Current Advances in Vehicles for Brain Gene Delivery

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Abstract: Gene therapy is a novel and promising treatment strategy for brain diseases. Yet, its development is largely obscured by various *in vivo* transport hurdles, especially the special BBB structure of brain. Developing an ingenious delivery vehicle can provide a great solution. Conventional vehicles for brain gene delivery are viral and non-viral vectors. With inherent superiority of gene transfection, researches on viral vectors are mainly focused on problems of brain cell targeting and global brain delivery. Non-viral vectors are more studied for better brain cell entrance either directly delivered to brain or systemically delivered to the body. Novel vehicles are cell vehicles (genetically engineered or nanoparticle-carrying cells) and exosomes. They exhibit distinct and unique features compared to viral and non-viral vectors. This review gives a summarization of current advances in these four kinds of brain gene vehicles. The merits and demerits of them are also pointed out respectively. We are hoping to give a clue to the future development direction of vehicles for brain gene delivery.

Keywords: Brain gene delivery, cell vehicles, exosomes, non-viral vectors, vehicles, viral vectors.

1. INTRODUCTION

Brain diseases (e.g. glioma, stroke, Alzheimer's disease, *etc*.) are usually devastating because of the brain's central role in body. Besides conventional therapies such as chemotherapy, radiotherapy and surgical operation, gene therapy has emerged as a novel treatment strategy for brain diseases. In gene therapy, genetic materials or silencing nucleic acids (e.g. siRNA, miRNA) are introduced into cells. They take effect on certain signal pathways and specific targets either directly [1] or after being expressed [2, 3], and then the disease progress can be selectively slowed and reversed. Compared to other therapy methods, gene therapy has a high selectivity for specific molecular targets in diseases. From 1989 to 2010, more than 1700 clinic trials of gene therapy had been initiated, and nearly all kinds of common brain diseases were covered. As for Parkinson's disease (PD) alone, 11 gene therapy methods have already been translated into clinical trials [4]. With further understanding on molecular mechanisms of various brain disorders, gene therapy would play an extremely significant role in brain disease.

Though promising, the development of gene therapy for brain disease is still largely hindered by the transport to brain. Similar to chemical drugs, a barrier for gene delivery to brain is the low permeability of brain blood vessels due to the BBB (Blood Brain Barrier). BBB is formed primarily by tight junctions of the cerebral capillary endothelium and surrounding perivascular elements, restricting the influx of molecules from blood stream into brain [5]. It is impermeable to approximately 98% of small molecules and nearly all large molecules including nucleic acids [6]. Besides the BBB, other biologic hurdles like *in vivo* degradation by nuclease, immune clearance, difficulty of cell entry and off target deposition also array on the road of effective brain gene delivery [7]. Moreover, different brain diseases require different delivery regions. For example, in Alzheimer's disease with entire brain lesions, global brain gene delivery is necessary, but in glioma, delivery to a restricted brain region is preferred. To address all challenges mentioned above in brain gene delivery, a forceful strategy is to develop an ingenious delivery vehicle.

Vehicles employed in brain gene delivery at present are various, and everyone has its own peculiarity. The differences are in sizes (nano [8], micro [9], *etc*.), materials (synthetic [10], biologic [11]), shapes (globular [12], rodlike [13], *etc*.), delivery routes (brain injection, intravenous injection, *etc*.) and so on. These lead to distinct *in vivo* behaviors and different applications. Conventional vehicles for brain gene delivery include viral and non-viral vectors. There also come the relatively novel ones consisting cell vehicles and exosomes which exhibit unique merits. This review will focus on recent advances of conventional and novel vehicles in brain gene delivery. Also, we will try to provide some clues for the trend of their future development.

2. VIRAL VECTORS

Viruses are natural biological gene delivery vehicles. They possess inherent superiorities of gene transfection especially high transduction owing to the evolution for steering virus genes to host cells [14, 15]. They have been studied for a long time and are relatively mature vehicles for gene delivery. Accordingly, they are the most widely used vehicles in

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clinic trials of brain gene therapy now [4]. In a phase I trial, intraputaminally injected AAV2s were used to deliver neurturin for the treatment of PD. The procedure was well tolerated, and no clinically significant adverse event was monitored in all patients at 1 year. Motor function was significantly improved. The initial data supported the safety, tolerability, and potential efficacy of AAV2 as vehicles for brain gene delivery [16]. Another clinic trial for PD also used subthalamically injected AAV2s to deliver glutamic acid decarboxylase (GAD). Good results about AAV2s were obtained again [17]. Even if many viral vectors are proved to be safe and effective, there are also numerous preclinical studies on viral vectors for better brain delivery. Adenovirus [18], adeno-associated virus (AAV) [19, 20], herpes simplex virus (HSV) [21], and retrovirus [22] are the most common viral vectors in researches about brain gene delivery (see Table **1**). With regard to brain gene delivery, researches on viral vectors recently are mainly focused on two objects. One is to achieve brain cell targeting, and the other is to achieve global brain delivery when transduction to the whole brain is needed.

2.1. Brain Cell Targeted Delivery

A main hurdle for brain gene delivery is how to get to target brain cells without affecting other tissues and cells. Some viral vectors have particular tropism. For example, AAV2 would selectively transduce neurons after brain infusion [23], while, rAAV4 prefers ependymal cells, and rAAV5 exhibits a broader transduction spectrum including astrocytes and neurons [24]. Whereas, they can also transduce various cells since receptors for viral endocytosis are not uniquely located on brain cells. Most systemic delivered viral vectors will firstly accumulate in liver [10]. To solve this problem, modification of viral vectors is required [25]. Since viral vectors are biologic, nearly all kinds of modification are finished with genetic reconstruction [26]. Generally, genetic reconstruction of viral vectors is either on structural genes or genes related to transgene expression.

It is known that different viruses have distinct tropisms for cells and tissues. The distinct cell tropisms are related to moieties on the capsids or envelops. Thus, reconstruction of surface moieties could be an easy choice to get specific transduction (Fig. **1.B**). Only when surface moieties match with receptors on cells can transduction happens. For example, Anliker *et al.* [27] fused coding sequences of scFvs specific for neuronal AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) glutamate receptor subunits GluA2 and GluA4 to the reading frame of lentiviral surface proteins. Adding targeting moieties artificially, lentiviral vectors could achieve more than 94% specificity for neurons when injected into adult mouse brain. For some virus, it is the original surface moieties that lead to the off-target deposition. Thus, modification of the original surface moieties can be essential. Researches revealed that adenovirus serotype 5 has preferential infection of astrocytes to a greater extent than neurons. It is a result from surface fiber proteins binding to CAR (coxsackie and adenovirus receptor) expressed on astrocytes and other non-neuronal cells [28]. Substantia nigra pars compacta (SNc) has a relative paucity of CAR expression. Dopamine neuronal transduction in SNc is important for gene therapy of PD. Thus, alteration of surface fiber proteins is rational. On the whole, genetic reconstruction on structural genes of viral vectors can get tropism-modified vectors thus achieving transductional targeting. Transductional targeting can avoid ubiquitous deposition of viral vectors at the first step after entering the body.

Genetic reconstruction on genes related to transgene expression is another aspect of brain cell targeting. The most common approach is employing a specific promoter or enhancer element to obtain different gene transcription in pathologic and physiological cells (Fig. **1.C**). Regev *et al.* [29] employed the 5'- flanking region of the corticotropin releasing factor receptor type 2 (CRFR2) gene as the choroid plexus specific promoter in lentiviral vectors. Transgenes could not be expressed in neurons or glial cells except in the choroid plexus cells. As cell biology and pathology of brain diseases progress, more intelligent promoters would be resorted for transcriptional targeting. Yet, loading capacity of virus is limited and sometimes specific promoter or enhancer element is hard to find. To overcome the potential obstacles, new approaches other than transcriptional targeting have been explored. The technique of gene silence with miRNA is a good example (Fig. **1.D**). Off-target organs or cells can express specific miRNA themselves. If transgenes are coupled with complementary sequence of selected miRNA, transcriptional product of transgene in off target cells can be degraded. Xie *et al.* [30] demonstrated that if complementary sequence of miR-1, miR-122 were packaged into the rAAV9 genome, endogenous miR-1 in the heart and miR-122 in liver could effectively silenced transcriptional products of transgenes. This is a new method for "post-transcriptional targeting" of viral vectors. Both transcriptional targeting and post-transcriptional targeting are achieved with genetic reconstruction of genes related to transgene expression. Transcriptional targeting can obtain brain cell targeted transcription, while post-transcriptional targeting can get brain cell targeted transgene expression.

2.2. Global Brain Delivery

Viral vectors are commonly injected or infused directly to brain since the majority of viral vectors can't conquer the BBB with systemic delivery. Nevertheless, transduction is limited to the injection site and direct delivery is invasive to brain tissue. It is an impediment when gene transduction to the whole brain is inclined in circumstances such as lysosomal storage disorders [31] and leukodystrophies [32]. Thus, global brain delivery is a pursuit in virus mediated brain gene delivery. Multiple injections [33] and convection enhanced delivery [34] are usually chosen at present to expand the distribution of viral vectors, but they are still invasive and need skillful surgical technique. Other powerful and witty solutions to this problem are appearing continuously.

Since the BBB is an interface that restricts the exchange of substances between brain and blood, a direct approach for global brain delivery is to find viral vectors which can cross over the BBB. A major leap forward in this field is the new use of AAV9. Foust *et al.* [35] first demonstrated that after intravenous injection of AAV9 into neonatal animals, extensive transduction could be achieved including dorsal root ganglia, motor neurons throughout the spinal cord and neurons throughout the brain such as the neocortex, hippocam-

Table 1. Examples of Viral Gene Delivery for Some Common Brain Diseases

Disease	Virus	Transgene	Delivery Routes	Ref
Stroke, Ischemic Brain Injury	AAV, rAAV, lentivirus, adeno-virus	GLT-1, BDNF, MMP-9 shRNA	ICV injection, right caudate putamen injection, intracerebral injection	[144-147]
Sandhoff Disease	rAAV2/1, Adenovirus	Hexosaminidase β	Striatum inection, intracerebral injections	[148, 149]
Gliomas	Adenovirus, retrovirus, bacu-lovirus, AAV2,	stTRAIL, mutated human IL-13, PDGF-B, p53 shRNA, HSVtk, hIFN-β	Intracranial injection, striatum injection, brain intratumoral injections, ICV injection	[150-155]
Parkinson's Disease	Lentiviral, AAV2, AAV9	GAD67 specific interfering RNA, neurturin, GDNF, domi-nant-negative TNF, EPO	Striatum injection or infusion, intraputaminal administration, putamen injection, intracranial injection	[156-161]
MPSI, MPSIIIA, MSPIIIB, MPSVII	AAV8, rAAV9, lentiviral, CAV-2	α-L-iduronidase, α-NAGLU, β-glucuronidase, N-SGSH	ICV infusion, intravenous injec-tion, ventricular injection, thalamus injection	[162-165]
Pain	HSV-1, rAAV2/6,	TH in antisense orientation, encephalin	Dorsal reticular nucleus injec-tion,caudal ventrolateral medulla injection, intrathecal delivery	[166-168]
Alzheimer's Disease	rAAV2/1, AAV2, Recombinant Sendai virus, adenovirus, HSV	Anti-Aβ scFv, mouse IL-4, Aβ1-43 and mouse IL-10, quadrivalent foldableAβ(1-15), 11 tandem repeats of Aβ1-6, APP shRNA, neprilysin	Intramuscular delivery, hippocampus injection, intrnasal delivery,	[169-174]

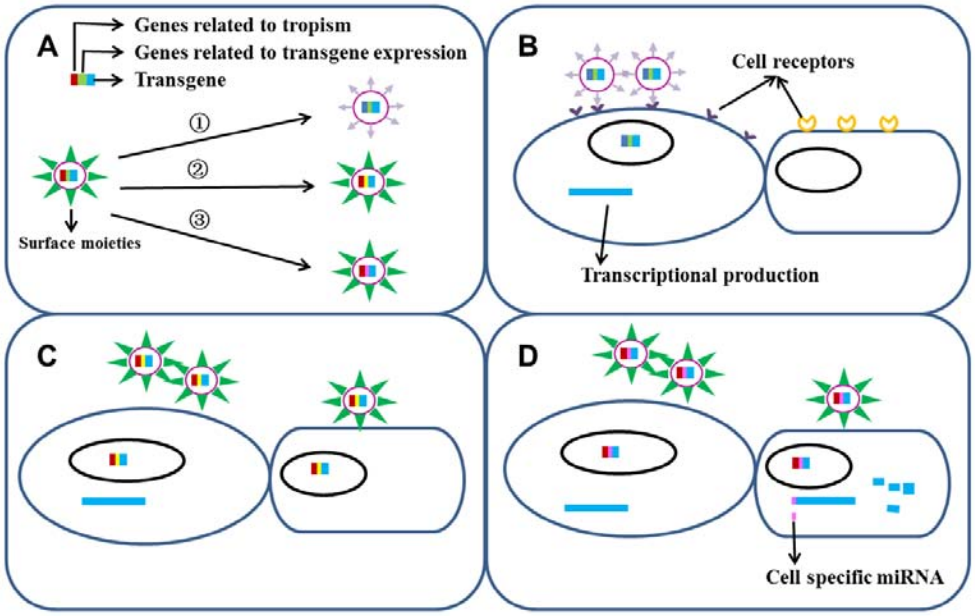

Fig. (1). Common reconstructions of viral vectors for brain gene delivery **A**, Genetic modification is often applied to genes related to tropism or transgene expression, ①Modification of genes related to surface moieties; ②Employment of cell specific promoters; ③Employment of complementary sequences of cell specific miRNAs. **B**, Transgenes will be delivered to target cells with certain receptors of surface moieties, and other cells will not be transduced since the receptors are not suitable. **C**, Transgenes will be delivered to cells indiscriminately, yet they will not be transcribed in non-target cells because of the lack of specific promoters. **D**, Transgenes will be delivered and transcribed indis-criminately, but cell specific miRNAs in non-target cells will mediate post-transcriptional gene silence.

pus and cerebellum. In adult mouse, neuronal transduction was only in hippocampus and dentate gyrus, but transduction of astrocytes was throughout the entire CNS. Further experiments proved that AAV9 could cross the BBB, but the exact mechanism needs more study. The capacity of AAV9 to transduce different types of brain cells in neonatal and adult mice may be result of developmental changes in the structure of BBB and brain extracellular matrix [36]. Similar conclusions could be seen in the work of Duque *et al.* [37]. AAV9 is the first viral vector that found to be able to cross the BBB and achieve non-invasive gene delivery to the whole brain [38]. Given the success of AAV9, it has been investigated in brain diseases where globe transduction is necessary, such as MPS IIIB and spinal muscular atrophy (SMA) [39, 40]. Besides, Gray *et al.* [41] created a library of chimeric AAVs with shuffled capsid DNA from AAV serotypes 1–6, 8, and 9. After directed evolution, they discovered two new chimeric AAVs (clone 32 and 83) which could transduce cells localized to the piriform cortex and ventral hippocampus. Further, these two new chimeric AAVs lost the parental tropism for non-CNS organs, such as the heart, lung, and liver. With the ability of crossing BBB, these vehicles could achieve widespread and brain specific transduction. Now, more viral vectors which can cross BBB and achieve widespread dispersal of therapeutic gene are still coming up [3, 42].

Another striking way for global brain delivery is to utilize brain capillary endothelial cells. To avoid confrontation with the BBB directly, transgene is integrated with endothelial genome. Then its expression products will be released continuously from endothelial cells. As endothelial cells are ubiquitous in brain, they can serve as cellular reservoirs for transgene throughout the whole brain [43]. To realize transduction of brain endothelia *in situ*, Chen *et al.* [44, 45] were the first to identify peptide motifs of high affinity for cerebral vasculature in diseased mice. After that, AAVs were modified with these specific peptide motifs. After peripheral injection, the epitope-modified AAVs distributed throughout the brain, and improved disease phenotypes in mice with MPS VII or LINCL (late infantile neuronal ceroid lipofuscinosis). The whole process is complicated and time-consuming, and it needs accurate understanding of the molecular signatures of vascular endothelia in diseased mice. With progress in molecular biology of brain endothelia and mechanisms of brain diseases, global brain delivery utilizing brain endothelia can be more practicable.

Besides finding ways concerning the BBB, some researches are striking out a whole new direction with the anterograde/retrograde ability of viral vectors. Anterograde transport makes transduction of cells in a region of brain that is not directly injected but receives axonal projections from the primary injection site target possible. In contrast, with retrograde transport, neurons away from the site of injection, but that directly innervate the site of delivery can be transduced. It is known that some viruses can undergo anterograde or retrograde transport in neurons [46-50]. Axonal transport is depended on serotype of viral vectors [51]. Kells *et al.* [46] showed putaminal delivery of AAV2 lead to anterograde transport in the primate brain with GDNF expression observed in the substantia nigra, and direct midbrain delivery of AAV2-GDNF lead to extensive anterograde transport to multiple brain regions and significant weight loss. Thus, anterograde can result to diffusion of viral vectors in brain [49]. Diffusion of viral vectors in brain with retrograde has also been proved to be possible [52]. Particularly, there were also studies demonstrating retrograde transport made gene delivery from peripheral nervous net to brain possible [53]. Given the ability of some AAV serotypes to transport along neuronal projections [54], Cearley *et al.* [55] studied if an injection into an area with multiple dispersed projections could allow distribution of therapeutic gene to a larger volume of brain. The results indicated a single 1 μl injection of AAV9 in the ventral tegmental area (VTA) resulted in distribution to many regions of the brain known to be projection sites of the VTA. Further, after a single injection of AAV9 into the VTA of MPS VII animals, complete correction of the storage lesions throughout the entire brain was achieved. Injection into a certain area is probably a potential new direction of global brain delivery [56]. It is a great improvement compared to multiple injections which needs more than 50 injection tracts to achieve widespread dispersal of a lysosomal enzyme in an infant brain [57]. However, this method is still invasive and delivery result will mainly depend on the neuronal net.

Viral vectors have been relatively mature and attractive vehicles for brain gene delivery. They have natural tropism for different brain cells, and they can be genetically manipulated for brain cell targeting in different levels. They are able to cross the BBB, inborn or after modification. After integration of viral genome to brain capillary endothelial cells, globe brain gene delivery can be achieved. Specially, viruses can undergo anterograde and retrograde transport in nervous net, bringing virus-specific administration routes to brain. Yet, they have other intrinsic limitations in the aspect of gene delivery as well. The biologic viral vectors need complex production procedures and it is hard to produce them on a large scale. Viral vectors may have limited packing size and not all therapeutic genes can be packed up. More seriously, viral vector may trigger immune response and they are proved to have potential genetic safety problem [58, 59]. For example, Mingozzi *et al.* [60] proved response of CD8(+) T cells to AAVs lead to the failure of transgene expression in humans. Li *et al.* [61] found that after retroviral infection in mice, leukemia was induced by the combination of insertional oncogene activation with signal interference evoked by transgene product. This statement was proved later in a clinic trial. Two leukemia patient were found after gene therapy of severe combined immunodeficiency-X1 (SCID-X1) with retroviral vectors [62]. Researches on these problems will certainly promote the development of viral vectors in brain gene delivery.

3. NON-VIRAL VECTORS

Non-viral vectors are popular in researches of gene delivery [63]. Lipofectamine™ 2000, a kind of liposome, is the most versatile transfection reagent and a standard for comparison of nucleic acid vehicles during most researches [64, 65]. Other non-viral vectors like polymer nano/microparticles, dendrimers, quantum dots, carbon nanotubes and *etc.* are also popular in researches of gene delivery. Nucleic acids could be physically adsorbed onto, entrapped into or

directly attached to vectors [66]. Different loading styles can get different release patterns *in vivo*. At present, most of these carriers could delivery gene to brain with either direct delivery or systemic administration.

3.1. Direct Delivery

Generally, a large portion of non-viral vectors get to brain directly with intracranial injection or infusion. Though invasive, direct delivery is convenient for dodging the BBB, avoiding the off-target effect and targeting brain regions selectively. Lacking the ability of anterograde and retrograde transport like viral vectors, it is harder for non-viral vectors to diffuse in the brain. Consequently, direct delivery is more seen in local brain diseases [67]. CED (convection enhanced delivery), using an external pressure gradient to induce fluid convection in the brain, is often adopted for a greater volume of distribution. It also prevents potential backflow of direct injection or infusion. Without the concern of getting into brain, after direct delivery, improving intracellular transport ability is the main strategy to promote non-viral gene delivery in brain. Since non-viral vectors are artificial, materials can be carefully chosen and vehicles can be particularly designed. Recently, carbon nanotube has been used to delivery siRNA into brain. It enters cells efficiently by different mechanisms [68, 69], such as phagocytosis, endocytosis, and nano needle mechanism (penetrate the plasma membrane following translocation), improving brain cell transfection efficiency. Al-Jamal [70] *et al.* illustrated that carbon nanotube could successfully deliver caspase-3 siRNA into neurons of the rodent motor cortex and led to protection against ischemic insult. More novel materials are tried to obtain a safe and effective gene delivery vehicle. Besides novel non-viral vectors like carbon nanotube, conventional non-viral vectors are also under improvements. Dendrimers are macromolecules with a regular and highly branched three-dimensional architecture. Agrawal *et al.* [10] combined polyamidoamine (PAMAM) dendrimers with magnetic nanoworms (cross-linked iron oxide nanoparticles) to form dendriworms. Convection-enhanced intracranial injection was chosen for this delivery. This new dendriworms enhanced endosomal escape than both nanoworms and dendriworms independently and induced a 2.5-fold stronger gene silence in human primary glioblastoma cells *in vivo* compared with commercial cationic lipids. The higher transfection efficiency mainly comes from the well-known "proton sponge effect". Positively charged dendrimers in dendriworms can lead to more effective endosomal escape, thus avoiding degradation of nucleic acids. Polyvalent conjugation of dendrimers onto an elongated magnetic nanoparticle host could generate a construct that would induce high "proton sponge effect". With more deep study on cellular uptake process of non-viral vectors, non-viral vectors with higher brain cell transfection efficiency will surely come up and non-viral vectors with direct delivery to brain will still occupy an important place in the near future.

3.2. Systemic Delivery

Systemic delivery is used more frequently for its non-invasive nature. Skillful surgical technique for direct delivery is not necessary any more. After systemic delivery, vehicles will soon undertake non-specific clearance by the mononuclear phagocyte system (MPS). Suitable size and masked surface can avoid fast clearance in the body and elimination by immune system. Carriers around 50-150nm are proved to possess a long circulation time *in vivo*. Choosing endogenous materials like protein for vehicle construction or PEGylation [71] of vehicles is often adopted to escape immune surveillance and extend circulation time.

Then these vehicles will confront the problem of how to penetrate the BBB and get into the brain. In fact, even with typical BBB, there is also a chance that a fraction of vehicles can squeeze through it. In some brain diseases, the BBB is compromised itself [72]. After artificial disruption of the BBB, the vehicles will accumulated passively in brain from cracks of the disrupted BBB. Now, more rational methods to open the BBB are coming up like focused ultrasound (FUS) [73, 74] and altering adenosine receptor (AR) signaling [75, 76]. It is a purely physic method of BBB disruption with FUS. FUS can enhance permeability of BBB by creating transient pores (sonoporation) on cells constructing the BBB. Microbubble/nanobubbles can significantly decrease the ultrasound intensity. Activation adenosine receptor (AR) on the BBB could lead to decreased transendothelial electrical resistance, increased actinomyosin stress fiber formation, and alterations in tight junction molecules. It is a biologic process of BBB disruption with AR activation. These new methods can incite dose-dependent and temporally discrete modulation of BBB permeability thus decreasing side effects of BBB disruption. Yet, non-viral vectors can also accumulate in other organs, and mere passive accumulation from compromised BBB is not sufficient. In brain gene delivery, magnetic particles are now often combined with BBB disruption for concentrated brain delivery [77]. For example, Liu *et al.* [78] combined FUS and magnetic guidance to deliver magnetic nanoparticles to brain. Magnetic vectors have the ability of brain targeting granted by external magnetic field [79-81]. With microbubbles, FUS temporarily disrupted the BBB and lead to influx of non-viral vectors into brain. Magnetic guidance increased deposition at brain synergistically. Combination of BBB disturbing and external magnetic force could greatly enhance the deposition in brain. While cytotoxic epirubicin was the cargo conjugated on magnetic nanoparticles in this research, the same nanoparticle has been used for siRNA delivery for brain tumor therapy *in vitro* [82]. Vehicles delivered with BBB disruption will surely get a large spread and deposition. Nevertheless, penetration degree of vehicles in brain will depend on many factors like time, the degree of BBB disruption and properties of vehicles [83, 84]. In conclusion, both BBB disruption and vehicle design should be paid attention when non-viral vectors get into brain with passive disposition.

In addition to passive disposition, non-viral vectors can also enter brain actively via receptor-mediated transcytosis [5]. Non-viral vectors can be functionalized with molecules that have certain receptors over or solely expressed on capillary endothelial cells. After specific binding with the receptors, they will be actively transported across the BBB. These common receptors on the BBB are transferrin receptors (TfR) [85], insulin receptors and transporters for low-density lipoprotein, leptin, and insulin-like growth factors. With the ability of active targeting, vehicles can get into brain even after systemic administration [86, 87], avoiding side-effects

brought by local administration directly and BBB disruption. Non-viral vectors with active targeting are attracting more interests in brain gene delivery now. Some receptors involved in BBB are also expressed on target brain cells [1]. With such ligands, non-viral vectors might be modified to accomplish both BBB crossing and cell targeting. For instance, low-density lipoprotein receptor-related protein-1 (LRP1) is expressed on brain capillary endothelial cells and glioma cells. Dendrimers conjugated with its substrate angiopep-2 can accomplish higher distribution in glioma [88]. Accordingly, when the ligand is only sufficient for BBB conquering, further modification is needed to improving brain cell targeting. Combination of different targeting ligands is an obvious choice for sequential targeting [89]. Ding *et al.* [90] employed anti-mouse mAbs to cross the mouse endothelial host system and anti-human TfR mAbs to target implanted human tumor cells. This system could realize higher brain accumulation compared with attaching only one kind of ligand. Compared to passive disposition, entering brain actively via receptor-mediated transcytosisis noninvasive and more efficient. Since modification of non-viral vectors is convenient, decoration of non-viral vectors with BBB conquering and brain cell targeting ligands will draw more attentions.

There are already many clinic trials which employ non-viral vectors as drug carriers for brain diseases. They haveless immunogenicity and little carcinogenicity, and they are easy to be produced on a large scale. Especially, the convenient modification of non-viral vectors makes them favored in brain delivery. With more elaborate design, they are becoming more and more intelligent and rational. Therapeutic genes like miRNA and siRNA need to be expressed after delivered with viral vectors, while they can be sent directly into brain via non-viral vectors straightly [91]. At present, non-viral vectors occupy a small proportion in gene therapy clinical trials, less than 15% [4, 92], and most involved are liposomes. For example, in a phase I/II clinical study, stereotactically guided intratumoral delivery of herpes simplex virus thymidine kinase (HSV-TK) gene bearing liposomal vectors and systemic ganciclovir were used to treat recurrent glioblastoma multiform [93]. The treatment was well tolerated and effective. Another phase I clinical trial for glioblastoma multiforme and anaplastic astrocytoma also proved the safety and efficiency of cationic liposomes [94]. The basic reasons for their unpopularity than viral vectors in clinical trials may be their low transfection efficiency and uncertain *in vivo* toxicity. With more gene delivery studies with non-viral vectors, they will surely proceed from bench to bedside for brain gene delivery owing to the apparent merits.

4. CELL BASED DELIVERY SYSTEMS

Cells are emerging as brilliant gene delivery vehicles during the past decade [95, 96]. Cell vehicles exhibit great potential for gene delivery. They are totally biocompatible and nontoxic, with no genetic insertion problem of viral vectors. Autologous cell vehicles can even exclude the problem of immune response completely. Cells can also prevent genes from degradation and serve as depots for sustained release of genes or their products. In addition, some cell vehicles exhibit therapeutic ability like immune regulation and tissue repair themselves thus enhancing the curative effects of gene cargos. All these lead cell vehicles to be an extraordinary strategy for brain gene delivery. Now, cells involved in brain gene delivery cells are mainly neural stem cells (NSCs), mesenchymal stem cells (MSCs), and macrophages. They are either genetically engineered or nanoparticle-carrying. These are two strategies for gene delivery.

4.1. Genetically Engineered Cells

In most researches now, cell vehicles are genetically engineered to deliver genes. That is, therapeutic genes are integrated into cell genome. Distinct to viral and non-viral vectors, cell vehicles have their own gene expression systems. Therapeutic genes can utilize the expressing system to achieve a long time release which is benefit for chronic brain diseases. Using vehicles' expressing system can prevent the possibility of incorrect gene inserting to brain cells from viral vectors, thus exhibiting a more secure system for gene delivery. Genes delivered with genetically engineered cells include cytokine, enzyme, secretable antibody, and *etc*. Engineered cells can provide a great choice for brain gene delivery, effectively and safely.

Currently, genetically engineered neural stem cells (NSCs) [97, 98] and mesenchymal stem cells (MSCs) [99-105] are mainly used for brain gene delivery. NSCs are progenitors of brain cells. They have the capability of tracking primary tumor and metastases [106]. The exact mechanisms of these tropisms are still under research. They are mainly used in gene delivery to glioma. Yet, sufficient NSCs are often got from fetal brain, adult brain or embryonic stem cells. The source of NSCs may cause logistic and ethical problems which give rise to deadly menace to the deeper researches. Another kind of stem cell MSC comes opportunely. They source from bone marrow, adipose tissue [107], umbilical cord blood [108], *etc*. MSCs also have intrinsic tropism for primary tumor and metastases [109]. Recent studies showed that MSCs are pericyte-like. They will localize primarily to perivascular niches within tumors. The fascinating "neoangiotropism" of MSCs could be exploited for antiangiogenic therapy of brain tumors. Yin *et al.* [110] wielded amniotic fluid derived MSCs to concurrently deliver genes coding endostatin and carboxylesterase. The pericyte-like MSCs could exert the inhibitory effects of endostatin on tumor angiogenesis to higher limits. Importantly, both NSCs and MSCs have the ability to cross the BBB [111]. Yong *et al.* [112] demonstrated that intracarotid given human mesenchymal stem cells (hMSCs) could successfully migrate to brain glioma. Besides NSCs and MSCs, other cells like macrophages [113], microglia [114], glial precursor cells [115] have also been engineered for brain gene delivery. Compared to stem cells, these cells are further differentiated and diminish the threat of certain differentiation from stem cells. In brain, cell vehicles can integrate well within the brain parenchymain a non disruptive manner [116]. As demonstrated by Hingtgen *et al*. [117], after implanted intracranially in mice together with glioma cells, mNSCs provided robust and stable delivery of TRAIL for at least 12 days. Postmortem immunohistochemical analysis performed 4 days postimplantation confirmed mNSCs remained in an undifferentiated state, and did not proliferate for a period. Danielyan *et al.* [118] showed after intranasal delivery into a rat model of Parkinson disease, 24% of MSCs survived

within the brain for at least 4.5 months. Thus, genetically engineered cells can integrate well with brain parenchyma and achieve a long time therapeutic release. With the development of molecular and cell biology, more suitable cells can be genetically engineered for brain gene delivery.

The most obvious deficiency of genetically engineered cells is that the kind of nucleic acids delivered is limited. Distinct from viral vectors, therapeutic nucleic acids will be expressed by cell vehicles. Hence, nucleic acids delivered with cells are often coding genes of secretory therapeutics. Nucleic acids like siRNA and miRNA have not been seen delivered with genetically engineered cells yet. Given the fact that cell can secrete nucleic acids out, genetically engineered cells that serve as reservoirs for siRNA and miRNA will emerge in future [119]. Further, genetic engineering of cell vehicles is tough and time consuming. Secondary gene vehicles like virus for the genetic engineering of cell vehicles are needed. With the development of genetic engineering technology, acquisition of genetically engineered cells can be easier. Cells can be further genetically engineered for better brain tropism, spatial and temporal-specific gene expression and so on.

4.2. Nanoparticle-Carrying Cells

Recently, researchers employ nanoparticle-carrying cell vehicles for brain delivery. Therapeutic genes are first loaded onto nanoparticles which are then carried by cells in some way. Along with movement of cells, genes can be delivered to disease sites. As combined vehicles, this delivery system can fully utilize the delivery advantages of different vehicles. Cells can shield particles *in vivo* and transport particles to destinations effectively. Particles can protect genes from degrading and they can be freed from cells afterwards. Distinct to genetically engineering of cells directly, this strategy can avoid complex genetic engineering process and relative genetic safety problems. Better than previous genetically engineered cells, nanoparticle-carrying cells can effectively deliver genes like miRNA and siRNA. This vehicle shows a bright future in brain gene delivery.

Nanoparticles can be loaded into cells in different ways and exhibit exciting therapeutic effects. Currently, the most convenient way is cell endocytosis. With the great ability of endocytosis and exocytosis, macrophages are widely employed for particle carrying [120]. Batrakova *et al.* [121-123] prepared antioxidant catalase loaded cationic nanoparticles with polyethyleneimine-polyethyleneglycol (PEI-PEG) which were then taken up by murine bone marrow derived macrophages (BMMs) after *ex vivo* cultivation. Actually, the cationic nanoparticles can prevent antioxidant catalase from the degradation in lysosome by "proton sponge effect". Nanoparticle loaded BMMs could slowly release active catalase over 20 days *in vitro*, and that is benefit for chronic diseases. After injected intravenously to mice with neuroinflammation, 2-fold increase in the amount of the antioxidant catalase was detected in the brain compared to injecting nanoparticles alone. This result could rely heavily on the movement of macrophages. Compared to the nanoparticles administered alone, this delivery system increased area under the curve (AUC), half-life, and mean residence time in blood circulation of antioxidantcatalase [124]. Besides macrophages, Roger *et al.* [125] used MSCs carrying lipid nanocapsules for glioma therapy. It proved that even if engulfing and liberating particles is a superiority of macrophages, other cells can also carrying nanoparticles by simple endocytosis [126]. The exact process remains to be further studied. In addition to direct cell endocytosis, nanoparticles can also be loaded to the surfaces of cell vehicles. This method can avoid potential cargo degradation inside cells and cargo-related side effect to carrying cells. Li *et al.* [127] modified silica nanorattles with antibodies which could recognize MSC specific membrane proteins CD73 and CD90. The antibody-bioconjugated silica nanorattles could be loaded to the MSCs by antibody–antigen recognitions or cellular endocytosis. The glioma targeting MSCs could then send doxorubicin to glioma effectively. Thus, particles can be loaded to cells in different patterns obviating the possibility that endocytosis and exocytosis abilities can be different in cells.

Despite the attraction of nanoparticle-carrying cells, issues of them are mostly still concealed. They are at the initial stage of development. Cargoes delivered to brain are mainly chemotherapy drugs, antibodies or enzymes. It might be related to hurdles of gene loading to nanoparticles and gene degradation. With advance in particle loading technique, nanoparticle carrying cells would certainly find a special nichein brain gene delivery. Further, influence of particles and cargoesto cell vehicles during and after the loading process is unknown. Different loading patterns can lead to different release of nanoparticles and genes. The exact results need deeper study. Only after detailed study of the exact release behavior of nanoparticles and genes, can the right particles, cell vehicles and loading pattern be chosen. Accordingly, in the future, cells carrying nanoparticles can be elaborately designed. Nanoparticles can be further modified for better gene loading and reservation. Cell vehicles can also be genetically manufactured for better brain delivery. No matter how particles and cells are modified, the ultimate goal is to fully utilize both particles and of cell vehicles for better gene protection, storage, and delivery.

5. EXOSOMES

Exosome is a kind of extracellular vesicles about 30-100nm in diameter (Fig. **2.D**), secreted from most cells under both normal and pathological conditions (Fig. **2.A**) [128]. As natural transporters of biologic signal substances including nucleic acids, researches show that exosomes can facilitate intercellular communication [129]. The naturally occurring exosomes are newborn gene vehicles between biologic and non-biologic ones. They are biocompatible and can be obtained from autogenous cells. The double membrane structure of exosomes is much like that of liposomes [130], whereas exosomes are derived from cells and totally biologic. Exosomes can fuse to recipient cells and unload nucleic acids directly into the cytoplasm (Fig. **2.B**). This makes them particularly useful for siRNA, miRNA and antisense oligonucleotide delivery. Derived from parent cells, exosomes would inherit cell specific molecules and exhibit similar self-tropism. Along with deeper comprehension of exosomes, their talent of nucleic acid delivery might provoke a great interest in the field of gene delivery.

Alvarez-Erviti *et al.* [131] are the first to apply exosomes for gene delivery (Fig. **2.C**). In this research, brain targeted exosomes were used to deliver BACE1 siRNA for Alzheimer's disease. Brain targeting was achieved by genetic engineering of parent cells to express brain targeted peptide RVG. During the process of biogenesis, membrane proteins on the surfaces of exosomes could retain the same topological orientation as on the cell surface (Fig. **2.A**) [132]. Fused with exosomal membrane protein Lamp2, RVG was localized on the external surfaces of exosomes. Exogenous siRNAs were then loaded into exosomes by electroporation. Compared with Lipofectamine 2000, exosomes could achieve more satisfying cell delivery efficiency *in vitro.* After intravenous administration, exosomes could achieve a strong mRNA (60%) and protein (62%) knockdown of BACE1 predominantly in the midbrain, cortex and striatum. The ability of exosomes to cross the BBB is much related to RVG mediated transcytosis. Besides, it has also been proposed exosomes can be internalized into the MVBs (multivesicular bodies) of recipient cells and then released again [133]. It may be another mechanism of exosomes to cross the multiple layers of the BBB. The exact mechanism needs more evidence. Overall, this research is a compelling proof of principle for the rosy prospects of exosomes in brain gene delivery.

As gene delivery vehicles, exosomes are in an initial stage. There are critical obstacles to the clinical translation of exosome mediated gene delivery. First, as an intermediator of signal transfer, exosomes possess their own biological functions. Despite an endocytic origin, contents of exosomes are not exactly the same with parent cells. The contents of exosomes from different cells or cells in different stages will be specific and distinct. The influence brought by the contents of exosomes should be considered carefully. It has already been approved that exosomes from murine mammary carcinoma [134], activated platelets [135] and glioblastoma [136] can promote tumor growth. The content and the biological function of exosomes are mainly depended on their cellular origin. Thus, choosing an appropriate and safe cell source is vital for exosomes mediated gene delivery. Second, a rational way of incorporating gene into exosomes is necessary. In the research of Alvarez-Erviti *et al.*, siRNA is loaded into exosomes and the electroporation efficiency was not satisfying. Unlike hydrophobic drugs like curcumin which can be physically entrapped into exosomes directly [137], gene loading is much harder. Given the lipid bilayer of exosomes similar to liposomes, it is conceivable to find a better loading strategy similar with liposome researches. Third, the abilities of brain targeting and BBB crossing are currently obtained by genetic engineering of the cells. Never-

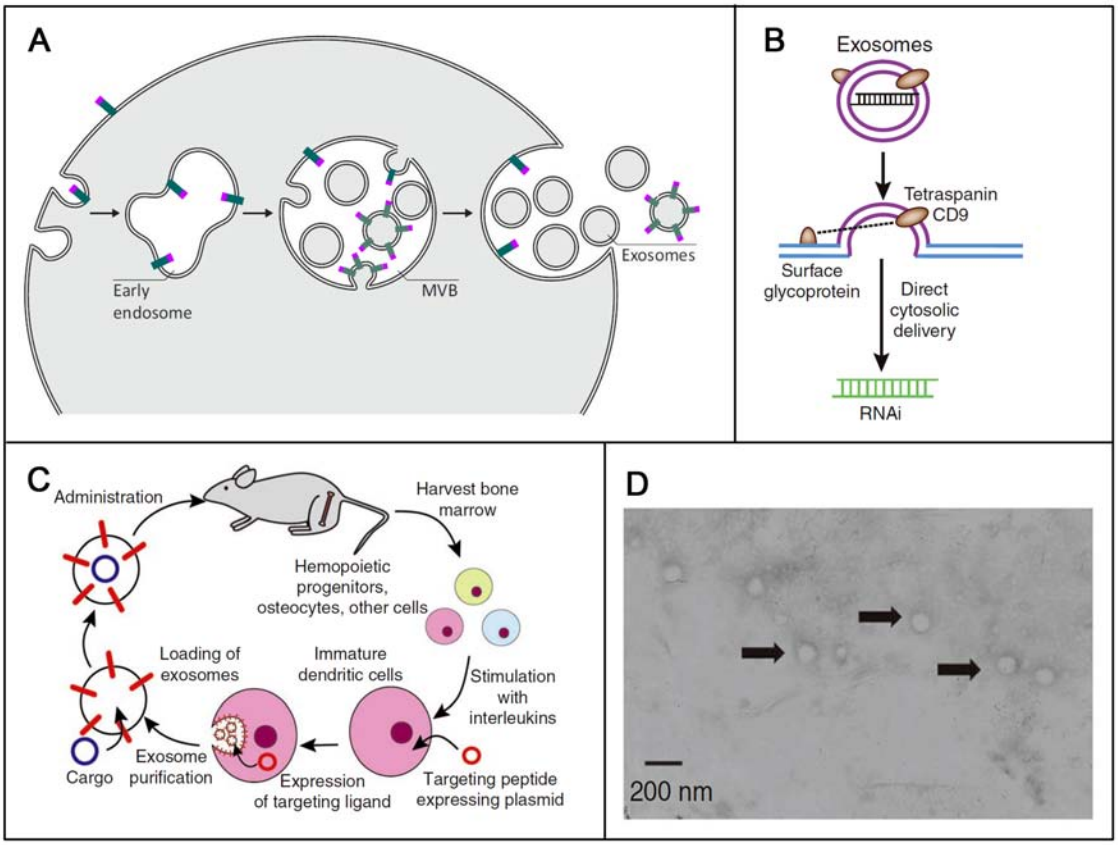

Fig. (2). A Biogenesis of exosomes: Exosomes are formed through three steps: first, early endosomes are formed by invagination of the plasma membrane; second, exosomes are formed by inward budding of endsomal membranes, giving rise to multivesicular bodies (MVBs); exosomes are secreted through the fusion of MVBs with cells. (modified from ref. [132]). **B** Process of exosomes entering cell: Membranes of exosomes are fused with cell membrane, and then cargoes inside exosomes are secreted into the cytoplasm directly [130]. **C** Diagram of research from Alvarez-Erviti *et al.* [131]. **D** Electronmicrograph of exosomes [131].

theless, it is labor-intensive and needs careful design in order to make targeting moieties localized on external exosomal surfaces. Fourth, preparation and purification for a large scale of exosomes are still waiting for more improvement. Current method of ultracentrifugation would induce a heterogeneous mix of exosomes, other vesicles and macromolecular complexes. Therefore, novel methods to obtain sufficient exosomes are required, possibly based on exosome specific markers or else. On the whole, further understanding of exosomes would greatly accelerate development of exosomes for gene delivery and provide inspired therapeutic potential for brain diseases.

6. CONCLUSION AND FUTURE PERSPECTIVE

Gene therapy for brain diseases holds great promise. But gene delivery to the brain is extremely sticky due to the unique structure of brain, *in vivo* delivery hurdles and specific delivery demands of different brain diseases. In a sense, delivering naked nucleic acids to brain is also advancing with the progresses of nucleic acid design [138], delivery technique like electroporation [139, 140] *etc.* Yet, modification of nucleic acids may have effect on the efficacy of therapeutic genes, and bring more safety concerns. Electroporation technique can enhance cell membrane permeability with an external, pulsed electric field. But, damage to brain tissue from insertion of needles for injection or electroporation, toxic diffusion of external media into cells, extreme motor spasms by electric pulses could happen when electroporation is employed for brain plasmid delivery. Whereas, employing delivery vehicles offers much more extra advantages and is more flexible.

Up to now, vehicles appeared for brain gene delivery can be categorized into conventional viral vectors, non-viral vectors, novel cell based delivery systems and exosomes. Everyone has distinct advantages and limits in brain gene delivery (Table **2**). Viral vectors have high transfection efficiency. Some of them are neurotropic and can undergo retrograde transport to brain. It has been demonstrated that certain virus can cross the BBB. Yet, they are relatively unsafe, and the modification is time consuming. Improvements are often concentrated on objects of brain cell targeted delivery and global brain delivery. Through genetic modification of viral structure genes or genes related to transgene expression, brain cell specific transduction can be achieved. When globe brain delivery is demanded, approaches such as finding virus able to cross the BBB, utilizing the ubiquitous endothelial cells, and injection into certain brain area have been proposed. Non-viral vectors are easy to be modified to reach brain targeting, and can deliver most kinds of nucleic acids. However, low transfection efficiency hurdles their application. For directly delivered non-viral vectors, improving the transfection is the main object. While for those delivered systemically, strategies for more brain deposition are adopted. There also appeared the relatively novel vehicles, cells and exosomes. Cells are biocompatible and genetically safe. They show intrinsic tropism for disease site and great BBB crossing. More researches are necessary to understand their *in vivo* fate and behavior. Exosomes have just been not introduced for gene delivery until recently. They are derived from cell, whereas the structure is more like liposome. They seem to be ideal brain vectors being safe and effective, and they are a good supplementary for cells to send silencing nucleic acids. Whereas, exosomes are in an initial stage, and further comprehensive studies are needed.

On the whole, an ideal brain gene delivery system should meet the following primary requirements. It should be safe and biocompatible, and can protect genes from degradation effectively. Through the manipulation of vehicles, therapeutic genes can arrive at satisfying brain regions surmounting numerous obstacles and traps *in vivo*. High delivery efficiency and coveted release pattern are also wanted. To meet all these criterions, researches are turning to nature for inspiration. Some natural particulates are in born gene vehicles to brain such as Rabies virus. Some others like exosomes can circulate and steer genes to distant target cells. Substantial efforts have been taken to thrash out the key features of these natural carriers. With deeper recognition of the mechanism,

Table 2. Advantages and Limitations of Vehicles for Brain Gene Delivery

Vehicles	Advantages	Limitations	Ref
Viral Vectors	High transfection efficiency, intrinsic tropism, retrograde transport to brain, sustained expression of gene products, crossing the BBB	Immune response, threat of gene insertion, long time for gene expression, uneasy to be modified and manufactured	[14, 30, 35, 144, 151, 155, 164, 175, 176]
Non-Viral Vectors	Easy to be modified for brain targeted delivery, easy to be manufactured, no genetic safety problems, rapid to take effect	Low gene delivery efficiency, immune response and toxicity, fast clearance, short duration	[1, 10, 71, 80, 90]
Cells	Intrinsic tropism, biocompatibility, natural therapeutic ability, crossing the BBB, sustained release of gene products, no genetic safety problems	Potential threat of uncertain differentiation, uncertain biologic function of cells, uneasy to be modified and manufactured, inconvenient to deliver siRNA, miRNA or antisense oligonucleotide	[95, 96, 103, 109, 123, 126, 177]
Exosomes	Intrinsic tropism, biocompatibility, relatively high delivery efficiency, natural therapeutic ability, no genetic safety problems, appropriate to deliver siRNA, miRNA or antisense oligonucleotide	Uncertain biologic function of exosomes, uneasy to be modified and manufactured, immature technique of gene loading	[128, 129, 131, 137]

future brain gene vehicles can be reconstructed from natural vehicles or artificial mimics of natural vehicles. That is reserving the dazzling properties of natural vehicles 4and trimming off the otiose parts. With more findings of attractive natural gene vehicles like exosomes, bioengineering of natural vehicle can propel the development of brain gene vehicles greatly. It is known the unique shape, mechanical properties and markers on surfaces of red blood cells (RBCs) lead to the ability of circulating and delivering oxygen for a prolonged period of time. Doshi *et al.* [141] prepared synthetic polymer particles that resembled natural RBCs in size, shape, mechanical flexibility, thus obtaining the oxygen-carrying ability. There are also virus-mimicking liposomes able to accomplish tumor targeted DNA delivery [142], cell-like compartmentalized carriers capable of drug delivery [143]. The researches mentioned above are all from fields related to drug delivery. Vehicles for brain gene delivery can gain some useful apocalypses from them. Overall, we can conclude that future vehicles for brain gene delivery will be more and more bio-inspired whether they are bioengineered or biomimetic.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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