Gene Therapy for the Nervous System: Challenges and New Strategies

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Abstract Current clinical treatments for central nervous system (CNS) diseases, such as Parkinson's disease and glioblastoma do not halt disease progression and have significant treatment morbidities. Gene therapy has the potential to “permanently” correct disease by bringing in a normal gene to correct a mutant gene deficiency, knocking down mRNA of mutant alleles, and inducing cell-death in cancer cells using transgenes encoding apoptosis-inducing proteins. Promising results in clinical trials of eye disease (Leber's congenital amaurosis) and Parkinson's disease have shown that gene-based neurotherapeutics have great potential. The recent development of genome editing technology, such as zinc finger nucleases, TALENS, and CRISPR, has made the ultimate goal of gene correction a step closer. This review summarizes the challenges faced by gene-based neurotherapeutics and the current and recent strategies designed to overcome these barriers. We have chosen the following challenges to focus on in this review: (1) delivery vehicles (both virus and nonviral), (2) use of promoters for vector-mediated gene expression in CNS,

and (3) delivery across the blood-brain barrier. The final section (4) focuses on promising pre-clinical/clinical studies of neurotherapeutics.

Keywords Gene delivery · Central nervous system · Virus vector · Nonviral gene delivery · Gene therapy

Introduction

Gene therapy to treat human disease has seen its ups and downs over the past 20 plus years. Through a dedicated research effort, many of the challenges to success are being overcome and there have been several promising clinical trials showing efficacy. The commercialization of Europe's first gene therapy product is also a testament to this progress. Preclinical models are continually yielding enhanced technology. For gene therapy for CNS disease, clinical efficacy has been obtained in both eye disease as well as neurodegenerative disease. We will not attempt to review the entirety of clinical, preclinical, and basic research into nucleic acid-based neurotherapeutics. There are excellent reviews on each of these topics [1, 2]. Instead, in this review our goal is to provide the reader with (1) an overview of the barriers to efficient gene delivery to the central nervous system (CNS) and (2) the state of the art in virus and nonviral-based neurotherapeutics. Each section is divided into “current challenges” and “new strategies” subsections in which we first present major issues that have hampered successful gene delivery in the past and then provide specific examples of new technology and approaches to solve these major challenges. Unfortunately, due to space limitations, we can cite only a subset of the literature in each section and we focus primarily on literature in the past 5 years.

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Delivery Vehicles

Challenges

Efficient and specific delivery to a target organ, such as the brain, is a rate limiting step in gene-based therapeutics whether the delivery vehicle is a virus, cell-based, or synthetic. Depending on the pathology of the disease and cells affected, either focal or widespread gene delivery may be preferred. Here we summarize many of the current challenges to effective delivery vehicles. As much of what we have learned about the barriers to *in vivo* gene transfer comes from studies of virus gene transfer vectors, this section will focus mainly on studies involving virus-based gene transfer systems.

Delivery Route and Gene Transfer Efficiency to Target and Non-Target Cells

The locale at which a delivery vehicle is administered greatly impacts its ability to transfer its genetic payload to the CNS. Due to the constraints imposed by the blood-brain barrier (BBB), the most common delivery route is direct injection into the target region in the brain, which bypasses this barrier. Adeno-associated virus (AAV) vectors injected into the brain parenchyma primarily transduce neurons around the needle track [3]. Similarly, other vectors, such as adenovirus (Ad) [4], lentivirus [5], and herpes simplex virus (HSV) [6] exhibit local transduction around the needle with varying degrees of efficiency. Barriers to transport after direct injection into the brain include diffusion through the extracellular matrix [7–9] and availability of primary receptors of the virus [10] at the site of injection, which can limit diffusion and cell type entry. For gene therapy of brain tumors, the increased interstitial pressure within the tumor is also a significant barrier [11]. Transduction can be improved using convection-enhanced delivery [12, 13], viruses that move by retrograde or anterograde transport within neuronal processes or use of cerebrospinal fluid (CSF) flow to transduce neuronal bodies in distant structures [14, 15], and alternate vector serotypes [16] to give high, yet focal levels of transgene expression. Although focal delivery has its limitations, for certain organs such as the eye and ear, and for certain diseases with localized pathology, such as Parkinson's disease, it remains a viable option.

Other routes of vector administration seek to achieve more widespread delivery for diseases of extensive pathology including glioblastoma multiforme (GBM), lysosomal storage diseases (LSDs), Alzheimer's disease, and Canavan's disease. Routes include intracerebroventricular/intrathecal [17–19], intranasal [20], and systemic (intravenous and intra-arterial) injections [21]. The systemic delivery route for gene delivery vehicles is very promising, but has major caveats. On the one hand, the vasculature has the potential to be a conduit to the entire CNS (every cell in the brain is a maximum distance of

40 μm from an endothelial cell [22]), while on the other hand the BBB blocks transport of most delivery vehicles (see section below on [Blood-Brain Barrier](#)), and the filtration systems of the spleen and liver are efficient at removal of viruses and virus vectors from the circulation before they can reach the brain [23–26]. Other non-target organs, such as muscle and heart, will also be transduced with potential off-target toxicities.

Once the delivery vehicle is in the CNS, for gene therapy to be successful, the gene must be expressed in the appropriate cells. Different virus vectors have different natural “tropisms” or affinities for certain cell types, which depend on various factors separated into pre- and post-entry events. The major pre-entry event is binding to a cell surface receptor on target cells. Post-entry events include endosomal escape, trafficking on cytoskeletal proteins to the nucleus, and nuclear import of the viral genome. For instance AAV, HSV and lentivirus vectors are neurotropic [16, 27, 28], while Ad vectors transduce primarily astrocytes [27].

Immune System

The process of evolution has enabled viruses to efficiently infect their hosts. By the same token, mammals have evolved multifaceted mechanisms to remove viruses from the body. One of the most effective and powerful of these mechanisms is the innate and adaptive immune response. Innate immune cells, such as macrophage and liver sinusoidal endothelial cells, can internalize and degrade virus vectors [29]; pre-existing antibodies against the wild type virus (from which the vector is derived) can eliminate vector from circulation; and cytotoxic T cells directed against viral antigens [30] or the transgene product [31] can eliminate transgene-expressing cells.

The route of vector administration also impacts the level of the immune response on transgene expression in the CNS. For instance, vector administered via the bloodstream is most vulnerable to elimination by antibody neutralization and opsonization [32–34]. Direct injection into the brain parenchyma [35] or CSF [36] can achieve transduction in the presence of antibodies in at least some cases as the antibody concentration is much lower in these sites than in the blood. The intensity of the innate and adaptive immune response is vector type related. For instance, first generation Ad vectors stimulate a robust inflammatory response by the innate immune system in the brain, leading to CD8 T cell-mediated destruction of transduced cells in the brain [37]. AAV vectors have shown long-term transgene expression in the brain, but it has been reported recently that, at least in some instances, even this vector type can induce a cytotoxic T lymphocyte (CTL) response against vector-transduced cells expressing an antigenic transgene [38].

Of all the targets for gene therapy in the CNS, the eye is likely the most “immune privileged” and accessible, and excellent transgene stability and therapeutic efficacy has been reported here in preclinical models [39, 40] and clinical trials [41, 42]. Figure 1 portrays the current limitations imposed by the immune response and also shows new strategies (next section) being used to overcome this issue.

New Strategies

The field of gene therapy has made major advances in the past 3 decades. Early on it was hampered by lack of efficacy and some serious adverse events [43, 44], but recently, with efficacy in clinical trials and Europe’s first commercial gene therapy product, progress forward is rapid [45]. Learning from early problems in the clinic, gene therapists have engineered viral vectors to be safer and more cell-type selective. Non-viral (NV) vectors have also become more efficient at gene delivery [46]. The following section focuses on new strategies that are leading to even more improved neurotherapeutics.

Virus-Based Systems

One of the most exciting finds for widespread gene delivery to the CNS has been with virus vectors which can cross the BBB. In 2009, Foust et al. [21] reported that AAV serotype 9 (AAV9) vectors could cross the intact BBB in neonates and adult mice and yield “widespread” transgene expression in astrocytes, neurons, and endothelial cells. This was followed by reports showing that this finding translated to non-human primates (NHPs) [47, 48] and could be achieved with other AAV serotypes [49]. The term “widespread” to describe the transduction pattern of intravenously injected AAV9 may be confusing to those not involved in the field. First, widespread refers to the distribution throughout the brain and not necessarily the cell types transduced (at least with the promoters utilized thus far). The vast majority (>90 %) of cells in the CNS transduced by intravenously injected AAV9 using strong promoters like CBA (see section below on [Use of Promoters for Vector-Mediated Gene Expression in CNS](#)) are astrocytes and neurons, with the exact ratios of each differing between studies [21, 48]. The distribution of vector is widespread with

Fig. 1 a–c The immune system challenge for gene therapies of the central nervous system (CNS). **a** Pre-existing antibodies against wild type viruses in the periphery can recognize the vector and neutralize it to prevent receptor binding and trafficking to the brain. Also, antibody binding to the vector surface can lead to opsonization by phagocytes. **b** Cytotoxic T cell-mediated killing of cells (e.g., neurons) that present viral antigens or antigens of the transgene product. **c** New strategies include: *i.* synthetic gene delivery vehicle (e.g., liposome and plasmid DNA) with

no pre-existing immunity; *ii.* vectors for which there is little to no pre-existing immunity (e.g., bacteriophage); *iii.* immuno-evasion using immunogenic vectors that are shielded by compounds or synthetics [e.g., polyethylene glycol (PEG)] or endogenous particles [e.g., extracellular vesicles (EVs)]. Alternatively, the patient may be immunosuppressed with drugs before therapy to dampen the immune response; and *iv.* altered delivery routes to avoid high titers of antibodies in the blood

transduced cells detected in all areas of the brain from the olfactory bulb to cerebellum. Depending on the dose as well as the region of brain, the transduction efficiency can range greatly from sparse to high percentages of astrocytes and neurons, although most of this data is qualitative from images and not stereologically quantified. However, Foust et al. [21] reported that, in adult mice, 64 % of glial fibrillary acidic protein (GFAP)-labeled astrocytes were green fluorescent protein (GFP)-positive in the lumbar spinal cord after a high dose of 4×10^{12} genome copies (g.c.) of self-complementary AAV9-GFP after i.v. administration. In neonates in the same study, the transduction efficiency of neurons ranged from 14 % to 71 % depending on the brain region [21]. Another interesting report in 2010 by Louboutin et al. [50] showed that, after transiently opening the BBB with mannitol, recombinant simian virus 40 (rSV40) vectors could achieve transgene expression in approximately 30 % of neurons after intravenous delivery. Remarkably, the authors reported there was no liver transgene expression under these conditions [50].

For brain tumors, particularly GBM, oncolytic vectors have made substantial progress in recent years. The list of types of viruses used for oncolytic virotherapy is extensive (for review see [51]). The more well-known oncolytic vectors, HSV and Ad have undergone significant modifications to improve their use. To decrease neurovirulence several genes have been deleted in current generation HSV oncolytic vectors [51]. Oncolytic Ad vectors have been engineered with an integrin binding peptide, RGD, on their fiber protein to increase affinity of the vector for tumor cells [52]. For viruses with broad cell tropism, efforts have focused on making them more tumor specific. For instance, the oncolytic measles virus has been targeted to specific receptors to increase tumor selectivity [53]. A distinctive approach to glioma treatment has been through the use of a non-lytic replicating retrovirus vector [54]. In this system the retrovirus carries a prodrug activating gene (cytosine deaminase) and replicates and spreads throughout the tumor mass (leaving most healthy cells uninfected), followed by administration of the prodrug 5-FC to kill the infected dividing cells (which in the brain will be primarily tumor cells).

One recent and promising trend in virus vector improvement for *in vivo* gene therapy has been achieved by using directed molecular evolution via DNA shuffling. This technique, originally described by Stemmer in 1994 [55], involves cutting a mixture of homologous cloned genes using DNase I and then reassembling the mixture using error-prone PCR. The resulting library contains full-length variants of the original gene with diversity created by point mutations from the PCR, as well as novel recombinations of the homologous genes. The next step is to submit the library to *in vitro* or *in vivo* selective pressure to isolate virus clones with a particular phenotype (e.g., immune evasion). This approach has been applied successfully with different virus vectors

including retrovirus and AAV [56–59]. Recently, several studies using directed molecular evolution have selected virus vectors useful for CNS gene therapy for glioma [60], seizures [61], and lysosomal storage disorder (LSDs) via glia [62] and for modification of neural stem cells (NSCs) [63]. Vectors that can evade neutralizing antibodies have also been selected using DNA shuffling [57, 64]. Use of RNA aptamer libraries and selection for tumor cell entry has proven effective for delivery of siRNA to tumors [65].

Another useful approach, which is in principal very different from the “unbiased” selection of directed molecular evolution, is the so called “rational design” method. In this case known targeting ligands are engineered into the capsid of non-enveloped virus vectors or into the glycoprotein domains of enveloped virus vectors to achieve cell specific targeting. Using receptor-specific single-chain antibodies fused to a truncated hemagglutinin envelope protein, Anliker et al. [66] achieved highly specific *in vivo* transduction of neurons with a lentivirus vector. In an innovative approach, Candolfi et al. [67] engineered an Ad vector encoding a GBM-specific cytotoxin by fusing a ligand specific for IL-13R α 2 (a tumor specific receptor) to *Pseudomonas* exotoxin and showed enhanced survival without neurotoxicity in a murine model of human GBM. The groups of Grandi and Glorioso reengineered glycoproteins on HSV including a single chain antibody to target to epidermal growth factor receptors on GBM [68].

Restricting transgene expression to cells of interest when your vector transduces many cell types can also be achieved through the use of tissue specific promoters (see below in [Use of Promoters for Vector-Mediated Gene Expression in CNS](#)). For example, in the brain, expression in neurons has been achieved using the neuron specific enolase promoter [69] and synapsin 1 promoter [70]. Glial promoters such as for GFAP have been utilized for astrocyte expression [71]. Retinal promoters have been used with lentivirus vectors in the eye to achieve selective expression [72]. An elegant approach to restricting transgene expression to certain cell types was demonstrated by Dr. Naldini’s group using endogenous microRNA (miRNA) machinery [73]. In this strategy, the goal is to “knock-down” transgene expression (at the mRNA level) in nontarget cells expressing high levels of a particular miRNA by including a binding site for this miRNA in the 3′-untranslated region (UTR) of the transgene message, leaving expression in target cells with low levels of this miRNA. This approach has been used to lower expression in nontarget cells such as heart, liver, and muscle and restrict it to the CNS [74] as well as to reduce expression in antigen-presenting cells to lower the immune response against transgene expressing cells [75].

As mentioned earlier, immune responses to virus-encoded proteins limits long-term transgene expression in the brain using certain virus vectors, including Ad. One approach to curtail this issue is the engineering of high capacity/gutless Ad

vectors. These vectors are deleted for all virus-specific genes and all the necessary genes are supplied *in trans* during vector production. The effect of deleting the viral genes is a longer expression profile in brain, as well as a greater capacity for transgene size [76].

Non-Viral Systems

Non-viral (NV)-based gene delivery systems have several advantages over virus vectors [46]. First, there is generally no pre-existing immunity to NV systems. Secondly, as they are not derived from pathogens, NV systems have fewer safety concerns than virus vectors, such as replication competency and insertional oncogenesis. However, in general, NV systems have been much less efficient at gene delivery *in vivo* than their virus-based counterparts. In recent years using new technologies and approaches, as well as virus peptides and nucleic acid components, the field is now generating some robust gene delivery vehicles.

The field of nanoparticles (NPs) for gene therapy continues to grow immensely with new types of polymers being described at a rapid pace. The definition of a NP is very broad to include uniform particles on a nanometer size scale. NPs can be comprised of a variety of compounds and materials and examples of NPs include liposomes, dendrimers, carbon nanotubes, magnetic NPs, and cationic polymers [e.g., polyethylenimine (PEI)]. Most NPs can be complexed with plasmid DNA, mRNA, siRNA, miRNA for gene delivery purposes. Synthetic lipid-based particles such as liposomes have been utilized for several years for gene delivery in cultured cells, and are now being used for *in vivo* gene transfer of the brain. Rungta et al. [77] used siRNA encapsulated in liposomes to knock-down specific mRNA targets in the brain after direct intracranial injection. Improvements in liposome brain-delivery properties have been accomplished by linking brain targeting ligands to the particle surface. Angiopep is a peptide that targets the low-density lipoprotein receptor related protein (LRP) found on the surface of the BBB and is capable of transporting drugs across the vasculature into the brain [78, 79]. Huang et al. [80] used an intravenously administered dendrimer conjugated to Angiopep and encapsulated DNA encoding a neuroprotective protein to improve disease symptoms in parkinsonian rats. Cationic polymers such as PEI have also transitioned from cell culture to *in vivo* gene delivery with commercially available reagents such as *in vivo*-jetPEI®. In an interesting approach to targeting NPs to the brain, Liu et al. [81] used focused ultrasound to temporarily disrupt the BBB and then positioned a magnet near the head in a mouse with a brain tumor to attract magnetic NPs loaded with chemotherapeutic drug to the tumor [81]. This technology may be adapted for nucleic acid delivery as well. Focused ultrasound has been employed with NPs termed microbubbles

that can burst at the site of focused ultrasound with efficient gene delivery to the immediate area [82].

A relative newcomer to the field of neurotherapeutics is extracellular vesicles (EVs). EVs encompass many types of membrane particles shed from cells into the extracellular environment including microparticles, microvesicles, exosomes, and apoptotic bodies. EVs are naturally secreted, membrane-encompassed structures known to deliver proteins, RNA, miRNA, and DNA to neighboring cells [83]. The size (generally in the 50–200 nm range), cell surface receptor expression, and ability to package nucleic acids and proteins, makes EVs much like an endogenous NP. EVs have been complexed with an anti-inflammatory compound or Stat3 inhibitor and these complexes have been injected intranasally in mouse models of brain inflammation and brain tumors, respectively [20]. In both models, a therapeutic benefit was achieved using the intranasally administered EVs, although the exact nature of the EV/drug association is unknown. In 2011, a study by Alvarez-Erviti et al. [84] stimulated much interest in the use of EVs as neurotherapeutics. This group used EVs isolated from murine bone-marrow-derived dendritic cells which had been engineered *ex vivo* to express a transmembrane receptor bearing a brain targeting peptide (rabies virus glycoprotein, RVG, peptide) that was presented on the EV surface. Post EV harvest, the researchers electroporated the RVG-targeted EVs with siRNA and showed specific knock-down of BACE1 mRNA (Alzheimer's therapeutic target) in the brain after systemic injection of siRNA-loaded EVs [84]. Recently, a follow-up report showed that the siRNA loading efficiency of EVs is, however, extremely low [85], suggesting that improvements need to be made in loading of EVs with RNA. Other strategies for loading RNA into EVs include both passive and active modalities [86]. Passive loading typically involves overexpression of the RNA in cells using an expression vector and relying on mass action to distribute some of that RNA into EVs during their biogenesis. It is widely assumed that ribonucleoproteins (RNPs) recognize sequences or configuration of RNA that leads to the selective enrichment of some RNA species in EVs [87]. Efforts are currently underway to improve the loading of EVs with RNAs. One recent report identified a short motif (GGAG) in certain miRNAs that directs them to be loaded into exosomes [88]. Furthermore, Bolukbasi et al. [89] reported that a motif in the 3' UTR of certain mRNAs leads to their enrichments in EVs.

Cell-Based Gene Therapy

Cell-based gene therapy has seen a rise in recent years. In fact, in 2009, the *American Society of Gene Therapy* changed its name to the *American Society of Gene and Cell Therapy* to reflect the large interest in this field. Much of the interest in neurotherapeutics with cell-based gene therapy has utilized NSCs and mesenchymal stem cells (MSCs). One of the

intriguing properties of NSCs and MSCs is their innate ability to traffic to regions of pathology in the brain. For instance, Munoz et al. [90] showed that MSCs transfected with an anti-miRNA specific for an endogenous miRNA [mir9, which upregulates expression of a drug efflux transporter, P-glycoprotein (P-gp)] could transfer the anti-miRNA to GBM cells (via EVs) resulting in decreased expression of P-gp with resulting chemosensitivity of the tumor cells. NSCs have been used therapeutically in several models including glioma [91]. Interestingly, it has been found that MSCs have immunosuppressive properties that lead to a more stable engraftment than NSCs [92].

Hybrid Systems

The goal of a hybrid car is to retain the robust aspect of the original technology (the horsepower of the gas engine) while reducing the undesirable high fuel consumption by way of a rechargeable battery. Similarly, in a hybrid gene delivery system, the aspect of the robust, original technology is often a virus vector, while the other component serves to enhance or dampen a particular aspect of the original vector system. One promising hybrid system is the hybrid bacteriophage vector (AAV/P), which contains genetic elements of AAV [93] packaged within a bacteriophage capsid. The benefit of using bacteriophage is that, in humans, there is little to no pre-existing immunity against it. However, on its own, the bacteriophage vector is inefficient at gene expression in mammalian cells. Incorporating genetic elements of AAV allows AAV/P to be efficient at transduction. Recently, Kia et al. [94] used AAV/P vectors to target and kill tumors in a mouse model of human GBM. Our group has recently reported use of another hybrid system comprised of AAV and EVs termed vexosomes (vector-exosomes) [95]. AAV is currently the pre-eminent vector for CNS gene transfer; however, it is still vulnerable to neutralizing antibodies as well as off-target liver uptake. We have shown that vexosomes are more resistant to human neutralizing antibodies than standard AAV in an *in vivo* murine model [86]. Vexosomes can also be redirected to organ targets by decorating the EV surface with transmembrane ligands [86].

Use of Promoters for Vector-Mediated Gene Expression in CNS

Challenges

The field of CNS gene therapy is evolving rapidly with the discovery of new viral vectors, mainly AAV capsids, with remarkable CNS tropism after vascular [21, 36, 49, 96–98], and CSF administration [18, 36, 47]. A large range of AAV capsids has been investigated for their CNS gene transfer

properties by direct intracranial injection [99–102]. The majority of these studies have used strong ubiquitous promoters to provide a broad overview of transgene expression in different tissues and cell types throughout the body after intravascular infusions, or CNS cell types after intracranial delivery. The CAG promoter (also referred to as CBA and CB) is the promoter most commonly used in these types of studies as it is demonstrably a strong promoter that mediates long-term gene expression in CNS and other tissues [103]. This hybrid promoter cassette was designed originally with the cytomegalovirus immediate early enhancer fused to the chicken β -actin promoter that includes 300 bp of the upstream region and a portion of intron 1 because it carries a strong enhancer conserved across species [104]. The downstream splice acceptor was replaced with the splice acceptor in exon 3 of the rabbit beta-globin gene [105].

The CAG/CBA/CB promoter is generally viewed as a ubiquitous promoter as it works in numerous tissues and cell types. The resulting assumption that it is highly active in every cell type may not be entirely warranted in the CNS. The CNS cellular tropism of numerous AAV capsids has been examined extensively in the context of intra-parenchymal delivery to different structures, and the universal finding is that most AAVs are exceptionally neurotropic with little evidence for transduction of glia, microglia or oligodendrocytes [16, 99–101, 106–109]. These studies have been carried out with AAV vectors carrying the cytomegalovirus (CMV) [16, 104, 111, CBA [99, 106], Rous sarcoma virus (RSV) [109], or human GUSB promoters [101, 108] to drive transgene expression.

However, the ongoing effort to develop/identify new AAVs with strong tropism to particular CNS cell types or structures after vascular or CSF delivery is based entirely on the ability to detect gene expression, most often GFP. If the vector is transcriptionally functional in a certain cell population or populations but, due to promoter restriction, the GFP reporter is not expressed, one would wrongly exclude this vector from further study. This could considerably influence the pre-clinical development process of therapeutic interventions for neurodegenerative diseases, which determines the AAV vectors that will be tested in human clinical trials. As discussed below in “[New Strategies](#)”, the apparent discrepancy in CNS transduction profiles of AAV vectors with the same capsid but carrying different CNS cell specific promoters or the CBA promoter [102, 110–112] suggests that the latter promoter may not mediate detectable transgene expression in all cell types in the brain.

The majority of AAV clinical trials for neurological diseases have used vectors carrying the CMV immediate early promoter followed by a chimeric intron composed of a CMV splice donor and a human globin splice acceptor [113–116], or a version of the CAG/CBA promoter, described above [117–119]. Two other clinical trials have tested AAV vectors carrying mammalian promoters such as the mouse

phosphoglycerate kinase (*PGK*) promoter [120], or the rat neuron-specific enolase (*NSE*) promoter [121]. The choice of promoters is based on the availability of extensive data from pre-clinical studies in different animal models showing that AAV-mediated transgene expression under these promoters is stable and long lasting in the mammalian brain [122–127]. The stability of AAV gene expression in the human brain has been evaluated in clinical trials using an AAV2 vector encoding aromatic amino acid decarboxylase (AADC) injected into the putamen of Parkinson's disease patients [116], or children afflicted with AADC deficiency [115]. Positron emission tomography (PET) of AADC activity has shown newly expressed enzyme in the injected structures and stability of expression over a long period [116]. Also a recent report shows evidence of nerve growth factor (NGF) expression in the post-mortem brain of Alzheimer's patients enrolled in a trial using an AAV2-NGF vector [117].

An indisputable fact about AAV-CMV or AAV-CAG/CBA vectors is their ability to drive strong gene expression in many neurons in the CNS of multiple species. The disparate CNS transduction results between cellular promoters and strong non-mammalian promoters (discussed below in “*New Strategies*”) raises the possibility that the current generation of AAV vectors express transgenes at exceptionally high levels in a small percentage of all cells that harbor transcriptionally ready vector genomes in the nucleus. This raises a central question for CNS gene therapy as to whether the goal should be to achieve some level of gene expression in as many cells as possible, or high levels of therapeutic protein expression in a small percentage of cells. Most likely the answer is that it will depend on the neurological disease and therapeutic mechanism. For instance, in LSDs where the entire CNS is involved, the ideal approach is to have dispersed expression of the missing lysosomal enzymes by systemic delivery of AAV9 vectors [128, 129]. Some degree of enzyme overexpression will be required to drive its secretion and uptake in non-transduced cells for correction of the lysosomal storage phenotype. The enzyme overexpression requirements may be higher for approaches that use direct injection of AAV vectors into different structures and where the enzyme produced locally is intended to distribute widely throughout the brain by diffusion [130], axonal transport [131, 132], and CSF flow in the perivascular spaces [131, 133]. The prevailing notion is that overexpression of most lysosomal enzymes is unlikely to trigger toxic responses. This is based on a large number of AAV CNS gene therapy studies in numerous mouse and large animal models of LSDs with neurological involvement. Results in beta-Glucuronidase (GUSB) transgenic mice suggest that massive overexpression of β -glucuronidase is tolerated in the brain to levels of 700-fold above normal, despite massive storage of enzyme in lysosomes [134]. The comparison with AAV gene delivery to brain is difficult to establish as the resulting level

of enzyme activity in different brain regions is measured in tissue blocks where the vast majority of cells are not transduced. Therefore the reported enzymatic activities of 10- to 100-fold above normal in different mouse [123, 135] and cat [126, 136] models of LSDs represent the activity of enzyme that is distributed throughout the brain from the target sites and now resides in non-transduced cells. Presently, there is no information on the degree of overexpression that takes place in AAV transduced cells in the target structures. A recent report documented considerable toxicity in non-human primates (NHPs) receiving intraparenchymal injections of an AAV2 vector encoding human acid sphingomyelinase [137]. It is possible that this toxic response is specific to this lysosomal enzyme due to a resulting imbalance in the sphingosine/sphingosine-1-phosphate metabolism [138]. Another safety study on intracranial delivery of an AAVrh10 vector encoding human tripeptidyl peptidase (TPP-I) in NHPs reported white matter edema and inflammation [139]. The interpretation of these results is complicated by the potential antigenic nature of human proteins expressed in the NHP brain, but a possible interpretation of the results is that these inflammatory responses are secondary to protein expression overload in AAV transduced neurons.

In other neurological diseases where the expressed proteins are not secreted, the need for high-level expression in AAV-transduced cells is less clear. In spinal muscular atrophy (SMA), systemic delivery of a self-complementary (sc) AAV9 encoding the survival motor neuron (SMN) protein rescues the phenotype in a mouse model [125]. Delivery of a scAAV9-SMN vector into CSF appears to also have a significant therapeutic effect, and the distribution and transduction of motor neurons appears reproducible in NHPs [140]. The first study by Foust et al. [125] used a scAAV9 encoding SMN under a version of the CBA promoter, while the study by Passini et al. [140] used a 0.4-kb fragment of the human GUSB promoter. The devastating nature of SMA, where some children die by 5–7 months of age, and the safety profile of both strategies in animal studies justifies moving the current approach to the clinic in an accelerated manner. The exact function of the SMN protein remains unclear, although studies suggest that it is involved in RNA splicing, and thus raises some concern about the potential long-term impact on neuronal function resulting from SMN overexpression, despite the astounding therapeutic benefit in a mouse model of SMA. Detailed studies of AAV9-SMN transduced spinal cord motor neurons for relative levels of vector-encoded to endogenous SMN mRNA levels and changes in the transcriptomic profile may be informative in understanding/predicting long-term outcomes and perhaps guide the design of next generation AAV vectors for SMA.

Development of an AAV gene therapy approach for Rett syndrome represents a significant challenge to the prevalent approach of simply using strong NV promoters. This disease

is caused by mutations in the MeCP2 gene on the X-chromosome. Interestingly, duplication of this gene also leads to neurodevelopmental delays [141, 142], which suggests that expression of this gene in the CNS has to be tightly controlled, which is a challenge to current AAV gene therapy. Two recent articles report on the therapeutic effect of two different scAAV9 vectors encoding MeCP2 under a CBA promoter [143], or the minimal MeCP2 promoter [144] previously shown to express in neurons [145]. Surprisingly, both reports show a significant therapeutic benefit with no evidence of toxicity [143, 144]. This may represent a slightly different biology in mouse, or that overproduction of MeCP2 impacts only pre-natal development in humans.

New Strategies

The relatively large size of the original CAG promoter (1.9 kb) places considerable limitations on the size of transgenes that can be encoded in the context of AAV vectors where the maximum genome size is 4.7 kb. This has spurred independent work by numerous research groups to reduce its size while maintaining its gene expression properties with respect to strength and sustainability of expression. Naturally, as in any non-coordinated effort, many varieties of the CAG promoter have been generated over the years that differ primarily in the intronic sequence (different truncation sizes). These structural modifications were also accompanied by a diversification of its name and the designations CBA and CB have become common. The development of sc AAV vectors [146] with superior *in vivo* gene transfer properties [147] but half the genome size (2.3–2.4 kb) has fueled further modifications to the CAG promoter with the original intron being replaced with smaller viral [148], hybrid [149], or synthetic introns (*ibid.*). As a result of the prolific re-engineering of the basic promoter, the CAG, CBA and CB names have become generic designations to identify a version of these promoters being used to drive transgene expression, but the presence and origin of any introns is seldom described in research articles. The potential impact of different introns on the expression characteristics of the CAG/CBA/CB promoter in the CNS is illustrated in a recent study where expression kinetics were heavily dependent on the type of intron [149].

The notion (based on CAG promoter data) that most AAVs are almost exclusively neurotropic upon direct intracranial infusion into the brain should be re-examined in light of a number of studies comparing AAV vectors using the same capsid but different cell type specific promoters to drive transgene expression. Apparently, AAV vectors carrying the original 2.2 kb *Gfap* promoter [150], or a smaller truncated version (*gfapABC1D*) [151], transduce astrocytes in the mouse brain effectively and with a great degree of specificity [102, 111]. Similarly, AAV vectors carrying the myelin basic protein (MBP) promoter transduce oligodendrocytes at high

efficiency and with excellent cellular specificity [102, 110, 112]. These findings are important because oligodendrocytes are a key therapeutic target for demyelinating CNS diseases, such as Canavan disease, and they challenge the widely held perception that no presently available AAV is capable of mediating efficient gene transfer to this cell population. These findings with cell-type specific promoters raise the possibility that our current knowledge on the CNS tropism of systemically delivered AAVs is most likely incomplete as the studies carried out thus far have used scAAV vectors encoding GFP under different forms of the CBA promoter [21, 36, 49, 97]. For new AAVs that display strong CNS tropism after systemic delivery using some version of the CAG/CBA promoter, it may be useful to extend studies to include scAAV vectors encoding a marker gene (GFP most likely) under different cell specific promoters for neurons [152], astrocytes [102, 110, 111], microglia [153], and oligodendrocytes [102, 110, 112] to develop a more complete understanding of its potential for global gene therapy interventions.

The Pleiades Promoter Project (<http://www.pleiades.org>) aims at developing small promoters (MiniPromoters) compatible with the AAV transgene capacity to drive region and cell type specific gene expression in the mouse brain. This effort combines genome wide bioinformatic analysis of promoter regions of genes that display the desired expression profile to identify the minimal elements necessary to achieve specificity followed by experimental validation using a large-scale knock-in transgenic approach [154, 155], and AAV somatic transgenesis using neonatal vascular delivery (personal communication, E.M. Simpson, Centre for Molecular Medicine and Therapeutics at the Child and Family Research Institute, University of British Columbia). This approach has yielded a veritable trove of validated mini-promoters to target gene expression in a regional and cell type specific manner [154, 155]. Recently AAV vectors carrying retinal ganglion cell (RGC) MiniPromoters were shown to mediate RGC-specific gene expression in adult mouse retina [155].

Targeting AAV-mediated gene delivery to specific neuronal populations in the CNS has been a challenging proposition. The AAV transgene capacity limits the size of promoters, and most transgenic mouse models target gene expression to a specific subset of neurons, for instance to drive Cre recombinase expression, and are generated using large genomic regions spanning the promoter region of a gene of interest. Some degree of success has been achieved in targeting AAV gene delivery specifically to one of the two populations of medium spiny neurons in the striatum. Striatonigral neurons projecting to the internal globus pallidus and substantia nigra pars reticulata (direct pathways) express D1 receptor, dynorphin (*Dyn*) and Substance P (*SP*). While striatopallidal neurons projecting to the external globus pallidus (indirect pathway) express D2 receptor and enkephalin (*Enk*). Functional studies examining the role of these pathways in

behavior and learning have shown a high degree of AAV-mediated gene expression in these two populations using vectors carrying the *SP*, *Enk*, or *Dyn* promoters [156, 157]. The small degree of transcriptional mis-targeting (5–6 %) may be due to some cells being transduced with large amounts of AAV vector, and a promoter not usually functional in a particular cell type may display sufficient basal activity to become detectable. Also it is important to consider that chromatin regulation mechanisms that may participate in restricting gene expression to particular cell types are unlikely to function effectively in an AAV genome that remains as an extrachromosomal element with a promoter that because of its size may not carry key elements involved in chromatin silencing. In addition, it is also possible that this small level of mis-targeting could be due to transactivation from the AAV2 inverted terminal repeat, which has promoter activity in the brain [158, 159], and at sufficiently high vector doses may become detectable. A similar finding of low percentage mis-targeting was reported for an AAV vector carrying an artificial dopamine beta-hydroxylase (PRSx8) promoter [160] to drive GFP expression in noradrenergic neurons in locus coeruleus for tracing of axonal projections [161].

The field of AAV mediated CNS gene therapy is accelerating rapidly towards clinical trials in a number of neurological diseases and the expedient approach has been to use promoters with a proven track record of mediating long-term gene expression given the desperate nature of many of these diseases, many of which are pediatric. It is possible that a more deliberate process of promoter selection may be beneficial in the long run by determining the minimum promoter activity that is necessary to tailor AAV-mediated gene therapy to a particular neurological disease. This will depend on the biology of the protein being expressed, target cell tolerance to protein overexpression, and the therapeutic principle being explored.

Blood-Brain Barrier

Challenges

By virtue of its location, structure, and function, the BBB represents a significant challenge to the treatment of CNS diseases. Both the absence of vascular fenestrations and presence of specialized tight junction complexes (TJCs) expressed by brain microvascular endothelial cells (BMVECs) create a physical barrier that restricts the paracellular diffusion of plasma proteins and small water-soluble molecules, as well as the migration of circulating leukocytes, from the blood to the CNS [162] (Fig. 2). These TJCs also maintain BMVEC polarity, which helps to define the expression of luminal surface proteins from those expressed at the basolateral aspect

of the cell membrane [163]. Organization of the BMVEC plasma membrane creates a transportation barrier that regulates the transcellular passage of select molecules from the blood to the CNS. Because the neurovascular unit develops so that no neuron is in direct contact with the cerebral vasculature, BMVECs express glucose transporter-1 (GLUT-1) at a certain apical to basolateral ratio to robustly deliver nutrients from the blood to neurons through intermediary astroglial cells, which facilitate this delivery [162]. Although some molecules may exit the blood vessel lumen and enter BMVECs, ATP-driven xenobiotic transporters like P-gp actively emit their substrates from the apical membrane back into the capillary lumen [164, 165]. Importantly, BMVECs concentrate the expression of P-gp, a member of the ATP-binding cassette (ABC) superfamily of proteins, to the luminal surface, where P-gp acts as an efflux transporter, removing a variety of diverse chemical structures away from the brain parenchyma [163]. The function of P-gp (and the redundant function of the related multi-drug resistant proteins) has been well defined as one of the most significant challenges to delivering chemotherapeutic agents to parenchymal brain cells and tumor cells in the CNS; furthermore, the BBB has proven no less challenging for the delivery of DNA-based therapies to CNS targets [165–167].

Enzyme replacement therapy (ERT) has been used successfully to treat non-neuropathic forms of Gaucher's Disease—an LSD caused by a glucocerebrosidase deficiency; however, for those patients who suffer from the less common form of the disease with neurological complications, ERT has been ineffective due to the function of the BBB, which restricts large molecules, like the infused glucocerebrosidase, from reaching the CNS [168]. Similarly, intravascular administration of most viral vectors has proven unsuccessful in the treatment of neurological diseases, potentially because these gene delivery platforms cannot negotiate their way across the BBB to access their target cells [21]. Because TJCs greatly reduce paracellular diffusion between endothelial cells, BMVECs are tasked with regulating the transcellular routes that shuttle materials across the BBB endothelium. Lipophilic molecules (such as steroid hormones) readily pass through the BMVEC plasma membrane and across the cell to access targets in the CNS [169, 170]. As mentioned earlier, transporter proteins like GLUT-1 selectively transport their substrates from the luminal to abluminal plasma membrane, while other transporters like P-gp function to restrict potentially harmful molecules from crossing the basal, endothelial plasma membrane [163]. Another process, adsorptive endocytosis recycles bound/internalized blood products back into the vessel lumen, which occurs when plasma proteins like heparin, albumin, immunoglobulin G, or wheat germ agglutinin (WGA) interact with the apical plasma membrane and bind to the cell surface or enter the cell within an endosome [163, 171–173]. A series of outcomes may arise from adsorptive

Fig. 2 A simplified schematic of the common challenges associated with blood-brain barrier (BBB) restriction of gene therapy delivery to the CNS. Unlike the vasculature in other organ systems, the brain endothelium forms intercellular tight junctions (TJs) that limit greatly the paracellular access of blood-borne components to the tissue parenchyma. As a consequence of this “physical barrier” generated by TJs, gene transfer vehicles attempting entry into the CNS are forced to go through the transcellular route. Specialized lumen facing efflux transporters, which include both the ABC family of transporters and multidrug resistance-related proteins, contribute to the formation of the “transport barrier” that prevents xenobiotic substances from gaining access to the neuronal environment. In the case of non-viral (NV) gene delivery constructs, efflux transporters can eliminate stabilizing molecules and induce early

decapsulation, hence lowering the odds of intact transendothelial passage into the brain. Another obstacle that partially prevents endothelial crossing of gene-therapy vehicles is the transcytotic/endosomal sorting, which can lead to degradation. Although the endothelium is the greatest obstacle in restricting access to the CNS, the presence of a complex extracellular space, molecular composition and geometry also significantly impede diffusion of NV and viral-based gene delivery systems. Shown on the *left* of the schematic are new developments in viral construction that take advantage of receptor mediated endocytosis, discovery of viral serotypes [such as AAV serotype 9 (AAV9)] with enhanced CNS penetration, and methods that transiently breach the barrier are exemplified as means to increase efficiency of gene transfer to the brain

endocytosis: (1) these endosomes may be recycled back to the apical plasma membrane, where their contents are released back into the circulation; (2) the internalized endosome may fuse with a lysosome, where the endosomal contents will be degraded preventing any unwanted, harmful blood-borne products from contacting the brain parenchyma; (3) adsorptive endocytosis can lead to delivery of materials at the basal plasma membrane and the release of endosomal contents into the CNS; and (4) endosomes may invaginate forming

multivesicular bodies which then release EVs (exosomes) containing cell contents into the extracellular space [163, 172, 174]. Of course, the first two of these endpoints present challenges to delivering therapeutic products to the brain; however, and importantly from the standpoint of a viral vector delivery mechanism, the process of adsorptive endocytosis could offer favorable outcomes considering that this may be the primary method for which blood-borne (a.k.a. cell-free) human immunodeficiency virus (HIV-1) crosses the BBB to

infiltrate the CNS [171, 172]. Better understanding of the molecular biology mediating the process of adsorptive endocytosis may elucidate opportunities for delivering viral vectors across the BBB endothelium. Yet an alternative transcellular route, known as receptor-mediated transcytosis, may be utilized to deliver gene therapy products across the BBB endothelium [163]. BMVECs express a variety of cell surface receptors that have the potential of binding with molecules (and other cells) in the blood. Once bound to its receptor, these molecules may be directed through the BMVEC and released at the basal lamina into the brain parenchyma. Some of the BMVEC receptors commonly targeted by researchers for the delivery of therapeutic technologies include the insulin, leptin, and transferrin receptors [173, 175]. However, even if a therapy can be designed to target one of these receptors and traverse the BMVEC cytoplasm to be released at the basal cell membrane, only the first of many guardian mechanisms at the BBB has been overcome. If neurons are the target cell type, once the BBB endothelium has been passed, the basal lamina, pericytes, microglial ramifications, and the glial limitans comprised of astrocytic endfeet still have to be navigated before surpassing all of the checkpoints at the BBB and encountering a neuron [162]. Therefore, multiple obstacles presented by the BBB have led to the exploration and development of novel delivery platforms to transport gene therapies into the CNS.

New Strategies

Bypassing the BBB through improved direct intraparenchymal injection of viral vectors is a common means for the administration of gene therapeutics to the brain. Challenges to this method of administration potentially include: (1) repeated neurosurgical procedures, which are required for intraparenchymal injection; and (2) a high probability of only achieving focal delivery of the viral vector [176]. Global delivery to the brain would be advantageous in neurological diseases that affect multiple areas of the CNS; however, due to the tortuosity of brain extracellular space (ECS) (which has a diffusion coefficient of 1.6, reduced by approximately 2.6 of the free diffusion coefficient of tetramethylammonium, and an ECS that accounts for approximately 15–20 % of the brain's total volume), as well as the tremendous surface area of the cerebral microvasculature, a focal administration treatment model would require repeated intraparenchymal injections at multiple sites [177]. The benefit of an intravenous therapy that can traverse the BBB would achieve the necessity for global delivery of gene therapeutics to the brain in many neurologic diseases [176].

Recently, new viral vectors and molecular engineering techniques have emerged that exhibit the potential for achieving global delivery across the BBB [176, 178, 179]. As mentioned in the [Introduction](#), several AAV serotypes have

been shown to deliver genes across the BBB after intravenous injection. The exact mechanism by which this occurs is not known; however, it has been speculated that it is an active-transport mechanism, as transient mannitol disruption of the BBB did not greatly enhance AAV transduction of the brain after intravenous injection [48]. A variety of techniques have been investigated for therapeutic delivery across the BBB including those developed to bypass the barrier (i.e. intranasal administration and intracranial NSC transplantation), those that use NP technology to cross the barrier (i.e. Trojan horse liposomes and cell-based NP systems), and other methods that transiently permeate the BBB using osmotic manipulation [167, 175, 180–183] or magnetic resonance imaging guided focused ultrasound (MRIGFUS) to selectively open the BBB and allow therapeutics to pass into the parenchyma [184]. However, because the integrity of the BBB is essential for maintaining neuronal homeostasis, any therapy that potentially disrupts the BBB may have untoward effects. Importantly, a great deal of recent research has sought to characterize the degree and significance of BBB dysfunction in a host of nervous system diseases [185]. Depending on the pathological characterization, data from these investigations may provide a novel strategy to treat nervous system diseases by targeting therapies to the BBB itself, potentially to rescue a dysfunctional process and attenuate disease symptomatology.

Amyotrophic lateral sclerosis (ALS) presents an excellent example for exploring the notion of targeting a therapy to the BBB endothelium, as multiple reports have noted the breakdown of both the BBB and the blood-spinal cord barrier (BSCB) in human patients and animal models of the disease [186–190]. Moreover, a mutation in the angiogenin gene has been linked to patients with ALS and may represent an interesting gene therapy target to investigate for the purpose of attenuating disease symptoms and progression potentially related to BBB dysfunction [190]. Other gene targets to consider that are directed at the BBB for the treatment of neurological disease include over-expression of P-gp or anti-oxidant enzymes in neurodegenerative diseases (i.e. Alzheimer's disease, Parkinson's disease, and HIV-associated dementia), and the expression of short-interfering RNAs to disrupt matrix metalloproteinases in cerebral adrenoleukodystrophies and GBM brain tumors [191–196].

Alternatively, the characterization of BBB dysfunction in various nervous system diseases may lead to the discovery of novel peptides expressed on the BMVEC apical plasma membrane in affected patients. The possibility of identifying target receptors uniquely expressed on the pathologically, activated BMVEC plasma membrane (which may be specific or common to a host of neurological diseases) may be exploited to assist in the process of receptor-mediated transcytosis and the delivery of DNA-based therapies. For example, Chen et al. [197] identified peptides that bound selectively to the brain

endothelial cells in LSDs and used them to target AAV to the brain vasculature.

Finally, peptide modifications may lead to the discovery of novel therapeutics for nervous system diseases that can cross the BBB. Apolipoprotein E-derived peptides targeting the low-density lipoprotein (LDL) and other related receptors have been shown to cross the BBB and reduce *in vitro* models of neuroinflammation using mouse BV2 microglial cells and lipopolysaccharide-activated whole human blood, as well as murine models of traumatic brain injury and cerebral ischemia [198]. Likewise, although ERT has been ineffective at treating neuropathic Gaucher's Disease, more recent evidence suggests that phosphorylated enzymes (i.e. phospho-beta-glucuronidase and phospho-sulfamidase) in other neuropathic LSDs, such as mucopolysaccharidosis type IIIA (MPS IIIA), are capable of crossing the BBB in neonatal mice using mannose 6-phosphate receptor-mediated transport, providing new avenues of investigation in ERT [199].

Despite the formidable challenges presented by the location, structure, and function of the BBB, advances in brain endothelial receptor targeting, usage of AAV serotypes with CNS tropism and directed (transient) BBB disruption strategies may one day prove clinically valuable for the delivery of genetic therapies for the treatment of nervous system diseases.

Promising Pre-Clinical/Clinical Studies

Challenges

Ten years ago concerns about gene therapy for neurologic disease focused on possible disruption of brain function through delivery methods and potential inflammatory and immune responses (e.g., [200]). These concerns have been alleviated largely through the safety profile of vectors used and the skill of neurosurgical procedures [1, 201]. In fact, there have been essentially no severe, adverse events associated with the most commonly used AAV vectors, or even oncolytic viruses for brain tumors, in clinical trials. And surprisingly, AAV vectors can confer gene expression in the human brain for many years. Still, there is no clinically approved gene therapy procedure for any neurologic disease in the United States. So where do the challenges lie and what strategies are being used to overcome them?

In an excellent review of the frustrations encountered in developing gene therapy for Parkinson's disease [201], one is struck by the different issues that confront clinical trials for gene therapy versus drug therapy. How is vector dosing established? How can success be measured? And how can you determine the level and location of transgene expression in the brain? These issues are compounded, as for all therapies, by the variability in symptoms among patients, the

frequently insufficient natural history for the disease, the lack of knowledge of the molecular etiology of neurodegenerative diseases for many individuals, and the poor translatability from animal models to human patients. In the case of genetic diseases, one has the possibility of targeting the direct cause by gene replacement of a defective allele(s) or knock-down or out a dominant mutant allele. In the case of etiologic unknown origins, the focus is on alleviation of symptoms, but without knowing the cause, the disease process may continue and confound evaluation of therapeutic effects.

Obviously the goal is to have pre-clinical animal studies translate into effective gene therapy in patients. By far the most widely used pre-clinical animal is the mouse. Mice are easy to breed, easy to handle (usually), and inexpensive to house. It is easy to create transgenic knockouts, and, because they are inbred, lower inter-animal variability is acquired in experimental data. However, for many reasons (highlighted nicely in the review "*Large animal models and gene therapy*" [202]), mice are not always the ideal candidate to translate neurotherapeutics to humans. First the human brain is well over 1000 times larger than the mouse brain, making it impossible to test multi-injection procedures and other surgical techniques. Secondly, the physiology differences between mice and humans can lead to unpredicted results in clinical trials. For instance, cell mediated immune responses to the AAV capsid were observed in a clinical trial for hemophilia that were not predicted in gene therapy models in the mouse [203].

On the other hand, large animal models can have brains the size of a small child, and often develop diseases phenotypically and genotypically similar to ones afflicting humans. For example, dogs develop hemophilia, LSD, and congenital blindness. This fact has been instrumental in translating gene therapy to clinical trials, with no better example than that of Leber's congenital amaurosis [40–42].

Mouse models are certainly useful and there are many examples where they have served as effective pilots for translation to gene therapy trials, although based on these findings alone one must be wary of possible unforeseen phenomenon when proceeding to larger animal models and to clinical trials. That being said, modern mouse models for gene therapy are taking some of these mouse to human discrepancies into account. Recently Lisowski et al. [204] developed a xenograft mouse model with chimeric livers comprised of mouse/human hepatocytes that allowed selection of more clinically relevant AAV's directed to human liver cells for gene therapy.

Clinical trials must always weigh risk versus benefit. Although in the case of AAV vectors, for example, risk appears to be minor, is the perceived benefit worth taking any risk? In the case of replication competent viral vectors for treatment of brain tumors, the risk has turned out to be minimal in the face of the magnitude of the disease. However, one must always consider the chance of generating a new epidemiologic variant

through recombination between the virus vector and endogenous viral sequences, as in the case of retrovirus vectors and human endogenous retrovirus sequences [205]. The ability to achieve therapeutic benefit depends on many factors, including how far the disease has progressed at the time of treatment and whether defects are reversible, or whether it is therapeutically sufficient to stay the course and prevent further deterioration. Most diseases are not strictly focal, even if the primary symptom may implicate a specific brain region, and it still remains a challenge to achieve “global” gene delivery in the brain. A major problem lies in setting the criteria for success in terms of quantification of symptom improvement and availability of biomarkers that monitor physical or metabolic benchmarks in the patient’s response to therapy. In addition to being confident that the therapeutic procedure is not toxic in its own right, it is important to know whether the therapy is “on the right track” and to set in place scientific assessments that inform modifications to the procedure to improve the chance of significant benefit.

Promising New Strategies

Here we will focus on a few recent “out of the box” ideas that are currently being implemented to improve gene therapy for neurologic diseases. They comprise a variety of tools including delivery modalities, cells, biologic and synthetic vesicles/NPs, and oligonucleotides. This section provides only a few examples of each; for a more comprehensive review see Simonato et al. [1] and Nagabhushan Kalburgi et al. [2].

Compartmental vs Global Delivery

With global delivery to the brain remaining a challenge, and most neurologic diseases affecting interconnected circuitries, there is an advantage to having a compartmentalized target. Both the eye and the ear offer confined spaces critical for sensory neurologic functions. One of the most striking recent successes in gene therapy is the recovery of vision in non-functional, but not dead photoreceptor cells in Leber’s congenital amaurosis [206]. The clinical arm of this study used direct intraocular injection of a standard AAV vector for gene replacement therapy and demonstrated the benefit of treating younger patients with less severe neurodegeneration. In addition to visual tests, which are quite quantitative, restoration of input into the visual cortex was also demonstrated by functional magnetic resonance imaging [207]. Although not translated into patients yet, success in mice has also been achieved for deafness caused by neuronal loss of function by injections of AAV vectors for gene replacement to neurons in the cochlea, again with the potential for highly sensitive quantitation of sensory function [208, 209].

A number of methods are being explored to achieve more global delivery in the brain, including: convection enhanced

delivery [210]; introduction into a fluid space, e.g., vasculature, brain ventricles [197] or intrathecal space in the spinal cord [211], such that cells lining the space produce the deficient protein; injection into regions of the brain that serve as a nexus of interconnections with retrograde transport of vectors, such as the thalamus, e.g., for AAV [126]; and/or by taking advantage of the “bystander effect” whereby proteins released from the producer cells can be taken up by the deficient cells [126, 197].

Using Cells to Deliver Genes

The migratory capacity of cells from the periphery into the nervous system and within the nervous system can also expand the range of gene therapy. Remarkably, gene correction using a lentivirus vector *ex vivo* in hematopoietic stem cells of patients with X-linked adrenoleukodystrophy serves to arrest neurologic degeneration caused by progressive demyelination [212]. The proposed therapeutic mechanism is quite remarkable, with monocytes derived from the stem cells migrating into the damaged brain from the vasculature and setting up residence as microglia-like cells, with production of the gene of interest, ABCD1 in these microglia leading to clearance of toxic storage products (very-long chain fatty acids, VLCFA) and allowing normal myelin maintenance. Again, this disease has the advantage that the metabolites can be measured by magnetic resonance spectroscopy in patients [213]. Another informative tale is the use of an NSC line, transduced with an Ad vector *ex vivo* to express a prodrug activating enzyme, carboxyesterase that can generate a chemotherapeutic agent from the prodrug irinotecan [214]. When injected into the brain, these stem cells migrate towards tumor foci [215] and release the activated drug in the vicinity of tumors cells following systemic treatment of animals with the prodrug [216].

Using Vesicles to Deliver Genes

One of the most promising ways to deliver oligonucleotides *in vivo* is through the use of liposomes or NPs [217]. Through biosynthetic variations the stability, efficiency of delivery and cellular targeting of these vehicles is continually being improved for delivery of RNAi or antisense oligonucleotides. Recent studies indicate that EVs produced by normal cells can be loaded with therapeutic RNAs *ex vivo* and be used to reduce levels of disease-associated proteins in the brain [84] or deliver prodrug activating mRNA/enzymes to peripheral nerve sheath tumors [218]. As described above, cell-derived vesicles can also be used to deliver AAV vectors with increased delivery efficacy and targeting capacity [95]. These EVs can be derived from autologous cells from patients, e.g., dendritic or MSCs, thus, having low immunogenicity and can be “mixed and matched” with components of liposomes or NPs to optimize delivery capacity [219].

Changing the Transcriptome or Genome of Sick Cells

Several truly revolutionary strategies have emerged in the past few years to use oligonucleotides to manipulate the genome and its transcription products as potential therapy (Fig. 3). For

instance, some repeat expansion diseases induce gain-of-function toxicity that can be mitigated by antisense oligonucleotides (ASOs). In myotonic dystrophy type 1 (DM1), the mutant expanded CUG repeat (CUG^{exp}) RNA sequesters muscleblind-like (MBNL) proteins, resulting in mis-

Fig. 3 Changing the genome or transcriptome. Different strategies are being developed to correct or compensate for mutations underlying hereditary diseases. *1.* Alter DNA genome. This includes using ZFNs, TALENs or CRISPR to target the mutant sequence in genome to either: *a.* disrupt it by non-homologous end joining of the double strand break so as to inactivate the allele (*triangle*) in the case of an autosomal dominant mutation; or *b.* correct the mutation by homologous recombination bringing in a normal (N) sequence covering the mutation as a guide [239, 240]. *c.* It is also possible to use a viral vector to bring in a new expression cassette for the normal gene (N) which can be extrachromosomal, as in the case of AAV vectors, or integrated at a random site in the genome, as for lentivirus vectors. *2.* Modulate RNA splicing. Here antisense oligonucleotides are designed to hybridize to precursor mRNA for the mutant gene. They can serve to: *a.* block a splice recognition site resulting in

skipping of a mutation-bearing exon and resuming the correct reading frame, as in the case loss of exon 52 in Duchenne's muscular dystrophy [230]; *b.* include a normal exon which is normally excluded due to a polymorphism in the splice site, as for exon 7 in SMN2 [228]; or *c.* for trinucleotide repeats either block their tenacious binding to splicing factors [218] or express artificial site-specific RNA endonucleases that specifically cleave them as for type 1 myotonic dystrophy [255]. *3.* Inhibit translation of mRNAs. This can be done by expressing small antisense RNAs (siRNA, shRNA or artificial miRNA) that hybridize to the mRNA and either: *a.* engage the RISC complex to cleave the mRNA; or *b.* block movement of the translational machinery. Other strategies include use of ASO gapmers which engage RNase H or a combination of a guide RNA sequence and RNase P to cleave the mRNA [256]

regulated alternative splicing of select transcripts. In DM1 mice, ASOs that prevent interaction of MBNL proteins with CUG^{exp} RNA, or that induce degradation of toxic CUG^{exp} transcripts, rescue mis-splicing in muscle tissue [220, 221]. ASOs that reduce RNA gain-of-function toxicity also have been effective in fibroblasts and iPSC neurons derived from patients with ALS and frontotemporal dementia caused by a hexanucleotide repeat in the non-coding region of C9ORF72 [222–224]. In mouse models of Huntington's disease and spinal and bulbar muscular atrophy, ASOs can reduce polyglutamine protein gain-of-function toxicity by targeting mutant RNA containing CAG repeat expansions [225–227]. In the case of the fatal motor neuron disease, SMA ASOs are being used to activate a duplicate gene copy (*SMN2*) of the defective *SMN1* gene by precursor mRNA splice modulation. *SMN2* is intact except for a defect in splicing, which can be corrected with an ASO that silences a negative intronic splicing sequence, thus restoring production of fully functional SMN2 [228]. Other oligonucleotides have been designed to promote exon skipping to remove a commonly duplicated exon 2 [229] or to re-establish a correct reading frame in patients with a deletion in exon 52 [230] in Duchenne's muscular dystrophy. Although delivery of ASOs to cells *in vivo* is challenging the development of chimeric delivery vehicles combining properties of viruses, vesicles, liposomes and NPs may surmount these obstacles [219, 231]. These ASO therapeutic strategies are being tested in Phase I clinical trials, e.g., for ALS (to reduce SOD1 mRNA and mutant SOD1 protein toxicity; [232]), SMA (splice-shifting strategy to upregulate expression of SMN2; [233]), and Duchenne muscular dystrophy (splice-shifting to restore the DMD reading frame and increase expression of functional dystrophin isoforms; [234]).

Taking Down the Good with the Bad

Although seemingly counterintuitive, an effective strategy to reduce disease symptoms in dominantly inherited disease states can be achieved using RNAi or shRNAs that knock-down levels of both mutant and wild-type proteins. In fact, haploinsufficiency (i.e. only one of two alleles being functional) for many genes is fully compatible with a healthy life. In animal models of two trinucleotide repeat diseases, Huntington's disease and spinal cerebellar ataxia type 1 delivery of antisense RNAs, e.g., short inhibitory (si), short hairpin (sh) RNA or artificial miRNA that target mRNAs for huntingtin and ataxin, respectively, are effective in reducing levels of both wild-type and mutant mRNAs in animal models for up to 7 weeks after intracranial injection with symptomatic benefit [235, 236]. A single injection of an siRNA for transthyretin delivered to the peritoneal cavity via lipid NPs has proven effective at reducing liver production of both normal and mutant forms of transthyretin in the circulation

by over 50 % for up to a month with a good safety profile, making this a strong strategy for gene therapy of amyloid neuropathy that compromises function of peripheral nerves [237].

Engineering the Genome/Transcriptome

Another “dream coming true” is the ability to physically correct defective gene sequences in the genome in living cells. This technology was based initially on identification of protein sequences in zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) that could be designed to target specific DNA sequences and fused to a DNase to cause a site-specific double strand break in genomic DNA [238–240]. Cells then either make a disruptive repair (non-homologous end joining), which can be used to knock out the function of a dominant-negative allele, or, with the help of a corrected DNA fragment use homologous recombination, to correct the DNA defect [241]. This technology has been effective in correcting a mutation in LRRK2 in induced pluripotent stem cells (iPSCs) from Parkinson's disease patients [242]. Newer technology uses a guide RNA and microbial Cas9 nuclease (CRISPR) to recognize and cleave specific DNA sequences [239, 243]. Although challenges remain to deliver these DNA corrective machineries to cells *in vivo*, especially in the nervous system, success has been achieved *in vivo* both in HIV patients by disrupting the viral entry receptor protein CCR5 in hemopoietic stem cells (genetically modified *ex vivo* with an Ad vector encoding targeted ZFNs) reinfused into patients (for review see Manjunath et al. [244]), and by hydrodynamic tail vein injection of expression plasmids for a guide RNA and Cas9 to correct a genetic hepatic disease in mice [245]. This technology continues to expand with recent papers describing methods to regulate gene expression through modulation of histone acetylation and methylation of promoter regions [246, 247].

Changing the Brain Environment

Given the wide variety of neurotrophic factors known to support different populations of neurons in model systems, it seems logical that their delivery in neurodegenerative diseases would sustain neurons. Thus, for example, AAV vectors injected into brains to deliver NGF in Alzheimer's disease [248], neurturin or glia cell-derived neurotrophic factor (GDNF) in Parkinson's disease [249, 250], and insulin-like growth factor I in ALS [251] have proven safe, but with no demonstrable benefit. This apparent lack of benefit may relate to the late stage of the diseases at the time of treatment, the progressive nature of the diseases and/or the inability to quantitate minor benefit with lack of a robust biomarker [201]. *In vivo* metabolic imaging offers a potential biomarker for individual response, as demonstrated in patients with

Table 1 Clinical trials for gene therapy to treat neurological disorders. Data obtained from <http://clinicaltrials.gov/>. *GDNF* Glial cell-derived neurotrophic factor; *AADC* aromatic L-amino acid decarboxylase; *NGF* nerve growth factor; *SGSH* N-sulphoglucosamine sulphonydrolase;

SUMF1 sulfatase modifying factor 1; *ARSA* arylsulfatase A; *ABCD1* ATP-binding cassette, sub-family D (ALD), member 1; *HSV-TK* herpes simplex virus-thymidine kinase; *CD* cytosine deaminase

Disease	Vector	Transgene	Phase	Status
Parkinson's disease	AAV	GDNF	1	Recruiting
Parkinson's disease	AAV	AADC	1	Recruiting
Leber's hereditary optic neuropathy	AAV	Human mitochondrial ND4 gene	1	Recruiting
Alzheimer's disease	Fibroblasts	NGF	1	Completed
Sanfilippo Type A syndrome	AAV	SGSH and SUMF1	1/2	Completed
Metachromatic leukodystrophy	AAV	ARSA enzyme	1/2	Recruiting
X-linked adrenoleukodystrophy	Lentivirus <i>ex vivo</i> transduced stem cells	ABCD1	2/3	Recruiting
Glioma	Adenovirus	p53	1	Completed
Glioma	Oncolytic HSV	—	1	Recruiting
Glioma	Adenovirus	HSV-TK + ganciclovir	1	Completed
Glioma	Replicating retrovirus	CD	1	Recruiting

Huntington's disease and Parkinson's disease [252, 257]. A variation on this approach is to express growth factors in the brain in order to stimulate generation of neurons from endogenous NPCs lining the ventricles [253]. The logic of this approach to modify the brain environment to withstand insult is an appealing one. A recent study by Hudry et al. [254] demonstrated that AAV-mediated delivery of the APOE isoform that is protective in human Alzheimer's disease, APOE2 to the brains of transgenic mouse models of this disease resulted in shrinking of amyloid plaques and reduced neuronal damage.

Ongoing Gene Therapy Clinical Trials for Neurological Disorders

There are many gene therapy-based clinical trial in humans to treat neurological disease planned, ongoing, or completed. Table 1 (data obtained from <http://clinicaltrials.gov/>) gives a small sampling of these trials, the diseases they are trying to correct, and the gene therapy strategies being employed. It can be appreciated from the table that, for the same the disease (e.g., glioma), different vectors and different transgenes are being tested in parallel trials. This is a good strategy as it increases the likelihood of finding a viable therapeutic strategy to move forward.

Conclusions

Despite the myriad of challenges faced, gene therapy directed to the CNS has recently made a great deal of progress. This has been accomplished through both serendipitous discoveries

(e.g., virus vectors found to naturally cross the BBB), as well as harnessing knowledge accumulated from basic neuroscience research (e.g., rationally targeting receptors expressed on the BBB). Further development of viral and NV systems that encompass all of the features identified empirically that are necessary for selective and efficient transgene expression in the CNS should result in clinically applicable neuro-gene-based therapeutics. This field is driven by the lack of current effective neurotherapies for many devastating neurologic diseases.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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