437059 and NCT01559077). Although RNAi is durable for weeks, the need for continuous exposure to the miRNA for target suppression necessitates repeated dosing of synthetic oligonucleotides, which is particularly challenging for CNS diseases. By contrast, artificial miRNAs or shRNAs expressed from a single administration of AAV gene therapy can provide durable gene silencing and represent an extension of the clinical experience with synthetic siRNAs. The endogenous miRNA biogenesis pathway can be harnessed at multiple points (for example, at the level of the pri-miRNA or shRNA) to inhibit gene expression via the RNA-induced silencing complex (RISC)[144]. Multiple head-to-head comparisons demonstrate that artificial miRNA cassettes that enter the pathway at the pri-miRNA level result in a safer and/or more efficient production of the inhibitory siRNA of interest than shRNAs that enter the pathway at the shRNA level[7,9]. shRNAs have been shown to disrupt miRNA biogenesis, whereas artificial pri-miRNAs do not[9]. Notably, in vivo, shRNAs caused striatal and Purkinje cell toxicity in mice after intrastriatal and intracerebellar administration, respectively, whereas artificial pri-miRNA expression did not[7,9]. Artificial pri-miRNA cassettes have provided safe and efficacious CNS expression of mature miRNAs targeting HTT[7], ataxin 1 (REF.[145]) and ataxin 3 (REF.[146]) in animal models of Huntington disease, spinocerebellar ataxia type 1 and spinocerebellar ataxia type 3, respectively, demonstrating proof of concept for this approach to harness RNAi for therapy. Thus, expressed RNAi using artificial pri-miRNAs is the preferred approach for an AAV gene therapy targeting inhibition by RNAi.

Regulating expression

Transgene and RNAi expression from AAV genomes is highly customizable through the use of an expanding set of transcriptional and post-translational regulatory elements. However, the regulatory elements discussed below are all constitutive and therefore provide no post-administration control over transgene 'dosing' in the event of transgene-related adverse effects. The holy grail of regulated gene expression would be to achieve drug-regulatable control over gene expression without a protein coding regulator. Efforts to date have not yet yielded a translatable approach, but such a regulatable system would be a monumental advance for the field of gene therapy and should be investigated further.

Controlling transcription with gene regulatory elements. Promoters and associated enhancers are the principal drivers of transgene transcription and thereby control how much, in what cell types and under what conditions the transgene is expressed. Owing to their ability to drive high levels of transcription across most cell types, strong ubiquitous promoters such as the cytomegalovirus (CMV) immediate-early enhancer and promoter,

Self-complementary AAV
An AAV genome that has been modified by elimination of the 5′ terminal resolution site and can fold into double-stranded DNA without the requirement for DNA synthesis.

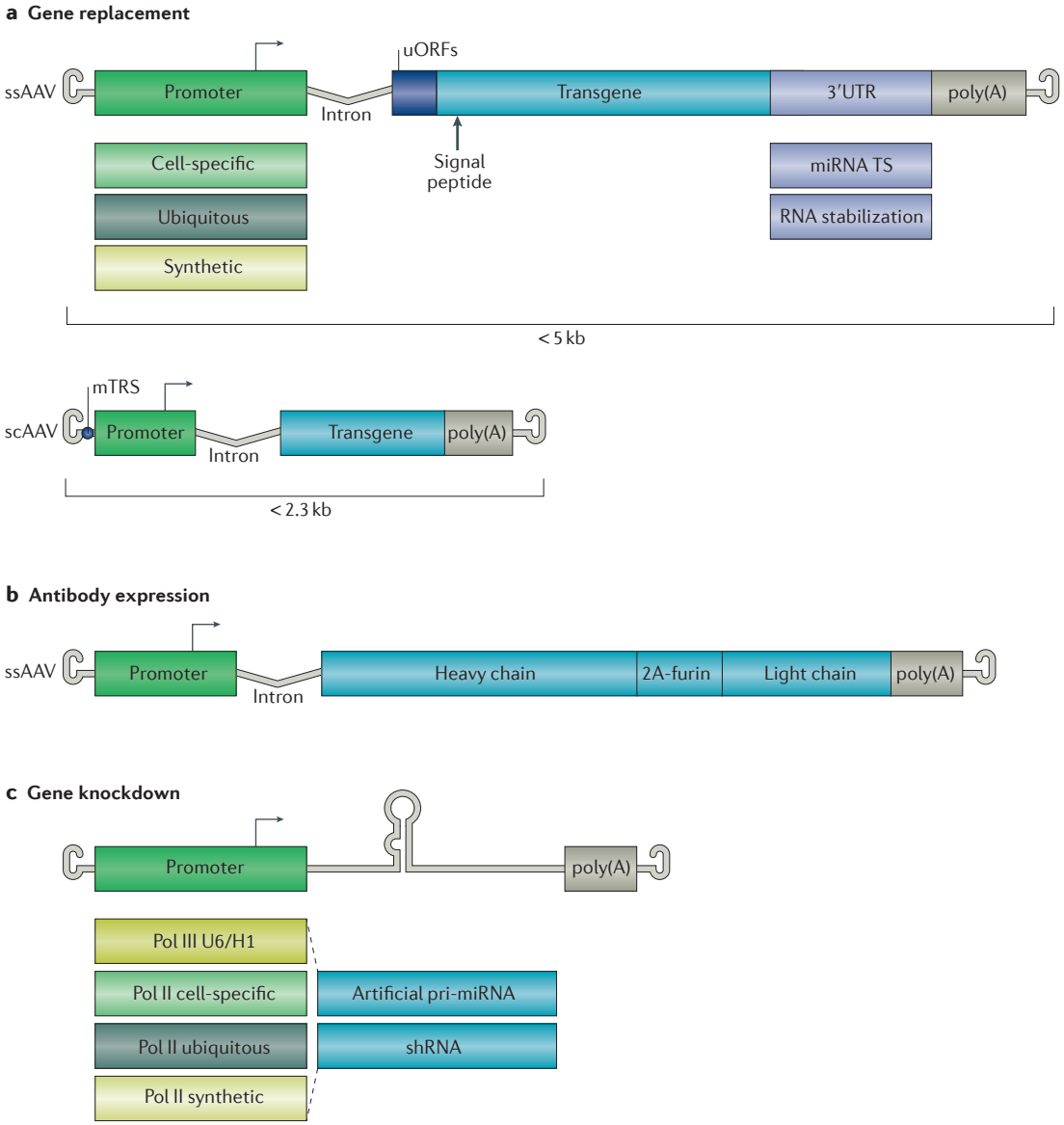

Figure 2 | **Recombinant AAV genome design.** The schematics show the basic design parameters for a therapeutic recombinant adeno-associated viral (AAV) genome for transgene expression (parts **a**,**b**) and gene knockdown (part **c**). Modular elements including enhancer promoters (green), introns, upstream open reading frames (uORFs; navy), transgenes (blue) and 3′ untranslated regions (UTR; purple) and polyadenylation signals (poly(A); grey) can be chosen to optimize where, when and how much transgene is expressed. **a** | AAV vectors can package single-stranded AAV (ssAAV) genomes that are up to nearly 5 kb in length or self-complementary AAV (scAAV) genomes up to 2.3 kb in length, which fold back on themselves to form a double-stranded genome through the mutation of one of the terminal resolution sites (mTRS). **b** | ssAAV genomes can carry full-length antibodies by expressing the heavy and light chains from a single multicistronic transcript through the use of 2A ribosomal skipping sequences and furin protease cleavage sites. **c** | To treat diseases with toxic gain-of-function mutations, artificial primary microRNAs (pri-miRNAs) and short hairpin RNAs (shRNAs) can be used for gene knockdown. Both pol III and ubiquitous, cell-type-specific or synthetic pol II promoters can be used to control the expression of the pri-miRNA or shRNA. miRNA, microRNA; TS, terminal sequence.

artificial CMV-chicken β-actin promoter with β-globin splice acceptor (CAG) and chicken β-actin (CBA) promoter have been chosen for gene transfer applications[10]. However, strong promoters may provide artificially high levels of expression, especially in cells transduced by multiple copies of the AAV genome resulting in potentially toxic levels of even a self-protein[19]. For many applications (for example, *FXN* delivery for Friedreich ataxia or *SMN1* for SMA), transgene expression that more closely matches the endogenous protein levels should be sufficient and would likely be better tolerated. Several trials have used the comparatively weaker phosphoglycerate kinase (PGK) promoter (MPS IIIA and B)[147]. Beyond controlling expression levels, gene regulatory elements can be used to restrict expression to target cell types for optimum therapeutic benefit and reduced off-target

expression. Enhancer-promoter elements that restrict expression to several neuronal and glial cell types in the CNS are available[148–150]. For expression of longer transgenes, several gene regulatory fragments and synthetic promoters have been developed, including a shorter CBA promoter (CBh)[151], an *MECP2* fragment[151] and an artificial JeT promoter[152], which is being used in the case of gigaxonin expression in the ongoing clinical trial. In addition, cytokine-responsive and hypoxia-responsive enhancer-promoter elements have been used to modulate expression in response to disease states[153–157]. The development of transcriptional regulatory elements remains an active area of research that will continue to expand the options available for optimizing transgene expression.

Post-transcriptional regulation. Transgene expression control can be further refined using elements that affect RNA (for example, by modulating splicing, nuclear export and stability), translation or protein stability. The transgene is often provided as a cDNA owing to the limited AAV packaging capacity, but the inclusion of an intron, when possible, can improve mRNA processing[158,159]. The strength of the polyadenylation signal can be used to affect expression[160].

Transgene expression in an off-target cell population can be dramatically reduced by including sequences complementary to endogenous miRNAs that are selectively expressed in off-target cell populations. For example, incorporating three miRNA target sequences that are complementary to miR-122, which is expressed in the liver, and miR-1, which is expressed in skeletal muscle, reduced expression by >30-fold in these 'off-target' organs[161] while having only negligible effects on expression in the CNS and pancreas, where miR-122 and miR-1 are not highly expressed. Incorporating miR-142-3p target sequences, which selectively degrade the transgene mRNA in antigen-presenting cells, reduced transgene immunogenicity following IM delivery[162]. Because these sequences are short, they can be multiplexed for greater refinement of expression[161].

Translational control. An additional level of control that may be precisely fine-tuned is the efficiency of translation of the open reading frame (ORF). Translational start sites that conform to consensus sequences first described by Kozak provide maximum likelihood for translation initiation in the absence of competing ORFs. Intriguingly, Ferreira et al.[163] have described a panel of two amino acids upstream of the ORF and transgene ORF pairs that can be used to fine-tune expression of the transgene over three orders of magnitude. This technique has a fairly unexplored potential for improving the safety of transgenes that may function at low expression levels. Transgene expression can also be optimized through codon modification[2,164,165].

Dosing optimization

Along with the AAV capsid and genome elements, the route of administration and dosing paradigm dictate levels and homogeneity of expression across different tissues and regions of interest. For the CNS, multiple routes of delivery have been evaluated in preclinical studies, including IPa, IT, ICV, subpial, IV and IM, and several of these are being used in ongoing clinical trials. Each route of administration has pros and cons that are context-specific, depending on the particular CNS disease application, tissue or cell type of interest for transgene expression and the level and homogeneity of transgene expression required.

Direct CNS delivery

IPa, IT, ICV and subpial routes of administration have been evaluated preclinically for biodistribution of AAV gene therapy to the CNS with direct delivery. Each route of administration, together with the choice of capsid, may be best suited for certain disease applications, depending on the targeted CNS region of interest and the specific cell types within this region.

Intraparenchymal delivery. Initial CNS gene therapy trials in humans have leveraged IPa administration to bypass the BBB and deliver genes directly into the brain region of interest (FIG. 3). The one-and-done feature of AAV gene therapy is essential given the required surgical procedure necessary for delivery. Most importantly, direct IPa administration of AAV vector to the CNS has been well tolerated[49,120,121,166]. Additional advantages of this route of administration include minimal biodistribution to peripheral organs, which can reduce immunogenicity, and substantially lower vector doses than required for other routes of administration.

IPa administration distributes drug directly to the brain region and neurons of interest. Substantially higher local levels of AAV vector and vector genomes can be achieved compared with IV[67] or IT[69] dosing in NHPs, both of which show relatively reduced levels of transgene delivery to CNS neurons owing to low penetration into the adult brain parenchyma. IPa delivery of AAV vector in multiple preclinical studies has demonstrated robust delivery of transgene to striatal medium spiny neurons and cortical pyramidal neurons following infusion into the putamen[167] and thalamus[168]. Direct administration not only exposes putaminal and thalamic neurons and glia to AAV vectors, but also other regions of the brain through axonal transport via extensive topographic connections to the cortex and more distant sites, thereby achieving more widespread distribution of AAV vectors[169–171]. In NHPs, IPa delivery of AAVrh.10 encoding human arylsulfatase A has similarly demonstrated transgene expression and safety at therapeutic doses[172]. Importantly, IPa delivery has been used safely in clinical trials in patients with MPS IIIB with 16 intracerebral injections per subject of AAV5 encoding the transgene for human α-*N*-acetylglucosaminidase[173].

Convection enhanced delivery (CED), which involves directly infusing molecules into the parenchyma under positive pressure, can be used to increase the distribution volume throughout the desired region of the brain[174]. A number of drugs, including AAV2, have not only been distributed to a substantially greater extent by CED IPa dosing than passive diffusion, but are also well tolerated when administered directly into the putamen of patients with advanced PD (NCT01973543).

Convection enhanced delivery
(CED). Infusion of adeno-associated viral vectors or other molecules into the parenchyma under positive pressure to increase the distribution volume.

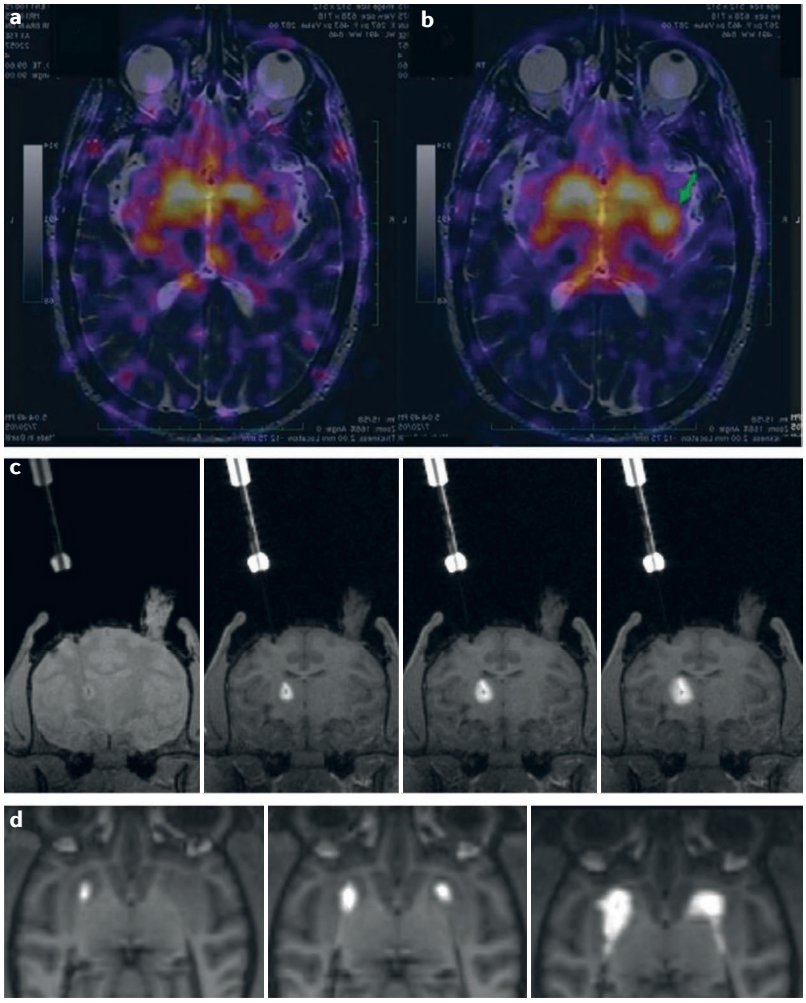

Figure 3 | **Delivery of AAV gene therapy with intraparenchymal administration.** Before (part **a**) and after (part **b**) aromatic-L-amino-acid decarboxylase (AADC) gene transfer images using [^{18}F]-6-fluoro-L-meta-tyrosine positron emission tomography (PET) as a measure of AADC enzyme activity from an early clinical trial using standard stereotactic delivery. Focal increases in enzyme activity (green arrow) are visible. Parts **a** and **b** are reproduced from Valles, F. et al. Qualitative imaging of adeno-associated virus serotype 2–human aromatic L-amino acid decarboxylase gene therapy in a phase I study for the treatment of Parkinson disease. *Neurosurgery* 2010, **67**(5): 1377–1385, by permission of Oxford University Press (REF.[228]). Subsequent developments in magnetic resonance guided imaging allow for greater physical coverage of the putamen and the potential for more widespread transgene expression. Coronal images showing progression of thalamic infusion (part **c**) and axial images showing progression of a putamen infusion, using a trajectory through the occipital lobes (part **d**), demonstrate diffuse coverage of the target structures evident in the magnetic resonance images acquired during infusion of a solution containing dilute magnetic resonance contrast in nonhuman primate putamen. Parts **c** and **d** adapted with permission from *Journal of Controlled Release*, **240**, Bankiewicz, et al. AAV viral vector delivery to the brain by shape-conforming MR-guided infusions, 434–442, Copyright (2016), with permission from Elsevier (REF.[229]). AAV, adeno-associated viral.

The success of the IPa route of administration with CED is volume-dependent. A sufficient volume of infusate that contains the AAV vector needs to be administered to cover the anatomical region of interest. This volume dependency has been an important issue in AAV-based clinical trials in PD in which suboptimal volumes were delivered into the putamen, which is >4,000 mm^3 in size in patients with PD. CED is based on pressure-induced temporal expansion of the extracellular space of the brain parenchyma that allows fluid containing AAV vector to fill a tissue volume on average two to three times the volume of the infusate. Thus, to cover 80% of the human putamen, at least 1,500 µl of fluid must be administered. The volume of infusion used in previous gene therapy trials in PD ranged from only 40 µl to 150 µl and therefore had no chance of providing gene expression beyond 10% of the putamen, most likely resulting in substantial underdosing. With the development of magnetic-resonance-guided AAV delivery methods and near-real-time monitoring of vector infusion in the putamen using a magnetic resonance imaging (MRI) tracer, much larger volumes are currently being investigated, ranging from 450 µl to 1,500 µl, which have resulted in substantially greater coverage of the putamen[47].

The choice of capsid can also be used to tailor how focused or broadly the AAV vectors are distributed within the brain following IPa delivery. Several AAV vectors, including AAV2, AAV6 and AAV-DJ[88], bind heparin sulfate proteoglycans (HSPGs) and, as a consequence, diffuse over smaller areas of the brain than AAV vectors lacking HSPG binding (for example, AAV1, AAV5, AAV8, AAV9 and rh.10), which results in expression over larger areas of the mouse brain[169]. For certain disease applications such as AAV2–AADC for PD, the restricted spread of AAV2 is highly desirable to limit the expression of AADC to the putamen. In an early clinical trial for Canavan disease, AAV2 carrying *ASPA* was injected into six white matter sites to increase the extent of transduction[119]. Although some evidence of efficacy was noted in this trial, the coverage provided by this multiple-injection strategy was probably inadequate for maximal therapeutic benefit. Recently, an AAV2 variant lacking the ability to bind HSPG has been described that results in greater IPa spread than AAV2 in rats[175].

Intrathecal, intracerebroventricular and subpial delivery. Other routes of direct CNS administration include injections into the CSF compartment. IT[33,67,68,70,71], IC[68–70] and ICV[11,12,16,70] dosing have been tested in multiple preclinical studies and have begun to be evaluated in the clinic. Subpial injections have also been studied, but only preclinically to date[41]; therefore, the translation of this dosing paradigm for AAV vectors remains to be seen. IT administration of AAV to the CNS has been well tolerated in multiple preclinical studies[33,67,68,70,176] and is particularly useful for delivering AAV vectors to motor neurons and sensory neurons of the dorsal root ganglia. Previous studies have demonstrated that AAV vectors, including AAV9 and AAVrh.10, distribute primarily to motor neurons in the spinal cord after IT administration in NHPs. IT dosing regimens can be varied by adjusting the volume delivered and the rate of infusion as well as the site of infusion and concentration of vector. Distribution within the brain after CSF dosing of AAV vector in NHPs has a notable perivascular distribution and distribution to certain cell types such as Purkinje neurons of the cerebellum[69]. Compared with IPa dosing, IT or ICV dosing results in lower tissue levels of the vector genome and transgene and a more

heterogeneous distribution. The limited clinical experience to date (TABLE 2) comprises a Batten disease trial with IT AAV9 to deliver a transgene encoding ceroid-lipofuscinosis neuronal protein 6 (CLN6; NCT02725580), a giant axonal neuropathy trial with IT AAV9 to deliver a transgene encoding gigaxonin (NCT02362438) and an expanded access study with IT AAVrh.10 to deliver an miRNA targeting SOD1 (R. Brown, personal communication). Total doses are generally higher than those used with IPa administration but considerably lower than those used with IV administration.

Intravenous delivery

In contrast to IPa, IT or ICV administration, IV administration of AAV vectors has the potential to transfer genes to the entire CNS non-invasively and with a more uniform distribution. The reports by Foust et al.[72] and soon thereafter by Duque et al.[73] that AAV9 can cross the BBB and transduce mouse neurons and glia in neonatal mice initiated a wave of interest in IV delivery of AAV gene therapy for CNS transduction. The finding was soon replicated and extended to other species[67,74–76], and several other naturally occurring AAV vectors, including rh.10 and rh.8, were also found to transduce the neonatal[177] as well as adult CNS[178] after IV administration, although to a much more limited extent in adult than in neonatal animals.

Despite the promise of non-invasive gene delivery throughout the CNS via engineered AAV vectors, several notable aspects must be kept in mind. First, tropism differences across species may pose particular challenges for capsid engineering and selection for human therapeutic use. IV administration of self-complementary AAV9 led to transduction of mouse and NHP CNS, although the latter was less efficient and largely directed towards astrocytes[75]. Second, IV injection exposes the virus to potential antibody neutralization in subjects who have been pre-exposed to natural AAV infections. An estimated 90% of adult humans have been exposed to AAVs, and a smaller but substantial fraction harbour neutralizing antibodies against AAV capsids, which can have a profound negative impact on AAV vector transduction. Modifying capsid surface epitopes through capsid engineering may enable the identification of capsids that better evade neutralizing antibodies[87,92,179]. Third, IV administration typically requires higher total doses to achieve efficient transduction than IPa, IT or ICV administration; therefore, the scale of manufacturing capacity must be sufficient to support these greater material requirements. Fourth, IV administration results in broad and substantial vector distribution to multiple peripheral tissues and organs, which comes with the associated biological impact of the potential for immune responses against the capsid, nascent unpackaged genome and transgene that needs to be considered from a safety standpoint. In this regard, Wilson and colleagues have recently reported that high-dose IV AAV administration in NHPs with two capsids similar to AAV9 — PHP.B and AAVhu68 — caused life-threatening hepatotoxicity and internal haemorrhage[105,180], although we have not observed such severe toxicity with similar capsids at comparable doses in adult NHPs (D.W.Y.S., unpublished data)[107,108]. The exact reason for these discrepant safety findings in NHPs will require more investigation. In addition, in juvenile NHPs and piglets, Wilson and colleagues reported that high-dose IV AAV administration with the AAVhu68 capsid and a transgene encoding human SMN1 caused axonopathy, dorsal root ganglion toxicity and ataxia[180]. More importantly, however, clinical trials of IV AAV9 encoding SMN have demonstrated safety as well as remarkable efficacy[1]. In our view, the recent progress in capsid engineering and manufacturing technologies (described below) has the potential to enable widespread gene delivery to the adult CNS via IV delivery and provide safe and effective AAV therapies for neurodegenerative diseases that affect multiple regions of the brain, such as Alzheimer disease or frontotemporal dementia.

Intramuscular delivery

IM delivery of AAV vectors may provide an alternative approach for delivering antibody therapeutics targeting the CNS. Local AAV transduction of muscle could provide a depot for transgene expression (that is, for sustained release of the antibody of interest), thus reducing the need for monthly IV administration as is the case with current passive immunization protocols[44,45]. Although the BBB still represents a considerable barrier, the major advantage of such an approach would be the durability of antibody expression following a single injection of AAV vector.

Manufacturing

Successful AAV gene therapy for the treatment of neurological disorders requires a production platform that reproducibly provides vector that is of sufficient and comparable quality across multiple batches and is scalable for late-stage clinical development as well as commercialization. A major challenge for AAV gene therapies has been the scalability of any manufacturing process to meet these material demands. Traditional production systems in mammalian cells such as HEK293 cells result in high-quality vector but rather limited yields. By contrast, production of AAV vectors using baculovirus–Sf9 insect cells has been shown to increase vector yields by approximately 10-fold to 100-fold[52]. However, with some capsids, potency of baculovirus–Sf9-produced vectors may be lower than vectors produced in mammalian cells, but this can be successfully overcome by molecular engineering[181]. For CNS applications that require limited quantities of vector such as an intraparenchymally administered AAV vector, mammalian systems may suffice. However, for IV and possibly IT administered AAV vectors, it has been a major challenge for mammalian systems to provide the quantities needed for clinical development, let alone scale up for commercialization. Production systems that can be scaled to address these needs include the baculovirus–Sf9 system[52], the recombinant HSV-based system[182–184] and the adenovirus–HeLa cell system[185–187].

Disease and target selection

There is a growing list of neurological disorders and targets for which AAV gene therapy offers potential therapeutic application. TABLE 1 shows a list of disorders

and associated targets compiled on the basis of criteria that support successful translational work and drug development and that have a favourable benefit-to-risk profile. These criteria are similar to what would be required for any neurological drug development programme, but can be operationally defined for AAV gene therapy: strong target validation; the ability to safely achieve therapeutic tissue levels of vector at the target CNS location as well as transduction of the relevant cell type or types; an adequate therapeutic index for pharmacological effects and safety; high unmet medical need; biomarkers and clinical assessments that will allow for measurement of pharmacological activity and clinical outcomes; and the ability to identify patients and importantly intervene at an appropriate time in the course of disease progression.

Rare, monogenic disorders still dominate this development landscape. The most clinically advanced and successful programmes in neurological, ocular and systemic disorders are for monogenic disorders[188]. The success and safety profile of AAV gene therapy in these disorders has broadened interest in the range of potential targets. These include genetic risk factors such as the *APOE* allele for Alzheimer disease[189] and pharmacological targets such as AADC for PD[47,49,120]. Targets without strong genetic, pharmacological or pathophysiological validation, such as trophic factors in PD, have shown less promise in clinical trials, although delivery challenges and the timing of intervention may have limited their potential efficacy[190].

Delivery

The potential to successfully deliver an AAV vector to the CNS is one of the most important questions in choosing a disorder and target to pursue with gene therapy. Direct delivery to discrete brain regions has markedly improved with the advent of MRI-guided imaging, offering the ability to tailor and optimize dosing in near real time for each subject. Cell therapy clinical trials also suggest the capability of safe delivery directly to the spinal cord[191]. Although direct delivery has upfront risk and costs, it circumvents the issues of pre-existing immunity and potential adverse effects related to systemic distribution and expression in non-target tissues. Broader CNS delivery may require CSF-based dosing using IT or ICV routes. IV dosing has been successful to date in infants with type I SMA in whom very modest levels of transgene expression are likely sufficient for therapeutic benefit[1]. Systemic dosing for CNS diseases in older children and adults needs to take into account the high doses required for gene transfer to the adult CNS and pre-existing immunity to AAV capsids[192–194].

Safety

After the acute dosing period, the concept of therapeutic index in AAV gene therapy is a function of both the transgene and the mechanism of action of the vector. Most clinically advanced AAV gene therapy programmes involve gene replacement, and only modest increases of expression and functional protein may be adequate for clinical benefit[1]. Potential toxic effects from gene replacement may occur owing to expression in non-target tissues or overexpression of the therapeutic transgene within the targeted cells. For example, mutations in *MECP2* (encoding methyl-CpG-binding protein 2) cause Rett syndrome in females, but duplications also cause a developmental disorder with intellectual impairment in boys[195–200]. MeCP2 overexpression in mouse models causes behavioural abnormalities, learning impairments and seizures[201]. A successful therapy for Rett syndrome would therefore require that transgene expression stays within a very narrow therapeutic window. Potential toxic effects related to gene silencing will also depend on the function of the endogenous protein, timing of the intervention and the level of expression of the miRNA and resultant silencing. HTT is involved in neurogenesis, and disruption of HTT results in embryonic lethality in mice[202,203]. However, silencing of both wild-type and mutant *HTT* by approximately 45% in the striatum of adult rhesus macaques appears safe and is not associated with neuronal pathology or motor deficits[204].

Although AAV gene therapy has been well tolerated to date in a range of diseases, the inability to regulate expression remains an important safety consideration for target and disease selection. Additionally, immune responses and durable expression may preclude subjects from participating in other clinical trials after receiving gene transfer. Disease states with high unmet clinical need and a favourable benefit-to-risk profile therefore remain most appropriate for AAV gene therapy.

Clinical development challenges

The clinical development challenges for neurological disorders are not specific to AAV gene therapy. A relative lack of biomarkers or the ability to biopsy affected CNS tissue has traditionally presented a challenge in assessing target engagement and pharmacodynamic effects. Advances in CSF biomarkers and brain imaging such as positron emission tomography (PET) tracers for amyloid-β and tau have enabled earlier detection of disease and measurement of pharmacodynamic effects that may facilitate clinical trials. However, even relatively rapidly progressive neurological disorders of interest for gene therapy, such as monogenic forms of ALS, must still be evaluated clinically or with neurophysiological measures and may require ≥12 months to assess treatment effects[205]. The window to intervene is also uncertain in many neurological disorders. Adult-onset neurodegenerative disorders, in particular, may progress for decades preclinically (that is, without symptoms or clinical signs manifesting)[206,207]. Therefore, potential biological targets must be carefully vetted for the feasibility of clinical development, including the ability to identify subjects who can be treated early enough in the course of their disease for potential benefit from AAV therapy, and the availability of assessments for pharmacological and clinical effects.

Clinical trials

Trial design

Clinical trials with AAV gene therapy address most of the same drug development questions as trials with other treatment modalities: appropriate patient population,

safety, dose selection, choice of end points and efficacy and ultimately overall benefit-to-risk ratio. AAV gene transfer clinical trials do not have a particular paradigm but have some common features shaped by the generally rare patient populations of interest, routes of delivery and expectations about the durability of expression and magnitude of the treatment effect. Key features include a dose range that is limited to doses that are expected to be pharmacologically active, because re-dosing may not be possible, and the ability to assess long-term safety and efficacy from the first subjects treated due to long-term expression of the therapeutic transgene.

Most completed AAV gene therapy trials in neurological disorders have had small sample sizes of <30 participants, with the exception of PD[10]. The small sample sizes are due to small incident and prevalent populations for many of the diseases of interest to date and the early stage of development of the interventions. Small sample sizes are also supported by expected large treatment effects with monogenic disorders as has been observed with type I SMA (REF.[1]). Many of the completed and ongoing trials are open-label, uncontrolled trials, which are appropriate for early trials in which safety and delivery methodology may be the key questions or for diseases with a uniform natural history or progression. These factors are not unique to gene therapy interventions. As the field progresses into larger populations with various functional, cognitive and behavioural outcomes, there may be a need for more rigorous, placebo-controlled trials. Careful consideration of the type of control group necessary for each individual clinical trial will be required.

The expected durability of gene therapy has also blurred the lines of the traditional phase I, II and III clinical trials. Because neurons in the adult brain are terminally differentiated, cell turnover, except through disease progression, should be minimal. In theory, subjects are thus continually 'treated' once dosed and, in practice, emerging clinical data support long-term, durable expression of transgenes. There is therefore little justification for a traditional single-ascending-dose or multiple-ascending-dose trial, and subjects treated in the first-in-human study will necessarily provide valuable long-term data on safety, pharmacology and efficacy. The evolving paradigm has an initial phase I study that assesses safety and dose selection over a limited range of vector doses followed by a trial or trials to address efficacy. The current inability to re-dose or clinically regulate expression has limited the initial dose range to doses that are expected to be pharmacologically active but well tolerated. A first-in-human trial therefore typically involves only two to three dose levels separated by half-log increments, and thus dose range must be defined by preclinical pharmacology, efficacy and toxicology studies.

Ongoing trials

Several AAV gene therapy clinical trials for neurological disorders are ongoing (TABLE 2; see ClinicalTrials.gov website, accessed 5 June 2018), including trials in SMA, PD, MPS and Batten disease. Clinical trials in SMA and PD illustrate the range of patients, doses, routes of administration, end points and trial designs used, and some examples and experiences will therefore be discussed in detail below.

Type I SMA. Type I SMA is a progressive, fatal, monogenic motor neuron disease with an onset in infancy[208]. It manifests in failure to achieve motor milestones and results in mechanical ventilation and death by 2 years of age. In one recently completed trial (NCT02122952), 15 infants received high or low doses of AAV9 carrying self-complementary DNA encoding the SMN protein by IV administration[1]. The mean age was 6.3 months in the low-dose group and 3.4 months in the high-dose group. Transient increases in hepatic transaminases occurred in four subjects post-dose, which were attributed to immune responses to the AAV9 capsid. Prophylactic prednisolone treatment was used in subjects 2–15 after aminotransferase elevations were seen in the first subject. As of August 2017, all 15 patients in this study were alive and event-free at 20 months of age, exceeding the expected survival from a natural history study. Some dose-dependent improvements in motor function and motor milestones were observed, although the higher-dose group was also treated at a younger age of ≤6 months.

This trial illustrates the potential safety concerns associated with the high doses of vector that are needed with systemic delivery of AAV, approximately 10^{15} vg in infants. Increases in hepatic transaminases, also seen in haemophilia trials, were readily managed with prednisolone. The safety at higher doses required to treat adults or older children is unclear, and the therapeutic index for acute adverse effects may be capsid-serotype-specific. The effects of a higher dose and lower age in the high-dose cohort cannot be separated, but the data suggest not surprisingly that earlier treatment of SMA is important. The objective outcomes of mortality or need for ventilation, uniform natural history and apparent large treatment effect support the open-label design. However, a trial with an antisense oligonucleotide in the same population used a randomized controlled trial design[209].

Parkinson disease. Unlike SMA, clinical trials with PD use functional and patient-reported outcomes and have generally been placebo controlled. The placebo procedure must be sufficient to blind participants, family members and study staff who may influence outcomes and ratings, but it should minimize risk to placebo participants, who do not receive brain cannula placements or infusions.

AADC is the final enzymatic step in the production of dopamine. Trials of AAV2 carrying single-stranded DNA encoding human AADC (AAV2.hAADC)[49,120] with intraputaminal infusion represent a pharmacological approach to treating advanced PD using relatively small amounts of vector with targeted administration. Gene transfer occurs primarily into medium spiny neurons of the putamen, which does not normally produce dopamine. Patients can then regulate the amount of

levodopa they take to control the amount of dopamine that is produced by this new depot that bypasses degenerating dopaminergic neuronal projections from the substantia nigra. In early dose escalation trials, AAV2.hAADC was administered using stereotactic infusions into each putamen. Several of the early patients in this trial experienced intracranial haemorrhages, which were clinically minor or asymptomatic. The procedure was changed to move the entry point more lateral, away from the central veins, with no subsequent bleeds. Importantly, the AAV2.hAADC vector itself was well tolerated with no vector-related safety events observed. No systemic adverse effects have been observed with intracranial administration of AAV2, which is thought to have limited systemic biodistribution. Dose-related, durable increases in AADC enzyme activity were measured over 5 years using the tracer [^{18}F]6-fluoro-L-metatyrosine and PET, demonstrating that enzyme activity can be restored and maintained using AAV2.hAADC gene transfer[49].

A more recent clinical trial (NCT03065192) has further optimized vector delivery using real-time MRI to guide the infusion, thereby maximizing the safety and coverage of the putamen and minimizing distribution to non-target brain areas. Vector is mixed with gadoluminate, an MRI contrast agent, for visualization of infusate distribution (FIG. 3c,d). This method has enabled a more than tenfold increase in the volume and total dose of vector administered to translate into a mean coverage of >50% of the putamen[47], a further increase in AADC enzyme activity as measured by ^{18}F-DOPA PET scans (FIG. 3a,b) and improved clinical outcomes.

PD trials with AAV gene therapy to deliver AADC and other therapeutic transgenes[49,120–122] have illustrated the safety of intracranial administration of AAV, the low doses required for pharmacology and efficacy and the limited systemic exposure with this approach. The increasing experience and progressive evolution of intracranial infusion technology will support AAV dosing not only for PD but also for other neurological disorders.

Summary and prospects

AAV vector-based therapies are a rapidly emerging approach for potentially treating or even preventing neurological diseases. AAV gene therapy is particularly well suited for highly validated CNS targets, especially genetic targets that are not readily druggable with conventional therapies. Multiple examples of successful proof of concept for AAV gene therapy exist in animal models of disease for both gene replacement to address loss-of-function mutations and gene silencing to address gain-of-function mutations. Clinical trials are underway to evaluate AAV vectors for delivery of therapeutic proteins and miRNAs to the CNS. To date, both safety and long-lasting pharmacology (over multiple years) have been demonstrated in clinical studies for neurological diseases such as PD, AADC deficiency and SMA. The durability of AAV gene transfer to non-dividing cells such as neurons after a single administration provides an important advantage for more complex dosing paradigms in which repeat dosing may be inconvenient or not feasible. In our view, the emerging database of clinical safety and efficacy of AAV gene therapy for CNS disorders illustrates the remarkable potential of AAV gene therapy to treat neurodegenerative diseases.

The greatest immediate consideration for realizing the full potential of AAV gene therapy for neurological diseases is delivery, governed primarily by the capsid, which dictates the tissues and cell types transduced. For the CNS, IPa, IT, ICV, subpial and IV administration have shown promise in preclinical studies for achieving the targeted distribution, and all of these routes of administration except subpial delivery have progressed to clinical trials. Each of these routes of administration, together with the capsid, has particular attributes and considerations, including total dose requirements and the impact of pre-existing immunity on vector transduction and capsid or transgene immunogenicity. It is likely that no one route of administration will be ideal for all CNS applications, but rather that the preferred route of administration for a particular target and disease will be driven by achieving the required target biodistribution profile and minimizing exposure to non-target tissues. Clearly, these different routes of administration will be associated with very different drug requirements and therefore require different production and manufacturing processes. Recent progress in AAV manufacturing using both mammalian (HEK293) and insect (Sf9) cells has enabled the production of AAV gene therapies at commercial quality and scale for a range of applications, including those in which larger drug quantities are needed.

In summary, the recent progress towards realizing the remarkable potential of AAV gene therapy for neurological disorders has resulted in a tremendous increase in interest and effort in this area. As advances in optimizing AAV capsids, vector delivery and transgene design continue, the breadth of CNS disease applications and targets will undoubtedly expand.

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Competing interests statement
The authors declare competing financial interests in the form of funding from Voyager Therapeutics, employment by Voyager Therapeutics and/or personal financial interests in Voyager Therapeutics.

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