

Review

How to Deliver Cas9 into Brain

Honghao Bi¹¹Department of Neurology, the Johns Hopkins University School of Medicine

How to deliver therapy into brain remains a headache-causing question for hundreds of years. Even with modern gene therapy, the delivery through blood brain barrier is still challenging. Here, I reviewed recent development in gene therapies, especially CRISPR/Cas9, and their delivery method into brain. AAV9 and exosomes are introduced as the two most promising tools. By comparing the two systems, some advantages and disadvantages of each system were talked. I also raised several possible new directions in gene delivery research.

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Introduction

Since Feng Zhang devised CRISPR/Cas9 system in 2012, people rapidly realized how powerful this genome-editing tool was[1]. Naturally, clinical gene therapy was gaining even more attentions. Several different general methods were proposed to insert genome-editing technology in gene therapy. Methods like directly editing embryo's genes would face enormous ethical challenge and potential risks[2]. Instead, editing patients' genes in non-germline cells by introducing CRISPR systems into target organs are more acceptable, both ethically and from risk considerations.

The introduction of CRISPR/Cas9 system into certain cells would require a safe and efficient delivery system. This challenge stimulated scientists' inspiration and ambitions. In the past few years, different strategies were developed to deliver CRISPR/Cas9 or other genome-editing tools into cells. The strategies are

generalized into two categories: viral and non-viral delivery system.

Viral delivery system takes advantage of viral infection ability and insert genome editing fragment into viral genome. The genome editing tools will be taken into cells as virus infecting cells and expressed in the cells, which in turn will edit target genes. Common viruses used in delivery include Adeno-associated virus (AAV) and Lentivirus. Viral delivery system was devised under the idea "one and done" treatment style. Instead of multiple injections in non-viral delivery system, viral delivery system tried to achieve the treatment goal by one shot. Supposedly the virus would replicate and infect human cells and finally achieve our goals.

On the other side, non-viral delivery system used chemical or physical measures like electroporation, microinjection, or nanoparticles to introduce DNA, RNA or proteins into target cells. Comparing to viral delivery system, non-viral delivery system has less safety concerns and larger capacity.

* Correspondence: Honghao Bi, The Johns Hopkins University School of Medicine, Department of Neurology. E-mail: bihonghao@gmail.com.

The delivery to central nerve system (CNS) raised additional challenges due to the existence of blood brain barrier (BBB). AAV becomes the most prominent virus used in brain gene editing, as it is capable of crossing BBB. The non-viral strategy usually requires special measures like brain injection or pre-natal processing[3-5]. Such operation causes obstacles in future clinical usage. In my personal opinion, breaking through BBB with physical force or deliver before BBB is fully developed does not count as going through BBB. This leaves AAV our only candidate to penetrate through BBB.

However, I do want to talk about exosome as a potential measure to deliver CRISPR/Cas9 into brain. Exosomes are 30-100nm vesicles secreted by all kinds of cells. The small vesicles can bear limited number of molecules including proteins and miRNAs. A hybrid of exosome and liposome was proven capable of delivering larger fragment like CRISPR/Cas9 system into mesenchyme stem cells (MSCs)[6]. Exosomes are also known to regulate brain functions when taken up by neurons. Taken together, it is reasonable to consider exosome as a potential candidate to deliver gene-editing tools to brain.

Adeno-associated virus (AAV) delivery and gene editing therapy

AAVs are single strand DNA dependoviruses that only infect human with the existence of viruses like adenoviruses or herpes simplex viruses (HSVs). Without the helper viruses, AAVs would go through a lysogenic cycle and integrate its genome fragment into Chromosome 19[7,8]. Under the lysogenic cycle, AAVs have limited gene replication and expression, which makes it with low infectivity and safe as delivery system.

Invasive administrations like Intraparenchymal (IPa), Intrathecal (IT) or Intracerebroventricular (ICV) were used in the earlier times and successfully delivered genes into CNS. Later, intravenous (IV) delivery of AAVs was proven capable to deliver genes into CNS, providing AAVs

capability to cross BBB[9,10].

The idea of delivering genes into CNS by systemic injection is certainly attracting. Among the 12 serotype AAVs, AAV2 is most studied and has been used in multiple clinical trials, while AAV9 displayed the greatest ability to penetrate BBB[11,12]. By systemic injection, AAV9 is observed with the highest distribution in CNS, as well as tropism in liver, heart and skeletal muscle. Although AAVrh.10 was also reported later to have extensive distribution in brain, AAV9 remains the most studied AAV in CNS delivery. IV (intravenous) injection or rAAV9 (recombinant AAV9) is capable to rescue most of ASPA deficiency by delivering *ASPA* gene into CNS[13,14]. IV rAAV9 mediated therapy for spinal muscular atrophy has also been entered into clinical research and showed promising results[15].

The distribution of AAVs is determined by the penetration of AAVs and its distribution in the system. Besides increasing the transduction strength, research into decreasing peripheral accumulation and off-target toxicity was conducted in parallel. Strategies including tissue specific promoters, miRNA inhibition by inserting miRNA recognition site in 3' UTR, and capsid engineering have been used to decrease peripheral targeting activity. Recently, Wang etc. reported a capsid engineered AAV9 that can significantly reduce transduction in peripheral tissues[16].

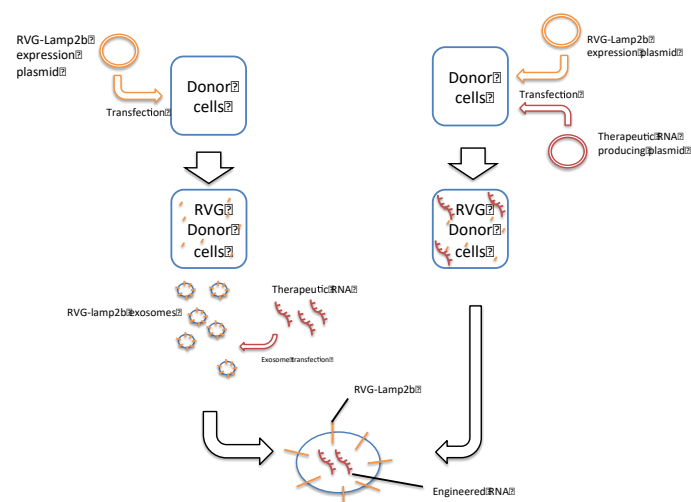


Fig.1 Methods to deliver RNA cargoes into exosomes. The RNAs can be delivered either by exosome transfection or through natural method by expressing therapeutic RNA in donor cells.

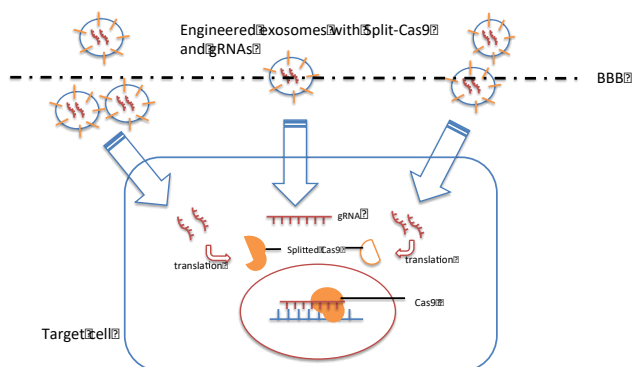


Fig 2. Split Cas9 delivered into CNS by exosomes

The idea to treat CNS disease in one IV injection is irresistible, although certain disadvantage cannot be ignored in AAV mediated therapy. The maximum capacity of AAV is around 4.7kb, which limits its usage in gene editing therapy (spCas9 is 4.2kb). Also, AAVs showed a delay in gene transduction. Because the AAVs need a second-strand synthesis, the vector would need ~2 weeks to reach maximum expression. Although it could be corrected by using double stranded AAVs, the double strand method would in turn reduce capacity to 2.2kb[16]. Also, AAV9 distributes in multiple tissues besides CNS, and its accumulation in CNS is largely determined by total viral load and organ distributions. This would inevitably cause non-specific toxicity in systemic injections.

Exosome mediated gene delivery

Exosomes are small extracellular vesicles secreted by all kinds of cells in human body. Initially people thought exosomes were just garbage, but the following researches showed that exosomes function as transporters between cells carrying various cargoes both inside and on the surface of the vesicles[17]. Besides the lipids and proteins carried on its surface, exosomes also contain macromolecules like lipid, protein and RNA. The composition of exosomes cargo reflects their parental cells state. The secreted exosomes can selectively enter certain cells by endocytosis through certain cell surface receptors. In a recent report, exosomes endocytosis was shown mediated by cell filopodia. The filopodia “grab and drag” extracellular exosome to the endocytic hot spot on cell surface and internalize the exosome. After internalization, the exosomes mainly move and stop in ER region, suggesting its function in post-transcriptional regulation[18].

Exosomes are exempt from immunity, opsonins, coagulation factors, and capable to penetrate BBB. All the characteristics make exosome a perfect tool for drug delivery into CNS. By incubation, electroporation, or sonication, drugs can be delivered into exosomes and administered as exosome vehicle drug. For example, co-incubation of curcumin with exosome can produce curcumin-exosome, which was proven to alleviate cell junction proteins[19]

The RNA cargo of exosomes can be delivered either by exosome transfection or natural pathway (**Fig. 1**). Exosomes are natural miRNA carriers protecting miRNAs from RNase[20,21]. As miRNAs are gaining attentions in gene therapy, scientists began to engineer exosomes into miRNA delivery tools. Ohno etc. reported that by engineering exosomes to carry GE11 or EGF (epidermal growth factor), proteins specifically combining to EGFR (epidermal growth factor receptor), miRNA let-7a was successfully delivered into breast cancer cells and inhibit tumor growth in vivo[22].

Table 1. Current clinical trials with AAV9 (Data is from ClinicalTrials.gov)

ClinicalTrials.gov Identifier	Study Title	Conditions	Interventions	Administration route	Starting date	Phase
NCT03770572	Gene Transfer Study of AAV9-CLN3 for Treatment NCL Type 3	CLN3-Related Neuronal Ceroid-Lipofuscinosis	AAV9-CLN3	IT	10-Dec-18	Phase1, Phase2
NCT03952637	Intravenous Gene Transfer With an AAV9 Vector Expressing Human α -Galactosidase in Type II GM1 Gangliosidosis	Lysosomal Diseases	AAV9-GLB1	IV	16-May-19	Phase1, Phase2
		Gangliosidosis				
		GM1				
NCT03882437	Gene Therapy for Male Patients With Danon Disease Using RP-A501; AAV9.LAMP2B	Danon Disease	RP-A501	IV	20-Mar-19	Phase1
NCT02240407	Re-administration of Intramuscular AAV9 in Patients With Late-Onset Pompe Disease	Pompe Disease	Recombinant Adeno-Associated Virus Acid Alpha-Glucosidase	IM (intramuscularly)	15-Sep-14	Phase 1
NCT02725580	Batten CLN6 Gene Therapy	Batten Disease	scAAV9.CB.CLN6	IT	1-Apr-16	Phase1, Phase2
		CLN6				
NCT03381729	Study of Intrathecal Administration of AVXS-101 for Spinal Muscular Atrophy	Spinal Muscular Atrophy	AVXS-101	IT	22-Dec-17	Phase 1
NCT02122952	Gene Transfer Clinical Trial for Spinal Muscular Atrophy Type 1	Spinal Muscular Atrophy 1	AVXS-101	IV	25-Apr-14	Phase 1

Besides miRNA, siRNA was also proven eligible to be delivered by exosomes. Alvarez-Erviti etc. used electroporation to load siRNAs into modified exosomes and successfully knocked down target genes in specific CNS tissues. The modified exosomes were equipped with CNS specific RVG-Lamp2b (rabies viral glycoprotein fused Lamp2b protein, Lamp2b is a membrane protein of exosomes), which shows highly selection to accumulate exosomes in CNS[22].

Due to strict limit of load volume, exosomes were usually considered not capable to carry CRISPR/Cas9. Lin etc. used exosome-liposome hybrid nanoparticles to contain CRISPR/Cas9 plasmid and successfully delivered CRISPR/Cas9 system in mesenchymal stem cells (MSCs)[6]. It is still not reported whether this hybrid nanoparticle can deliver CRISPR/Cas9 system into brain.

Future directions

rAAVs have shown promising potential in delivering genes into CNS, with AAV2 and AAV9 already entered into clinical trials (**Table 1**). Exosomes, on the other hand, are now a new star in CNS drug delivery.

Besides the advantage of less risk in immunity reactions, exosomes also show highly tissue selection by fusing different signals with Lamp2b on the membrane. The highly specificity provides treatment method with less potential side effect and non-specific toxicity.

However, the small loading size seriously limits exosomes use in gene editing therapy. Methods like

exosome-liposome seem to be a good idea to overcome this disadvantage, although it is not sure whether the large vesicle can cross BBB.

Besides increasing load size, a split-Cas9 system reported in 2015 might also provide a solution. The system used split and fused Cas9 protein with FK506 binding protein 12 (FKBP) and FKBP rapamycin binding (FRB) domains. Such design was intended to control Cas9 activity, but it would also reduce Cas9 plasmid size[23,24]. It is possible to use the split version Cas9 in exosomes and perform gene editing in the brain (**Fig. 2**).

The combination of different technologies, like combined use of rAAV9 and exosomes, is also an interesting topic. Providing that rAAV9 can bear Cas9 and exosomes can target specific organs, combination of two different tools might lead a good strategy to conduct gene editing in brain. For example, using AAV9 to express Cas9 protein in the brain and later transducing guiding RNAs with exosomes specifically in brain. Or use exosomes to deliver miRNAs or siRNAs inhibiting AAV9 in peripheral organs.

Conclusion

AAVs and exosomes are the two most prevalent tools used in gene therapy delivery. AAVs are especially attractive for its one shot and all effect. The replication of virus would finally reach a maximum effect of treatment. However, AAVs also face challenge in safety and non-specific toxicity. Exosomes, on the other side, can overcome the disadvantages of AAVs, but are difficult to prepare and require multiple shots to achieve treatment goal. Both tools show great potential and we expect more tools developed in the future.

Acknowledgement

None.

Conflict of interest

None.

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