Review

Next-Generation Gene Therapy for Parkinson's Disease Using Engineered Viral Vectors

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Abstract. Recent technological and conceptual advances have resulted in a plethora of exciting novel engineered adeno associated viral (AAV) vector variants. They all have unique characteristics and abilities. This review summarizes the development and their potential in treating Parkinson's disease (PD). Clinical trials in PD have shown over the last decade that AAV is a safe and suitable vector for gene therapy but that it also is a vehicle that can benefit significantly from improvement in specificity and potency. This review provides a concise collection of the state-of-the-art for synthetic capsids and their utility in PD. We also summarize what therapeutical strategies may become feasible with novel engineered vectors, including genome editing and neuronal rejuvenation.

Keywords: Genetic therapy, Parkinson's disease, clinical trial, gene editing, rejuvenation, neuroprotection, dependovirus

Viral vector technology for the treatment of Parkinson's disease (PD) has undergone three significant generations of improvement over the last two decades. What was initially *ex vivo* gene transfer using retroviral vectors was developed into proof-of-concept *in vivo* gene expression using adenoviral, herpes simplex (HSV), or lentiviral vectors [1]. One lentiviral vector has even reached clinical trials for enzyme replacement in PD [2]. However, a significant breakthrough was seen with the application of adeno-associated viral (AAV) vectors for gene therapy in the CNS [3]. The AAV virus has a few unique features, making it a near-instant success as the clinical gene therapy vector of choice in non-dividing cells. As a dependovirus (i.e., requiring Adenovirus or HSV

co-infection for replication), it had already outsourced an intricate component of the replication process to other viruses [4]. These mechanisms could, fortunately, be replaced by transient transfection of plasmids in the production cell line. This approach left the need for only two major genes (and two minor genes expressed from the same genetic sequence) to be expressed from the AAV genome. They are required for viral genome replication and packaging (the replication gene Rep) and viral capsid synthesis (the Cap gene).

Like the helper-genes derived from the Adenovirus, the Rep and Cap genes could be extracted from the AAV genome and placed in a third plasmid during production. The resulting transient transfection system produces a replication-deficient, gutted AAV vector where only the inverted terminal repeats (ITRs) remain in the viral vector's genome [4]. For a more complete picture of AAV biology and AAV life cycle see Vance et al. (2015) [5].

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The space generated from removing the Rep and Cap genes (between the ITRs) is filled with the therapeutic gene of choice, including promoter and required regulatory sequences for mRNA stability. To date, AAV vectors have been utilized in 241 clinical trials (planned, ongoing, and performed, clinicaltrials.gov), including trials in PD (15 trials), and over 10,000 patients have received the vector (over 400 in PD). So far, only three AAV-based therapies have reached approval, two in the US and Europe and the third only in Europe. While encouraging, the progress has not been clear cut and many therapies have failed or reached a standstill at different stages of preclinical [6] and clinical testing [7, 8]. However, clinical trials are still being evaluated for the AAV-mediated AADC pro-drug approach, both in PD [9] and in children with AADC insufficiency (NCT02852213) and GDNF neuroprotection, in PD and multiple system atrophy (NCT01621581 & NCT04680065 at clinicaltrials.gov).

Although the AAV capsid and the gutted genome remain one of the lowest immunogenic gene therapy vectors developed to date, it is not without risks of complications [10]. Most patients have neutralizing antibodies against one AAV serotype or another. The common AAV seropositivity has two significant implications [10]. The primary implication is that not all patients are equally suitable for a particular AAV-based therapy, and a considerable fraction of the patient cohort may have to be excluded. The second implication is that this shows that our immune system reacts to the AAV infection and can develop strong immunity against the AAV capsid [11]. Thus, we need to monitor immune reactions carefully and not expect a therapeutic vector to be re-administered in the same patient without modifications. Circulating anti-AAV antibodies have not been extensively reported in the clinical trials using AAV for PD, but some data have been reported in the context of the AAV2 based GAD Phase I Trial (NCT00195143) [8]. In this trial seven out of twelve patients showed detectable levels of anti-AAV antibodies with two patients showing very high levels. This did not negatively impact the safety profile of the therapy, but it is still unknown if it had any impact on the transduction efficacy. A preclinical study suggests that circulating anti-AAV antibodies may not be the sole determinant to inhibit brain transduction [12].

Fortunately, genetic engineering provides new avenues to address these issues and has the potential to revolutionize the gene therapy field, not least in PD [13].

METHODOLOGIES TO ENGINEER THE CAPSID SURFACE

Genetic engineering, economical and accurate multiplexed gene synthesis [14], deep sequencing, and rapid production techniques [15] have opened up an entirely new avenue for AAV engineering. In a single round of *in vivo* screening, we can perform experiments that previously required years of directed evolution and iterative improvements [16].

With the new generation of AAV evolution, the capsid surface regions responsible for cell adhesion and infectivity can be accurately tailored for cell-type specificity or novel functional properties (Table 1). Such properties can be to traffic across the blood-brain barrier (BBB) [17] or transport retrogradely in axons [16, 18]. However, capsid engineering does not have to be limited to tropism or transport. The same approach can be taken to systematically modify each amino acid over the entire capsid and thereby potentially allow for the identification of immune-evading variants or capsid variants with increased packaging capacity (a significant limitation with wild-type AAV variants) [14]. However, increased packaging capacity may come with altered transduction efficiency and infectivity profiles, and further work is needed to answer these questions.

One AAV-evolution approach has led to the PHP.B/eB AAV variant, which show highly efficient transport over the BBB in C57/BL6 mice [17, 19, 20] (Fig. 1). While being a very useful research tool, it lacks clinical applications using systemic injections because it depends on the Ly6A receptor expression, which is not found in higher species (e.g., rats and primates) and is also absent in many other mouse strains [21]. However, recent publications utilizing PHP.B/eB have showed efficient transduction in the CNS in rats and nonhuman primates (NHP) following intrathecal injections [22, 23]. Due to this unfortunate revelation, the search is still on to find an equally potent capsid variant for systemic injections, suitable for clinical translation [24–28]. A promising development is the TRACER 9P801 capsid developed by Voyager therapeutic. At the time of completion of this review, this capsid was only presented at the American Society for Cell and Gene therapy meeting but shows significant improvement in BBB crossing and CNS transduction in the NHP [29].

We have taken a different approach in the BRAVE (Barcoded Rational AAV Vector Evolution) methodology of capsid engineering [16]. With this approach, we computationally devise novel capsid variants

Table 1
Novel AAV capsid variants to keep an eye on for research and clinical therapies in PD

Methodology	Capsid	Origin	Transduction target	Injection	Species used for screening	Other species tested	Noteworthy characteristics	Ref	Year
BRAVE	MNM008	AAV2	Retrograde infectivity of DA neurons	Direct	Sprague Dawley rats	Humanized rat	Retrograde transport to human DA neurons *in vivo*	[16]	2019
BRAVE	MNM004	AAV2	Retrograde infectivity of neurons	Direct	Sprague Dawley rats	Humanized rat	Similar retrograde efficiency as AAV2-Retro	[16]	2019
TRACER	9P31	AAV9	Neurons, spinal cord & heart	IV	C56Bl/6J & BALB/c mice	–	–	[27]	2020
TRACER	9P801	AAV9	Neurons, spinal cord	IV	NHP	–	Identified from screening in NHP	[29]	2021
iTransduce	AAV-F	AAV9	Neurons, spinal cord & astrocytes	IV	C57Bl/6J and BALB/c mice	–	Fetal-derived primary human neurons	[26]	2019
iTransduce	AAV-S	AAV9	Neurons, astrocytes & muscle	IV	C57Bl/6J and BALB/c mice	–	Peripheral organs	[26]	2019
SCHEMA	SCH9	AAV9	CNS, NSCs in the SVZ	Direct	C57Bl/6J, Cre & tdTomato mice	–	Efficiently transduces Purkinje Cells	[28]	2018
M-CREATE	CAP-B10	AAV9	CNS - neurons	IV	C57Bl/6J & Cre mice	Marmosets	Peripheral detartgeting	[24]	2020
M-CREATE	CAP-B22	AAV9	CNS - broad transduction	IV	C57Bl/6J & Cre mice	Marmosets	–	[24]	2020
M-CREATE	PHP.V1	AAV9	Brain vascular cells	IV	C57Bl/6J & Cre mice	–	Astrocytes (less efficient than PHP.eB)	[25]	2020
CREATE	PHP.eB	AAV9	Neurons & astrocytes	IV	C57/Bl6J mice	BALB/cJ & NHP	Limited to C57BL/6J mice	[19]	2017
CREATE	PHP.S	AAV9	DRG, cardiac & enteric neurons	IV	C57/Bl6J mice	–	Liver, lung, heart stomach	[19]	2017
Directed evolution	AAV2-retro	AAV2	Retrograde infectivity of neurons	Direct	C57/Bl6J mice	Macaque & rats	Efficient retrograde transport (not DA neurons)	[18]	2016
Rational design	AAV9-retro	AAV9	Neurons	IV & direct	C57/Bl6J mice	–	Similar retrograde efficiency to AAV2-Retro	[59]	2020
Rational design	T2 3Y+T+dH	AAV2	Neurons	Direct	Sprague Dawley rats	–	Increased transduction volume	[60]	2017
Rational design	AAV-TT	AAV2	Neurons	Direct	SD rats	C57Bl/6J	Transduces photoreceptors and astrocytes	[61]	2018
Rational design	AAV8 THR	AAV8	Neurons & Periphery	IV	C57/Bl6J mice	–	Decreased clearance with THR binding	[62]	2018
Rational design	AAV2.5	AAV2	Muscle cells	IM	BALB/c mice	Human	The only one used in a clinical trial	[63]	2012
DNA shuffling	AAV-DJ	AAV2/8/9	Liver (and CNS after direct injection)	IV	FWB mice	C57BL/6J	*In vitro* infectivity	[64]	2008
DNA shuffling	AAV-B1	Numerous	CNS, skeletal muscle & heart	IV	C57Bl/6J	Cat	Efficient in pancreas and lung	[65]	2016
Peptide insertion	AAV-AS	AAV9	CNS - neurons	IV	C57Bl/6J	Cat	Spinal cord	[66]	2016

Intranigral AAV delivery

Suitable capsids:
AAV2, AAV9, AAV-TT, MNM008

Applications in PD:
• Neuroprotection
• Disease modification
• *In vivo* reprogramming

Pros & cons:
+ low dose required
+ ease of target specificity
– requires stereotactic injection
– does not reach the striatal output region

Striatal AAV delivery

Suitable capsids:
AAV5, AAV9, AAV-TT, MNM008

Applications in PD:
• Neuroprotection
• Enzyme/dopamine replacement
• *In vivo* reprogramming

Pros & cons:
+ intermediate dose required
+ ease of target specificity
+ MNM008 variant can reach both SN and Str
– requires stereotactic injection

Intrathecal/glymphatic AAV delivery

Suitable capsids:
PHP.eB *(in C57/BL6)*, Clinical candidate missing

Applications in PD:
• Neuroprotection
• Disease modification
• α-synuclein knock-down

Pros & cons:
+ can reach large regions in the CNS
+ requires lower dose than systemic administration
– no clinical candidate vector available
– target specificity is challenging

Systemic AAV delivery

Suitable capsids:
PHP.eB *(in C57/BL6)*, AAV-F, Cap-B10,
Cap-B22, 9P31, 9P801

Applications in PD:
• Neuroprotection
• Disease modification
• α-synuclein knock-down

Pros & cons:
+ can reach the entire body
+ least invasive of all delivery routes
– requires a very large dose of vector in humans
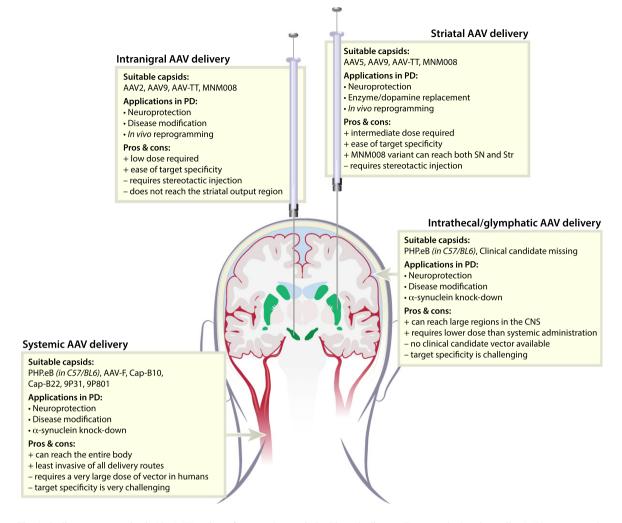– target specificity is very challenging

Fig. 1. Delivery routes and suitable AAV variants for gene therapy in Parkinson's disease. To target the basal ganglia, AAV vectors can be delivered through four different routes; Intranigral, striatal, intrathecal, or systemic. All routes have advantages but also present challenges. Fortunately, we now have engineered AAV-vector capsids suitable for most routes.

inspired by other viral proteins or endogenous proteins/ligands with desired functions such as cell type tropism or transportation characteristics. With this approach, we can be sure that the protein sequence selected will be efficiently synthesized in human cells, and the potential for being infective cross-species is increased. The same library can then be characterized across numerous model systems such as hESC-derived dopamine (DA) cells, organoids, transplanted human neurons in the rat brain, and many more [30, 31]. The readout from each model system can then be integrated to identify novel capsid variants to be highly efficient and translational to the human brain.

With this approach, we have generated (among many others) the MNM008 AAV capsid variant with

a unique capacity to reach DA neurons from the terminals (Fig. 1). However, the potential of the system does not stop there. It has the potential to allow us to identify capsid variants that could potentially traffic from the glymphatic system (through the intrathecal delivery route) [32] or over the BBB (after i.v. injection) [33] and home to the DA neurons. Thanks to the novel multiplexed gene synthesis approaches, such improvements can be made iteratively and systematically.

However, making such improvements will also require advancement in our ability to predict function and extrapolate from large sets of data. This is where recent advances in machine learning (ML) and machine-guided design can prove to be essential. In an initial exploration, ML has been applied to predict

capsid assembly with training data generated from AAV libraries [34]. In a very recent study by Bryant et al., ML has been used to construct AAV variants that can differ significantly from any known AAV serotype without losing the capacity to assemble and infect [35]. One area where such large changes are essential is immune evasion.

AVOIDANCE OF INNATE IMMUNE RESPONSES

The adaptive immune system with preexisting neutralizing antibodies and recruitment of capsid-specific cytotoxic T cells is only one hurdle for administration or re-administration of AAVs. Furthermore, studies also show varying levels of neutralizing antibodies to different serotypes, both in humans and NHP [12, 36].

More work on how AAVs can evade circulating antibodies is needed. However, several studies have addressed the question successfully and provided valuable information on the subject [37, 38].

The innate immune system can also recognize the AAV infectivity by triggering the Toll-like receptor 9 (TLR9) mediated by the AAV genome. Chan et al., have recently shown an exciting proof-of-concept for incorporating TLR9 inhibition into the therapeutic AAV genome itself [39]. This is achieved by using a short repeat structure inspired by mammalian telomeres with the motif TTAGGG. This small change alone was observed to significantly reduce the innate immune activation in both mice and pigs but with a smaller effect in NHP. While requiring further improvements before being clinically relevant it provides important insights into AAV immunity.

ENGINEERING THE AAV GENOME FOR TARGET SPECIFICITY

The engineering of the AAV capsid holds great promise for improving gene therapy efficacy in PD. Still, it will not be able to achieve every goal of specificity and regulation. Not every cell type or cell state will have an external representation on the cell surface which may be permissive to AAV targeting. Furthermore, as AAV-based gene therapy is irreversible in its current form, there are many scenarios where a regulated therapeutic approach is modulated, e.g., by inflammation state or apoptotic cascade.

Earlier attempts have focused on the inclusion of cell-type-specific promoters into the AAV-genome to drive the transgene expression. However, taking the promotor out of its genomic context and truncating it to fit the limited space of the AAV has led to disappointing results [40]. Specificity is usually low and unpredictable and can even change dramatically, e.g., from glial cells to dopamine neurons [41].

A promising alternative avenue is to incorporate short enhancer sequences upstream of minimal promoters. Recent advances in the sequencing of chromatin accessibility in bulk tissue and single nuclei have enabled us to accurately identify short enhancer elements with high cell-type and cell state specificity [42, 43]. Importantly, this mapping can be achieved at single-nuclei resolution also from human post-mortem brain samples [44]. The identified enhancer elements have been used to generate AAV genomes with high specificity for cortical neuronal subtypes [45]. It is highly conceivable that a similar approach can be taken to identify enhancer elements relevant to PD. Such vectors could sense both relevant dopamine neuron subtypes and disease state and only express the therapeutic transgene in the right cell at the right time and at a physiological dose. A major challenge will be translatability as chromatin state and enhancer function are not guaranteed across species and disease states. Humanized animals, accurate disease models, and efficient screening techniques will here be instrumental.

In scenarios where it is not feasible to restrict the therapeutic transgene expression through capsid or promoter engineering, improvements in delivery may provide improved target specificity instead. One such approach is targeted ultrasound where the BBB is transiently opened in a confined region of the brain to allow for passage of the AAV from the blood stream [46, 47].

TOWARDS CLINICAL UTILITY IN PD

To date, very few engineered AAV have been utilized in the clinic [48] but many are now on the horizon. AAV engineering has the potential to radically improve the on-target specificity and thereby reduce the dose needed and the immune response. The CRISPR toolbox is evolving at a very rapid pace, with exciting tools for gene perturbation arising. One such development is the CasRx RNA-guided RNA nuclease [49]. What this system allows for is the possibility for gene perturbations at the RNA level without the modification of the genome or the formation of unpredictable protein variants

formed by NHEJ (non-homologous end-joining). CRISPR perturbation has been shown to have a much higher on-target specificity than previously used shRNA/microRNA-based RNA-interference [49], which further strengthens its translational appeal. Recently, Zhou et al., achieved *in vivo* reprogramming of resident striatal glia into functional dopamine neurons through the CasRx mediated silencing of the PTB gene [50]. If this is reproduced in other modeling systems, it has intriguing potential.

Another area in clinical translation where engineered AAV vectors have great potential is in combination with cell therapy utilizing extrinsic cell sources such as ESCs or IPS cells. AAV vectors have the potential to facilitate accurate modulation of DA graft function to potentiate the therapeutic outcome [51], modulate any potential adverse events [52] and tailor the cells to avoid immune rejection [53]. In a future where autologous cell sources have become a reality (being it from *in vivo* reprogramming or transplantation), an important application of viral vectors is to correct any disease-causing gene changes (e.g., a-synuclein overexpression) in the host's cells [54].

Further in the future, engineered AAVs may become so safe, targeted, and efficient that we can start to explore them for neuroprotection and disease prevention in broader masses, even in pre-symptomatic PD or in people with increased risk of developing PD. While very early days, such therapies could target macroautophagy [55], slow down neuronal aging [56, 57] or even rejuvenate the dopamine neurons [58].

CONCLUDING REMARKS

Technological advances have had a remarkable impact on the AAV capsid development. The bar has been raised for what is possible to achieve using viral vector-based gene therapy, not least in the CNS. Engineered AAVs and genome editing are a perfect match for novel clinical therapies. We expect that we will see some of the herein presented capsids moving to the clinic for multiple indications, not least PD. While a challenging road ahead, the future is bright for gene therapy, and we will see many more capsids emerging with even greater potential.

CONFLICT OF INTEREST

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