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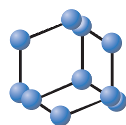
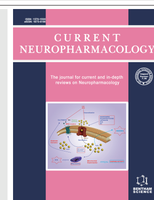
REVIEW ARTICLE





Cell-Based Delivery Approaches for DNA-Binding Domains to the Central Nervous System

Peter Deng[1], Julian Halmai[1], Jennifer J. Waldo[1] and Kyle D. Fink[1,*]

[1]*Department of Neurology, Stem Cell Program and Gene Therapy Center, UC Davis Medical Center, Sacramento, CA, USA*

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Abstract: Advancements in programmable DNA-Binding Proteins (DBDs) that target the genome, such as zinc fingers, transcription activator-like effectors, and Cas9, have broadened drug target design beyond traditional protein substrates. Effective delivery methodologies remain a major barrier in targeting the central nervous system. Currently, adeno-associated virus is the most well-validated delivery system for the delivery of DBDs towards the central nervous with multiple, ongoing clinical trials. While effective in transducing neuronal cells, viral delivery systems for DBDs remain problematic due to inherent viral packaging limits or immune responses that hinder translational potential. Direct administration of DBDs or encapsulation in lipid nanoparticles may provide alternative means towards delivering gene therapies into the central nervous system. This review will evaluate the strengths and limitations of current DBD delivery strategies *in vivo*. Furthermore, this review will discuss the use of adult stem cells as a putative delivery vehicle for DBDs and the potential advantages that these systems have over previous methodologies.

Keywords: DNA-binding domains, artificial transcription factors, zinc finger, CRISPR/Cas9, mesenchymal stem cell, Angelman syndrome, lipid nanoparticle, gene therapy.

1. INTRODUCTION

Pharmacological interventions can be broadly classified into small molecule drugs and biologics. Biological systems contain four types of macromolecules that act as drug targets, namely proteins, polysaccharides, lipids, and nucleic acids [1]. Toxicity, specificity, and inability to obtain potent compounds against the latter three macro-systems have largely directed drug development toward protein targets. Proteins function as a druggable substrate for the 20,000-25,000 expressed genes in the human genome [2-4]. Advances in protein imaging, libraries of tens of thousands of small molecule drugs or biologics, and rational design have made this paradigm highly successful in the treatment of acute diseases such as infections, inflammation, and more chronic disorders such as cancer or neuropathies. However, there remains a limitation in the overall scope of what has been targetable by conventional drug therapies. Computational analysis of known genes suggests that the total number of druggable targets is ~3,000 based on protein structures that are conducive to favorable ligand-protein interactions [5]. As of 2017, there are 1,371 FDA-approved drugs targeting human macromolecules, 667 of which target human proteins, demonstrating a log scale discrepancy between available drugs and the number of expressed human proteins. Furthermore, a majority of approved drug targets have remained focused on the same well-characterized, privileged class of druggable protein families [1, 3, 4] over the last 20 years which consist of G-protein coupled receptors, ion channels, protein kinases, and nuclear receptors with an average of 2 approved drugs per target [4]. Small molecule drugs have an estimated average of 11.5 alternative molecular targets at therapeutic doses (IC_{50}), demonstrating high intrinsic polypharmacology. Together this data indicates that approximately ~11% of known druggable proteins are currently targetable with conventional therapies, less than ~1.3% to 1.6% of the total known genes are targetable, and there are high-intrinsic off-target effects of conventional drug therapies.

*Address correspondence to this author at the Department of Neurology, Stem Cell Program, and Gene Therapy Center, UC Davis Medical Center, Sacramento, CA, USA; E-mail: kdfink@ucdavis.edu

With regards to the CNS, only 240 of 1,669 unique drugs (including anti-malarial) have CNS indications. Despite this, neurologic or neurodegenerative disorders are responsible for more years lost to disability than any other medical condition [6, 7]. Common neurologic disorders such as those related to autism spectrum disorders, schizophrenia, and Parkinson's may be the result of complex gene-environment factors as well as a wide variety of genetic risk factors [8-10]. When further considered in the context of genetically-linked disorders such as Huntington's Disease (HD) or Angelman Syndrome (AS), all clinically approved drug therapies are palliative in nature [11, 12]. Despite major strides in understanding the causative mutation for these disorders [13, 14], conventional drug therapies do not target the underlying cause for genetic diseases, involving mutations in DNA.

The paradigm of conventional drugs for the treatment of genetic disorders has shifted in recent years. Advances in

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molecular therapies through the development of DNA-Binding Domains (DBD) like zinc finger, transcription activator-like effectors, and Cas9 proteins have broadened the "druggable" genome from protein substrates to targeting the substrate for gene expression - DNA. The field of somatic cell genome engineering, with the ability to create designer gene regulatory proteins that can alter the expression of specific genes, has undergone explosive growth in recent years. The mechanism of action of these DBDs is the programmable targeting of nucleic acids to either induce corrective genome editing events [15-17] or manipulate endogenous gene expression using nuclease-free DBDs known as Artificial Transcription Factors (ATF) [18-21]. The specificity of these DBD arrays and the ability to recognize unique loci in the genome with the known disease-causing variants, provide significant value for individuals suffering from "undruggable" genetic disorders, particularly those with neurologic diseases that often involve severe developmental and degenerative consequences [22]. Furthermore, *in silico* and *in vitro* methods such as RNA-seq, ChIP-seq, and amplicon sequencing provide stringent off-target analysis of DBD binding [23, 24].

At the time of this review, pre-clinical and clinical works with DBD gene therapies have generated considerable excitement in their application toward human disease [25]. However, it is important to consider that an effective delivery platform for the CNS is vital for the translational success of these DBD gene therapies and remains a major translational hurdle. The search for a system that effectively crosses the blood-brain barrier to reach target cell types is of particular interest. Given the recombinant nature of DBD proteins, potential immunogenicity in response to these proteins are also a potential translational concern. Thus, ideal characteristics for an effective gene therapy require three components for success: 1) Effective delivery into both the CNS and cells of interest, 2) Reduction of potential toxicities to the delivery vehicle, and 3) protection of payload DBD from being detected by the circulating immune system. Presently, we discuss preclinical and clinical delivery methodologies for DBDs such as adeno-associated virus, lipid nanoparticles, and recombinant protein systems in the CNS landscape. Lastly, we will discuss a novel means of *in vivo* DBD delivery through the use of adult stem cells (Fig. **1**).

Systemic administration of specific AAV serotypes can cross the blood-brain barrier and transduce neurons. AAV propagates in a retrograde manner along axons, endocytosed into neurons, whereby genetic material translocates into the nucleus of the host cell and exists as an episomal vector.

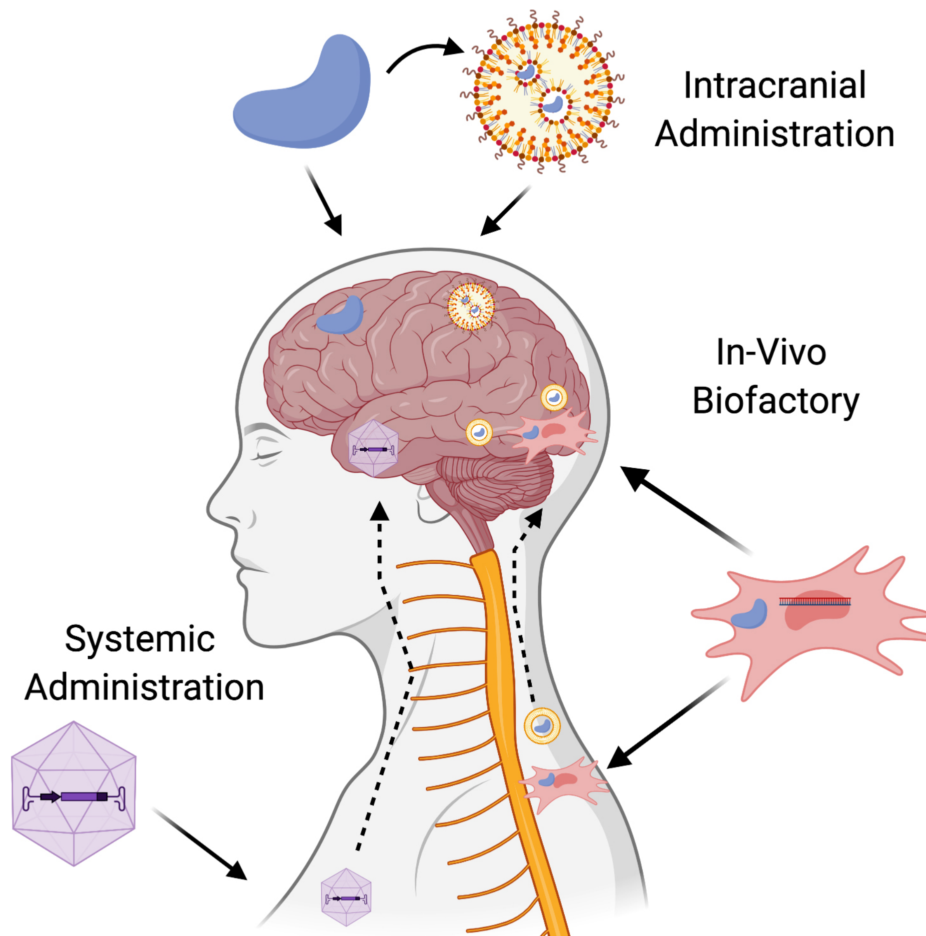

Fig. (1). Delivery systems and routes of administration in the central nervous system. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Purified DNA-Binding Domains can be intracranially injected into CNS tissue or packaged within lipid nanoparticles to improve cellular uptake and protect from scavenging glial cells. Cell-based systems such as hemopoietic stem cells or mesenchymal stem cells can be injected systemically and may interact with the CNS in a number of ways and act as *in vivo* biofactories. HSC replaces endogenous microglia cells and can secrete deficient proteins to neighboring cells. MSCs may travel into the CNS in neuroinflammatory states where they cross BBB and actively secrete trophic factors and exosomes that can influence the CNS microenvironment. Direct injection of stem cell therapies into the brain allows for engraftment and secretion of therapeutic factors in the CNS.

2. ADENO-ASSOCIATED VIRUS IN THE CENTRAL NERVOUS SYSTEM

The brain is comprised of a complex network of multiple cell types (neurons, oligodendrocytes, astrocytes, microglia) that create a highly connected system that drives complex behaviors. Entry to the brain is highly regulated by the presence of the Blood-Brain Barrier (BBB), a series of non-fenestrated endothelial cells that form tight junctions and work in concert with astrocytes and pericytes [26]. The efficacy of the BBB to filter macromolecules has made CNS drug development a traditionally difficult task [27]. The majority of molecules that passively cross the BBB are <300 dA in size [28, 29] and are highly lipid-soluble while retaining low hydrogen bond interaction (<8 surface hydrogen bonds) [30, 31]. These characteristics disqualify all biologics including DBD proteins [32]. Thusly, identifying delivery systems and strategies to circumvent the BBB has been an intensely focused field of study [33].

Viral-mediated approaches, such as an Adeno-Associated Virus (AAV), have been the most thoroughly explored and successful method of expressing biologics in the clinical space [34-36]. AAVs are 20 nm ssDNA non-integrating viral vectors with selective cell tropism depending on the specific serotype and capsid used [37, 38]. AAV2, AAV5, and AAV9 have demonstrated robust *in vivo* neuronal transduction potential. In particular, AAV9 has demonstrated strong pre-clinical transduction of neurons and has been optimized with hybrid capsids to create the more effective AAV-PHPB that has demonstrated robust transduction in deep brain regions of C57BL/6 mice following tail vein injection [39, 40]. Regardless of serotype, AAV is thought to disperse in a retrograde manner along transduced cell axons [41] to engage with various cell receptors to be endocytosed into the cell and translocate to the nucleus to release ssDNA that undergoes second-strand synthesis and exist as an episomal transgene for years [37]. Clinically, Luxturna was the first FDA-approved AAV drug in which an AAV2 vector expressing the normal *RPE65* gene is delivered directly into the retina to treat a genetic form of dystrophy [36]. This was shortly followed by the clinical approval of intravenous infusion of Zolgensma, an AAV5 expressing the missing *SMN1* for spinal muscular atrophy in children. In the context of DBD, the specific use of AAV for *in vivo* delivery has been evaluated extensively [42-45]. Currently, there are four ongoing clinical trials where patients have been infused with an AAV expressing a DBD nuclease; three ZF trials for the correction of malfunctioning genes in Hemophilia B, MPSI, and MPSII and one Cas9 trial for the correction of retinal cells in Leber congenital amaurosis 10 [25].

Despite the meteoric rise of AAV for its ability to effectively transduce tissue, there are still a number of caveats to its use as a delivery system. AAV has a maximum packaging size of 4.9 kilobase pairs [46, 47]. This is large enough to fit zinc fingers (0.6 kbp), TALEs (2.5 kb), and some Cas9 variants with their respective gRNAs (2.7 kb - 4.2 kb) [48] and the necessary transcriptional machinery such as promoters and polyA signals. These packaging limitations only consider nuclease-competent forms of DBDs, a potentially limiting therapeutic strategy compared to ATF-DBDs that utilize effector domains that can alter gene transcription through manipulation of DNA methylation, surrounding chromatin landscapes, or recruitment of transcription factors to bound DNA. AAV size limitations for ATF-DBDs have been a concern as these proteins become particularly large due to the complexity and potential need for combinatorial approaches that necessitate multiple effector domains [18, 19, 49]. For example, a DBD-ZF consisting of the core CMV promoter (0.2 kb), a ZF DBD (0.6 kb), a kruppel-associated box (0.3 kb), and a polyA signal (0.06 kb) is approximately 1.36 kb in size when not accounting for intervening sequences. Further alterations such as the inclusion of nuclear localization signals (0.02 kb), epitopes such as HA or FLAG (0.02 kb), additional effector domains such as DNMT3A (1.1 kb), or switching to an alternative DBD protein such as SaCas9 (3.7 kb) would rapidly reach and exceed the capacity of an AAV. With this consideration, much work has been done on the identification of smaller Cas9 variants that would allow for AAV packagings such as *Parvibaculum lavamentivorans* (pl)Cas9's (3 kb) [50] or CasX's (2.9 kb) [51] with the use of truncated or reduced promoters with observed *in vivo* success [42, 52]. However, Cas9 variants typically have different PAM recognition sites that may limit DNA targets or require the identification and characterization of new guide RNAs for therapeutic development [53].

Beyond technical size limitations, there are a number of considerations for biological responses to AAV following *in vivo* administration. Both developed and pre-existing immunogenicity towards AAV and DBDs remains a challenge in the field. A significant portion of the population possesses a pre-existing immunity to some AAV serotypes [54], reducing the potential patient population for these otherwise very rare disorders or necessitating temporary immunosuppression for the delivery of these viruses. The use of steroids has been shown to spare AAV transgene expression in some hemophilia trials [55-57], however, their use often coincided with a decline of transgene expression [58-61]. Another limiting component of AAV gene therapies is related to its mode of retrograde transport. Retrograde movement of AAV appears to be hindered in the degenerating brain as seen in an induced model of Parkinson's disease [41], potentially necessitating earlier intervention in neurodegenerative disorders. While AAV has demonstrated sustained expression of upwards to 12-15 years [62, 63], safety concerns persist in regards to potential genotoxicities from the sustained expression of nuclease-competent ZF and Cas9 as they have demonstrated off-target effects [64] or unwanted genome

integrations of AAV [44, 65]. Beyond AAV immunogenicity, adult mice have also been shown to develop an immune response towards a long-term expression of these expressed DBDs [44, 66, 67]. Mice that were immunized for Cas9 and then infused with an AAV-Cas9, designed to edit the *Ldlr* gene in the liver, demonstrated an initial gene corrective event, elevated CD8+ T cells, and an eventual complete loss of gene-corrected hepatocytes [68].

While there have been multiple proposed strategies to address the aforementioned issues, including human codon optimization of DBD proteins [66], split Cas9 vectors to account for AAV packaging limits [67, 69], alternative AAV or Cas9 orthologues to ameliorate immune responses [70] or thorough immune screening and preparation in patients [71] where in some cases parents have kept their children in extended isolation to avoid the development of AAV neutralizing antibodies [72], there remain potential safety concerns inherent with AAV vectors that will continue to challenge the field.

3. ALTERNATIVE DELIVERY STRATEGIES

Similar to enzyme replacement therapies, direct administration of a recombinant protein is the simplest and most direct way of applying DBDs therapeutically. Effective delivery of these "naked" proteins to the CNS is confined by certain limitations: avoidance of the host immune system in circulation, effective trafficking across the BBB, and uptake into target cells [73]. There has been considerable work that addresses that the former such as utilizing the existing anionic properties of ZFs to allow for intrinsic uptake into cells [74] or that the inclusion of nuclear localization tags may allow for improved cell penetration [75, 76]. The former two considerations may be avoided through direct, parenchymal injection into the brain. In a study by Staalh *et al. a* single administration of Cas9 Ribonucleic Protein (RNP) into the brain parenchyma has been shown to induce *in vivo* gene corrective events while avoiding microglial response in mice [76]. Direct RNP approaches for gene editing are potentially advantageous as they have been shown to induce less off-target editing due to the temporal nature of these proteins [77] when compared to constitutively expressing systems [78, 79]. However, the *in vivo* correction efficiency of RNP approaches may be limited in post-mitotic cells, such as the neurons, as these cells will not propagate new, "corrected" cells to replace the surrounding dysfunctional neurons. This approach may not be scalable for complex neurologic imprinting disorders such as AS, where there is a large deletion of maternal *UBE3A.* Alternatively, putative approaches using ATF-DBDs have focused on activating the epigenetically silent, but intact paternal *UBE3A*. Repeated intracranial administration of any recombinant protein poses considerable safety concerns, however, manipulation of RNPs through the inclusion of cell-penetrating peptides can allow for systemically administered proteins to cross the BBB [80, 81]. Bailus *et al.* demonstrated that purified HIV-derived TAT peptide-fused ZF protein effectively crossed the BBB and increase the Ube3a protein in the whole brain of the AS mice after intraperitoneal administration, following tri-weekly administrations over the course of 1 month [21]. A caveat to this approach is that the efficacy of deliverable proteins may be attenuated due to pre-existing immunities to DBD proteins such as SpCas9 and SaCas9 [82]. Multiple re-administration of DBD proteins may also result in a progressive decline in efficacy as the host immune system develops neutralizing antibodies towards the recombinant DBD [68], though these issues may be attenuated by utilizing different DBD orthologues as seen in Moreno *et al.* work with Cas9 [70].

Lipid Nanoparticles (LNP) that encapsulate purified DBDs or nucleic material that encodes for DBDs are able to shield putative payloads against circulating proteases [83], RNAses [84], DNAses [85], and immune cells [86]. LNPs are small particles (100-1000 nm) in size that are composed of ionizable cationic lipids that interact with charged payloads. A nanostructured core contains amino lipids that interact with nucleic acid phosphates with their choline heads and work to stabilize the DNA-lipid complex. Cholesterol is evenly distributed throughout both the core and the surface of the LNP, and polyethylene-glycol conjugates are found throughout the surface of the LNP. Neuronal cells are an extensively explored *in vitro* cell type for LNP-based delivery due to an inherent interaction observed between LNPs and apolipoprotein E that facilitates LDL-mediated endocytosis of LNPs [87]. In the context of packaging DBDs, LNPs have been readily assembled with Cas9 RNP for *in vivo* gene correction [88-90]. The synthetic nature of LNPs offers a number of advantages over recombinant protein and AAV approaches - 1) each component of the LNP is highly controllable and can be manipulated to reduce immunogenicity [91] or cell specificity [92, 93] and 2) the choice of payload allows for considerable control of dosing. LNPs have sustained gene expression for weeks to months following injection of plasmid DNA [94] before becoming undetectable which provides an intermediate between the transient nature of recombinant proteins and the long-lasting durability of AAV vectors. Furthermore, LNPs have been shown to be re-doseable as repeated systematic administration of LNP-Cas9 mRNA demonstrates a cumulative increase in editing efficiencies (>50%) of *Ttr* compared to single LNP administration in transgenic mice (>20%), albeit this was in liver-specific correction [95]. Clinically, multi-dose administration of an *IL-12* plasmid expressing LNP demonstrated sustained exogenous gene expression 5-weeks following the final injection in human cancer patients [96]. *In vivo* administration of LNP has been extensively explored, however, a major hurdle in applying LNPs towards CNS disorders is their general inability to cross the BBB. Cas9 delivery with LNPs to specific organs appears to be highly controllable [92], however effective LNP-DBD delivery to the brain have been done through direct intracranial injections [88, 90]. There is growing sentiment within the nanoparticle field that temporal disruptions of BBB permeability or coating of the nanoparticle membrane with Rabies virus glycoprotein (*Rvg)* [93] may allow LNPs to cross the BBB [97, 98]. In regard to LNP-specific toxicities, they are variable and appear to be dependent on the specific composition of the particle itself. Toxicities from systemic administration of LNP are commonly the result of hepatotoxicity and reduction in blood cell counts [96, 99].

Ultimately the choice of delivery methodology is highly contingent on the end goal of the deliverable DBD. Immu-

Table 1. Summary of putative delivery systems toward the CNS.

-	Purified Protein	Lipid Nanoparticle	Adeno-Associated Virus	Cell Therapies
Package Size (kb or kDa)	ZF - 70 kDa TALE - 120 kDa Cas9 ~ 180 kDa	7 kb (nucleic acids) [100] 180+ kDa proteins [101]	4.6 kb - 4.9 kb. **9.2 kb - 9.8 kb in split-AAV systems	8.9 kb (per lentiviral vector)
Mechanism of Neuronal Cell Engagement	Cell-penetrating peptides [80], nuclear localization signals [76]	Engineered tropisms of a surface membrane with small molecules or recombinant proteins [93, 97]	Serotype specific, Engineered capsid proteins [40, 102]	Direct cell engraftment, Cell migration to CNS, Uptake of secreted factors, Engineerable tropisms of extracellular vesicles [93]
Routes of Administration (Preclinical/ Clinical)	Intracranial, Intravenous	Intracranial [88], Systemic [95, 98]	Intracranial, Intrathecal, Systemic	Intracranial, Systemic
	Systemic [103]	Systemic [96]	Intracranial [104], Systemic [35]	Intracranial [105, 106], Intravenous [107-109]
Duration (Preclinical/ Clinical)	Variable	2-Months (plasmid) [110] 3-8 days (mRNA) [95, 111]	12 years (canine) [63], 15 years (NHP) [62]	HSC: Permanent engraftment in circulation MSC: 75 days [112]
	Variable [113]	5-weeks (plasmid) [96]	10+ years [104, 114]	Fetal cell transplants: 15-24 yrs [105, 106] HSC: 13+ years [107, 108] MSC: 87 days [115]
Toxicity	Pre-existing immune response [82], Developed immune response [70]	Nanoparticle aggregation [86] Hepatotoxicity, reduction in blood counts [96]	Liver toxicity, pre-existing immunities, Immune responses [71]	Potential immunization and rejection of transplanted tissue [116, 117] Potential immunogenicity of secreted protein. *Potential amelioration of inflammation with MSC factors

*Packaging size can be increased by using multiple AAV systems.

nogenicity towards both the therapeutic and delivery vehicle, ability to cross the BBB, and durability of effect are all important considerations when selecting a delivery vehicle system (Table **1**) [100-117]. An ideal approach would encompass some balance of all these principles.

4. STEM CELL DELIVERY SYSTEMS AND REGENERATIVE MEDICINE

The use of stem cell therapies has become increasingly attractive as a putative therapy for CNS disorders. The ability to work synergistically with the endogenous microenvironment to upregulate intrinsic cell proliferation or neuroprotection *via* trophic factor secretion potentially enhances the overall regenerative potential of the transplanted tissue. Stem cell-based therapeutics for CNS disorders have been studied clinically for more than 30 years as a cell replacement strategy in neurodegenerative disorders [118]. Cell transplantation approaches can be broadly divided into three categories 1) cell transplants from dissected fetal tissue, 2) pluripotent stem cells derived from embryonic stem cells or from reprogrammed adult somatic cells such as fibroblasts, and 3) adult multipotent stem cells such as hemopoietic stem cells or mesenchymal stem cells [118]. Fetal tissue has been studied in the neurodegenerative field, demonstrating engraftment for up to 10 years following transplantation of fetal basal ganglionic eminence and a corresponding improvement in disease phenotypes at early time points in HD [105]. Persistent engraftment of fetal ventral mesencephalon in a PD patient was observed 24 years after surgery, also associating with phenotypic improvements for 10 years [106]. The use of fetal tissue remains both a technical and controversial issue. Thus, the development of induced pluripotent stem cells by Yamanaka in 2006 marked a major milestone in regenerative medicine by providing an alternative source of pluripotent stem cells and reducing reliance on fetal tissue. In regard to CNS disorders, iPSCs have been evaluated for cell replacement in the neurodegenerative [119, 120] and modeling in the neurodevelopmental field [121-125]. Clinically, iPSCs have been injected for macular degeneration disorder [126] and Parkinson’s disease [127].

Stem cells have historically been utilized as a method of cell replacement; however, their properties provide a unique alternative to AAV, RNP, and LNPs. Stem cells are metabolically active entities that can engraft for some period of time and are able to actively produce biologics *in vivo*. Gene replacement of ADA-SCID through the retroviral manipulation of Hemopoietic Stem Cells (HSC) was clinically evaluated in the 1990s [128, 129], and the summation of this field was the clinical approval of Strimvelis in Europe. A similar platform utilizing highly purified CD34+ HSCs pioneered by Don Kohn’s group is progressing towards clinical trials in the United States [130].

More recently, data on Lysosomal Storage Diseases (LSD) have elucidated an alternative approach towards treat-

ing CNS disorders. Sandhoff's, MPSI, MPSII, and metachromatic leukodystrophy are different types of genetic LSDs that result in the build-up of toxic metabolites within lysosomes, resulting in cognitive impairments, developmental delays and declines, and reduced life-span [131]. Lifelong enzymatic replacement strategies are able to attenuate pulmonary and cardiac phenotypes associated with LSDs, however, these enzymes are not able to cross the BBB [132]. Interestingly, allogeneic transplantation of healthy CD34+ HSCs demonstrates improved neuronal outcomes in patients by providing a stable, endogenous source of the deficit proteins [108, 133, 134]. Newly derived healthy microglia and macrophages are able to cross the BBB to locally deliver the deficient proteins in the brain due to their myeloid origin [135]. Long-term follow-ups in the first child transplanted with CD34+ HSCs for MPS1 demonstrated sustained improvement in cognitive performance 13 years after surgery [107]. Additionally, overall attenuation of brain atrophy (11/17 patients) and reduction in cognitive decline (6/7 patients) were observed 10 years following CD34+ HSC transplantation for MPSII [108]. Moreover, therapeutic efficacy can be improved by increasing the levels of the deficient proteins in LSDs. Beegle *et al.* have recently shown that human CD34+ HSCs transduced to over-express *HEXA*/*HEXB* improved phenotypic outcomes and ganglioside aggregates in immune-deficient mouse models of Sandhoff's disease [136]. *Ex vivo* modification of CD34+ HSC with lentivirus to produce supraphysiological levels of the deficient enzyme is currently being evaluated in ongoing clinical trials [137, 138], while Cas9-edited CD34+ HSCs have been preclinically shown to improve phenotypes in mice [139].

Thus, the use of stem cell therapies as a delivery system provides an *in vivo* "biofactory" for sustained delivery of a biologic. The utilization of CD34+ cells as a delivery system for DBDs may not be advantageous due to the potential self-recognition of these proteins as well as potential genotoxicities associated with constitutive expression of DBDs for the life of the reconstituted transplant. However, there are other cell-based therapeutics that may be more suitable as a delivery system.

5. MESENCHYMAL STEM/STROMAL CELLS

Mesenchymal Stem/Stromal Cells (MSCs) have generated great interest in regenerative medicine and immunotherapy due to their unique biological properties. MSCs are multipotent stem cells derived from a broad subset of adult tissue such as adipose, bone marrow, and umbilical cord that are readily available for clinical use [140]. Due to their immune-modulating potential, MSCs do not require immunosuppression following allogeneic transplantation and have demonstrated a strong safety profile in clinical trials across multiple disease indications in the last twenty years and over 900 reported clinical trials [141]. Despite not being neurologic in origin, there have been 120 clinical trials using MSCs for a diverse set of CNS disorders such as spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, stroke, Alzheimer's, and Parkinson's [141].

MSC therapeutic potential is mediated by their robust anti-inflammatory abilities [142-150], ability to disperse to a wide range of tissues following administration [112], and secretome of bioactive molecules [151]. MSCs modulate the adaptive immune system by suppressing T-Cell activation [142, 150], T-Cell proliferation [145], and differentiation. Umbilical cord-derived MSCs are able to inhibit monocyte to macrophage differentiation [143] and reduce transcriptional expression of inflammatory cytokines such as IL6, IL8, TNFa, and MFP-a [144]. Importantly, these immunomodulatory effects appear to be paracrine in nature as Le Blanc *et al.* description of MSCs expressing low levels of MHC-I and no MHC-II on surface proteins, suggesting that there is no direct interaction between MSCs and T-Cells [147]. In induced rodent stroke models, systemic administration of MSCs demonstrated migration of cells toward the hemisphere with the infarction [152, 153]. This homing is thought to be mediated as a response to chemical stressors [154]. Direct striatal administration of allogeneic umbilical-cord-derived-MSCs in the R6/2 transgenic mouse model of Huntington's disease demonstrated sparing of striatal atrophy, greater density of Darpp32+ cells, and improvements in spatial memory tasks 6-weeks following injection [155]. Striatal co-administration of allogeneic bone-marrow-derived MSCs with allogeneic adult NSCs has demonstrated functional improvements in motor coordination tasks in transgenic rat models of Huntington's. Interestingly, adult NSCs have been shown to improve phenotypes in the degenerating HD brain [116, 156], however, these inflammatory microenvironments are often non-conducive for favorable engraftment of NSCs. Co-administration of MSCs improved engraftment size of NSCs 20-weeks after surgery in 17-months old transgenic HD rats, likely due to secretion of anti-inflammatory cytokines that attenuated the presence of reactive astrocytes around NSC transplant [156]. MSC cocktails have been evaluated clinically in human umbilical cord blood cell transplants. Autologous human umbilical cord blood IV-infusion in young children with cerebral palsy in a double-blind, placebo-controlled, crossover phase 2 clinical trial demonstrated motor score improvements in hi-dose patients in relation to low-dose and placebo groups as well as improvements in whole-brain connectivity imaging scores post-treatment [109].

Human MSCs have been shown to persist for up to 75 days following systemic administration in immune-deficient mice in the spleen, pancreas, kidney, liver, muscle, art, and brain [112]. Previous work by Rossignol *et al.* demonstrated the persistence of rat bone-marrow-derived MSCs in the striatum of transgenic HD rats for up to 20-weeks following administration [156]. Bahr *et al.* evaluated the long-term engraftment of both haplo-matched and mismatched bone-marrow-derived MSCs in a small set of hematologic cancer patients receiving bone-marrow engraftments [115]. A total of 5 out of 15 haplo-mismatched, HLA-mismatched MSC patients demonstrated positive engraftment of MSCs following intravenous infusion as compared to 1 out of 6 haplo-matched, HLA-mismatched MSC patients. The longest observed engraftment was 87-days following infusion in one individual. Histological identification of MSCs at these later times is often infeasible due to the low presence of cells and more sensitive molecular techniques such as PCR are necessary to detect these cells.

The relatively short persistence of MSCs within the body following administration [157-160] and low *in vivo* proliferative potential would suggest that the therapeutic effects of MSCs are not due to engraftment and cellular replacement as would be seen with NSCs, HSCs, hESC, or iPSC-derived cell types. Recent studies from the last decade have suggested that the therapeutic potential of MSCs is mediated by a "hit-and-run" effect that is mediated by its robust secretome of bioactive factors such as proteins, nucleic acids, and lipids. Interestingly, conditioned media from MSCs have been shown to be as equally effective as MSCs in improving outcomes in multiple disease indications [161]. While MSCs have been known to secrete both trophic and immunomodulatory factors for decades, many of these factors including nucleic acids do not have signal peptides to promote their extrusion from their host cells [162, 163]. This gap in understanding was addressed in landmark work by Trimmer *et al.* that revealed a 50-200 nm biologically active fraction from the conditioned media of human ESC-derived MSCs. Succeeding characterization studies on MSC conditioned media have revealed a population of extracellular vesicles within the MSC secretome that include exosomes and microvesicles with a similar membrane as their donor MSC such as CD63+, CD81+, CD90+, and CD105+ markers. Ragni *et al.* use of electron microscopy on both human bone and umbilical cord blood-derived MSCs revealed the presence of extracellular vesicles of diverse sizing (40-50 nm and 600-700 nm), indicating the presence of exosomes and microvesicles.

Extracellular vesicles are lipid-covered nanoparticles that are secreted from many cell sources as a mode of intracellular communication. Microvesicles (100-1000 nm) are released from the cell surface through fission of the plasma membrane and are known to contain proteins and RNA molecules. Exosomes (40-100 nm) are endosomal in origin and are the result of segregating intraluminal vesicular bodies within these endosomes which are released into the extracellular space upon fusion with the apical side of the plasma membrane [164].

Ragni *et al.* further demonstrated the presence of mRNA transcripts encoding for immune regulators Il-10 and IL-4 within these exosomes. Isolated exosomes containing IL-10 mRNA were able to be uptaken and expressed in HKC8 cells. Deep sequencing in MSC-derived exosomes reveals an enrichment of miRNA and tRNAs which provide an insight into the use of exosomes as both intracellular communication tools and potential diagnostic biomarkers [165]. A recent analysis by Toh *et al.* suggested that the therapeutic effects of exosomes are more likely due to the presence of packaged protein and not due to the horizontal transfer of nucleic material due to low abundance of both mRNA or biologically important miRNA [166]. Proteomics data from Wharton's Jelly-derived MSCs have shown a dynamic protein secretome with over 400 discrete proteins ranging from 10-500 kDa. The majority of these secreted proteins (74.1%) have been shown to fall within traditional secretion pathways *i.e.* known signal peptides that induce protein secretion through the ER and Golgi apparatus which stands in contrast to the majority (61.4%) of published proteins found in exosomes, which appear to be intracellular proteins that do not demonstrate traditional secretion dynamics. Exosome-specific proteins also demonstrate a dynamic size range of 10 kDa to 200+ kDa [167]. Taken together, this data suggests that the paracrine effects of MSCs can be mediated by multiple mechanisms of action from a direct secretion of proteins or extravesicular packaging of non-secretable proteins of dynamic sizes.

The identification of exosomes as a mediator for the therapeutic effects has expanded the MSC field of study towards "cell-free systems" where isolated MSC-derived exosomes are directly infused into biologic systems. Tail vein infusion of allogenic exosomes isolated from bone-marrow-derived MSCs improved neuroinflammation, neurogenesis, and attenuated behavioral deficits in a rat TBI model [168]. Xenogeneic exosomes from human bone-marrow-derived MSCs have demonstrated improvements in neuroinflammation markers and cognitive function in rat TBI models [169], in neurogenesis, inflammation, and motor behavior in induced mouse stroke models [161], as well as a reduction in hypomyelination and seizure activity in a fetal ischemic ovine model [170]. Interestingly, the role of inflammation *via* dysfunctional microglia may be a contributor to schizophrenia [10]. Intravenous injection of human umbilical MSCs reduced molecular markers and presence of hyperactive microglia in amphetamine-sensitized schizophrenic mice [171]. This data was further corroborated by improvements in cognitive behavior tasks of phencyclidine-induced schizophrenic mice following intranasal administration of exosomes from human MSCs [172]. More directed research revealed that isolated exosomes from dendritic cells expressing the *Rvg*-fused to LAMP2 for specific exosomal targeting could be complexed with siRNAs *ex-vivo* and silence endogenous genes in the brain following tail vein injection [173]. Similar approaches have been utilized in Parkinson's disease [174] and morphine addiction [175]. LNP particles that have been coated with *Rvg*-expressing exosomes have also demonstrated uptake in the brain following tail vein injection in healthy mice [93], suggestive that engineered exosomes can potentiate uptake into the brain. The exact mechanism of how exosomes cross the BBB is not fully understood. The BBB can be permeabilized due to sudden insults such as a TBI or have a "leaky" state due to chronic neuroinflammation as in the case of neurodegenerative disorders that allow the diffusion of nanoparticles into the brain [176]. *In vitro* work in BBB models has shown that the ability of exosomes to cross the BBB may be endocytosed on one side of brain endothelial cell and then secreted on the other monolayer of the BBB [177].

The composition of secretable factors differ depending on the source of MSCs and can be further modulated for desired cell-specific effects through pre-conditioning regiments such as incubation in hypoxic environments that can increase Vascular Endothelial Growth Factor (VEGF) [178, 179] or pre-treatment with small molecule drugs can improve the therapeutic outcomes and homing potential of MSCs towards the site of damage. MSCs from different donor origins have differing secretome compositions (adipose MSCs vs BM-MSCs) and overall exosome quantity production. It is interesting to note that there is inherent variation within the same MSCs as passage number influences survivability and expression of trophic factors [155]. Production of

bioactive factors within MSCs is still constrained due to natural limits in gene transcription. The translational outcome of this may be the overall amount of produced bioactive particles that may not be physiologically relevant when accounting for the entire organ of interest. Fortunately, MSCs are relatively easy to transduce using viral vectors to produce genetically engineered stem cells that are capable of producing supraphysiologic levels of trophic factors, miRNAs, or transcription factors [180]. Human bone-marrow-derived MSCs that were engineered to produced increased amounts of BDNF improved anxiety phenotypes in the immune-suppressed YAC128 HD mice as well as survivability in immune-suppressed R6/2 HD mice as compared to non-engineered MSCs controls [181]. Rat MSCs engineered to overexpress a hyperactive *Hif1a* transcription factor increased endogenous *Vegf* expression and reduced infarction size compared to MSCs alone in an induced MCAO model [182].

6. MESENCHYMAL STEM CELLS AS PUTATIVE DELIVERY VEHICLE FOR DBDS

Cell-based production systems for the encapsulation of DBDs into vesicles have been a novel field of study in the last decade. Our group has been highly interested in these systems as a delivery modality for DBD and we received a California Institute for Regenerative Medicine Quest Grant for “MSC delivery of an artificial transcription factor to the brain as a treatment for Angelman Syndrome” [183]. DBDs such as zinc fingers, TALEs, and dCas9 protein can be effectively packaged into lentiviral vectors (~9 kb total size) to be used to engineer cells to act as bio-factories for endogenous protein secretion, bypassing AAV size limitations and the need to produce vast amounts of protein *ex vivo* for administration as seen in RNP studies. Presently our group has demonstrated the secretion of both ZFs and TALEs from reprogrammed MSCs through the addition of eukaryotic signaling peptides. The inclusion of cell-penetrating peptides and nuclear localization signals allowed for uptake and biological activity of DBDs in AS models (in preparation) [184]. A caveat to applying this same system towards Cas9 has been effectively packaging Cas9 with its obligate gRNA.

This challenge has been addressed by a number of groups utilizing Viral-Like Particles (VLPs) to package Cas9 protein and gRNAs within extracellular vesicles with high transduction potential [185, 186]. Emerging VLP systems are exciting, cell-free systems of delivering DBDs that may allow for direct packaging of DBD proteins into particles with controllable tropism and cell uptake. Mangeout *et al.*’s Nanoblades system offers a VLP system capable of engineerable tropisms which may allow for repeated administration of VLP and wide cellular transduction potential [187]. Gee *et al.*’s NanoMEDIC system provides a chemically inducible system for producing high yield DBD VLP for xeno-free clinical applications [188]. VLP approaches and other cell-free systems such as MSC-derived exosomes are favorable in a manner that they reduce the need for the isolation of scarce stem cell types and avoid potential tumorigenicity of transplanted cells, though they may still require repeatable dosing of therapeutic as seen in both direct protein and LNP approaches.

Conversely, the combination of elegant VLP production strategies with stem cells with existing robust secretion abilities such as MSCs may be the “best of both worlds” in regard to an effective *in vivo* delivery system. MSCs can be readily expanded and modified *ex vivo* to express proteins such as DBDs [189]. Potential immunogenicity towards delivered protein may be ameliorated by the production of anti-inflammatory cytokines inherently produced by MSCs to ameliorate local activation of immune cells. Furthermore, expressed DBDs can potentially be shuttled to VLP-exosomes with engineered tropisms and can be uptaken by cells with high efficiency. Using gene-modified MSCs as a delivery strategy addresses multiple caveats to conventional delivery of proteins, LNPs, and AAV. These MSCs can persist for a while within injected tissue, actively produce a VLP-exosome that encapsulates DBD from circulating immune cells and secrete anti-inflammatory cytokines that reduce innate or induced immunogenicity towards the DBD payload, creating a microenvironment that is highly conducive to gene modification with minimal immunogenic toxicity.

CONCLUSION

Ultimately the choice of delivery vehicle is highly contextual, whereby a single administration of gene editing particles may be sufficient to achieve the desired effect, though this requires a transient system such as a purified protein injection or LNP. *In vivo* gene editing is inherently risky due to the potential off-target editing that may be difficult to reverse. In comparison, DBD proteins fused with effector domains that remodel chromatin structure or DNA-methylation serve as candidate biologics that can act with increased pharmacological duration in comparison to conventional drugs while not permanently altering the genome [21, 190, 191]. AAV serves as an ideal candidate as its durable persistence in the tissue would assuage concerns of re-administration, though there are persistent immune concerns to AAV and the impact of long-term expression of DBD modifying proteins is not yet fully understood. The limits of choice between long-lasting or highly transient delivery systems are changing as key effector domains that result in durable epigenetic change may not necessitate repeatable dosing as the effect persists even when the protein is gone [18, 192]. An epigenetic approach would likely necessitate a delivery system that would persist just long enough for these durable effects to take place. Regardless, identifying an ideal delivery system for DBDs toward the CNS remains a major goal within the gene therapy space. Advancements in synthetic LNP strategies may provide viable alternatives to the current dominance by AAV. Adaptation of VLP approaches with either cell-based or cell-free systems may provide highly potent *in vivo* biofactory approaches that overcome shortcomings in both LNP and AAV. Additional refinement in the current delivery technologies, understanding of how these systems function *in vivo*, and reducing potential toxicities will be necessary to advance the field forward.

LIST OF ABBREVIATIONS

AAV = Adeno-associated Virus

AS = Angelman Syndrome

ATF	=	Artificial Transcription Factor
BBB	=	Blood-Brain Barrier
CM	=	Conditioned Media
CNS	=	Central Nervous System
DBD	=	DNA-Binding Domain
HD	=	Huntington's Disease
HSC	=	Hemopoietic Stem Cell
iPSC	=	Induced Pluripotent Stem Cell
LNP	=	Lipid Nanoparticles
MSC	=	Mesenchymal Stem/Stromal Cell
VLP	=	Viral-Like Particles

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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