Next-generation strategies for gene-targeted therapies of central nervous system disorders: A workshop summary

Jill A. Morris,[1] Chris H. Boshoff,[1] Nina F. Schor,[1] Ling M. Wong,[1] Guangping Gao,[2] and Beverly L. Davidson[3]

[1]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA; [2]Horae Gene Therapy Center, UMass Medical School, Worcester, MA 01605, USA; [3]Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, PA 19104, USA

The National Institute of Neurological Disorders and Stroke (NINDS) held a workshop titled "Next generation strategies for gene-targeted therapies of central nervous system (CNS) disorders" in September 2019 in Bethesda, MD, USA. The meeting brought together a multi-disciplinary group of experts in the field of CNS-directed gene-targeted therapy delivery from academia, industry, advocacy, and the government. The group was charged with identifying the key challenges and gaps in this evolving field, as well as suggesting potential solutions. The workshop was divided into four sessions: (1) control of level and location, (2) improving delivery and distribution, (3) enhancing models and manufacturing, and (4) impacting patients. Prior to the workshop, NINDS established working groups of key opinion leaders (KOLs) for each session. In pre-meeting teleconferences, KOLs were tasked with identifying the research gaps and key obstacles that delay and/or prevent gene-targeted therapies to move into the clinic. This approach allowed for the workshop to begin with problem-solving discussions and strategy development, as the key issues had been established. The overall purpose of the workshop was to consider knowledge gaps and potential strategies to inform the community around CNS gene-targeted therapies, including but not limited to researchers and funders.

INTRODUCTION

Recent advances in the field of gene-targeted therapies have contributed to increased interest in gene addition, gene silencing, and genome-editing strategies as approaches to treatment of neurological disorders. The National Institute of Neurological Disorders and Stroke (NINDS) held a workshop titled "Next generation strategies for gene-targeted therapies of central nervous system (CNS) disorders" on September 26 and 27, 2019 in Bethesda, MD, USA. This workshop brought together a multi-disciplinary group of experts from academia, industry, advocacy, and government to identify key challenges and gaps, as well as opportunities in gene-targeted therapies for CNS disorders. In addition, the discussions provided insight into how NINDS could facilitate these next generation strategies for developing therapies for patients with neurological disorders. CNS-directed gene-targeted therapies involve unique challenges, including delivery into the spinal cord and brain, distribution throughout the

CNS or in specific brain regions, cell type-specific expression, spatiotemporal specific expression for neurodevelopmental disorders, timing of delivery before neurodegeneration is irreversible, and effects on CNS circuitry. The workshop was held in a roundtable format with the overall goal of having a highly interactive forum focused on therapeutic strategy development.

The workshop objectives were to determine what is needed and how to accelerate progress in four key areas: (1) Control of level and location, including issues with overexpression, the need for cell type-specific capsids and regulatable promoter/enhancers, and the potential of off-target effects; (2) Improving delivery and distribution, including understanding and evading the immune response upon delivery of the gene-targeted therapy, enabling second administration, new tools to detect and evaluate an immune response, and tracking distribution; (3) Enhancing models and manufacturing, including preclinical models, animal models of immune responses, and manufacturing challenges; and (4) Impacting patients, including clinical trial readiness, innovative/adaptive trial design, standardized clinical procedures/measurements, ethics, and clinical trial networks.

In preparation for the workshop, NINDS convened pre-meeting working group teleconferences for each session during which key opinion leaders (KOLs; see supplemental information) were tasked with brainstorming research gaps and key obstacles to be discussed during the in-person meeting. This approach enabled more productive, problem-solving dialogue during the workshop. By the start of the workshop, key issues had been established and the participants were poised to devise strategies for addressing these obstacles and identify pathways through which to move forward.

The in-person workshop was videocast and virtual attendees were encouraged to participate by sending their comments to the

organizers. To facilitate strategy development, participants were instructed to think high level and big picture and to propose solutions: across CNS diseases (i.e., not disease specific); for both common and rare diseases; considering all types of gene-based strategies, including gene replacement, antisense oligonucleotides (ASOs), RNA therapies, and genome editing; considering all delivery methods (e.g., viral, non-viral) as well as specific delivery routes (i.e., systemic or local); identifying the key gaps (e.g., in knowledge, infrastructure); and providing input on what NINDS needs to know about these obstacles and strategies. To frame strategy implementation, participants were tasked with considering the following: realistic goals and specific deliverables; prioritization and timeline; roadblocks that are immediately and addressable; and ways that the community, across academia, government, industry, and non-profit organizations, could collaborate to address these obstacles.

SESSION 1: CONTROL OF LEVEL AND LOCATION

Session one focused on the challenges of transgene overexpression, the need for cell type-specific capsids and regulatable promoters/enhancers, the development of an adeno-associated virus (AAV) CNS atlas, and how to assess and minimize off-target effects.

Overexpression

Multiple participants noted that too much gene expression or overexpression in the wrong cells can be deleterious. For example, in early GM2 gangliosidoses preclinical studies it was determined that AAVrh8 vectors encoding species-specific α- or β-hexosaminidase subunits at a 1:1 ratio were tolerated when delivered by intracranial (i.c.) infusion in the mouse, cat, and sheep models. However, a comparable dose to that used in the cat model delivered by bilateral infusion into the thalamus and left ventricle in nonhuman primates (NHPs) resulted in neurotoxicity.[1] In other studies, aimed at treating frontotemporal dementia and neuronal ceroid lipofuscinosis, AAV9 vectors containing the progranulin (GRN) gene were delivered via the lateral ventricles to obtain widespread distribution of GRN overexpression in a Grn null mouse model. The investigators found region- and cell type-specific T cell-mediated toxicity due to GRN overexpression causing degeneration of hippocampal neurons and glia.[2]

In many cases, overexpression might not even be necessary to achieve clinical benefit. Workshop participants discussed the finding that the same efficacy can be obtained using a weak promoter (e.g., the JeT promoter) as using a strong promoter (e.g., the CAG promoter) to drive transgene expression in mouse models of disease. The distribution of AAV throughout the CNS is more important than getting high levels of expression for some diseases. The key is to get the right level of expression in enough target cells to provide clinical benefit. For example, it was asserted that in lysosomal storage disorders, it is typically thought that just 10% of endogenous protein expression levels can correct cellular dysfunction.

It became clear during the discussion that widespread and uniform cellular distribution of viral transduction is a critical objective in AAV gene therapy. If AAV is delivered by intrathecal (i.t.) adminis-

tration, the cells close to the injection site will be transduced by numerous virions, resulting in a high level of transgene-encoded protein expression while other cells farther away will have low levels of expression. For example, there could be 500 copies of an AAV-delivered transgene in one cell and 5 copies in another cell and, therefore, there could be 100-fold higher expression in one cell than another. Furthermore, a recent publication examining ASO distribution after i.t. delivery reported similar issues with differences in intracellular ASO concentrations.[3]

It was also noted that expression levels should be considered in the context of normal neurodevelopment, as endogenous expression levels may vary at different developmental time points. For example, some genes may require higher levels in specific cell types during early postnatal development, and decreased expression in adulthood. This links directly to the idea of regulatable gene therapy. This is complicated even more by cell-autonomous effects, that is, the need to manage expression levels on a per-cell basis. The understanding of the natural expression profile of the gene of interest in the context of lifespan and in the right intracellular environment (i.e., cell autonomous) is critical to the proper tailoring of gene therapies. For example, for numerous neurodevelopmental disorders, including Rett syndrome, overexpression of the gene product is clinically known to be deleterious.[4] Some groups, therefore, are trying DNA or RNA editing approaches, but there are concerns about low efficiency and off-target effects of these therapies.

Alternatively, there are situations where high levels of transgene expression may be needed. For example, gene therapy approaches to shrink tumors use AAV vectors containing the caspase transgene that are injected into the tumor, and high levels of caspase expression are required for tumor volume reduction. High expression levels may also be needed for *ex vivo* gene therapy. For example, to treat some lysosomal storage disorders, cells are harvested from the patient, then lentiviral vectors transduce the cells *ex vivo*, and the cells are then transplanted back into the patient to secrete the transgene-encoded protein for cross-correction of other cells.

In summary, improved methods and technologies should ideally provide sufficient intracellular concentrations of AAVs or ASOs to many cells across the CNS (Table 1). In addition, for neurodevelopmental disorders, it is critical to first understand the endogenous expression levels in specific cell types throughout development. Therefore, there is also a clear need to develop regulatable gene therapy approaches. This would include the generation of regulatable promoters/enhancers that mimic the endogenous expression levels in the correct cell type throughout the lifespan and especially during neurodevelopment (Table 1).

Developing cell- and organ-specific capsids

To prevent overexpression and to achieve the appropriate spatiotemporal expression, it is critical to develop vectors to target the right cells, with expression at appropriate levels (Table 1). The workshop attendees identified specific considerations that need to be kept in

Table 1. Session 1: Control of level and location

Topic	Needed knowledge and strategies
Addressing overexpression	Advance methods and technologies that will result in desired intracellular concentrations of AAV or ASOs across the CNS or to targeted cell types.
	Understand the natural expression of the gene of interest in the context of lifespan and in the right intracellular environment (cell autonomous), particularly for neurodevelopmental disorders.
Developing cell- and organ-specific capsids	Develop AAV capsids that will (1) transduce specific CNS cell types, including different neuronal and glial cell subtypes; (2) transduce the various neuronal and glial progenitor cells at different stages of CNS development; and (3) de-target specific cell types or organs that contribute to an immune response or toxicity when they are transduced.
	Develop accessible methods to determine whether AAV capsids identified by rational design or directed evolution in animal models translate to cell- and organ-specific expression in humans.
	Explore other viral vector delivery options including lentivirus, nonpathogenic herpes virus, rabies, measles, and other RNA and DNA viruses or non-viral delivery methods such as liquid nanoparticles.
Developing regulatable vectors: Promoters/enhancers	Develop new cell type-specific promoters/enhancers in combination with AAV capsids (and potentially miRNAs) that will express the transgene in specific CNS cell types, including the different types of neurons as well as glia, and result in the appropriate expression levels in the correct cell type.
	For neurodevelopmental disorders, design regulatable or spatiotemporal promoters/enhancers that mimic the endogenous expression levels in the correct cell type throughout neurodevelopment.
	Understand how the *cis* elements (e.g., ITRs) from viral vectors are contributing to the promoter/enhancer activity and whether ITRs can be insulated to prevent interaction with the promoter/enhancer.
	Examine other factors that may influence transgene expression, including sex differences, circadian rhythm differences, and environmental factors.
AAV CNS atlas for cell specificity and biodistribution	Produce a public resource that includes data on both cell specificity and biodistribution for the various AAV vectors, including the capsid, promoter/enhancer, transgene, and miRNA combinations; in addition, it should integrate data on the methods/processes of rAAV production and capture the vector quality, including the full/empty capsid ratio.
	Multiple variables should be considered, including species, age, sex, and environmental factors; the data could include advances in imaging modalities, single cell expression, and post-mortem analysis.
Off-target effects and toxicity	Investigate the mechanisms and determinants of AAV integration into the host genome.
	Understand the long-term effects in patients of CNS-directed AAV gene-targeted therapy, including AAV integration.

mind when generating regulatable vectors or modifying capsids for targeting. First, there is an insert size constraint of approximately 4.7 kb for AAV vectors. Second, the generation of AAVs engineered for specific transduction properties or expression in cell lines, mice, and/or NHPs may not translate to the *in vivo* human situation. This is a key concern and challenge.

Participants discussed different elements of AAV-based gene therapeutics that can be modified. For example, the capsid itself may be engineered to enhance cell type specificity. The two main approaches used to modify AAV capsids are rational design and directed evolution. Rational design is the genetic modification of the AAV capsid based on current knowledge of AAV structure and function. Directed evolution allows the alteration of the capsid landscape by generating capsid libraries through error-prone polymerase chain reaction (PCR), random peptide insertions, domain-focused and saturated mutations, and DNA shuffling. The library is subsequently screened for the desired cell type-specific expression in cells and/or animal models.[5]

One example of a capsid-directed evolution screening method uses Cre recombination-based AAV targeted evolution (CREATE)[6] and the Multiplexed-CREATE platform.[7] For the latter, the researchers performed a positive and negative screen of a 7-aa substitution library at the AA455 loop of AAV-PHP.eB by intravenous (i.v.) administration in the mouse. Their goal was to find variants that bind preferentially to specific cells. They identified an AAV variant (AAV.CAP-B10) that transduces CNS neurons with high efficiency and transduces peripheral organs, including the liver, with reduced efficiency. De-targeting transduction of the liver is important to reduce the likelihood of an immune response and toxicity. Importantly, the cell and organ specificity of AAV.CAP-B10 was replicated in the adult marmoset following i.v. administration.[8]

Often, directed evolution is performed with a multi-systems approach. The AAV library is first screened in one species and a pool of AAV variants is selected for cell and organ specificity. The resulting pool of variants is then screened in another species with the objective of identifying capsids that show the same specificity in multiple species. This is necessary as some AAV variants may be highly efficient at CNS transduction in one species but not in others. For example, the AAV variant AAV-PHP.B was found to be highly efficient at transducing cells throughout the CNS by i.v. administration.[6] However, this efficacy did not translate to NHPs.[9] Nonetheless, there

is still the possibility that using directed evolution in rodents or NHPs may not translate to humans, in particular for patients with CNS disease where the extracellular matrices, cell surface receptors, or other cellular properties important for vector-mediated gene transfer are impaired. Understanding how engineered vectors transduce cells (e.g., identifying the receptors required for binding and entry) would help inform their translatability. The general consensus was that the field would benefit from using primates, or human tissue-based organoids or systems, to gain increased confidence to predict which capsids identified by directed evolution approaches are most likely to translate to cell- and organ-specific expression in humans (Table 1).

The overarching goals of capsid engineering include discovering AAV capsids that can transduce specific CNS cell types, including neuron and glia subtypes, better than currently used serotypes. As we advance to treating neurodevelopmental disorders, AAV capsids that transduce neuronal and glial progenitor cells at different stages of CNS development are essential. Moreover, the identification of variants that de-target specific cell types or organs that may contribute to an immune response or toxicity are needed (Table 1). Finally, the field should also consider revisiting other viral vector delivery options, including lentivirus, nonpathogenic herpes virus, rabies, measles, and other RNA and DNA viruses, as well as non-viral delivery methods such as lipid nanoparticles (Table 1).

Developing regulatable vectors: Promoters/enhancers

The field lacks the ability to specifically express transgenes and would benefit from an array of available small cell type-specific promoters and/or enhancers for the various CNS cell types, including neurons and glia as well as their progenitors. As with developing vectors for treating neurodevelopmental disorders, promoters and enhancers that function in specific spatiotemporal patterns are critical (Table 1). These promoters/enhancers can be identified using similar types of approaches described for capsids. For example, barcoded libraries of promoters/enhancers can be inserted into an AAV vector and screened *in vitro* or *in vivo* for cell type-specific expression. However, the cell type or temporal specific expression of the promoter/enhancer needs to be identified in combination with the AAV serotype of choice because viral *cis*-regulatory elements can affect transgene expression.

Moreover, attendees suggested that there should be a dedicated effort to unravel how the viral *cis*-regulatory elements (e.g., inverted terminal repeats [ITRs]) are contributing to the promoter/enhancer activity because it is the combination of these elements that constitutes the therapeutic product (Table 1). Promoters and enhancers inserted into a viral vector can have crosstalk or work independently of the ITRs. It was suggested that the use and testing of cell type-specific promoters/enhancers needs to be done in the context of the viral serotype or variant capsid, as the ITRs can have promoter activity.[10] For example, a liver-"specific" promoter was shown to very efficiently drive Cre expression in spleen, kidney, brain, and other tissues.[11] Therefore, insulating the ITRs may be necessary (Table 1).

It was also noted that another approach to regulate expression of the transgene is to use cell type-specific microRNAs (miRNAs). Cell type-specific miRNA binding sites are placed in the 3′ untranslated region (UTR) of the transgene in the viral vector construct. The transgene expression levels will be regulated by the binding of the miRNAs to the mRNA transcript, resulting in its destabilization.[12] Multiple miRNA binding sites can be used to de-target expression in certain cell types where transgene expression could be problematic. The miRNAs have the advantage of being very space efficient and can also allow for the use of a smaller, more promiscuous promoter. More recently, studies show that oral drugs can be used to modulate translation to varying levels.[13] This approach, in combination with 3′ UTR-embedded miRNA sequences or enhancers/promoters, may provide cell-specific and expression-level control.

Thus, as the field develops additional tools for gene therapy, goals should include expanding our capability to approach disorders where refined expression (at many levels) is needed. These should work in concert with AAVs that transduce specific cell types, as neurodegenerative and neurodevelopmental disorders require regulatable and/or spatiotemporal-specific promoters/enhancers that will work in combination with AAV serotypes to mimic the endogenous expression levels in the correct cell type throughout disease or development. The KOLs also noted that there are other factors to be considered that may influence expression, including sex-specific differences, circadian rhythm differences, and environmental factors (Table 1).

AAV CNS atlas for cell specificity and biodistribution

It was suggested by KOLs that an AAV CNS atlas that includes data on cell specificity and biodistribution for current and emerging capsids would be of enormous use. Data should include the capsid, regulatory elements, and transgene, as the expression patterns will differ based on capsids and payloads. Therefore, two separate approaches are needed. One part of the atlas would contain information about the physical distribution of the capsid, which would be the blueprint. The second part would add expression patterns that emerge with varying payloads of elements between the ITRs. Researchers could use the atlas to identify the capsid/expression cassette combination that optimally matches the desired gene expression distribution (Table 1).

In addition, biodistribution may not only be affected by the capsid specificity but also by the manufacturing method and quality of the recombinant AAV (rAAV). Therefore, the atlas should integrate data on the method of rAAV production (e.g., baculovirus-*Sf*9 and human HEK293), as it may affect the viral properties such as trafficking and receptor binding.[14] It should also capture the vector quality, including the full/empty capsid ratio.

To facilitate translation, the atlas should integrate data from multiple species. Moreover, it was suggested that multiple biological variables should be considered in conjunction with the vector-related data, including age, sex, and environmental factors. With the accumulation of data in the atlas, these variables can then be examined for any effect

on efficacy. When available, patient data could be added, which would allow researchers to determine the value of the atlas and its continued support. The data could include positron emission tomography (PET) imaging, single cell expression, and post-mortem analysis (Table 1). It would be of great value if both academic and industry partners contributed data to the atlas, as both would benefit. The atlas would be useful for both basic, translational, and clinical research.

Off-target effects and toxicity

There is a gap in our knowledge about the effects of having AAV episomes residing in the nucleus of transduced cells for long periods of time. From a hemophilia B gene therapy clinical trial, there are data demonstrating the presence of the AAV vector DNA and expression of factor IX (*FIX*) 10 years after intramuscular administration. Post-mortem muscle biopsies were obtained from one patient who died from an unrelated cause. The investigators found that *FIX* was still being expressed by both RT-PCR and western blot, and they identified the AAV vector sequence in the whole genomic DNA extraction.[15] In this case, the long-term effect must have been tolerated, as the nuclei remained. If this holds true for other tissues is unknown.

It was discussed among panel members that a better understanding of the long-term outcomes in patients who receive AAV gene-targeted therapy for CNS diseases would be useful to guide further development (Table 1). For example, we need to better understand the extent of AAV integration after gene addition strategies or gene editing. Some tissues, such as the the liver,[16,17] may show a higher prevalence of AAV integration than does the CNS, but when active nucleases provide free DNA ends, AAV integration can occur.[18] Therefore, determining the mechanism of recombinant AAV integration into the host genome is critical so that we can develop methods to avoid these potential adverse events (Table 1). Furthermore, it needs to be determined whether AAV integration is occurring in the currently ongoing CNS-directed human gene therapy trials (Table 1).

Importantly, note that a recent meta-analysis study determined that dorsal root ganglion (DRG) pathology occurs after high-dose AAV administration in the cerebrospinal fluid (CSF) or blood in NHPs.[19] A strategy has been developed that avoids AAV pathology in the NHPs by downregulating the expression of the transgene in the DRG using the binding sites of a DRG-specific miRNA in the 3′ UTR,[20] or using capsids with reduced DRG transduction patterns. Patients who receive AAV gene-targeted therapy need to be clinically monitored for DRG function and toxicity. In the case of death, it would be beneficial to perform post-mortem analysis of the DRG to identify any pathology.

SESSION 2: IMPROVING DELIVERY AND DISTRIBUTION

Session 2 focused on understanding and evading the immune response upon delivery of the gene-targeted therapy. This includes understanding and detecting both systemic immune responses as well as responses in the CNS. In addition, the discussion for the second half of the session focused on tracking distribution and axonal

transport of viral serotypes as well as the importance of understanding the disease state.

Immune response after delivery

Immune responses after the delivery of gene-targeted therapies may affect both their safety and efficacy. In addition, they may prevent the ability to readminister the therapy at a later time point. For CNS-targeted gene therapies, there are numerous options for vector delivery, including i.v., i.c., i.t., intracerebroventricular (i.c.v.), intraparenchymal (i.p.), and focused ultrasound (fUS) administration after i.v. delivery. Notably, the method of delivery, in addition to what is being delivered and expressed, can impact systemic and/or local immune responses.

The panel discussed that when characterizing an immune response, investigators should distinguish between immune responses to the capsid and to the protein expressed, between innate and adaptive immune responses, and between the different components of the immune response (e.g., activated T cells and neutralizing antibodies). In addition, the route of administration should be considered in that, relative to i.v. administration, administration directly into the brain may produce lower systemic immune responses; however, this is variable. Also, if delivery protocols employ blood-brain barrier (BBB) opening, such as mannitol or fUS, there needs to be consideration of the length of time the BBB is open and the possibility of an increased immune response. Finally, the disease state may impact the integrity of the BBB.

Other considerations are the differences between an immune response in a child versus an adult as well as the immune response in a relatively healthy brain versus that of a neurodegenerative disorder where the immune system may already be in an activated state. Degeneration often involves inflammatory processes as cells that are dying may attract immune cells to the site, which could affect the overall efficacy of the therapy.

It was also discussed that immune responses may depend on the cell specificity of the serotype. Serotypes that transduce multiple cell types may transduce immune cells that then act as antigen-presenting cells (APCs). Also, engulfment of AAV particles by APCs may drive immune responses.

It is important to determine both the cause and the character of an immune response to appreciate its clinical significance and understand how to it is best addressed. For example, after CNS AAV administration, testing for an immune response in the blood against the capsid and/or expressed protein may not be indicative of what is happening in the CNS. Microglia reside in the CNS, and if activated they can recruit inflammatory cells to the site of administration; other immune responses could be more systemic (e.g., due to neutralizing antibodies or lasting T cell responses to the capsid or transgene product).

Overall, there needs to be a better understanding of the CNS immune response to gene-targeted therapies to improve their safety and

Table 2. Session 2: Improving delivery and distribution

Topic	Needed knowledge and strategies
Immune response after delivery	Better understand the CNS immune response to gene-targeted therapies with consideration of all the variables, including route of delivery, transgene, dose, age at administration, neurodegenerative status, AAV serotype, AAV DNA sequences, presence of neutralizing antibodies, and purity of the capsid preparation.
Evading immune responses	Strategies to evade an immune response to the capsid, AAV DNA sequences, and the transgene-encoded protein need to be developed for CNS-directed gene-targeted therapies.
	Strategies that enable the re-administration of a CNS gene-targeted therapy are necessary, because efficacy may decline after a period (e.g., 10 years), and another dose may be required to maintain therapeutic benefits.
Cross-reactive immunological material (CRIM) to the transgene-encoded protein	Design new assays to determine when immune tolerance is reached in humans.
	Create a public database that includes immune response data as well as immunosuppression protocols from clinical trials.
New tools to detect and evaluate an immune response in the CNS	Develop new noninvasive tools and methods to detect and characterize the CNS immune response *in situ* in live animal models as well as in the clinic.
Tracking distribution	Development of new methods and technologies such as tracers are needed to determine the biodistribution of the vector as well as the transgene expression in real time; a significant advancement would be establishing methods that enable determination of the specific cell types transduced.
	New noninvasive technologies, including imaging tracers, are needed to determine in real time whether transgene-encoded proteins are being expressed and functioning; this is particularly critical for the long-term follow-up of gene therapy patients; these would be necessary for each specific recombinant protein.
	For CNS gene-targeted therapies that affect circuitry function, innovative minimally invasive tools need to be designed to measure specific circuit activity over time.
AAV transport	Develop methods to track the expression or function of the capsid and/or the transgene-encoded cargo throughout the CNS.

efficacy (Table 2). As stated above, variables that may influence the immune response include delivery route, the protein expressed and if it is foreign, dose, patient age at administration, neurodegenerative status of the patient, the capsid type if an AAV and previous exposures, AAV vector genome sequences, and pre-existing neutralizing antibodies. Importantly, note that animal models often have different immune responses that may not represent what will happen in humans (discussed in Session 3: Enhancing models and manufacturing). Finally, the purity of the capsid preparation, as well as the nature of impurities based on different manufacturing methods, could alter the immune response.

Strategies to evade an immune response

Workshop participants discussed concerns over the immune response to AAV vector DNA sequences and methods to avoid it. Toll-like receptor 9 (TLR9) functions to detect foreign DNA and stimulates immune responses. TLR9 binds to unmethylated CpG motifs, which are frequently enriched in codon-optimized transgenes and strong ubiquitous promoters. Modification of AAV vector sequences to decrease CpG motifs can reduce innate immune responses and help with long-term transgene expression in the mouse skeletal muscle.[21] Also, AAV vector sequences can be engineered to include short DNA oligonucleotides that inhibit TLR9 activation, reducing immune responses in the mouse liver, muscle, and retinas, as well as in pig retinas.[22]

As AAVs are often capable of transducing dendritic cells (DCs; a type of APC), immune responses to the expressed protein products occur. To circumvent this, researchers used a trick that was developed more than 15 years ago to restrict gene expression to certain cell types by embedding miRNA binding sites in the 3′ UTR of the transgene; miRNAs expressed in undesired cell types would turn the gene off. Because those miRNAs were not expressed in desired cell types, there would be no inhibition of desired gene expression.[23] More recently, AAV vectors were engineered to contain a miR-142 and other immune cell-specific miRNA-binding sites in the 3′ UTR of the transgene to improve muscle gene therapy. The miRNA motifs lowered expression in DCs and reduced cytotoxic T cell responses.[24,25] These strategies are being used similarly to prevent or mitigate immune responses to transgene-encoded proteins for CNS therapies (Table 2).

In addition to CpGs, it was also noted that the woodchuck hepatitis virus post-transcriptional regulatory elements (WPREs) can elicit immune responses and should be avoided. Finally, strategies that enable the re-administration of a CNS gene-targeted therapy could become necessary, because efficacy may decline after a period (e.g., 10 years), necessitating re-dosing for sustained therapeutic benefits (Table 2).

Cross-reactive immunological material (CRIM) to the transgene-encoded protein

In addition to an immune response to the AAV capsid, there can also be an immune response to the transgene-encoded protein. CRIM-negative patients are those who are naive to the transgene-encoded protein and therefore may elicit an immune response to what, to them, is foreign. CRIM-positive patients express some level of endogenous protein and should be tolerant. In many monogenic diseases, the patients may have deletion mutations or mutations that result in no protein expression. Therefore, because these patients' bodies have never encountered a functional version of the protein, a significant proportion of the disease population may be CRIM-negative.

There were two challenges noted by KOLs. One challenge is correctly identifying patients who are CRIM-negative before initiating gene therapy. The other is prophylactically managing an immune response against a transgene-encoded protein. However, it was also noted that the importance of CRIM status depends on the protein. In Pompe disease, it is critical to know CRIM status prior to administering enzyme replacement therapy (ERT) or gene replacement therapy, as an antibody response to transgene protein has been shown to decrease the efficacy and can be life threatening.[26] However, in other lysosomal disorders, it has been seen that a patient with a negative CRIM status may exhibit an antibody response that may reduce efficacy, but it is not a life-threatening response. As immune responses to recombinant proteins can be very distinct in different diseases, it would be helpful to have a public database for the human clinical trials data to help compare and predict the potential for an immune response to a gene-targeted therapy as well as the effectiveness of various immunosuppression protocols (Table 2). Such a database, which would evolve as new data emerge, would complement the AAV CNS atlas described in Session 1: Control of level and location.

To prevent immune responses to foreign proteins, immunomodulation strategies to induce tolerance can be developed. These could be mirrored after immunomodulation protocols developed for ERT for CRIM-negative patients with Pompe disease.[26] Finally, participants indicated the need to develop new assays to determine when immune tolerance is reached in humans (Table 2).

New tools to detect and evaluate CNS immune responses

Workshop participants stated that more tools and methods to detect and evaluate CNS immune responses *in situ* in both animal models and humans are needed. Edema and inflammation after AAV administration can be detected by neuroimaging, but it would be helpful to have methods with higher sensitivity to detect the types of immune responses. In addition, there were questions about pleocytosis and its induction as well as the function of the white blood cells in terms of activity and antigen specificity. Therefore, new noninvasive tools and methods to detect and characterize CNS immune responses *in situ* in both animal models and humans would be advantageous for the field (Table 2).

Tracking distribution

During session 1, the issue of overexpression, and specifically the need to develop methods and technologies that will result in more uniform intracellular concentrations of AAV or ASOs across the CNS were discussed (Table 1). As these methods are developed, the field needs accurate technologies to quantify both vector distribution and transgene expression levels. Currently, the most common tools are neuroimaging with tracers and post-mortem analysis. Imaging and tracers have been proven to be quite powerful noninvasive tools over the years in clinical medicine, but they would be especially useful for gene therapy. Magnetic resonance imaging (MRI) has successfully been used both in NHPs[27] and humans[28] to track rAAV distribution. In NHPs, an AAV9 dose that included the contrast agent gadoteridol was administered by i.t. delivery into the cerebromedullary cistern or lumbar space to monitor distribution patterns by MRI. This cistern delivery resulted in broad distribution of the contrast agent in the brain and spinal cord, and this distribution was enhanced when combined with lumbar injection.[27] Furthermore, the kinetics of the CSF vector concentration was determined in real time. In addition, a quantitative method has also been developed for the whole-body imaging using iodine 124 (I-124) as a PET tracer. I-124-labeled AAV (AAV9 or AAVrh.10) was administered by i.v. and i.c. injections in NHPs. The tropism of the virus was measured by dosimetry organ-specific biodistribution for 72 h after administration.[29] These methods allowed for AAV vector tracking immediately after administration. However, they only show where vector can go, and would benefit from being coupled with methods to assess expression *in situ* (Table 2). This could include the development of non-substrate PET ligands. Noninvasively determining the specific cell types transduced is extremely challenging, but the ability to do this would be a significant advancement to the field (Table 2). Additionally, postmortem analysis of tissues at the single cell level should be done. These data could be added to the atlas describing biodistribution together with noninvasive imaging data, providing a summary of where the vector particles distribute, and the outcome after delivery.

In addition to knowing where vectors go and what cells they transduce, it is important to assess the functional output of the gene therapy product, as in the case for AAV delivery of aromatic L-amino acid decarboxylase (AADC) for Parkinson's disease. After *AAV2-AADC* administration, dopamine synthesis levels increase due to recombinant AADC expression, which can be determined using 6-[^{18}F]fluoro-L-3,4,-dihydroxyphenylalanine (FDOPA) and PET imaging.[30] This allows for the detection of a functional recombinant protein, localization of expression, and assessment of the level and longevity of expression. This approach should be applied to other disorders, which would require new tracer development. These and other novel noninvasive technologies would enable analysis of gene expression in real time for the long-term follow-up of gene therapy patients (Table 2). In a similar vein, CNS gene-targeted therapies that affect circuitry function require innovative minimally invasive tool development to measure specific circuit activity temporally (Table 2). The technologies could be validated in large animal models and eventually applied to patients to help assess therapeutic efficacy.

AAV transport via neuronal processes

The CNS has long neuronal projections that extend into different CNS and peripheral nervous system (PNS) areas. Participants discussed that different AAV serotypes can move anterograde and/or retrograde along the axons. Therefore, the primary transduction can be targeted to a certain part of the brain and the virus can be transported to other parts of the brain by axonal projections. Also, note that if the transgene-encoded protein can be released into the synapse by the pre-synaptic neuron, post-synaptic uptake could occur. However, to date, most cell-to-cell protein transfer of gene therapy products overexpressed in CNS cells occurs from release and uptake by the endosomal-lysosomal system, a process known as cross-correction. Methods to non-invasively track the transgene

expression (e.g., mRNA) as well as the transgene-encoded protein throughout the CNS (Table 2) would be advantageous to better understand the penumbra of cross-correction that can occur after focal gene delivery.

The pathology of each disease may impact intercellular movement of particles and the ability for cells to secrete and take up gene therapy products. It may be possible to bypass the pathways that are not functioning and target those that have an available detour around the affected pathway. This highlights the importance of knowing the neurodegenerative status of the animal model or human during the disease course.

Disease states
The KOLs noted that the disease state throughout the course of the disease needs to be understood. For example, cell surface receptors may be affected that will influence transduction. The pathology of each disease may impact intercellular movement of particles and the ability for cells to secrete and take up gene therapy products. It may be possible to bypass the pathways that are not functioning and target those that have an available detour around the affected pathway. Also, CSF flux may be affected by disease state, affecting distribution. As mentioned above, there are other variables to consider, including neurodegeneration, inflammation, and status of the BBB. Therefore, it is important to understand and determine the disease state of the animal models or patients prior to gene-targeted therapy administration.

Session 3: Enhancing models and manufacturing
For the first half of session 3, the focus of the discussion was on animal models, including advantages and disadvantages of rodents compared to large animal models, as well as how to model the immune responses to therapies. For the second half of session 3, participants discussed AAV manufacturing and batch production challenges, especially for ultra-rare diseases.

Animal model scale
Participants discussed the importance of using animal models to address issues that have been identified from clinical trials. Despite the known differences between species, much of the preclinical research on gene-targeted therapies is conducted in the mouse. This is due to the numerous advantages of the mouse model, including the relatively short breeding time, large litter size, and low animal care cost, as well as the ability to make genetic disease models and the availability of genetic and cellular tools for analysis.

Despite these advantages, the gene therapy field cannot rely exclusively on mouse studies. The human brain is approximately 3,800 times larger than the mouse brain,[31] such that with CNS-directed and localized administration, a focal delivery that is effective in the mouse will cover only a fraction of the target brain tissue in humans. There are also significant differences in diffusion and cellular transport not only because of size but also differences in the cellular and extracellular architecture. In addition, this difference in scale makes it difficult to extrapolate from mouse studies to ascertain appropriate dosing and optimal distribution for patients. Is an accurate dose being used in the clinic? Is underdosing an issue from mice to humans?

For brain-directed gene therapies, the larger animal models, including dogs, pigs, sheep, and NHPs, are better models for determining dosing and delivery due to their relatively more similar size to humans. This is especially important when targeting deep brain areas. When AAV is administered i.t., i.c., or i.c.v., it can be difficult to reach the entire human brain, in particular the deep brain regions such as the midbrain. A noninvasive method to deliver vector to the deep brain regions could be developed in larger animal models. Another advantage of using large animal models is that their neocortex is gyrencephalic (i.e., has convolutions) as in humans, whereas the mouse brain is lissencephalic (i.e., is smooth). Parallel studies conducted in both a mouse and a large animal model using the same capsid, promoter, vector preparation, delivery route, and normalized dose so that all the variables are standardized would facilitate translation across species (Table 3). Such studies would be important building blocks for the AAV CNS atlas described in Session 1: Control of level and location. Additionally, as we learn more about virus receptors, we can learn what models to apply to current and emerging capsids, as different species have varying glycosylation patterns and extracellular matrix proteins that may impact vector binding, uptake, and, therefore, transduction.

Another advantage of the large animal model is that transgene expression can be followed for years to determine efficacy and long-term safety. Moreover, such a model can be used as for research into the therapeutic re-administration. Based on ongoing clinical studies, re-administration may not be needed for many years (e.g., 10 years), but large animal models could help inform questions such as if, when, and how often to re-administer gene therapy in patients (Table 3).

Animal models of immune response
Another concern identified in clinical trials that can be evaluated in animal models is the immune response to the therapeutic product. In the clinic, if a patient develops anti-transgene or anti-capsid immunity, they may lose transgene expression, which diminishes any beneficial effects. The scientific community requires better animal models that reflect what happens in the human immune response. This is vital for both determining whether an AAV capsid or vector payload (expressed or DNA sequences) will elicit immune responses prior to going to the clinic as well as for developing immunosuppressive regimens for preventing or mitigating those responses.

While the mouse model offers many advantages to examine immune response to foreign antigens, including genetic and cellular tools, they are problematic because they are typically inbred and immunologically naive. In addition, the mouse immune system is significantly different from humans, including the balance of leukocyte subsets, Toll-like receptors, antigen-presenting function of endothelial cells, and antibody subsets.[32]

Table 3. Session 3: Enhancing models and manufacturing

Topic	Needed knowledge and strategies
Animal model scale	Large animal models may be more informative than rodent models to assess vector distribution and estimate appropriate dosing in humans; parallel studies conducted in both mice and a large animal model using the same capsid, promoter, vector preparation, delivery route, and normalized dose so that all the variables are standardized would facilitate translation across species.
	Take advantage of the increased lifespan of large animal models to understand how to enable re-administration of CNS-directed gene-targeted therapies.
Animal models of immune response	Identify or develop the appropriate animal models that predict the human immune response to capsids and transgene-encoded proteins; a prerequisite might be a better understanding of the immune systems of larger animal models to determine whether they are an appropriate representation of the human response.
	Develop better tools to detect and monitor the immune response in large animal models.
	Testing AAV administration in NHPs paired with immunosuppression protocols that may be used in clinical trials could yield new important information.
AAV manufacturing	Address manufacturing challenges, including capacity, cost, complexity, and diversity of the available production systems and standards for the identification and measurement of critical quality attributes of the final product.
Batch production for ultra-rare diseases	Consider the unique complexities to developing gene-targeted therapies for ultra-rare diseases and how to advance the therapeutics in a timely way where they are held to the same safety standards as those for common disorders, but maybe not the same regulatory standards in terms of the requirement for multiple manufacturing runs for licensing; this could possibly be accomplished through collaboration with the FDA.
	Develop a CMC platform for ultra-rare disorders.

The rat was also discussed as a model to determine immune response to a therapeutic agent. The rat immune system is more like that in humans than is the mouse immune system.[33] For example, rats and humans have an active complement system that is not as active in mice.[34] In addition, rats and humans both have activated T cells that express major histocompatibility complex (MHC) class II molecules and CD4+/CD8+ macrophages.[33] Finally, genetic engineering tools are increasingly available in non-mouse models. For example, the CRISPR-Cas9 system can be used to modify the rat to make a disease model for preclinical studies.[35]

Large animal models should also be considered for immune response studies, including dogs, pigs, sheep, and NHPs. However, the limitations with large animal models relative to rodents are the extended time for breeding, the smaller litter size, and the increased animal care cost. Furthermore, in comparison to mouse models, there is the lack of genetic and cellular tools to characterize the immune response in-depth. It was also noted that large animal models do not always predict what will happen in humans. For example, liver-directed AAV gene therapy in the hemophilia B dog model resulted in long-term expression of canine factor IX, as intended. However, in the clinic it resulted in an immune response to the AAV capsid and low efficacy.[36]

Clearly more research is required to identify or develop more appropriate animal models for predicting human immune responses to viral capsids and the transgene-encoded proteins or other viral and non-viral elements. A better understanding of the immune systems of larger animal models would be a first step toward determining their utility in predicting human immune responses (Table 3). Better tools to detect and monitor immune responses in large models are needed

(Table 3), as the repertoire of immunosuppressive agents that are used in humans might not be applicable to other large animal models (e.g., dogs, sheep, or pigs) due to interspecies differences. Perhaps for this reason, NHPs are best, but they are expensive and becoming increasingly more so for academic research in this space.

Finally, it was discussed that because immunosuppression protocols are used in clinical trials, lack of use of similar protocols in preclinical studies may misinform the ability for proper clinical translation. Therefore, it was suggested that the testing of clinical immunosuppression protocols in NHPs paired with AAV administration may yield new important information (Table 3).

AAV manufacturing

There were many challenges identified pertaining to manufacturing of gene therapies, including capacity, cost, production systems, and product quality attributes. Limited capacity has been a constraint for AAV gene-targeted therapies, and the ability to manufacture investigational products in a timely manner to support clinical development and eventual commercial launch is a common problem. In addition, cost poses serious challenges, especially for academic centers, small biotechnology companies, and foundations. One cause of this is that multiple batches of a product may need to be manufactured before a process is fully optimized and compliant to be used in a clinical trial setting. The complexity and diversity of the available production systems is a further challenge, and efforts are being made to simplify these procedures. A question was also raised about whether manufacturing systems and quality release assays could be standardized and made publicly available. This becomes increasingly difficult if multiple capsids are emerging, each with their own nuances and differing transduction capabilities in cell lines. In addition, the

necessary quality attributes of the product must be established, including the identification of the most critical steps to ensure safety and potency and the determinants of innate and adaptive immune response (e.g., CpG motifs, AAV empty capsids). Characterizing these quality attributes will help guide vector design and manufacturing methods and may improve cost and timelines to product release for patient application (Table 3).

Batch production for ultra-rare diseases

Much of the discussion focused on one of the challenges raised in the pre-meeting call: the need to address the issues of batch production for the ultra-rare diseases. Based on the Rare Diseases Act of 2002 (Public Law 107-280), a rare disease is defined as a condition affecting fewer than 200,000 individuals in the United States (~0.06% of the population). There is no legal definition of ultra-rare disease in the United States; however, in Europe the National Institute for Health and Care Excellence defines an ultra-rare disorder as one that affects 1 in 50,000 persons (0.002% of the population).[37] Therefore, this definition will be used to define ultra-rare diseases for the purpose of this workshop summary.

The participants pointed out that for an ultra-rare CNS disorder, it may be possible to make one batch of the therapeutic product for all of the good laboratory practice (GLP) studies, investigational new drug (IND)-enabling studies, stability studies, and to treat all of the patients for the foreseeable future. One of the difficulties in developing gene-targeted therapies for ultra-rare diseases is the cost associated with manufacturing multiple batches to satisfy regulatory requirements, particularly for commercialization of a product. However, there may be no need to commercialize products for the ultra-rare CNS disorders but rather use the product as an open-ended IND, best sponsored by the National Institutes of Health (NIH) or other government agencies. This is because for-profit industry and biotech groups are unlikely to be interested in developing a therapeutic with a low likelihood of commercial viability. Thus, KOLs concluded that the complexities of gene-targeted therapies for ultra-rare diseases need special consideration. One possibility to advance the therapeutics in a timely way is to hold them to the same safety standards as those for common disorders, but perhaps not to the same regulatory standards in terms of the requirement for multiple manufacturing runs typically required for licensing (Table 3).

For some ultra-rare CNS disorders, systemic AAV delivery may be used to treat the brain, spinal cord, and other peripheral organs. With currently available AAV capsids, a very high dose of the vector would be required, and one manufacturing run may be insufficient. Work is needed to improve capsids with these capabilities so that reasonable doses can be applied to limit the toxicities that would likely emerge from these high-dose exposures. Advances in this space would reduce the amount of product required for many patients as well as improve safety measures.

In addition, attendees discussed the feasibility of establishing standardized processes and procedures for manufacturing (Table 3).

One possibility is to develop a chemistry, manufacturing, and controls (CMC) platform for ultra-rare disorders in which only one component is changed in the vector product (e.g., the transgene). It is a "plug-and-play" concept where the capsid, enhancer, and promoter would stay the same and one would "plug-in" a different transgene. Therefore, all of the other data that are generated from prior AAV batch productions using the vector backbone could be applicable and referenced by investigators, manufacturers, and regulatory groups. These standardized data would accelerate manufacturing and regulatory approval processes and be a public resource upon which new clinical trials for different diseases could be more readily launched. At the present time, no given capsid satisfies the requirements for a plug-and-play modality. As discussed in Session 1: Control of level and location, most diseases require sufficient but not too much transgene expression, and broad coverage of many different brain areas. For the CNS, an additional consideration is the substantial heterogeneity in the cell types and brain regions that may be affected in the various disorders. The plug-and-play approach would be applicable to disorders that would require the transduction of the same cell type or brain region. For example, CNS disorders that require transgene expression throughout the brain and spinal cord could all use the same vector backbone.

Session 4: Impacting patients

The focus of session 4 was on how gene-targeted therapies can impact patients, including learning from models of collaborative networks, establishing a shared database of preclinical and clinical data, implementing innovative/adaptive trial design, standardizing clinical procedures/measurements, improving diagnostics and clinical trial readiness, and considering ethics in all aspects of research.

Collaborative network

Dr. Nita Seibel from the National Cancer Institute (NCI) presented the Children's Oncology Group (COG; https://childrensoncologygroup.org/) as an example of how to address a medical area such as childhood and adolescent cancers through collaboration among clinicians and research scientists in academic centers, advocacy groups, industry partners, and NCI officials. The COG is a multi-center cooperative initiative that aims to prevent as well as cure pediatric cancers. The COG has approximately 200 research sites across the United States, where more than 90% of children with cancer in the country are treated.

Participants discussed the potential of establishing a similar type of collaborative effort for gene-targeted therapies for both rare and ultra-rare neurological disorders. It was emphasized that therapies are needed for the ultra-rare disorders that are unlikely to be commercially viable for industry. Establishing such a network would require a concerted effort from academic researchers, clinical trialists, patient advocates, industry researchers, ethicists, members of the NIH, and regulatory professionals at agencies such as the US Food and Drug Administration.

Furthermore, it was suggested that a network of Gene-Targeted Therapies Centers of Excellence (COEs) for Rare Neurological Disorders

Table 4. Session 4: Impacting patients

Topic	Needed knowledge and strategies
Collaborative network	Establish a network of Gene-Targeted Therapies Centers of Excellence (COEs) for Rare Neurological Disorders with expertise in preclinical research, diagnostics, natural history studies, outcome measures, imaging, manufacturing, ethics, and clinical trials for delivery of gene-targeted therapies; the network could also provide local clinical sites for patients to minimize travel for the therapy administration and follow-up.
	Create a database that contains the relevant preclinical, manufacturing, and clinical data that could be shared across a network.
Natural history, product delivery, and outcome measures	Develop innovative methods for characterizing natural history, distribution to patients, patient access, and identifying outcome measures.

should be established (Table 4). Multiple components were suggested for these centers, including expertise in preclinical research, diagnostics, natural history studies, patient-centered outcome measures, imaging, manufacturing, and clinical trials for delivery of gene-targeted therapies. There could be a centralized Institutional Review Board (IRB) as well as a Data Coordinating Center (DCC). The DCC could host a database that contains the relevant preclinical, manufacturing, and clinical data that could be shared across the network (Table 4). The DCC could also assist in developing clinical protocols and regulatory documents. In addition, training could be offered for coordinators, research nurses, and clinicians.

With the collection and sharing of both preclinical and clinical data, the field can learn from each therapy development step and clinical trial to inform the next cycle for the same or a different disease. This could include the AAV vector, route of delivery, immunosuppression protocols, outcome measures, and long-term follow-up. There also is the opportunity to standardize clinical procedures and measurements. For example, the standardization of methods to measure the immune response will allow for comparability across trials. In addition, adaptive trial designs for extremely rare disorders are of prime importance because there are very few patients to determine efficacy and, for AAV gene therapy, the patients are currently unable to undergo a second administration.

The COEs should be geographically distributed throughout the United States to make it convenient for patients and families. For some disorders, it can be logistically arduous for patients and families to travel to a clinical center, supporting a need for innovation in patient enrollment and post-therapy delivery monitoring methods. In addition, for some therapies such as ASOs that must be re-administered frequently (e.g., every few weeks), geographic distribution would allow patients access to a local COE without having to relocate their entire family to one clinical center. Having access to local COEs would ease family burdens for long-term follow-up (Table 4).

Diagnosis, natural history, and clinical outcome assessments (COAs)

The early diagnosis of patients was identified as a critical challenge. For neurological disorders, and especially neurodegenerative disorders, it is predicted that early intervention will result in better clinical outcomes. In addition, it was discussed that when identification of ge-

netic disorders through newborn screening is implemented, many diseases may be more prevalent than was previously thought.

Clinical trial readiness should also be a shared goal. Researchers and advocacy members should collaborate to establish a patient registry for identification of patients for natural history studies of disorders and to recruit for clinical trials. The natural history of ultra-rare disorders and the identification of fit-for-purpose outcome measures are critical to determine efficacy of gene-targeted therapies. The characterization of the natural history and identification of COAs need to be initiated before or in parallel with gene-targeted therapies.

It was suggested that the COEs could provide a shared infrastructure, but also develop innovative methods for characterizing natural history and identifying outcome measures (Table 4). As it will be difficult to obtain robust, prospective natural history data for ultra-rare disorders (e.g., 10 patients in the world), retrospective analysis should be considered to complement prospective studies.

The question of how to identify efficacy measures for Ns of 1 or 2 or even Ns of 5 clinical trials was raised and is an important problem to solve moving forward. As N of 1 trials are becoming more prevalent for ASO therapies, the FDA recently released guidance for IND submissions for these individualized therapies (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/ind-submissions-individualized-antisense-oligonucleotide-drug-products-administrative-and-procedural) as well as discussed the implications in an editorial.[38] These trials are for disorders that are rapidly progressing with high morbidity and often fatal. It is suggested that as soon as a therapy development begins, the patient should be followed and assessed frequently to potentially identify COAs to measure efficacy.

Ethics

An important component of any clinical trial is the ethics of both the intervention itself and the design and conduct of the trial. There needs to be equal access across racial, ethnic, and socioeconomic groups. This includes equity in patient recruitment as well as transparency of the recruitment criteria. There also needs to be transparency of the outcome expectations and the potential risks and benefits to the patients. We need to better understand the constraints in our infrastructures that place burdens on different socioeconomic and ethnic

groups in cell and gene therapy clinical trials, and help all individuals gain access to these life-changing therapies.

CONCLUSIONS

This workshop summary provides an overview of key challenges in the field and suggests strategies to address them (Tables 1, 2, 3, and 4). While the field has made great strides forward in gene-targeted therapies during the last several decades for CNS disorders, much remains to be learned as we move to the clinic. Emerging technologies in delivery methods, vectors for delivery, and the payloads harnessed within them are invigorating the field and offer hope of addressing the unmet needs of thousands of patients. Major challenges will continue to emerge as we apply our learning, but with additional measures in place we hope to understand what roadmaps predict the correct approach and what early warning signs mean in terms of safety, efficacy, and broader utility. The enormity of the challenges and complexity of the technologies necessitate us to create a collaborative infrastructure that fosters synergy, maximizes efficiency, and expedites progress toward improved outcomes for patients.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.09.010.

AUTHOR CONTRIBUTIONS

J.A.M. and B.L.D. wrote the manuscript with contributions from all authors. J.A.M., C.H.B., B.L.D., and G.G. were co-chairs of the workshop. All authors contributed to the development and implementation of the workshop.

DECLARATION OF INTERESTS

B.L.D. is a co-founder of Spark Therapeutics and Spirovant Sciences. B.L.D. serves an advisory role and/or receives sponsored research support for her laboratory from Roche, NBIR, Homology Medicines, Triplet Therapeutics, Resilience, Intellia Therapeutics, Spirovant Sciences, Panorama Medicines, and Voyager Therapeutics. G.G. is a scientific co-founder of Voyager Therapeutics, Adrenas Therapeutics, and Aspa Therapeutics and holds equity in the companies. G.G. is an inventor on patents related to AAV-based gene therapy, some of which were licensed to commercial entities. The remaining authors declare no competing interests.

REFERENCES

1. Golebiowski, D., van der Bom, I.M.J., Kwon, C.S., Miller, A.D., Petrosky, K., Bradbury, A.M., Maitland, S., Kühn, A.L., Bishop, N., Curran, E., et al. (2017). Direct intracranial injection of AAVrh8 encoding monkey β-N-acetylhexosaminidase causes neurotoxicity in the primate brain. Hum. Gene Ther. 28, 510–522.

2. Amado, D.A., Rieders, J.M., Diatta, F., Hernandez-Con, P., Singer, A., Mak, J.T., Zhang, J., Lancaster, E., Davidson, B.L., and Chen-Plotkin, A.S. (2019). AAV-mediated progranulin delivery to a mouse model of progranulin deficiency causes T cell-mediated toxicity. Mol. Ther. 27, 465–478.

3. Monine, M., Norris, D., Wang, Y., and Nestorov, I. (2021). A physiologically-based pharmacokinetic model to describe antisense oligonucleotide distribution after intrathecal administration. J. Pharmacokinet. Pharmacodyn. 48, 639–654.

4. D'Mello, S.R. (2021). MECP2 and the biology of MECP2 duplication syndrome. J. Neurochem. , Published online February 26, 2021. https://doi.org/10.1111/jnc.15331.

5. Colón-Thillet, R., Jerome, K.R., and Stone, D. (2021). Optimization of AAV vectors to target persistent viral reservoirs. Virol. J. 18, 85.

6. Deverman, B.E., Pravdo, P.L., Simpson, B.P., Kumar, S.R., Chan, K.Y., Banerjee, A., Wu, W.L., Yang, B., Huber, N., Pasca, S.P., and Gradinaru, V. (2016). Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat. Biotechnol. 34, 204–209.

7. Ravindra Kumar, S., Miles, T.F., Chen, X., Brown, D., Dobreva, T., Huang, Q., Ding, X., Luo, Y., Einarsson, P.H., Greenbaum, A., et al. (2020). Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. Nat. Methods 17, 541–550.

8. Flytzanis, N.C., Goeden, N., Goertsen, D., Cummins, A., Pickel, J., and Gradinaru, V. (2020). Broad gene expression throughout the mouse and marmoset brain after intravenous delivery of engineered AAV capsids. bioRxiv. https://doi.org/10.1101/2020.06.16.152975.

9. Hordeaux, J., Wang, Q., Katz, N., Buza, E.L., Bell, P., and Wilson, J.M. (2018). The neurotropic properties of AAV-PHP.B are limited to C57BL/6J mice. Mol. Ther. 26, 664–668.

10. Earley, L.F., Conatser, L.M., Lue, V.M., Dobbins, A.L., Li, C., Hirsch, M.L., and Samulski, R.J. (2020). Adeno-associated virus serotype-specific inverted terminal repeat sequence role in vector transgene expression. Hum. Gene Ther. 31, 151–162.

11. Lang, J.F., Toulmin, S.A., Brida, K.L., Eisenlohr, L.C., and Davidson, B.L. (2019). Standard screening methods underreport AAV-mediated transduction and gene editing. Nat. Commun. 10, 3415.

12. Domenger, C., and Grimm, D. (2019). Next-generation AAV vectors—Do not judge a virus (only) by its cover. Hum. Mol. Genet. 28 (R1), R3–R14.

13. Monteys, A.M., Hundley, A.A., Ranum, P.T., Tecedor, L., Muehlmatt, A., Lim, E., Lukashev, D., Sivasankaran, R., and Davidson, B.L. (2021). Regulated control of gene therapies by drug-induced splicing. Nature 596, 291–295.

14. Rumachik, N.G., Malaker, S.A., Poweleit, N., Maynard, L.H., Adams, C.M., Leib, R.D., Cirolia, G., Thomas, D., Stamnes, S., Holt, K., et al. (2020). Methods matter: Standard production platforms for recombinant AAV produce chemically and functionally distinct vectors. Mol. Ther. Methods Clin. Dev. 18, 98–118.

15. Buchlis, G., Podsakoff, G.M., Radu, A., Hawk, S.M., Flake, A.W., Mingozzi, F., and High, K.A. (2012). Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. Blood 119, 3038–3041.

16. Chandler, R.J., Sands, M.S., and Venditti, C.P. (2017). Recombinant adeno-associated viral integration and genotoxicity: Insights from animal models. Hum. Gene Ther. 28, 314–322.

17. Donsante, A., Miller, D.G., Li, Y., Vogler, C., Brunt, E.M., Russell, D.W., and Sands, M.S. (2007). AAV vector integration sites in mouse hepatocellular carcinoma. Science 317, 477.

18. Hanlon, K.S., Kleinstiver, B.P., Garcia, S.P., Zaborowski, M.P., Volak, A., Spirig, S.E., Muller, A., Sousa, A.A., Tsai, S.Q., Bengtsson, N.E., et al. (2019). High levels of AAV vector integration into CRISPR-induced DNA breaks. Nat. Commun. 10, 4439.

19. Hordeaux, J., Buza, E.L., Dyer, C., Goode, T., Mitchell, T.W., Richman, L., Denton, N., Hinderer, C., Katz, N., Schmid, R., et al. (2020). Adeno-associated virus-induced dorsal root ganglion pathology. Hum. Gene Ther. 31, 808–818.

20. Hordeaux, J., Buza, E.L., Jeffrey, B., Song, C., Jahan, T., Yuan, Y., Zhu, Y., Bell, P., Li, M., Chichester, J.A., et al. (2020). MicroRNA-mediated inhibition of transgene expression reduces dorsal root ganglion toxicity by AAV vectors in primates. Sci. Transl. Med. 12, eaba9188.

21. Faust, S.M., Bell, P., Cutler, B.J., Ashley, S.N., Zhu, Y., Rabinowitz, J.E., and Wilson, J.M. (2013). CpG-depleted adeno-associated virus vectors evade immune detection. J. Clin. Invest. 123, 2994–3001.

22. Chan, Y.K., Wang, S.K., Chu, C.J., Copland, D.A., Letizia, A.J., Costa Verdera, H., Chiang, J.J., Sethi, M., Wang, M.K., Neidermyer, W.J., Jr., et al. (2021). Engineering adeno-associated viral vectors to evade innate immune and inflammatory responses. Sci. Transl. Med. 13, eabd3438.

23. Brown, B.D., Gentner, B., Cantore, A., Colleoni, S., Amendola, M., Zingale, A., Baccarini, A., Lazzari, G., Galli, C., and Naldini, L. (2007). Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat. Biotechnol. 25, 1457–1467.

24. Muhuri, M., Zhan, W., Maeda, Y., Li, J., Lotun, A., Chen, J., Sylvia, K., Dasgupta, I., Arjomandnejad, M., Nixon, T., et al. (2021). Novel combinatorial microRNA-binding sites in AAV vectors synergistically diminish antigen presentation and transgene immunity for efficient and stable transduction. Front. Immunol. 12, 674242.

25. Xiao, Y., Muhuri, M., Li, S., Qin, W., Xu, G., Luo, L., Li, J., Letizia, A.J., Wang, S.K., Chan, Y.K., et al. (2019). Circumventing cellular immunity by miR142-mediated regulation sufficiently supports rAAV-delivered OVA expression without activating humoral immunity. JCI Insight 5, e99052.

26. Desai, A.K., Li, C., Rosenberg, A.S., and Kishnani, P.S. (2019). Immunological challenges and approaches to immunomodulation in Pompe disease: A literature review. Ann. Transl. Med. 7, 285.

27. Ohno, K., Samaranch, L., Hadaczek, P., Bringas, J.R., Allen, P.C., Sudhakar, V., Stockinger, D.E., Snieckus, C., Campagna, M.V., San Sebastian, W., et al. (2018). Kinetics and MR-based monitoring of AAV9 vector delivery into cerebrospinal fluid of nonhuman primates. Mol. Ther. Methods Clin. Dev. 13, 47–54.

28. Bankiewicz, K.S., Pasterski, T., Kreatsoulas, D., Onikijuk, J., Mozgiel, K., Munjal, V., Elder, J.B., Lonser, R.R., and Zabek, M. (2020). Use of a novel ball-joint guide array for magnetic resonance imaging-guided cannula placement and convective delivery: technical note. J. Neurosurg. , Published online October 3, 2020. https://doi.org/10.3171/2020.6.JNS201564.

29. Ballon, D.J., Rosenberg, J.B., Fung, E.K., Nikolopoulou, A., Kothari, P., De, B.P., He, B., Chen, A., Heier, L.A., Sondhi, D., et al. (2020). Quantitative whole-body imaging of I-124-labeled adeno-associated viral vector biodistribution in nonhuman primates. Hum. Gene Ther. 31, 1237–1259.

30. Christine, C.W., Bankiewicz, K.S., Van Laar, A.D., Richardson, R.M., Ravina, B., Kells, A.P., Boot, B., Martin, A.J., Nutt, J., Thompson, M.E., and Larson, P.S. (2019). Magnetic resonance imaging-guided phase 1 trial of putaminal AADC gene therapy for Parkinson's disease. Ann. Neurol. 85, 704–714.

31. Van Essen, D.C., Donahue, C.J., Coalson, T.S., Kennedy, H., Hayashi, T., and Glasser, M.F. (2019). Cerebral cortical folding, parcellation, and connectivity in humans, nonhuman primates, and mice. Proc. Natl. Acad. Sci. USA 116, 26173–26180.

32. Mestas, J., and Hughes, C.C. (2004). Of mice and not men: Differences between mouse and human immunology. J. Immunol. 172, 2731–2738.

33. Wildner, G. (2019). Are rats more human than mice? Immunobiology 224, 172–176.

34. Ong, G.L., and Mattes, M.J. (1989). Mouse strains with typical mammalian levels of complement activity. J. Immunol. Methods 125, 147–158.

35. Chenouard, V., Remy, S., Tesson, L., Ménoret, S., Ouisse, L.H., Cherifi, Y., and Anegon, I. (2021). Advances in genome editing and application to the generation of genetically modified rat models. Front. Genet. 12, 615491.

36. Markusic, D.M., and Herzog, R.W. (2012). Liver-directed adeno-associated viral gene therapy for hemophilia. J. Genet. Syndr. Gene Ther. 1, 1–9.

37. Harari, S. (2016). Why we should care about ultra-rare disease. Eur. Respir. Rev. 25, 101–103.

38. Woodcock, J., and Marks, P. (2019). Drug regulation in the era of individualized therapies. N. Engl. J. Med. 381, 1678–1680.