GENE THERAPY IN THE CNS – PROGRESS AND PROSPECTS FOR NOVEL THERAPIES

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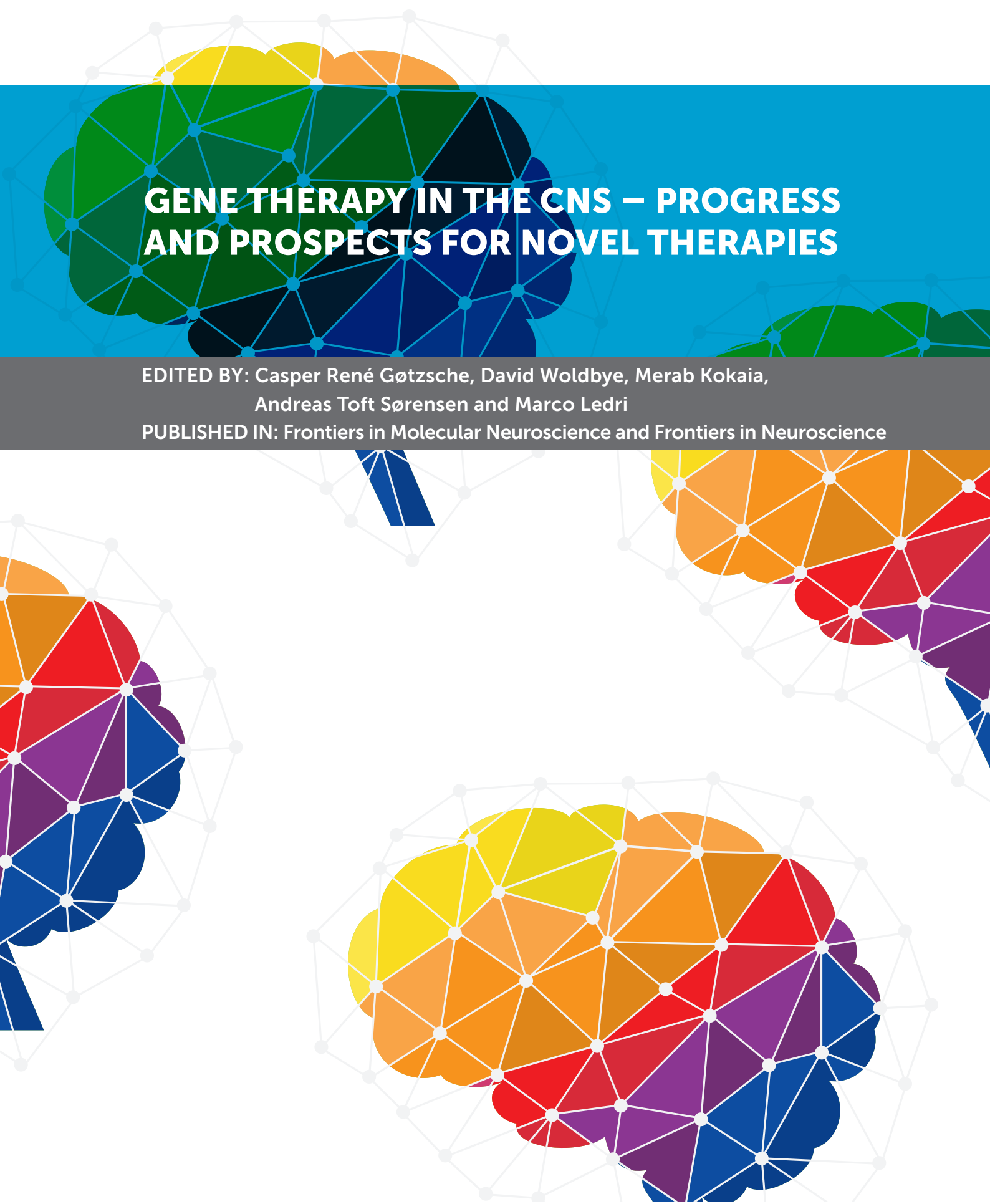

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GENE THERAPY IN THE CNS – PROGRESS AND PROSPECTS FOR NOVEL THERAPIES

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EDITORIAL
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Editorial: Gene Therapy in the CNS – Progress and Prospects for Novel Therapies

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Editorial on the Research Topic

Gene Therapy in the CNS – Progress and Prospects for Novel Therapies

Gene therapy for central nervous system (CNS) diseases holds a compelling potential for the development of novel therapies. It offers the prospective of transformative and disease-modulating treatment opportunities with potential long-lived therapeutic effects. Recent advances in the field have renewed the optimism for the possibility to develop new innovative solutions and especially certain CNS diseases could benefit greatly from novel gene therapies.

Gene therapy encompasses the administration of biological medicinal products containing recombinant nucleic acids, administered to a human to regulate, repair, replace, add, or delete a genetic sequence with the aim to treat or cure diseases[1]. This includes *in vivo* vector-mediated gene therapy, *ex vivo* cell transduction gene therapy, and genome editing (Brenner et al., 2020). In the previous decade the first gene therapies were approved in Europe and/or USA, including Glybera® (alipogene tiparvovec) for lipoprotein lipase deficiency (Watanabe et al., 2015), Strimvelis® (*ex vivo* hematopoietic stem and progenitor cell (HSPC) gene therapy) for adenosine deaminase deficiency-induced severe combined immunodeficiency (ADA-SCID) (Aiuti et al., 2017), Zynteglo® for β-thalassemia (Schuessler-Lenz et al., 2020), Luxturna® (voretigene neparvovec) for inherited retinal dystrophy (Gao et al., 2020), Zolgensma® (onasemnogene abeparvovec) for spinal muscular atrophy (Keeler and Flotte, 2019), and Libmeldy® (*ex vivo* HSPC gene therapy) for metachromatic leukodystrophy (Bulaklak and Gersbach, 2020). These successes could be the beginning of the discovery, development, and approval of many new gene therapies in the near future. To realize new CNS gene therapies, multiple challenges must be addressed including identifying beneficial therapeutic targets, developing efficient administration and distribution techniques, documenting sustained treatment responses and long-term safety aspects, and demonstrating proof-of-concept for clinical improvements over current available standard of care.

This Research Topic aims at collecting articles describing novel discoveries and technologies relevant for development of gene therapies targeting CNS diseases including neurological, neurodevelopmental, neuroimmunological, neurodegenerative, neuro-oncological,

[1]Based upon the definitions from the FDA (*Cellular & Gene Therapy Guidances, July 20, 2018*) and the EU commission (*Directive 2001/83/EC, Part IV of Annex I*).

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and neuromuscular disorders. We here present 11 unique articles covering a broad span of original research and scientific field reviews within these topics.

First, Jensen et al. provide an overview of rare genetic diseases in the brain and spinal cord, where gene therapy is being investigated as new viable treatment strategies. The review describes the major progress at both the preclinical and clinical levels within degenerative, developmental, lysosomal storage, and metabolic disorders. This field has reached unprecedented milestones with recent market approvals by the FDA and EMA, and here the authors provide an overview of what could be the next breakthrough therapies.

Belur et al. demonstrate how a vector based upon adeno-associated virus (AAV) of serotype 9 was developed for alpha-L-iduronidase (IDUA) gene delivery by different routes of administration in a mouse model of mucopolysaccharidosis type I. The vector-induced gene expression was shown to increase the IDUA enzyme levels, leading to normalization of glycosaminoglycan levels and restored cognitive performance in a spatial memory model.

Cattaneo et al. summarize available preclinical data on neuropeptide Y (NPY) gene therapy for treatment of epilepsy, and discuss the anti-epileptic effects and critical aspects still remaining to be thoroughly investigated before clinical testing.

Szczygiel et al. demonstrate how AAV vector-mediated combinatorial delivery of NPY and its antiepileptic receptor Y2 unilaterally into the hippocampus of adult rats provides sustainable and neuron-specific transgene expression as long as 6 months post-injection. No significant side effects were observed on body weight or memory performance.

Audouard et al. investigate the short-term effects of using a novel AAV serotype, AAVPHP.eB, to introduce the gene expression of human lysosomal enzyme arylsulfatase A (hARSA) in a mouse model of metachromatic leukodystrophy (MLD). Three months after treatment, brain and spinal cord sulfatide storage was significantly decreased, and improvement of astrogliosis and microgliosis in brain and spinal cord was evident. These results support the AAVPHP.eB-hARSA gene therapy to be further tested in symptomatic rapidly progressing forms of MLD.

Banerjee et al. provide an overview of current approaches for glioma gene therapy and virotherapy, highlighting the progress, prospects, and challenges. Even though we still remain to see clinical success with innovative gene-mediated therapies and oncolytic virotherapies, the implementation of better preclinical translational models holds the potential for clinical breakthroughs in coming years.

Lubroth et al. present and discuss the current progress within *in vivo* genome editing and modifying technologies, including the clustered regularly interspaced short palindromic repeats (CRISPR) systems, zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALEN), for translational neuroscience research and development of new treatments of CNS disorders. They also discuss the technical and commercial limitations as well as potential solutions to overcome these hurdles.

Rittiner et al. focus on the latest innovative solutions with delivery of therapeutic cargo to the nervous system using lentiviral and AAV vectors, overcoming problems associated with repeated drug administration and difficulties in delivering drugs across the blood-brain barrier. Centrally, they also describe how this technology can be applied in genome and epigenome-editing tools including CRISPR/Cas9 and the development of novel treatment of neurodegenerative diseases.

Zhu et al. describe how recent advances in gene sequencing and gene editing tools can be utilized for development of new therapies targeting neurodegenerative diseases. Not only is it possible to use gene editing for therapeutic approaches, it is also a valuable tool in research and to develop new experimental *in vitro* and *in vivo* disease models. Here the focus is on the progresses made in areas of Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.

O'Carroll et al. provide an overview of the potential and challenges for glial specific gene therapy, since different glial cell types are involved in nervous system pathology, playing roles in neurodegenerative disease and following trauma in the brain and spinal cord (astrocytes, microglia, oligodendrocytes), nerve degeneration and development of pain in peripheral nerves (Schwann cells, satellite cells), retinal diseases (Müller glia), and gut dysbiosis (enteric glia).

Finally, Tosolini and Sleigh outline how gene therapy can be administered with minimal invasiveness into skeletal muscles for extensive transduction of cells within the spinal cord, brainstem, and sensory ganglia, for treatment of neuronal conditions. In addition, they discuss optimization opportunities to the intramuscular administration route for improved gene delivery and therapeutic potential.

Taken together, the articles presented in this special issue of Frontiers in Molecular Neuroscience give a comprehensive overview of several important disciplines in gene therapy and provide novel insights into what could become viable treatments for CNS diseases.

AUTHOR CONTRIBUTIONS

CG drafted the manuscript. All authors contributed to the editing and finalization of the manuscript.

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REVIEW
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Intramuscular Delivery of Gene Therapy for Targeting the Nervous System

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Virus-mediated gene therapy has the potential to deliver exogenous genetic material into specific cell types to promote survival and counteract disease. This is particularly enticing for neuronal conditions, as the nervous system is renowned for its intransigence to therapeutic targeting. Administration of gene therapy viruses into skeletal muscle, where distal terminals of motor and sensory neurons reside, has been shown to result in extensive transduction of cells within the spinal cord, brainstem, and sensory ganglia. This route is minimally invasive and therefore clinically relevant for gene therapy targeting to peripheral nerve soma. For successful transgene expression, viruses administered into muscle must undergo a series of processes, including host cell interaction and internalization, intracellular sorting, long-range retrograde axonal transport, endosomal liberation, and nuclear import. In this review article, we outline key characteristics of major gene therapy viruses—adenovirus, adeno-associated virus (AAV), and lentivirus—and summarize the mechanisms regulating important steps in the virus journey from binding at peripheral nerve terminals to nuclear delivery. Additionally, we describe how neuropathology can negatively influence these pathways, and conclude by discussing opportunities to optimize the intramuscular administration route to maximize gene delivery and thus therapeutic potential.

Keywords: adenovirus (AdV), adeno-associated virus (AAV), axonal transport, lentivirus, motor neuron, neuromuscular junction (NMJ), peripheral nerve, sensory neuron

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INTRODUCTION

With thousands of clinical trials to date, gene therapy is a flourishing strategy with great promise for the treatment of diseases impacting the nervous system. Indeed, virus-mediated gene therapies have now been approved by the FDA in the US for *RPE65*-associated retinal dystrophy (*voretigene neparvovec* marketed as Luxturna) and *SMN1*-linked spinal muscular atrophy (SMA; *onasemnogene abeparvovec* marketed as Zolgenmsa), as well as non-neuronal conditions (High and Roncarolo, 2019). Gene therapy viruses are non-replicating, but still hijack host cell machinery to express transgenes of interest in the nucleus. Crucially, some viral vectors (i.e., viruses specifically used to deliver genetic material into cells) have the potential to circumvent the blood-brain- (BBB) and blood-spinal cord barriers (BSCB) when intravenously injected. Similarly, direct injection of viruses into the cerebrospinal fluid (e.g., *via* lumbar puncture in humans) also permits targeting of the peripheral (PNS) and central nervous systems (CNS). These two administration routes

for neuronal delivery have been extensively covered in recent reviews (Hocquemiller et al., 2016; Deverman et al., 2018; Hudry and Vandenberghe, 2019). A complementary, and perhaps sometimes superior (Benkhelifa-Ziyyat et al., 2013), method to introduce genetic material into select neuronal populations is by virus administration into muscle, which is the focus of this review. Muscles contain the synaptic connection between lower motor neurons and muscle fibers, i.e., the neuromuscular junction (NMJ), as well as specialized sensory nerve endings (e.g., muscle spindles). Viruses can be internalized into peripheral nerve terminals and subsequently retrogradely transported along axons to deliver viral payloads into corresponding motor and sensory neurons, with scope for widespread transfer to additional cells throughout the spinal cord and brain (Benkhelifa-Ziyyat et al., 2013; Chen et al., 2020).

The NMJ is a tripartite synapse comprised of a pre-synaptic motor nerve terminal, a post-synaptic muscle fiber, and several terminal Schwann cells (Li et al., 2018a). Moreover, the synaptic cleft consists of a complex and dynamic extracellular matrix (ECM) that contributes to receptor translocation and internalization of a variety of molecules (Heikkinen et al., 2020). Targeting muscles with viruses can transduce all three cellular constituents of the NMJ (Mazarakis et al., 2001; Homs et al., 2011)—by "transduction," we mean the introduction of genetic material into target cells. Furthermore, uptake at sensory nerve terminals can lead to transgene expression in dorsal root ganglia (DRG), trigeminal ganglia, and dorsal horn nerve fibers (Watson et al., 2016; Chen et al., 2020). When injected into a muscle, viruses are close to nerve endings for longer periods and at higher concentrations than when systemically injected. Moreover, limiting widespread virus distribution is likely to decrease safety risks due to immunogenicity or toxicity, while possible negative effects caused by central injections will be avoided. Hence, targeting muscle may prove to be a useful method to introduce viral vectors to certain central and peripheral neurons and/or glia.

For this strategy to be exploited, viruses must undergo several major processes, including host cell binding, internalization, intracellular sorting, and retrograde axonal trafficking to neuronal soma before nuclear entry. In this review article, we outline these mechanisms for major gene therapy viruses—adenovirus (AdV), adeno-associated virus (AAV) and lentivirus (LV; **Table 1**)—with a focus on peripheral neurons. We also comment on the impact of neuropathology on using intramuscular virus injection as an administration route. To conclude, we discuss opportunities to optimize gene therapy delivery to muscle for nervous system targeting.

TABLE 1 | Gene therapy virus characteristics.

	Adenovirus	AAV	Lentivirus
Size (nm)	≈90	≈25	80–120
Genome type	dsDNA	ssDNA	ssRNA
Packaging capacity (kb)	≈8*	≈4.7#	≈8
Enveloped	No	No	Yes
Integration	No	No	Yes
Expression	Transient	Persistent	Persistent
Immunogenicity	High	Moderate	Low

*Adapted from Worgall and Crystal (2014); Lee et al. (2017); Kariyawasam et al. (2020). *This is increased to ≈36 kb in the "helper-dependent" human AdV serotype 5. #This is halved in self-complementary AAV. ds, double-stranded; ss, single-stranded.*

GENE THERAPY VIRUSES

Adenovirus

First isolated in the 1950s, AdVs are non-enveloped, double-stranded DNA viruses with an icosahedral-shaped capsid comprised mainly of hexon and penton capsomeres (Greber and Flatt, 2019). *Adenoviridae* encompasses more than 300 different vertebrate-infecting types, including seven human AdV (HAdV) species (A to G) currently comprised of ≈80 types classified by serology or sequencing. HAdVs primarily cause ocular, gastrointestinal, or respiratory infections (Ghebremedhin, 2014). It is estimated that more than 80% of the human population has been exposed to HAdV and develop type-specific humoral and cross-reactive cellular immunity (Ahi et al., 2011), hence, for utilization as a gene therapy vector, strategies to circumvent the host immune response have been examined (Duffy et al., 2012). In the 1990s, AdV became the first gene therapy virus to be tested in human clinical trials and currently remains the most investigated (Lee et al., 2017). The more common human serotypes 2 and 5 belonging to species C have been the focus for gene therapy development. E1/E3-deleted AdVs have a relatively large packaging capacity of ≈8 kb, can transduce many different cell types, and form episomes rather than integrating into the host genome. Moreover, AdVs can be efficiently produced in large, concentrated quantities. In some hosts and some organs, transgene expression using AdV can be transient, likely due to host-specific responses, while in other cases, transgene expression remains robust for months (Li et al., 2016). In this regard, the transient expression can be advantageous for scenarios requiring short-term upregulation of therapeutic genes and for limiting deleterious consequences that may arise from long-term expression (discussed in Tosolini and Morris, 2016b). However, the transgene capacity of AdV can be increased up to ≈36 kb by removing essential elements and exogenously providing them for *in vitro* packaging, and with this approach, they lack the elements that usually activate host immunity, which can thereby facilitate prolonged-expression (Ricobaraza et al., 2020). Permitting much broader options for transgene incorporation, this expansive packaging capacity is one major advantage of AdV over other viral vectors.

AdVs display broad cell and tissue tropisms mediated by the interaction between their capsid and specific cellular receptors (Arnberg, 2012). Capsid modification, for instance by altering the virus genome or adding ligands, can widen or narrow tissue specificity depending on the required strategy (Worgall and Crystal, 2014). Direct intracranial injection of HAdV has been shown to result in the transduction of several different neuronal and non-neuronal cell types in the rodent CNS (Akli et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993). Furthermore, intramuscular administration of AdVs can result in their uptake at rodent NMJs and sensory terminals before retrograde transport to cell bodies (Finiels et al., 1995; Ghadge et al., 1995; Tosolini and Morris, 2016a), which is a viable strategy to counteract neuromuscular disease (Haase et al., 1998;

Acsadi et al., 2002) and peripheral nerve injury (Giménez y Ribotta et al., 1997; Baumgartner and Shine, 1998). Of note, the canine adenovirus serotype 2 (CAV-2; also known as CAdV-2), which can cause mild respiratory infections in *Canidae*, has become the AdV of choice for neuronal transduction (Del Rio et al., 2019). Due to possessing greater specificity in host cell receptor binding than HAdVs, CAV-2 preferentially targets neurons (Soudais et al., 2001). Furthermore, it is efficiently retrogradely transported along axons (Salinas et al., 2009), while a helper-dependent CAV-2 has been shown to drive transgene expression in the rodent CNS for over a year (Soudais et al., 2004). CAV-2 injection into craniofacial muscles of rhesus monkeys caused robust motor neuron transduction (Bohlen et al., 2019), while intramuscular administration in rats results in superior motor neuron uptake and transport compared to AdV serotype 5 (Soudais et al., 2001), which together highlight the potential of CAV-2 for motor neuron targeting *via* skeletal muscle.

Adeno-Associated Virus

Belonging to the *Dependoparvovirus* genus and thus needing factors from helper viruses (e.g., AdV) to replicate, AAVs are non-enveloped, single-stranded DNA viruses discovered as AdV preparation contaminants (Zinn and Vandenberghe, 2014). More than 100 natural AAV variants, including 13 serotypes from primates, have been identified, each with differing tissue tropisms, transduction efficiencies, and antigenicities, all resulting from their distinct protein capsids (Zincarelli et al., 2008; Srivastava, 2016). Additional synthetic AAV subtypes have been derived/engineered in the laboratory to optimize these features for gene transfer (Kotterman and Schaffer, 2014). Impinging considerably upon its tractability, the packaging capacity of AAV is limited to ≈4.7 kb, which is halved in the more rapidly expressing self-complementary AAV (for simplicity, we refer to single-stranded and self-complementary AAV as one), although DNA delivery across separate AAV particles is possible (Patel et al., 2019). In most cases, AAV vectors induce limited immunogenicity in naïve hosts (Ronzitti et al., 2020), and have a good safety record, although there may be toxicity issues when administered at high doses (Hinderer et al., 2018). However, the AAV vector effect on brain homeostasis has not been completely addressed and is an important consideration (He et al., 2019). Forming stable, non-replicating episomes for sustained transgene expression, AAV is largely non-integrating (Schnepp et al., 2005), although insertional mutagenesis has been reported (Chandler et al., 2017). These combined features have led to AAV becoming the premier clinical gene therapy vector and its recent regulatory approval for the treatment of several conditions (High and Roncarolo, 2019). However, AAV gene therapy is not entirely infallible, as wild type AAV infections have been linked with human disease (Nault et al., 2016); however, potential solutions to overcome these and other concerns to drive human AAV gene therapy are continuing (Colella et al., 2018). Nonetheless, many more clinical trials of AAV-mediated gene therapy are ongoing or planned, including several involving intramuscular administration (although not necessarily for neuronal transduction).

AAVs have been used for many years in the laboratory to drive transgene expression in the nervous system (Hudry and Vandenberghe, 2019). Due to its ability to cross the BBB, AAV serotype 9 (AAV9) has become the principal serotype for CNS-targeting upon systemic administration (Foust et al., 2009; Bevan et al., 2011; Samaranch et al., 2012), although superior serotypes, such as AAVrh10, have also emerged (Tanguy et al., 2015). However, cell binding and transduction can change with age (Chakrabarty et al., 2013), thus engineered serotypes with greater neuronal tropism, at least in mice, are being developed (Choudhury et al., 2016; Deverman et al., 2016). Nervous system delivery has also been achieved by AAV injection into muscle; intramuscular administration of several AAV serotypes (e.g., AAV2, AAV9) results in AAV uptake into the motor and sensory neurons in rodents (Hollis Ii et al., 2008; Zheng et al., 2010; Benkhelifa-Ziyyat et al., 2013; Jan et al., 2019; Chen et al., 2020) and motor neurons in non-human primates (Towne et al., 2010). Consequently, this method of gene delivery has proven beneficial in mouse models of motor neuron diseases amyotrophic lateral sclerosis (ALS), and SMA (Tosolini and Sleigh, 2017). Increasing the possible clinical applicability of AAV, single intramuscular injections of rAAV2-retro, a newly evolved variant with robust retrograde transport capacity (Tervo et al., 2016), were recently shown to result in broad transgene expression across ipsilateral and contralateral motor neurons along the length of the spinal cord, as well as brainstem motor nuclei, DRG, trigeminal ganglia and dorsal horn nerve fibers (Chen et al., 2020). Importantly, AAV targeting of peripheral neurons is therefore not limited to those cells innervating the injected muscle.

Lentivirus

Belonging to the *Retroviridae* family, LV possesses a single-stranded RNA genome and can infect both dividing and non-dividing cells (Parr-Brownlie et al., 2015). LV is an enveloped virus with a packaging capacity of ≈8 kb and it relies on reverse transcription of its single-stranded RNA genome to generate corresponding double-stranded DNA for integration into the host genome (Mátrai et al., 2010). This provides benefits of long-term transgene expression and inheritance of genetic material in dividing cells; however, integration also has the major disadvantage that it can disrupt host gene function through insertional mutagenesis, which poses a safety risk. Incorporation into the host genome is not random, as there are preferential sites and conditions for integration (e.g., highly expressed and intron-rich genes), but it is unpredictable (Lesbats et al., 2016). Nonetheless, this has not prevented several LV-mediated gene therapies being approved for human use, albeit being utilized for *ex vivo* modification of autologous immune cells (High and Roncarolo, 2019). For gene delivery, essential viral coding regions (e.g., *gag*, *pol*, and *env*) are removed from the LV genome, and instead provided by separate expression plasmids for *in vitro* packaging (Milone and O'Doherty, 2018). This removal of viral genes ensures that the immunogenicity of LV is relatively low, although not absent (Annoni et al., 2019).

LVs are typically derived from primate or non-primate immunodeficiency viruses [e.g., human immunodeficiency virus type 1 (HIV-1) or equine infectious anemia virus (EIAV)]. LV

tropism is mediated by the viral envelope, which is engineered to include glycoproteins from other enveloped viruses in a process called pseudotyping (Cronin et al., 2005). The most common virus used to pseudotype LV is the vesicular stomatitis virus (VSV), but heterologous envelope proteins from many other viruses have been used to target LV to particular cells and tissues, e.g., measles virus, murine leukemia virus and influenza viruses (Joglekar and Sandoval, 2017). The VSV glycoprotein (VSV-G) binds to a widely expressed receptor, leading to broad tropism when integrated into the LV envelope. In contrast, LVs pseudotyped with rabies virus (RV) display greater neuronal selectivity and have been shown to aid efficient transduction of neurons both *in vitro* and *in vivo*. Compared to VSV, LV pseudotyped with RV glycoprotein (LV-RV) shows superior neuronal transduction and transport when injected into the rat striatum and spinal cord (Mazarakis et al., 2001). A similar high efficiency has been reported when injected into the primate brain (Kato et al., 2007), while distal uptake and efficient retrograde trafficking occurs in rodent primary motor neurons (Hislop et al., 2014). Moreover, LV-RV administration into gastrocnemius muscle results in effective transgene expression in spinal cord motor neurons, while LV-VSV remains restricted to the muscle injection site (Mazarakis et al., 2001), which was confirmed with additional RV strains (Wong et al., 2004; Mentis et al., 2006). Pseudotyping with several different hybrid glycoproteins has since shown improved targeting of motor neurons when delivered to muscle, which can be further enhanced by the coupling of antibodies against NMJ receptors to the virus surface (Hirano et al., 2013; Eleftheriadou et al., 2014). As a consequence, numerous different LV-mediated therapeutic strategies that target motor neurons *via* muscle have proven successful in mouse models of ALS and SMA (Azzouz et al., 2004a,b; Ralph et al., 2005; Raoul et al., 2005; Benkler et al., 2016; Eleftheriadou et al., 2016).

FROM VIRUS BINDING TO NUCLEAR ENTRY

For viruses injected into a muscle to express transgenes in neurons, they must undergo a series of events: host cell binding and internalization, intracellular sorting, retrograde axonal transport, liberation from the transporting structure/organelle and nuclear entry (**Figure 1**). AdV, AAV, and LV rely on the same or similar mechanisms for several parts of this journey which are also shared by botulinum and tetanus neurotoxins (Surana et al., 2018). For instance, they all hijack retrograde axonal transport (Merino-Gracia et al., 2011), which is dependent on active, processive movement along microtubules by the motor protein complex cytoplasmic dynein-dynactin (Schiavo et al., 2013). By trafficking towards the stable minus ends of the microtubule, which are located at the cell body end of an axon, cytoplasmic dynein enables long-range retrograde delivery of cargoes, such as autophagosomes and neurotrophin-containing signaling endosomes. Additionally, the Rab (Ras-related proteins in the brain) GTPase protein family is specifically required for signaling endosome trafficking (Villarroel-Campos et al., 2018). Target tissue-derived (e.g., muscle) neurotrophins transition from early Rab5-positive endosomes into retrogradely transported Rab7-positive signaling endosomes (Deinhardt et al., 2006). Unlike in the canonical endolysosomal pathway, retrograde Rab7-endosomes within axons display a tightly regulated neutral pH value that is maintained during transport (Bohnert and Schiavo, 2005). All three gene therapy viruses have been shown to localize to these axonal Rab7-endosomes, indicating that they share a common compartment when voyaging to the nucleus. Retrograde trafficking is a rapid and constitutive process that delivers large quantities of endosomes to the motor and sensory soma; it is thus unlikely to be a rate-limiting step in virus transgene expression. Rather, idiosyncratic aspects of the journey of each virus, e.g., binding to specific receptors or endosomal liberation at the cell body, probably have a greater impact on overall transduction efficiency.

Highlighting similarities and differences, we now describe the individual journeys that each virus must take to migrate from muscle to peripheral nerve soma for transgene expression.

Adenovirus

Similar to most viruses, AdV is typically internalized in a two-step, receptor-mediated fashion that is dependent on the viral capsid, although non-specific, large-scale internalization has also been reported (Meier et al., 2002). Primary receptors that mediate AdV attachment to cells include, heparan sulfate proteoglycans, CD46, and sialic acid, which selectively interact with different serotypes (Arnberg, 2012); however, the appears to be the major initial binding partner for AdVs (Bergelson et al., 1997; Arnberg, 2012). Coxsackie and adenovirus receptor (CAR) is a widely expressed cell adhesion protein critical for heart development (Dorner et al., 2005), and is involved in neurogenesis through its synaptic expression throughout the mature brain (Zussy et al., 2016). CAR serves as the primary receptor for several different HAdV species (i.e., A, C-F) and serotypes, including 2 and 5, as well as CAV-2 (Arnberg, 2012; Loustalot et al., 2016). The second step of AdV internalization (i.e., entry) is facilitated by penton capsomere binding to members of the integrin receptor family, e.g., $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (Wickham et al., 1993). Facilitating cell-to-cell and cell-to-ECM interactions, integrins are expressed in a tissue-specific fashion and can in some instances mediate AdV attachment in the absence of CAR (Huang et al., 1996).

Despite extensive knowledge on AdV receptors, relatively little is known about the specific entry of AdV at the NMJ or sensory nerve terminals. Intramuscular injections of AdV result in the targeting of both muscle fibers and innervating motor neurons in juvenile and adult mice (Tosolini and Morris, 2016a), which is consistent with the reported expression of CAR in muscle fibers (Nalbantoglu et al., 1999) and at both mouse and human NMJs (Shaw et al., 2004; Sinnreich et al., 2005). However, one of the major issues with AdV-mediated gene therapy is the relatively poor transduction of neurons in adults compared to young mice, including upon intramuscular injection (Acsadi et al., 1994; Huard et al., 1995; Tosolini and Morris, 2016a). This is somewhat unsurprising as CAR is downregulated post-natally in several neuronal subtypes (Hotta et al., 2003) and muscle (Nalbantoglu et al., 1999). Indeed, CAR is highly expressed in

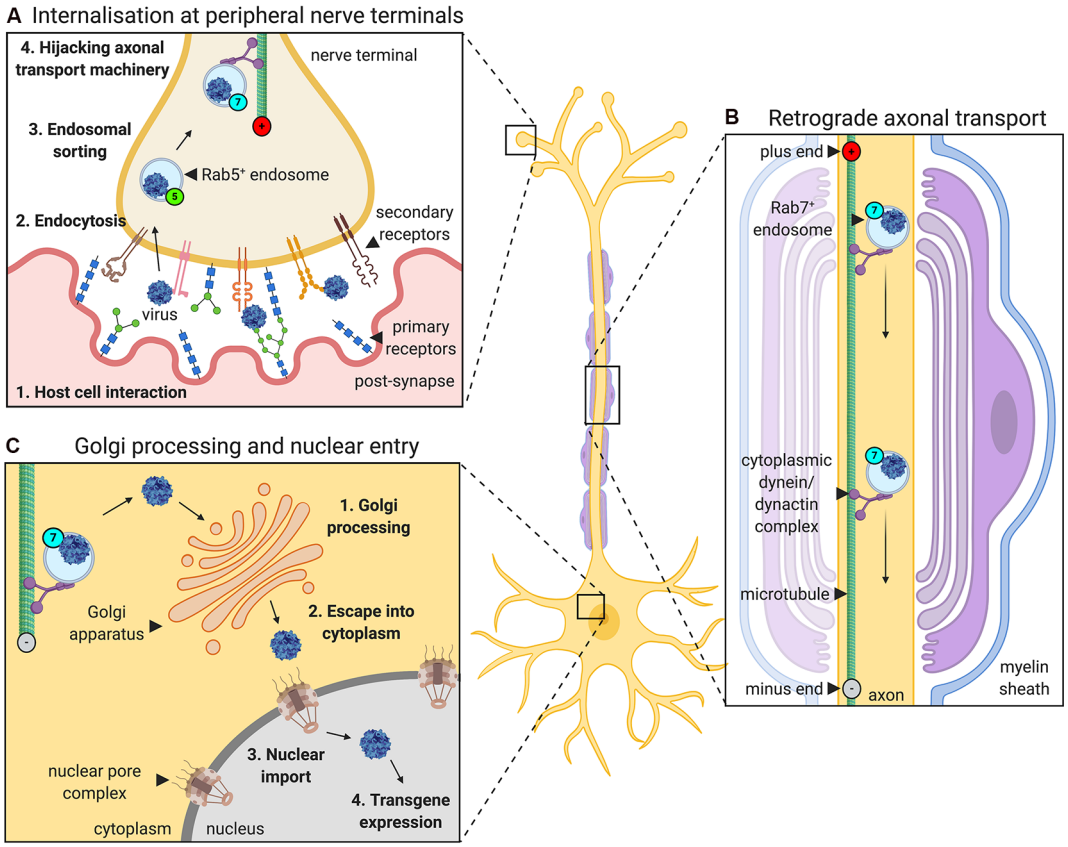

FIGURE 1 | The journey of gene therapy viruses from peripheral nerve terminals to the nucleus. Viruses used to deliver gene therapy must access cell nuclei to express their packaged genetic material. When administered into muscles for targeting of peripheral nerve somas, viruses such as adenovirus, adeno-associated virus (AAV) and lentivirus, undergo a series of processes that aid their transfer from the periphery to CNS (depicted here using AAV as an example). **(A)** First, the virus interacts with specific host cell surfaces. This entails primary receptor binding (e.g., glycans) followed by internalization, which is often mediated, at least in part, by a secondary receptor (e.g., AAV receptor, AAVR or fibroblast growth factor receptor, FGFR). Internalization at nerve terminals is regulated by a variety of endocytic pathways. Post-internalisation, viruses hijack the Rab GTPase-mediated endosomal sorting system, transitioning through Rab5-positive early endosomes to non-acidic Rab7-positive late endosomes. **(B)** Virus-containing Rab7-positive signaling endosomes are actively transported along microtubules by cytoplasmic dynein-dynactin complexes towards nerve cell bodies (i.e., retrogradely). **(C)** At the neuronal soma, viruses escape endosomes and are processed, sometimes through the Golgi apparatus, before entry into the nucleus (e.g., *via* the nuclear pore complex), where the virus can begin to drive transgene expression.

immature skeletal muscle fibers but is drastically downregulated after birth (Nalbantoglu et al., 1999) becoming restricted to the NMJ (Shaw et al., 2004; Sinnreich et al., 2005). Nevertheless, to better understand the limited uptake of AdV into adult motor neurons, further investigation is required to provide a thorough longitudinal assessment of CAR levels at post-natal neuromuscular synapses. Upon muscle damage caused by Duchenne muscular dystrophy or polymyositis, CAR expression increases within muscle fibers and co-localizes with markers of regeneration (Sinnreich et al., 2005); given the parallels between mechanisms of muscle development and regeneration, this suggests that CAR may indeed be developmentally regulated at the NMJ and serve in the synaptic response to regeneration (Sinnreich et al., 2005).

After binding to CAR, AdVs are internalized and processed in a cell type-dependent manner. Experiments in immortalized non-neuronal cells describe AdV internalization into endosomes *via* clathrin-coated pits (Meier et al., 2002) and subsequent endosomal liberation *via* acidification (Leopold et al., 1998). The intracellular domain of CAR plays a critical role in this by recruiting the endocytic machinery and influencing subsequent intracellular AdV trafficking (Loustalot et al., 2015). AdVs are then transported towards the nucleus by cytoplasmic dynein-mediated trafficking along with the microtubule network (Kelkar et al., 2004), impairments in which drastically disrupt this nuclear targeting (Suomalainen et al., 1999; Leopold et al., 2000). The AdV capsid directly interacts with cytoplasmic dynein *via* hexon capsomeres (Bremner et al., 2009), suggesting that in non-neuronal cells AdVs are transported as "naked particles" rather than in membrane-bound organelles (e.g., endosomes; Scherer et al., 2020). Moreover, this interaction appears to be dependent on exposure to low pH, suggesting that AdV binding to the motor protein is primed by transition through the early endosomal system (Bremner et al., 2009). AdV serotype 5 has also been shown to interact with the Kif5B subunit of kinesin-1, a motor protein that drives transport in the opposite direction to

cytoplasmic dynein (i.e., towards dynamic plus ends), possibly as an evolutionary strategy for increased cellular exploration (Zhou et al., 2018).

In primary neurons, AdVs are also internalized in a CAR-dependent manner (Loustalot et al., 2016), facilitated by CAR enrichment in actin-domains of neuronal growth cones as well as lipid rafts (Huang et al., 2007). Internalization occurs through a lipid microdomain-, actin- and dynamin-dependent manner before the receptors are eventually targeted for lysosomal degradation (Salinas et al., 2014). The major difference between neuronal and non-neuronal AdV trafficking is that in neurons, CAR does not undergo lysis during intracellular sorting, and is instead transported to the neuronal soma as part of non-acidic, Rab7-positive endosomes, thus preventing pH-induced conformational changes to the AdV capsid and restricting endosomal liberation (Salinas et al., 2009). CAR-positive organelles favor the retrograde direction but can also be anterogradely transported by kinesin motor proteins (Salinas et al., 2009). Again confirming the essential nature of transport to AdV migration, *in vivo* pharmacological blockade of microtubule dynamics inhibits the delivery of AdV to the neuron (Boulis et al., 2003). Once in the soma, AdV accesses the nucleus at the nuclear pore complex *via* histone H1 (Trotman et al., 2001) or the nucleoporin receptors (Trotman et al., 2001; Cassany et al., 2015), with the route also appearing to be cell type-dependent (Kremer and Nemerow, 2015).

Adeno-Associated Virus

AAV also gains cellular access *via* a two-step process involving primary cell surface receptors with a secondary receptor mediating entry. Negatively charged glycans or glycoconjugates serve as primary attractants with which AAVs initially interact allowing extracellular viral accumulation and co-receptor access. These include heparan sulfate proteoglycans for AAV2, AAV3, AAV6 and AAV13, N-terminal galactose for AAV9, and specific N- and O-linked sialic acid moieties for AAV1, AAV4, AAV5 and AAV6 (Huang et al., 2014). The wide expression of surface glycans, including in neuronal extracellular matrices (Broadie et al., 2011; Singhal and Martin, 2011), explains the broad infectivity of AAV, while glycan diversity and relative density likely dictates selectivity of AAV serotype tropism.

Several serotype-specific co-receptors have also been identified that after glycan binding, facilitate AAV uptake. These co-receptors include fibroblast growth factor receptor (FGFR) and hepatocyte growth factor receptor (HGFR) for both AAV2 and AAV3, platelet-derived growth factor receptor (PDGF) for AAV5, and epidermal growth factor receptor (EGFR) for AAV6 (Madigan and Asokan, 2016). Signaling through each of these receptors has been linked to NMJ formation/function (Zhao et al., 1999; Li et al., 2012; Taetzsch et al., 2018), consistent with their synaptic availability. Additional receptors have been identified for engineered serotypes contributing to distinct tropisms (Hordeaux et al., 2019; Huang et al., 2019). However, a common receptor required for endocytosis of most natural primate AAV serotypes was recently identified (Pillay et al., 2016). Originally called KIAA0319L and linked with dyslexia and functions of neuronal migration and axon guidance (Poon et al., 2011), the AAV receptor (AAVR) possesses an N-terminal MANSC domain, several immunoglobulin-like PKD domains, a C6 domain, and a transmembrane region before a short C-terminal tail (Poon et al., 2011). As expected given the broad cellular and tissue infectivity of AAV, AAVR is expressed across many human tissues, including muscle and nerve, and can be found as several spliced variants and post-translationally modified isoforms (Poon et al., 2011; Gostic et al., 2019). AAVR knockout rendered mammalian HeLa cells highly resistant to infection with AAV serotypes 1, 2, 3b, 5, 6, 8, and 9, with a similar finding in AAV9-injected AAVR knockout mice *in vivo* (Pillay et al., 2016). The removal of AAVR resulted in no obvious phenotype, suggesting that AAVR is non-essential or there is genetic compensation. In subsequent work from the same group and others, AAV serotypes have been shown to differentially interact with AAVR PKD domains (Pillay et al., 2017; Zhang et al., 2019), while AAV4 gains full cellular access in absence of the receptor, suggesting that some serotypes can utilize non-AAVR internalization pathways (Dudek et al., 2018). In immortalized cells, AAVR localizes to the cytoplasm and perinuclear region where it associates with the Golgi network (Poon et al., 2011; Pillay et al., 2016). Several hypotheses as to where exactly AAV interacts with AAVR have been put forward, including on the cell surface, in the endolysosomal system and at the Golgi apparatus; however, this requires further clarification (Summerford et al., 2016; Pillay and Carette, 2017).

Data are supporting several distinct AAV internalization mechanisms, including clathrin-dependent endocytosis (Uhrig et al., 2012), caveolar endocytosis (Sanlioglu et al., 2000), and the clathrin-independent carriers and GPI-enriched endocytic compartments (CLIC/GEEC) pathway (Nonnenmacher and Weber, 2011). However, not all routes result in an efficient delivery to the nucleus, rather they traffic AAV through unproductive paths leading to a viral cul-de-sac (Nonnenmacher and Weber, 2012; Pillay and Carette, 2017); only ≈30% of internalized AAV is estimated to enter the nucleus (Zhong et al., 2008; Xiao et al., 2012). Nonetheless, there are distinctions in AAV uptake depending on cell type and serotype (Weinberg et al., 2014), thus future work identifying neuronal-specific internalization mechanisms is required.

Upon cellular entry, AAVs have been reported to be retrogradely transported from the cell surface to Golgi in a syntaxin 5-dependent mechanism (Nonnenmacher et al., 2015), before escaping into the cytoplasm and entering into the nucleus *via* the nuclear pore complex (Nicolson and Samulski, 2014). However, before reaching the Golgi, AAV must transit through various acidic endosomal compartments to drive pH- and cathepsin-mediated conformational changes in the capsid (Akache et al., 2007; Salganik et al., 2012). Indeed, the passage of AAV through the endosome to Golgi system appears to be necessary for transgene expression, as AAV directly injected into cytosol do not migrate to the nucleus (Sonntag et al., 2006). AAV has been reported to localize to Rab5-, Rab7-, and Rab11-positive (recycling) endosomes (Berry and Asokan, 2016), and, as expected, requires a functioning microtubule network for transport (Xiao and Samulski, 2012). Nevertheless, its exact route

through the cell requires further elucidation, especially its transit through long and highly polarized peripheral nerves, as little data have been generated in neurons.

That being said, there is ample indirect evidence that AAVs are transported in axons *in vivo* both in peripheral (Hollis Ii et al., 2008; Towne et al., 2010; Zheng et al., 2010; Benkhelifa-Ziyyat et al., 2013; Jan et al., 2019) and CNS (Salegio et al., 2013; Castle et al., 2014a,b) neurons, suggesting the availability of AAV receptors and uptake mechanisms; however, observations of AAV being actively trafficked are limited. Nevertheless, peripherally administered AAV likely hijacks Rab-positive endosomes in peripheral nerves to reach the CNS, like that of AdV. Indeed, in primary cortical neurons grown in microfluidic chambers to separate axons and soma, AAV9 was shown to localize in a time-dependent fashion to several different endosomes/vesicles (e.g., Rab5-, Rab7-, Rab11-positive; Castle et al., 2014b). AAV9 internalized at axon tips was retrogradely transported in cytoplasmic dynein-dynactin-driven Rab7-positive endosomes and was subsequently capable of inducing transgene expression post-transition through the Golgi (Castle et al., 2014b). Moreover, in a companion study it was shown that AAV1, AAV8, and AAV9 share the same intra-axonal compartment when being transported in primary cortical neurons, indicating that once they have gained access to the endosomal sorting system, AAV serotypes harness common axonal transport mechanisms (Castle et al., 2014a). However, direct evidence from the motor and sensory neurons remains unavailable.

Lentivirus

LV tropism is dictated by the envelope glycoproteins with which it has been pseudotyped (Cronin et al., 2005). VSV-G interacts with the low-density lipoprotein receptor (LDLR; Finkelshtein et al., 2013). LDLR mediates uptake of cholesterol-rich LDL and is broadly expressed, thus LV-VSV is pan-tropic. A measure of cell-type selectivity can be achieved with cell/tissue-specific promoters, which is a strategy used with all three gene therapy viruses. For example, LV-VSV combined with an *hGFAP* promoter induces astrocytic expression, whereas LV-VSV with an *rNSE* promoter selectively expresses in neurons (Jakobsson et al., 2003). Alternatively, envelope modification coupled with surface antibody-mediated targeting can confer tissue specificity and improve virus uptake (Yang et al., 2006; Eleftheriadou et al., 2014). In contrast, LV-RV interacts with receptors that are predominantly expressed by neurons, including the pan-neurotrophin receptor $p75^{NTR}$ (Tuffereau et al., 1998), neuronal cell adhesion molecule (NCAM; Thoulouze et al., 1998) and nicotinic acetylcholine receptor (nAChR; Hanham et al., 1993). $p75^{NTR}$ non-selectively binds to all neurotrophins (i.e., BDNF, NGF, NT-3, and NT-4/5) and, depending on the active co-receptor, can activate both pro-survival or pro-death signaling (Gentry et al., 2004). NCAM is an immunoglobulin-like glycoprotein that mediates cell-to-cell contact and functions in adhesion, guidance, and differentiation during neuronal growth (Weledji and Assob, 2014). nAChRs bind to the excitatory neurotransmitter acetylcholine secreted into the synaptic cleft to facilitate depolarization of the postsynaptic cell. All three LV-RV receptors are integral constituents of the NMJ (although nAChRs are post-synaptic), explaining the efficient *in vivo* uptake into motor neurons of these RV pseudotyped viruses when injected into a muscle (Mazarakis et al., 2001; Azzouz et al., 2004a,b; Wong et al., 2004).

After receptor-mediated internalization, most likely in clathrin-coated pits as dictated by their neuronal receptors (i.e., $p75^{NTR}$; Bronfman et al., 2003), RV-LVs migrate through the endolysosomal system transitioning from Rab5-positive early endosomes to the non-acidic Rab7-positive compartment (Hislop et al., 2014). In non-neuronal cells, endosome acidification causes a conformational change in LV glycoproteins, which initiates membrane fusion between the viral envelope and endosome membrane to permit the escape of the virus into the cytoplasm (Gaudin et al., 1993; Gaudin, 2000). However, in neurons, LVs are retrogradely transported within neutral Rab7-positive signaling endosomes towards peripheral nerve cell bodies through the same motor protein-driven process as AdV and AAV. In rat primary motor neuron cultures, LV-RV was shown to co-localize in axons with all three receptors (i.e., $p75^{NTR}$, NCAM, and nAChR) with co-migration confirmed for $p75^{NTR}$ (Hislop et al., 2014). However, despite transport being rapid and effective, neuronal transduction was comparatively inefficient, suggesting that post-trafficking processes are suboptimal in neurons (Hislop et al., 2014). Upon arrival at the cell body, LV must undergo a process known as uncoating, in which several viral proteins (e.g., Gag structural proteins) are removed to permit reverse transcription of the viral RNA (Matreyek and Engelman, 2013). The resulting double-stranded DNA then complexes with virus proteins for entry into the nucleus *via* the nuclear pore complex, before integration into the DNA of the host neuron. Improving understanding of these processes in motor and sensory neurons will be key to optimizing the effectiveness of intramuscular virus delivery.

INFLUENCE OF PATHOLOGY

Neuropathology will impact most, if not all, major steps in the journey of viruses from the nerve terminal to the nucleus (**Figure 2**). Neurodegeneration of peripheral nerves results in the loss of axon terminals within muscles (**Figure 2A**). Motor neuron retraction from the NMJ, i.e., denervation, is an early feature of motor neuron diseases [e.g., ALS, SMA and Charcot-Marie-Tooth disease (CMT; Goulet et al., 2013; Moloney et al., 2014; Sleigh et al., 2014; Spaulding et al., 2016)], and will limit neuron-virus interactions within muscles. Sensory degeneration observed in conditions like CMT (Sleigh et al., 2017), will have a similar restrictive effect. Nonetheless, motor neurons branch frequently within muscles resulting in multiple contacts across the entire muscle; thus, if one or several NMJs become denervated, there is likely to be a window of time in which at least some neuromuscular contacts of a pathological neuron remain viable. In ALS mice, for instance, rather than all neuromuscular contacts of a single motor neuron denervating simultaneously, healthy synapses close to degenerating NMJs are more likely to denervate than those located further away, suggestive of localized pathological

transfer (Martineau et al., 2018). It is therefore conceivable that functional synapses may facilitate virus uptake and nuclear delivery to preserve the integrity of NMJs that remain. Moreover, once delivered, viral vectors encoding secretable proteins (e.g., neurotrophins) can influence central networks through both autocrine and paracrine mechanisms (Baumgartner and Shine, 1997; Benkhelifa-Ziyyat et al., 2013). NMJs resident in different muscles, and even within a single muscle, can show large differences in both pre- and post-synaptic structures (Mech et al., 2020) as well as levels of key synaptic proteins (Allodi et al., 2016), thus virus binding and uptake are likely to differ across motor nerve terminals.

Significantly, intramuscular injections of gene therapy viruses can result in efficient and extensive transgene expression within the neonatal and adult mouse spinal cord, brainstem, and sensory ganglia, likely *via* the cerebrospinal fluid (Benkhelifa-Ziyyat et al., 2013; Chen et al., 2020). This finding is particularly important, as it suggests that injecting one muscle can result in viral transduction of an array of central neurons (Chen et al., 2020), meaning that not all muscles require injection for potential widespread motor and sensory neuron transduction; although injecting more muscles can cause greater therapeutic benefit (Benkhelifa-Ziyyat et al., 2013). Furthermore, muscle transduction can be used to promote synaptogenesis and/or reinnervation after neuromuscular pathology (Darabid et al., 2014). In this regard, collateral sprouting and dynamic remodeling of the NMJ, as is observed in ALS mice (Martineau et al., 2018), may also be therapeutically targeted.

In addition to the loss of peripheral nerve endings in muscle, deficiencies in endocytosis (e.g., in SMA; Dimitriadi et al., 2016), endolysosomal sorting (observed in many conditions; Neefjes and van der Kant, 2014), Golgi processing (e.g., in ALS; van Dis et al., 2014), and nuclear import (e.g., in ALS; Dormann and Haass, 2011) would all likely reduce the efficiency of viral transgene expression (**Figure 2B**). As would pathology-associated restrictions in axonal transport (**Figure 2C**), which have been reported in many neurodevelopmental and neurodegenerative conditions (Sleigh et al., 2019), such as the signaling endosome transport deficits observed in ALS mice (Bilsland et al., 2010; Sleigh et al., 2020a). Nevertheless, Rab7-positive endosomes containing AAV have been shown in primary cortical neurons *in vitro* to increase retrograde transport speeds compared to non-AAV containing Rab7 organelles (Castle et al., 2014b), which could perhaps counteract transport dysfunction.

Only a few studies are have investigated the impact of disease on virus transduction after intramuscular delivery. Despite downregulation during development, CAR expression is upregulated in regenerating adult skeletal muscle in response to disease (Nalbantoglu et al., 1999; Shaw et al., 2004; Sinnreich et al., 2005), which will likely positively impact AdV uptake. Increased levels of sialic acid, a known AAV9 inhibitor, in the CNS of a mouse model of lysosomal storage disorder have been shown to severely limit the effectiveness of AAV9-mediated gene therapy (Chen et al., 2012). Nonetheless, the opposite may be true for particular AdV and other AAV serotypes, which use sialic acid as a primary attachment factor. Involved in pro-apoptotic signaling during development, but downregulated in the mature nervous system, the $p75^{NTR}$ receptor is also re-expressed in neurons after disease or trauma (Dechant and Barde, 2002), possibly impacting LV efficacy. For example, $p75^{NTR}$ expression is increased in $SOD1^{G93A}$ mice motor neurons and human ALS tissue (Lowry et al., 2001), and plays a key role in organizing and maintaining NMJ connectivity (Pérez et al., 2019). Moreover, NCAM expression is a major regulator of synaptic remodeling in pre-synaptic NMJ terminals (Chipman et al., 2014) and levels are dysregulated in ALS (Jensen et al., 2016), which could also affect LV binding. Also, the background of the experimental animal can influence the transduction efficiency of some vectors and must be carefully considered (He et al., 2019). Overall, these studies warn against the assumption of similar virus binding and uptake profiles between healthy and disease states and indicate that further studies in disease models at symptomatic stages are required.

Despite these hurdles, intramuscular injections of gene therapies have proved successful at symptomatic stages in ALS mice (Tosolini and Sleigh, 2017), hence the above-discussed effects of pathology do not abolish virus transduction. Furthermore, symptomatic SMA patients treated with *onasemnogene abeparvovec* to augment SMN protein levels respond positively to treatment (Mendell et al., 2017), albeit with AAV administered intravenously. Nevertheless, while it remains unclear precisely how and to what extent specific diseases and associated pathologies will impact the transduction of peripheral neurons, the described viral vectors have the undisputed potential for the treatment of neuromuscular disorders when delivered to skeletal muscle.

OPTIMIZING INTRAMUSCULAR GENE THERAPY

One of the biggest challenges facing gene therapy is achieving sufficient delivery to target cells/tissues to combat disease. This is particularly difficult for peripheral nerve disorders in which pathological cells are located deep within the spinal cord and behind the BBB and BSCB. Several investigator-independent factors such as nervous system maturity (Foust et al., 2009; Tosolini and Morris, 2016a) and pathology influence viral transduction and transgene expression, but these cannot be modified in a clinical setting. However, varied investigator-driven factors also impact the effectiveness and should be carefully considered when designing gene therapy for intramuscular administration. Differences in tropism, infectivity, and transport between viruses and their serotypes will impact the success of this delivery method; for example, in a side-by-side comparison, muscle injection of rAAV2-retro was shown to have superior capacity to transduce peripheral neurons compared to AAV serotypes 1, 2, and 5–9 (Chen et al., 2020). Similarly, superior LV pseudotypes based on hybrid glycoproteins have also been identified (Hirano et al., 2013; Eleftheriadou et al., 2016). Moreover, vector purity and concentration will impact transduction levels (Hollis Ii et al., 2008; Klein et al., 2008), as will the efficiency and specificity of the promoter (von Jonquieres et al., 2013; Borel et al., 2016), the choice of which can also reduce

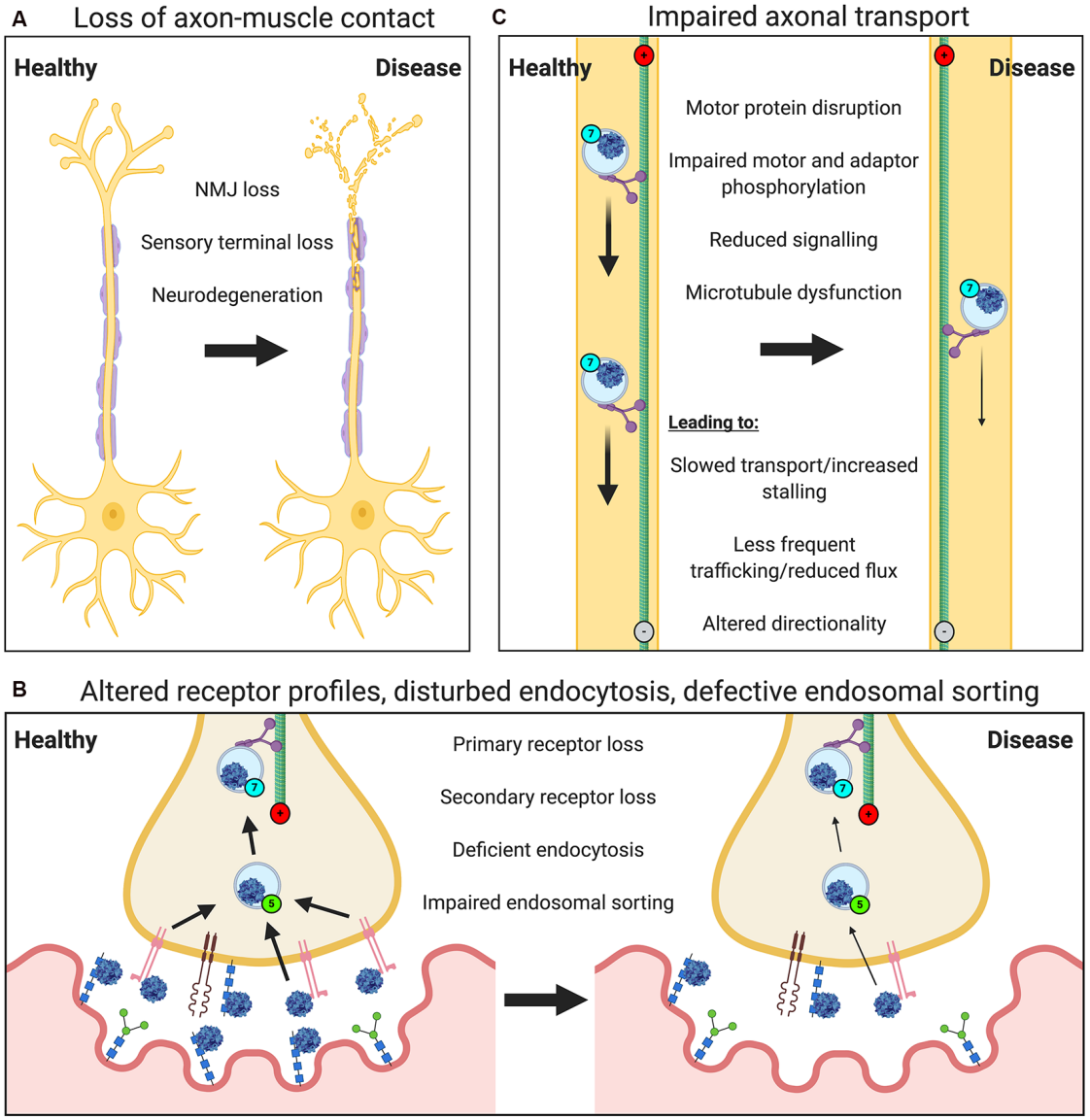

FIGURE 2 | Neuropathological events impair the viral transduction of peripheral neurons. Several general and virus-specific pathological events caused by neurological disease diminish the effectiveness of gene therapy delivery to the nervous system *via* muscle. **(A)** Loss of motor and sensory nerve endings due to neurodegeneration will restrict nerve-muscle connections and the frequency of virus-nerve interaction. **(B)** Alterations in the expression or availability of certain primary or secondary receptors will affect virus attraction and binding. Deficits in endocytosis, as seen in spinal muscular atrophy (SMA), or impaired endosomal sorting, as identified in amyotrophic lateral sclerosis (ALS) and some forms of Charcot-Marie Tooth disease (CMT), could reduce virus uptake into peripheral nerve terminals. Defects in Golgi processing and nuclear import may also decrease viral transduction (not depicted). **(C)** A variety of impairments affecting axonal transport machinery (e.g., microtubule dysfunction) are known to cause defects in cargo trafficking (e.g., slowed transport or reduced quantity/flux), which will limit viral delivery.

off-target expression, and hence further enhance therapeutic potential (Parr-Brownlie et al., 2015).

Several different methods have been pioneered that can enhance peripheral neuron transduction upon intramuscular virus administration. As may be expected, these techniques focus on enhancing virus uptake rather than other processes essential to transduction. For instance, a complementary viral strategy can be used to boost the expression of the virus receptor(s) at peripheral nerve terminals that can then be therapeutically targeted with a different virus, as has been demonstrated with AAV-mediated CAR expression for increased AdV binding and uptake (Larochelle et al., 2010; Li et al., 2018b). Receptor expression may also be selectively increased by genetic overexpression (Nalbantoglu et al., 2001) or administration of drugs that enhance transcription, albeit non-specifically (e.g., histone deacetylase inhibitors; Larochelle et al., 2010). Similarly, genetic screens are beginning to identify a variety of viral restriction factors (i.e., proteins that constrain uptake and transduction), which could also be genetically or chemically manipulated, perhaps in a tissue-

specific fashion, to aid uptake (Mano et al., 2015; Madigan et al., 2019). Alternatively, approaches are being developed in which recombinant viral receptor proteins are conjugated to biomaterials and pre-loaded with gene therapy viruses before injection. Indeed, intramuscular administration of recombinant cysteine-tagged AAVR chemically linked to polyester microspheres and pre-incubated with AAV resulted in local and prolonged gene delivery with reduced spread compared to AAV alone (Kim et al., 2019). However, it remains to be seen whether this system can be adapted to increase uptake into peripheral nerve terminals, which would require the release of AAV from the receptor microspheres. Similarly, viral capsids can be chemically modified with a variety of different substances that may aid peripheral nerve binding (e.g., conjugation with neuron-specific homing peptides; Terashima et al., 2009), or antibodies against key neuronal receptor proteins (e.g., $p75^{NTR}$ and CAR; Hedley et al., 2006; Eleftheriadou et al., 2014). Furthermore, motor neuron transduction efficiency upon intramuscular administration of AdV was shown to be enhanced by pre-treatment with flaccid paralysis-causing botulinum toxin type A (BoNT/A; Millecamps et al., 2002). Likely mediated by enhanced motor terminal sprouting, this enhancement was even greater in the $SOD1^{G93A}$ ALS mouse (Millecamps et al., 2001, 2002).

Unfortunately, many of these strategies are not currently a clinical possibility, for obvious reasons. Nonetheless, their implementation in the laboratory to deliver genes within the therapeutic range, along with the development of novel and improved tools to assess virus transduction and treatment efficacy (Han et al., 2019; Chen et al., 2020; Sleigh et al., 2020b; Surana et al., 2020; Ueda et al., 2020), will undoubtedly lead to improved understanding of disease mechanisms and assessment of potential gene therapy strategies.

CONCLUSION

Gene therapy injected into the skeletal muscle for delivery to neurons holds therapeutic promise for peripheral nerve disorders. Motor and sensory nerve terminals located within muscles can act as therapeutic conduits not only for the innervating neurons (**Figure 1**) but also neighboring nerve and glial cells *via* paracrine mechanisms. Moreover, some viruses can escape from the initially transduced neurons, resulting in widespread gene delivery throughout the spinal cord, brain stem, and sensory ganglia. Importantly, this indicates that not all muscles need to be injected to obtain broad cellular dosing. Unfortunately, neuropathology is likely to hinder the effectiveness of intramuscular gene therapy delivery (**Figure 2**); but innovative pre-clinical methods are being developed that will enhance peripheral neuron transduction *via* this method. Also, the intramuscular administration could be combined with, for example, intrathecal delivery to further enhance CNS uptake. However, due to the immune response, repeated successful dosing is unlikely, and hence such treatments need to be given within a short time frame to circumvent this impediment. Nevertheless, by factoring in a detailed understanding of the dynamics of viruses and host cell receptors, especially in the context of peripheral nerve biology and neuromuscular pathology, perhaps this minimally invasive delivery method can contribute to successful gene therapy in the future.

AUTHOR CONTRIBUTIONS

AT and JS wrote the manuscript and have approved the submission of this work.

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frontiers in Molecular Neuroscience

REVIEW
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Gene-Editing Technologies Paired With Viral Vectors for Translational Research Into Neurodegenerative Diseases

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Diseases of the central nervous system (CNS) have historically been among the most difficult to treat using conventional pharmacological approaches. This is due to a confluence of factors, including the limited regenerative capacity and overall complexity of the brain, problems associated with repeated drug administration, and difficulties delivering drugs across the blood-brain barrier (BBB). Viral-mediated gene transfer represents an attractive alternative for the delivery of therapeutic cargo to the nervous system. Crucially, it usually requires only a single injection, whether that be a gene replacement strategy for an inherited disorder or the delivery of a genome- or epigenome-modifying construct for treatment of CNS diseases and disorders. It is thus understandable that considerable effort has been put towards the development of improved vector systems for gene transfer into the CNS. Different viral vectors are of course tailored to their specific applications, but they generally should share several key properties. The ideal viral vector incorporates a high-packaging capacity, efficient gene transfer paired with robust and sustained expression, lack of oncogenicity, toxicity and pathogenicity, and scalable manufacturing for clinical applications. In this review, we will devote attention to viral vectors derived from human immunodeficiency virus type 1 (lentiviral vectors; LVs) and adeno-associated virus (AAVs). The high interest in these viral delivery systems vectors is due to: (i) robust delivery and long-lasting expression; (ii) efficient transduction into postmitotic cells, including the brain; (iii) low immunogenicity and toxicity; and (iv) compatibility with advanced manufacturing techniques. Here, we will outline basic aspects of LV and AAV biology, particularly focusing on approaches and techniques aiming to enhance viral safety. We will also allocate a significant portion of this review to the development and use of LVs and AAVs for delivery into the CNS, with a focus on the genome and epigenome-editing tools based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas 9) and the development of novel strategies for the treatment of neurodegenerative diseases (NDDs).

Keywords: neurodegenarative diseases, AAV vectors, lentiviral (LV) vector, CRISPR-Cas 9 system, gene editing, epigenetics (DNA methylation, histone modifications)

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INTRODUCTION

As of this year, more than seven million Americans suffer from neurodegenerative disorders, with the majority of cases due to Alzheimer's disease. By 2050, this number is projected to rise to nearly 14 million (Alzheimer's Dementia, 2020). In addition to the lost quality of life, these increasingly prevalent conditions impose a major financial burden on our society. Alzheimer's and other dementias will cost the U.S. economy an unbelievable $305 billion in 2020, with projected costs rising as high as $1.1 trillion/year by 2050 (Alzheimer's Dementia, 2020). As such, effective preventive and therapeutic approaches are desperately needed. Unfortunately, current pharmacological treatments provide only temporary symptomatic relief (if that), without addressing the underlying causes.

Virus-mediated gene therapy, on the other hand, is a viable long-term strategy for the disease-modifying treatment of several neurological and neurodegenerative disorders. Traditionally, "gene therapy" has entailed the introduction of an entire gene, which either compensates for a malfunctioning gene or provides a new function to cells which allows them to better combat a disease state. Recently, however, researchers have gained the ability to introduce constructs that can edit the genome—or alter gene expression by modifying the epigenome—with astonishing precision and flexibility. These recent advances are primarily the result of engineering a bacterial defense system called clustered regularly interspaced short palindromic repeats (CRISPR), which we will review in-depth. Indeed, the appealing prospect of treating diseases at the root of their cause has led to considerable efforts toward the development of viral vector systems for delivery into the central nervous system (CNS).

Due to the natural ability of viruses to efficiently transduce cells and tissues with foreign nucleic acid, they have attracted attention as a means of gene delivery since the 1980s (Friedmann, 1976). Viral vectors are engineered such that their wild type virus' genome is replaced with a transgene of interest. Production of said vectors is normally accomplished by co-transfecting cells with multiple plasmids. One plasmid contains the desired transgene adjacent to the required packaging signals, and the other plasmids encode and thus provide all proteins necessary for vector formation in *trans*. As of 2018, over 3,000 gene therapy clinical trials have been initiated worldwide (with ∼2% targeting neurodegenerative diseases; Ginn et al., 2018), and delivery *via* recombinant retro-, lenti-, or adeno-associated virus is employed in around 35% of these[1]. Simple recombinant retroviral vectors (based on γ-retroviruses) were used in the first gene therapy proof-of-principle study, aiming to correct a severe combined immunodeficiency disorder (SCID) in 1995 (Blaese et al., 1995). Tragically, the retroviral vector used in clinical trials induced severe T-cell leukemia in several children 2–5 years after gene therapy, and one of these children died. The insertion of the retroviral vector cassette in the proximity of a proto-oncogene, which then led to an uncontrollable expression of the gene, was determined to be the cause of leukemia, dramatically highlighting the limitations of γ-retroviral vector-based gene therapy (Kantor et al., 2014b). Furthermore, γ-retroviral vectors are not capable of transducing postmitotic cells, a huge disadvantage when targeting the CNS. Infection of slowly dividing cells is possible but is highly inefficient because these retroviral vectors rely on nuclear membrane disassembly for nuclear transportation (Miller et al., 1990; Lewis and Emerman, 1994). As such, simple retroviral vectors are not good candidates for gene therapy of neurodegenerative diseases.

[1]http://abedia.com/wiley/index.html

LENTIVIRAL VECTORS (LVS): BASIC BIOLOGY

Unlike γ-retroviruses, lentiviruses [a different genera in the retroviridae family, exemplified by human immunodeficiency virus type-1 (HIV-1)] evolved a mechanism that exploits host-protein machinery to achieve efficient nuclear import through the intact nuclear membrane (Lewis and Emerman, 1994). Subsequently, these viruses have been engineered into useful viral vectors, as they are capable of transducing nondividing or terminally differentiated cells (e.g., postmitotic neurons) with high efficiency (reviewed in Kantor et al., 2014a). Since the first publication demonstrating the efficient transduction of lentiviral vectors into post-mitotic neurons *in vivo* (Naldini et al., 1996), thousands of studies have probed the use of HIV-based vectors for gene delivery into the CNS (Azzouz et al., 2002; Bayer et al., 2008; Kantor et al., 2011). HIV-based vectors have been demonstrated to transduce most cell types of the brain, including neuronal stem cells, neurons, astrocytes, and oligodendrocytes (Blömer et al., 1997; Consiglio et al., 2001; Azzouz et al., 2002; Jakobsson et al., 2003). Furthermore, HIV-based vectors are capable of sustaining long-lasting transgene expression in the brain (Bayer et al., 2008; Kantor et al., 2011). This last point is of the utmost importance, as continuous, long-lasting production of the therapeutic gene-of-interest (thus providing permanent steady-state "dosing" after a single administration of virus) is essential for gene therapy applications in the CNS.

As mentioned above, lentiviral vectors (LVs) are derived from the HIV-1. The lentiviral genome occupies ∼10.7 kbs of positive-sense single-stranded RNA (**Figure 1A**), of which two copies are packaged inside a lipid-enriched viral shell that is ∼100 nm in diameter (**Figure 1B**). In recombinant LVs (which lack all the HIV-1 ORFs but retain several critical non-coding elements, detailed below), this results in a packaging capacity of approximately 10 kb. The genome encodes structural and enzymatic genes including *gag* and *pol*, respectively. The *gag* (group-specific antigen) encodes the viral matrix (MA), capsid (CA), and nucleoproteins (NC). The enzymatic machinery of the virus consists of reverse transcriptase (RT), protease (PR), and integrase (IN). The virus uses its envelope for attachment and entry into the host cell. Construction of heterologous envelope proteins for pseudotyping viral particles was one of the major steps in dramatically diversifying the tropism of lentiviral vectors. Furthermore, it greatly enhanced the safety profile of the vector (reviewed in Kantor et al., 2014a). Lentiviral vectors can be pseudotyped with a wide variety of envelope proteins; many of them, including Mokola virus (MV), Ross River virus

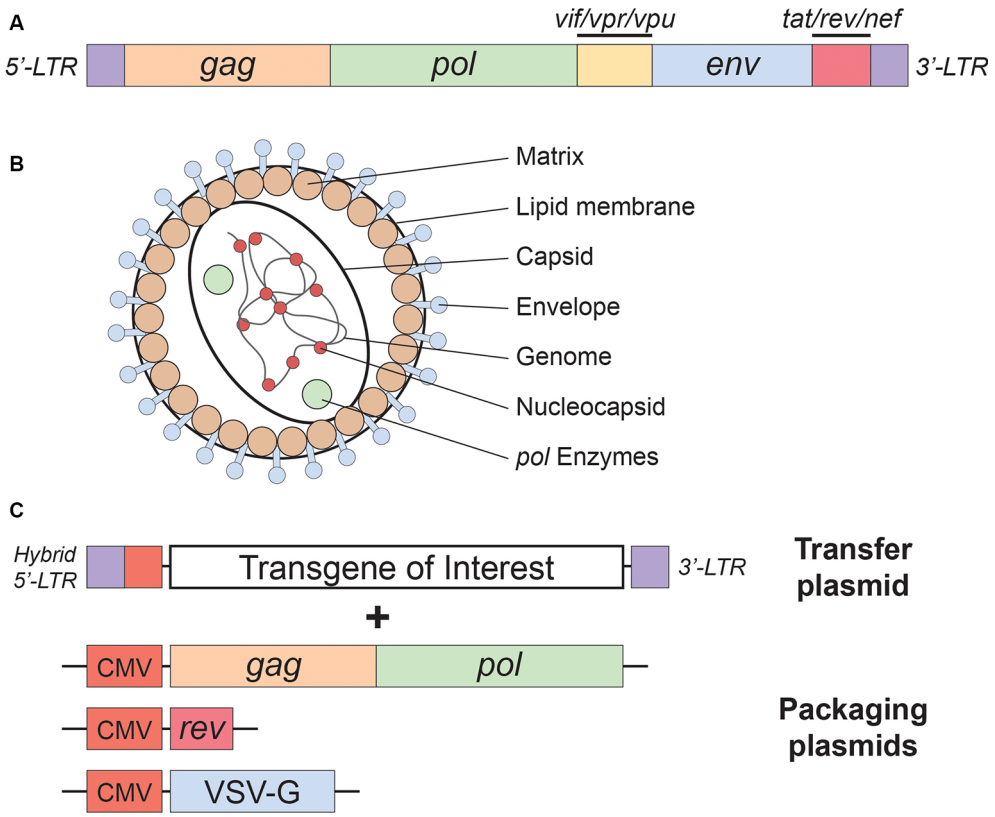

FIGURE 1 | Lentivirus basics. **(A)** Simplified schematic of the wild-type human immunodeficiency virus type-1 (HIV-1) genome. **(B)** Lentivirus particle structure. **(C)** Plasmids used in the current (3rd generation) lentivirus packaging system. See the main text for a detailed description of the lentivirus packaging system; see **Table 1** for lentivirus envelope proteins.

(RRV) and Rabies virus (RV) shown strong neurotropic tropism (**Table 1**; also reviewed in Cronin et al., 2005). However, the most commonly employed envelope is vesicular stomatitis virus protein G (VSV-G), characterized by its extremely broad cellular tropism.

Following the entry into host cells *via* receptor binding and fusion of the viral envelope with the cell membrane, reverse transcription (RT) reaction takes place in the cytoplasm (see **Figure 3**). The RT enzyme mediates a complex reverse transcription process which results in the generation of double-stranded (ds), linear, DNA. For this reaction to take place, the LV genome must include a primer binding site (PBS) and a polypurine tract (PPT). The PBS is responsible for RT initiation, as a $tRNA^{Lys3}$ binds to it and is used as a primer, and it is also critical in the second template exchange that occurs. The PPT contains a purine-rich stretch that survives RNase H—mediated degradation of the positive-stranded RNA, and thus acts as a primer for RT to create positive-stranded DNA (reviewed in Kantor et al., 2014a). The viral DNA corresponds with its genomic RNA but contains a duplicate of the U3 and U5 regions at the 5′LTR (long terminal repeat) and 3′LTR, respectively. The U3 region harbors the promoter sequence, while the U5 region carries the poly-A signal (reviewed in Kantor et al., 2014a). The linear dsDNA is then imported into the nucleus and serves as a precursor for integration. Integrase (IN) protein mediates this process by catalyzing binding and cleaving within the *att* sites located on both ends of the DNA (Colicelli and Goff, 1985; Craigie et al., 1990; Leavitt et al., 1992). Following integration, the viral DNA acts as a part of the host's DNA and is therefore replicated along with it, and passed on to the cell's progeny (Buchow et al., 1989). The RT and PR proteins are essential for LV production; contrarily, the vector can sustain its life-cycle without IN. Unsurprisingly, this fact has

TABLE 1 | Envelope proteins used for pseudotyping lentiviral vectors (LVs).

Envelope	Hosts	CNS Tropism
VSV-G	Mouse, rat, pig, dog, human	Non-selective
Mokola virus	Mouse, rat	Non-selective
Rabies virus	Mouse, rat	Prefers neurons; efficient axonal transport
LCMV	Mouse, rat	Prefers astrocytes; some expression in neurons
RRV	Mouse, rat, human cells *(in vitro)*	Non-selective

VSV-G, vesicular stomatitis virus G-protein; LCMV, lymphocytic choriomeningitis virus; RRV, Ross River Virus. Adapted from Kantor et al. (2014a).

been exploited and lead to the formation of integrase-deficient lentiviral vectors (IDLVs), which provide some significant advantages over the conventional integrase-competent lentiviral vectors (ICLVs), a topic that will be discussed later in this review.

In addition to the core proteins, *gag* and *pol*, lentiviruses such as HIV-1 harbor six additional genes: two regulatory (*rev* and *tat*), and four accessory genes (*nef*, *vif*, *vpr*, and *vpu*), involved in the viral entry, replication, and particle release (Coffin et al., 1997). The accessory products can be deleted from the packaging cassette as they are not necessary for LV production. Their exclusion not only enhances the safety of the vector but also creates a space for the insertion of transgenic sequences (Naldini et al., 1996; Blömer et al., 1997; Kafri et al., 1997; Dull et al., 1998). This realization led to the construction of second-generation packaging cassettes that harbor only the *tat* and the *rev* genes (Zufferey et al., 1997). The *tat* gene encodes a trans-activator of transcription *(Tat)* protein responsible for enhancing HIV-1 expression. The replacement of the endogenous HIV-1 promoter in the U3 region of the 5′LTR with a strong promoter, such as Rous sarcoma virus (RSV) or cytomegalovirus (CMV), creates independence of the virus from *tat*. Still, some of the second-generation packaging plasmids continue to harbor *tat*, as it seems to have a positive effect on the viral production titer. However, *tat* is excluded in third-generation packaging systems (**Figure 1C**), which are also characterized by the separation of the *gag/pol* and *rev* sequences into two different cassettes, and are the safest LVs to date (Dull et al., 1998). In contrast to *tat*, the *rev* gene is indispensable, as its protein product is responsible for exporting full-length and partially spliced RNAs from the nucleus to the cytoplasm (Cockrell et al., 2006). Another improvement present in the current (third-generation) packaging systems is the replacement of the virus' weak polyadenylation signal (poly-A) for either SV40 or bovine/human growth hormone (bGH/hGH), which potentiate mRNA stability (Dull et al., 1998; Cockrell et al., 2006). Also, the incorporation of a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a central polypurine tract (cPPT) into the expression cassette further up-regulated RNA stability, transcription efficiency, and viral titer (Zufferey et al., 1999; Zennou et al., 2000). Importantly, the above modifications neither reduced vector yield nor hampered the ability of LVs to transduce nondividing cells, such as terminally differentiated neurons (Dull et al., 1998; Zufferey et al., 1999; Zennou et al., 2000; Cockrell et al., 2006). Together, they significantly reduced the likelihood of generating recombination-competent retroviruses (RCR), thus contributing to the vector's superb safety.

Non-integrating Lentiviral Vectors

Despite the aforementioned advances in vector safety, employment of retroviral vectors in clinical trials is hampered by a relatively high risk of insertional mutagenesis (reviewed in Kantor et al., 2014a,b). It is important to note that the likelihood of insertional mutagenesis is considered to be lower in lentiviral vectors compared to their γ-retroviral vector counterparts. For example, in the tumor-susceptible mouse model, transplantation of γ-retroviral vector-transduced hematopoietic cells resulted in an accelerated tumorigenic process, whereas no additional adverse events were detected with lentiviral vectors (Montini et al., 2006). Moreover, it has been shown that a higher quantity of lentiviral vectors is necessary to cause an oncogenic risk similar to that of γ-retroviral vectors (Montini et al., 2009). Nevertheless, lentiviral vectors are not completely detached from this problem. An Equine infectious anemia virus-derived vector has been reported to be associated with the formation of tumors in the livers of mice following *in utero* and neonatal vector administration (Themis et al., 2005).

To avert insertional mutagenesis, integrase-deficient lentiviral vectors (IDLVs) have been developed (see **Figure 3**). The IDLVs can be generated by introducing non-pleiotropic mutations within the open reading frame (ORF) of the *int* ORF (Engelman et al., 1995). Such mutations have been shown to specifically target the integration process without significantly affecting other steps of the LV life cycle (**Figure 3** and **Table 3**). We previously reported that IDLV genomes are indeed capable of being expressed *in vitro* and *in vivo*, however, they do demonstrate lower expression levels compared to ICLVs (Bayer et al., 2008; Kantor et al., 2009). Still, these reduced expression levels are often sufficient for correcting genetic disorders in animals (Philippe et al., 2006; Yáñez-Muñoz et al., 2006). We demonstrated that the reduced level of IDLV expression is attributed to the formation of a repressive chromatin structure around the episomal DNA (Kantor et al., 2009). Furthermore, we showed that the reduced expression of IDLVs can be corrected by removing repressive factors such as histone deacetylases (HDACs) either *via in-cis* or *in-trans* methods. For example, we demonstrated that the deletion of negative transcription elements (NTE) located within U3-region of the 3′LTR resulted in significant activation of IDLV expression in both *in vitro* and *in vivo* experiments (Philippe et al., 2006; Yáñez-Muñoz et al., 2006; Kantor et al., 2011). More recently, we showed that the addition of the transcriptional enhancers, such as Sp1 within the viral expression cassette can further stimulate packaging efficiency and transgene expression *in vitro* and *in vivo* (Ortinski et al., 2017). Here, we carefully analyzed the levels and duration of transgene expression, the integration rate, and the overall therapeutic potential of IDLV vectors in comparison to their integrase-competent counterparts (Bayer et al., 2008; Kantor et al., 2011; Saida et al., 2014). Importantly, IDLV-mediated proviral integration into host's cell chromosomes occurred in approximately 1/3850 HeLa cells and approximately 1/111 mouse cerebellar neurons *in vivo* (Bayer et al., 2008; Kantor et al., 2011); that is ∼500-fold lower than the integration rate of ICLV. To examine the therapeutic potential, IDLVs and ICLVs carrying therapeutic cargo encoding an enhancer of the ubiquitin-proteasome pathway were injected into the cerebellum of spinocerebellar ataxia type 3 model mice (SCA3 mice). Remarkably, IDLV-injected SCA3 mice showed significantly improved rotarod performance even 1-year post-injection (Saida et al., 2014). Furthermore, immunohistochemistry at 1-year post-injection showed a dramatic reduction of mutant aggregates in Purkinje cells of both IDLV- and ICLV-injected SCA3 mice. Many other laboratories have also demonstrated efficient use of IDLVs for the transduction of most cell types in the brain

(Saida et al., 2014; Lu-Nguyen et al., 2016; Ortinski et al., 2017). More recently, we established and optimized IDLV vectors as a means for safe and efficient delivery of CRISPR/Cas9 components (Ortinski et al., 2017; Vijayraghavan and Kantor, 2017). Importantly, we reported that IDLV vectors are capable of attaining a strong and sustained CRISPR/Cas9 expression in dissociated post-mitotic neurons and in the rat brain in post-mitotic neurons *in vitro* and *in vivo*. Furthermore, we demonstrated that IDLV-CRISPR/Cas9 vectors are significantly less prone to induce off-target DNA perturbations, and as such are more specific and safe comparing with their integrase-competent counterparts (Ortinski et al., 2017). These studies altogether suggest that IDLVs may provide an effective and safe means of delivery of therapeutic transgenes into the CNS.

ADENO-ASSOCIATED VIRAL VECTORS (AAV VECTORS): BASIC BIOLOGY

Adeno-associated viral vectors are the most frequently utilized platforms for the delivery of therapeutic genes (reviewed in Kantor et al., 2014a). These recombinant AAV (rAAV) vectors were engineered from the wild type virus, which belongs to the *Dependovirus* genus of the Parvoviridae family. As indicated in the genus name, the virus depends on coinfection of another virus (adenovirus or HSV) for replication in host cells (reviewed in Lentz et al., 2012). The packaging-competent form of the AAV genome is represented by a 4.7 kb ssDNA (**Figure 2A**). The genome itself appears quite simple: two ORFs, *rep* and *cap*, flanked by a pair of 145 bp inverted terminal repeats (ITRs; Lusby et al., 1980; Srivastava et al., 1983; Sonntag et al., 2010). However, the wild-type AAV genome encodes eight proteins in total. The *rep* ORF encodes four isoforms of the Rep protein (each combination of two promotors and two splice variants). The long isoforms (Rep78/68; named for their molecular weight) are responsible for replication and integration of the viral genome, and the short Rep52/40 isoforms mediate genome packaging. The *cap* ORF encodes the structural capsid proteins VP1, VP2, and VP3. Through a combination of transcriptional and translational mechanisms beyond the scope of this review, VP1/VP2/VP3 are produced at a ratio of about 1:1:10, respectively (Kronenberg et al., 2001); Sixty copies of VP1/2/3 in the same ratio make up each icosahedral AAV particle. Lastly, assembly activating protein (AAP) is encoded by a cryptic, out-of-frame ORF contained within *cap*; AAP is involved in trafficking capsid proteins to the nucleolus (the site of virion assembly) and is also instrumental in the capsid assembly process (reviewed in Smith, 2008).

During infection, AAV enters cells through receptor-mediated endocytosis, which occurs *via* clathrin-coated pits (Bartlett et al., 2000). As AAV encodes no envelope protein, the viral capsid determines the tissue specificity or tropism. Once inside the cell, the virus escapes from the early endosome and translocates into the host's nucleus where virion uncoating is completed (**Figure 3**). The hairpin endings of the ssDNA genome are then recognized by a host DNA polymerase and are subsequently filled in to create dsDNA (Ferrari et al., 1996). At this stage, WT AAV is capable to efficiently and site-specifically integrate (onto chromosome 19 in humans) into the host cell genome (Deyle and Russell, 2009). The integrated form can be released from the host's genome following coinfection with a helper virus (Adenovirus or HSV-1) or cellular stress, which leads to a lytic cycle where AAV transcription and DNA replication are reactivated to produce AAV viral particles (Kotin et al., 1990; Samulski et al., 1991). In the absence of a helper virus, wild-type AAV DNA can also be retained in the nucleus in linear and circular episomal forms (Duan et al., 1998; Schnepp et al., 2005).

AAV is an ideal virus to modify into a delivery vector for several reasons. Most importantly, the virus has no known associated pathologies and causes a mild immune response in humans. Second, the AAV genome can be preserved for extended periods in episomal forms, and thus presents an opportunity for prolonged transgene expression. Furthermore, AAVs are common in nature, and as such many serotypes exist, with varied tropisms (**Table 2**). Lastly, the AAV genome is well-understood, so the consequences of genetic manipulations can reasonably be predicted. For these reasons, over the last 30 years, a substantial effort has been devoted to transforming AAV into one of the gold-standard platforms for gene therapy. In this time, several major milestones have been achieved towards creating a safe and efficient rAAV toolkit. First, it was found that the stem-loop-forming inverted terminal repeats (ITRs) are the only cis-acting elements required for both genome replication and packaging of the genome into virions (Lusby et al., 1980; Nash et al., 2008). Unsurprisingly, this led to the creation of a packaging plasmid which provides the *rep* and *cap* genes *in trans*. Thus, in recombinant AAVs, nearly the entire genome is replaced with a transgene of interest, yielding a functional packaging capacity quite close to the 4.7 kb WT genome size. Furthermore, the split of these genes from the vector plasmid is critical to prevent the formation of WT AAV during rAAV production (reviewed in Kantor et al., 2014a). As the necessary rep gene is no longer packaged, this separation of the viral cassette also causes rAAV to lose the site-specificity of its integration into human chromosome 19. Instead, rAAVs appear to integrate randomly at a low rate (integration occurs in 0.1–1% of cells), with the vast majority of DNA being maintained as episomes (reviewed in Kantor et al., 2014a). Second, the helper function needed for AAV replication and viral production was initially provided by co-infecting the production cells with Adenovirus or HSV-1. However, this method results in the contamination of rAAV preparations with Adenovirus or HSV particles. To solve this problem, researchers constructed a separate cassette carrying only the essential adenovirus helper genes: E1a, E1b, E2a, E4orf6, and viral-associated RNA genes (Xiao et al., 1998). Importantly, HEK293T cells, which are commonly used for rAAV production, already express E1a and E1b; as such, these genes have been excluded from the helper cassette (Xiao et al., 1998). The optimized rAAV production protocol (**Figure 2B**) thus utilizes three plasmids transiently transfected into HEK293T producer cells: the vector plasmid with the transgene-of-interest flanked by AAV ITRs, the packaging plasmid containing the *rep* and *cap*

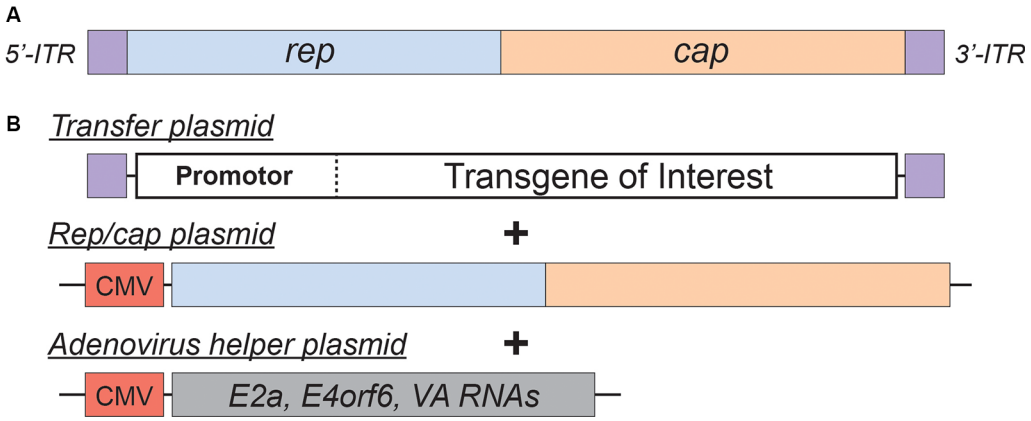

FIGURE 2 | Adeno-Associated Virus (AAV) basics. **(A)** Simplified schematic of the wild-type AAV genome. **(B)** Plasmids used in the current AAV packaging system. See the main text for a detailed description of the AAV packaging system; see **Table 2** for a comparison of common AAV serotypes.

genes from a specific AAV serotype, and the adenovirus helper plasmid (Xiao et al., 1998). These revolutionary advancements have enabled large-scale production of pure rAAV with low immunogenicity, which can be used for a variety of gene transfer applications, including human gene therapy.

More recently, researchers have developed second-generation rAAV vectors with modified capsids that enhance tissue selectivity as well as evading neutralizing host antibodies. An understanding of the biology of naturally occurring serotypes allowed scientists to create hybrids and then engineer these new vector capsids. AAVs use specific regions of their capsid proteins to bind to receptors on the host's cellular membrane; a virus's serotype is determined by the particular amino acid residues that make up these hypervariable loop regions. These variations affect which receptors the capsid proteins bind to, and thus different serotypes confer different tropisms. Furthermore, it has been demonstrated that serotype plays an essential role in viral trafficking from the host's cell membrane to the nucleus as well as in the virion uncoating process, which may in turn control the efficiency of transduction and expression (Keiser et al., 2011).

Over 100 AAV serotypes and variants have been described so far, with the most studied and utilized being AAV2 (Summerford and Samulski, 1998; Summerford et al., 1999; Gao et al., 2005; Wu et al., 2006), and reviewed in Mitchell et al. (2010). However, researchers have also contributed significantly to this remarkable variety by creating pseudotyped viral variants. AAV pseudotypes are usually created by altering the packaging plasmid such that it carries *cap* from the serotype-of-interest along with *rep* from AAV2 while keeping AAV2 ITRs in the transgene-carrying plasmid. The resulting viruses are denoted using a slash: for example, AAV2/5 indicates a virus containing the genome of serotype 2 packaged in the capsid from serotype 5 (reviewed in Mitchell et al., 2010). AAV2/5 in particular demonstrates improved affinity for neuronal cells that are not efficiently targeted by AAV2/2 and is distributed more widely in the brain, allowing for greater transduction efficiency (see below). Another method to expand AAV tropism is to create hybrid capsids derived from multiple serotypes (reviewed in Castle et al., 2016). Multiple groups have further engineered these second-generation AAV vectors using both rational design-based and directed evolution-based approaches (reviewed in Gray et al., 2010). Together, these newly engineered AAV vectors offer a broad range of tropisms to meet a variety of experimental and therapeutic needs.

Due to the advances described above, AAV is the platform of choice for viral gene delivery into the CNS (**Tables 2**, **3**; also reviewed in Gray et al., 2013). The following serotypes have been effectively used in the CNS: AAV2/1, AAV2/5, AAV2/6, AAV2/8, AAV2/9, and the recently engineered PhP.eB (Chan et al., 2017). When injected into the brain, AAV2/1 and AAV2/5 are more efficient than AAV2/2 at transducing both neurons and glial cells, in multiple brain regions of rats and nonhuman primates (Burger et al., 2004; Mandel and Burger, 2004). In contrast AAV2/7, AAV2/8, and AAV2/9 primarily transduce neuronal cells, with AAV2/9 exhibiting the widest spread from the site of injection (Cearley and Wolfe, 2006). Axonal transport varies amongst the AAV serotypes and can be exploited to infect both the directly-targeted cell types as well as the projection field of those cells. For example, when injected into the ventral tegmental area, AAV2/1 and AAV2/9 have shown a high level of spread in both directions along with axonal projections (Cearley and Wolfe, 2006). One of the challenges of targeting the brain is identifying vectors that can cross the blood-brain barrier (BBB) so that, ideally, gene therapy can be administered peripherally. To this end, Foust et al. (2008) and Duque et al. (2009) demonstrated that AAV2/9 administered intravenously crosses the BBB of mice and cats, in both neonatal and adult animals; similarly, Gray et al. (2013) showed that AAV2/8 was able to cross the BBB in mice, although to a lesser extent than AAV2/9 (Hester et al., 2009). Importantly, both neurons and astrocytes were transduced by intravenously injected AAV2/9 vectors, demonstrating that it is possible to deliver gene therapy to a large portion of the brain and spinal cord without

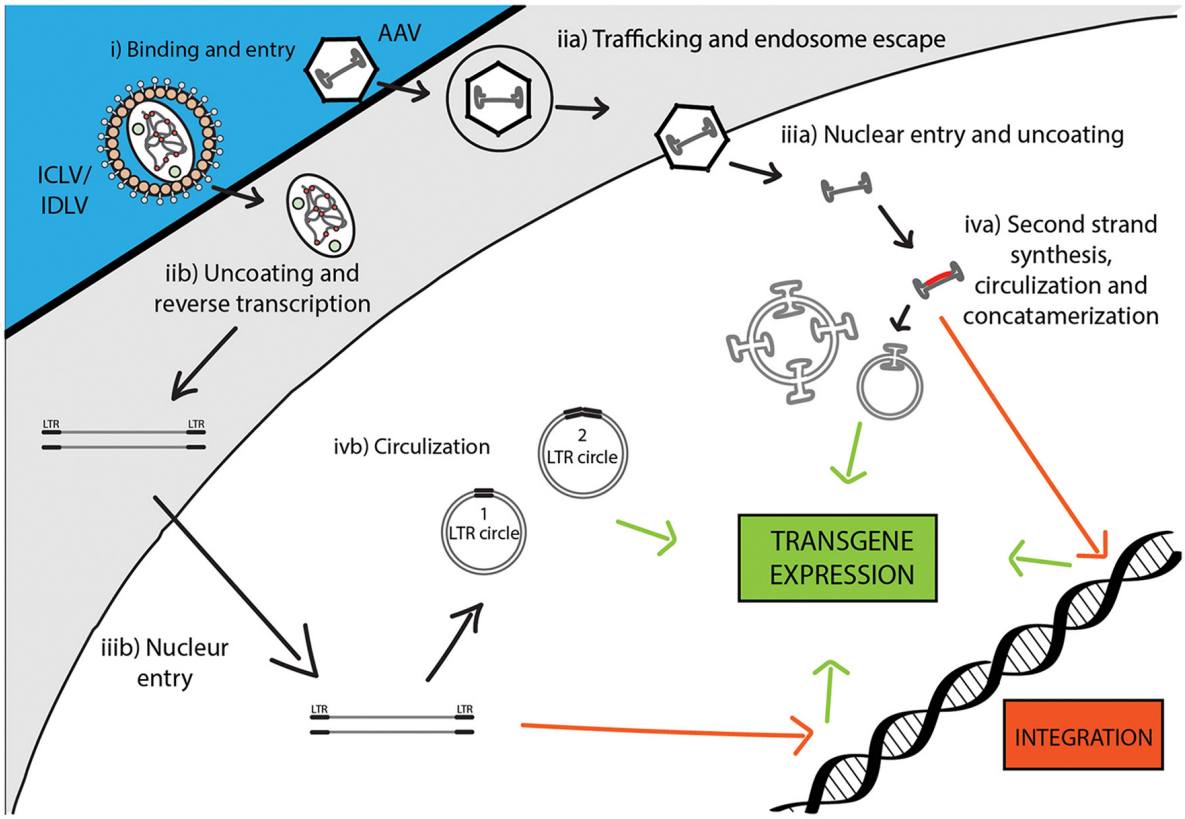

FIGURE 3 | Comparison of recombinant Integrase-Competent Lentivirus (ICLV), Integrase-Deficient Lentivirus (IDLV), and AAV life cycles. ICLV, IDLV, and AAV bind and enter target cells (i). AAV particles escape from endosomes into the cytoplasm (iia), then enter the nucleus and un-coat (iiia). After un-coating, a host polymerase performs second strand synthesis, leading to circularization and concatemerization of the AAV vector; a small percent integrates randomly into the host genome. Transcription occurs from all forms of the AAV transgene (iva). Uncoating and reverse transcription of ICLVs and IDLVs occur in the cytoplasm (iib). The dsDNA product is then imported into the nucleus (iiib). Some of this DNA integrates into the host genome, while the majority recombines into one- or two-LTR circles and remains episomal (ivb). Transcription occurs from all forms of the transgene, but rates of integration and circle formation differ between IDLV and ICLV (see **Table 3**).

having to inject directly into the CNS (Foust et al., 2008; Duque et al., 2009).

In addition to the options provided by simple AAV pseudotyping, a growing array of engineered AAV serotypes are now available, which display a range of useful properties (**Table 2**). These include Olig001 and TM6, which selectively transduce oligodendrocytes and microglia, respectively, when delivered to the CNS. As glial cells are known to play important roles in the neurodegenerative process, the ability to target glia selectively may prove critical for future therapeutic applications. Also notable is rAAV2-retro, a derivative of the AAV2 capsid (*via* directed evolution) which displays robust retrograde transduction across synapses. This is a particularly valuable tool for basic research into brain connectivity. Furthermore, selective delivery to sets of neurons defined by their downstream connectivity may prove to have therapeutic applications. Lastly, the recently engineered PHP.eB serotype consistently exhibits efficient transduction of the CNS *via* systemic delivery in adult animals (Chan et al., 2017). Furthermore, in *in vivo* studies, it has consistently shown higher transduction rates comparing to those of AAV2/9. Indeed, the intravenously injected (IV) PHP.eB-AAV found to be superior to AAV2/9 in both the expression level per cell and the number of transduced cells; its transduction has been reported to be close to 100% in neurons in the cortex and striatum, and over 75% in cerebellar Purkinje cells (Chan et al., 2017). Notwithstanding the enhanced CNS tropism in mice, AAV-PHPeB failed to efficiently transduce the CNS in nonhuman primates following intravenous infusion. Further investigation will be required to determine if the efficient transduction of AAV-PHPeB extends beyond the model in which it was originally tested (Hordeaux et al., 2018). Furthermore, the extent of pre-existing immunity towards this serotype shall be determined; as the presence of anti-AAV2/9 vector neutralizing antibodies (closely relating to PHPeB) in the human population presents a significant challenge for any AAV2/9-based gene therapy. One strategy for circumventing this potential problem would be to use alternate routes of administration. For example, delivery into CSF *via* intrathecal injection has been tested as an alternative route to IV injections (Federici et al., 2012; Samaranch et al., 2012; Gray et al., 2013). Although more invasive than an IV injection, intra-CSF administration has proven much more efficient for targeting cells in the

TABLE 2 | Common Adeno-Associated Virus (AAV) serotypes.

Serotype	Origin	Tissue Tropism	
		Mouse	***Primate***
AAV1	Human	CNS, retina, liver, heart, muscle, airway, pancreas	CNS, muscle
AAV2	Human	CNS, retina, liver, muscle, kidney	CNS, retina
AAV3	Human	Muscle	Liver
AAV4	Non-human primate	CNS, retina, lung, kidney	Lung
AAV5	Human	CNS, retina, muscle, airway	-
AAV6	Human	Heart, muscle, airway	Airway
AAV7	Rhesus macaque	CNS, retina, liver, muscle	-
AAV8	Rhesus macaque	CNS, retina, liver, heart, muscle, pancreas, kidney	CNS, liver
AAV9	Human	CNS, retina, liver, heart, muscle, lung, pancreas, kidney, testes	CNS, retina, heart
AAVrh10	Rhesus macaque	CNS, retina, liver, heart, muscle, lung, pancreas, kidney	-
AAV-AS	Derived from AAV9	CNS—transduction improved 6–15x vs. AAV9	
AAV-BR1	Derived from AAV2	Brain endothelium	
Olig001	Derived from AAVs1, 2, 6, 8, and 9	Oligodendrocytes	
TM6	Derived from AAV6	Microglia	
AAV-DJ	Derived from AAVs 2, 4, 5, 8, and 9	Liver	
AAV-DJ/8	Derived from AAV-DJ	Liver, CNS	
rAAV2retro	Derived from AAV2	CNS—efficient retrograde transduction	
PHP.B	Derived from AAV9	CNS—transduction improved ∼40x vs. AAV9	
PHP.S	Derived from PHP.B	Peripheral nervous system	
PHP.eB	Derived from PHP.B	CNS—transduction rate further improved vs. PHP.B	

Adapted from Grimm et al. (2008); Lisowski et al. (2015); Choudhury et al. (2016b); Deverman et al. (2016); Körbelin et al. (2016); Powell et al. (2016); Rosario et al. (2016); Tervo et al. (2016) and Chan et al. (2017).

spinal cord. Consistently, many groups have now demonstrated that intra-CSF delivery of AAV2/9 results in widespread transgene expression in large experimental animals (Haurigot and Bosch, 2013). Remarkably, it has been demonstrated that AAV-mediated transgene expression in the brain is long-lasting: more than a year in mouse (Klein et al., 1999), at least 6 years in primates (Rivera et al., 2005), and over 8 years in dogs (Niemeyer et al., 2009). Most importantly, a therapeutic level of expression has been detected 8 years post-transduction in the human brain (Leone et al., 2012). Significantly, clinical-grade AAV vectors have been routinely manufactured at the high titers for CNS delivery using human-suitable protocols. Furthermore, AAV-based treatments for CNS disorders are as of this moment finding their first success in the clinic: Zolgensma, an AAV9-based gene replacement therapy for spinal muscular atrophy was approved by the FDA in 2019. A detailed description of how AAV vectors have been developed into a CNS gene-transfer products can be found in (Kantor et al., 2014a).

OVERVIEW OF CRISPR/CAS9-BASED GENE-EDITING SYSTEMS

The CRISPR and CRISPR-associated protein (Cas) system has recently emerged as a revolutionary genetic tool for genome- and epigenome- editing in the CNS. CRISPR/Cas has already advanced our understanding of complex neurologic diseases by enabling the rapid generation of novel, disease-relevant animal models. Furthermore, as will be discussed comprehensively in this review, CRISPR/Cas-based editing provides us with an unprecedented tool to treat neurodegenerative diseases (NDDs). Here, we will review the development and use of CRISPR-mediated genome engineering.

TABLE 3 | Viral vector comparison.

	ICLV	IDLV	AAV
Insert size	10 kb	10 kb	4.7 kb
Integration rate*	∼30%	∼0.05%	Up to 1%
Risk of insertional mutagenesis	Medium	Low	Low
Cytotoxicity	Low	Low	Low
Pre-existing Ab	Low	Low	High
Immunogenicity	Low	Low	Medium
Neurotropism**	High	High	High
Titer	Medium	Medium	High

**in vitro. **With optimized pseudotype/serotype. ICLV, Integration-competent lentivirus; IDLV, Integration-deficient lentivirus; AAV, Adeno-associated virus. Adapted from McCarty et al. (2004) and Kantor et al. (2011).*

The CRISPR/Cas system offers notable advantages over earlier genome-editing technologies, the two most prevalent of which are zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs are relatively small, and once successfully designed can be highly effective, but targeting a ZFN construct to a specific DNA sequence is a non-trivial, time-consuming process. Targeting of TALENs, meanwhile, is relatively straightforward compared to ZFNs (though not as simple as CRISPR), but the size of an active TALEN construct (a two-protein heterodimer totaling ∼6 kb of coding sequence) often proves extremely challenging for delivery (van Haasteren et al., 2020). Nevertheless, while CRISPR has largely supplanted these technologies, its rapid development (particularly on the delivery front) was undoubtedly aided by previous work using ZFNs and TALENs. In particular, IDLVs have been employed to both map ZFN cleavage sites and deliver ZFN constructs *in vivo* (Yin et al., 2017).

TABLE 4 | Major CRISPR-associated protein 9 (Cas9) isoforms.

Name	Construct size	sgRNA length	PAM sequence*
Naturally occurring			
SpCas9	4.1 kB	20 bp	NGG
StCas9	3.4 kB	20 bp	NNAGAAW
NmCas9	3.2 kB	24 bp	NNNNGATT
SaCas9	3.2 kB	21 bp	NNGRRT
CjCas9	2.9 kB	22 bp	NNNNRYAC
Engineered from SpCas9			
SpCas9	∼4.1 kB	20 bp	NGG**
SpCas9-HF1	∼4.1 kB	20 bp	NGG**
HypaCas9	∼4.1 kB	20 bp	NGG**
evoCas9	∼4.1 kB	20 bp	NGG**
VQR	∼4.1 kB	20 bp	NGAN or NGNG
EQR	∼4.1 kB	20 bp	NGAG
VRER	∼4.1 kB	20 bp	NGCG
Cas9-NG	∼4.1 kB	20 bp	NGN
xCas9	∼4.1 kB	20 bp	NGN or GAW
SpG	∼4.1 kB	20 bp	NGN
SpRY	∼4.1 kB	20 bp	NRN > NYN

*Nucleotide codes: N—any base; W—A or T; R—A or G; Y—C or T. **Fidelity improved vs. WT SpCas9. Table adapted from Kleinstiver et al. (2015); Komor et al. (2018); Slaymaker et al. (2016); Chen et al. (2017); Adli (2018); Casini et al. (2018); Hu et al. (2018); Nishimasu et al. (2018) and Walton et al. (2020).*

In nature, CRISPR/Cas is a prokaryotic acquired-immunity mechanism that evolved to target and destroy the nucleic acid of phages, viruses, archaea, and other invading organisms (Barrangou et al., 2007; Sorek et al., 2008). The CRISPR/Cas system encompasses a variety of components that differ widely in the mechanism of action (reviewed in Makarova and Koonin, 2015; Makarova et al., 2015). The overall diversity of the system is tremendous, consisting of six Cas enzyme types (I–VI), and at least 29 subtypes (Koonin et al., 2017). Despite the complexity of the Cas family, all systems share CRISPR RNA [guide RNA (gRNA) and trans-activating RNA (tracrRNA)]-defined targeting specificity (Deltcheva et al., 2011; Jinek et al., 2012). The most attractive platform for gene-editing applications in humans derives from the class II CRISPR-associated enzyme Cas9, which acts as a single effector protein; in contrast, the class I Cas enzymes operate as multi-subunit protein complexes (reviewed in Shmakov et al., 2017). Herein, only Cas9-based systems will be discussed.

For gene editing applications, the two CRISPR RNAs mentioned above are combined into one small guide RNA (sgRNA), which greatly simplifies delivery. Cas9 itself can only bind to DNA at a specific sequence, known as its protospacer-adjacent motif (PAM). After PAM binding, the double-stranded DNA unwinds, allowing the Cas9-associated sgRNA to hybridize with the exposed DNA strand (the protospacer), assuming they are complimentary. If so, the catalytic domains of Cas9 then cleave both strands of the target DNA. Cas9's unprecedented specificity has been rapidly exploited by scientists to fit a great range of applications, from basic science to translational research and medicine (Hsu et al., 2014). In turn, this early progress has inspired further efforts to develop novel CRISPR/Cas systems and apply them for a range of diseases, including NDDs.

One constraint of Cas9 is its dependency on the aforementioned PAM sequence to bind DNA. For example, the canonical PAM associated with the Cas9 nuclease of Streptococcus pyogenes (SpCas9) is the sequence 5′-NGG-3′ (Anders et al., 2014). Many other Cas9 proteins have been (and continue to be) isolated from other prokaryotes in nature which have different PAMs (**Table 4**). However, the efficiency of these Cas9 proteins varies, and to our knowledge, none have surpassed SpCas9. Thus, to increase coverage of potential target sites, rational engineering and evolution-based approaches have been employed to create new Cas9 variants with altered PAM specificities (**Table 4**). For example, Kleinstiver et al. (2015) used a series of positive selection screens in bacteria to identify mutants of SpCas9. They evolved three variants (VQR, EQR, and VRER) that recognize the novel PAM sequences NGAN/NGNG, NGAG, and NGCG, respectively. Another example is the Cas9 of Francisella novicida, which has been engineered to recognize a non-canonical 5′-YG-3′ PAM (Hirano et al., 2016). Recently, however, a more groundbreaking solution to the PAM specificity problem was reported, again from the Kleinstiver lab. Through a multi-step process of rational design, two significant SpCas variants were engineered: SpG, which is capable of targeting an expanded set of NGN PAMs, and a near-PAMless variant called SpRY (Walton et al., 2020). Collectively, SpG and SpRY enable unconstrained targeting using CRISPR-Cas9 nucleases across nearly the entire genome, with single base-pair precision. Using SpRY, the authors were able to correct mutations associated with human diseases located in previously "un-editable" regions of the genome (Walton et al., 2020).

Another impetus for engineering Cas9 is to increase targeting specificity and minimize off-target effects (Mueller et al., 2018). Several studies have described Cas9 variants evolved to reduce off-target cleavages (Kleinstiver et al., 2016; Slaymaker et al., 2016; Chen et al., 2017; Kulcsár et al., 2017). Alternatively, an improvement in on-target CRISPR/Cas specificity can be achieved by modifying the secondary structure of the gRNA spacer region in such a way that it increases the thermodynamic barrier to gRNA binding at off-target sites (Kocak et al., 2019).

As mentioned above, when co-expressed with CRISPR RNA, active Cas9 endonuclease cuts both strands of the target DNA, introducing a double-stranded break (DSB). Eukaryotes predominantly repair DSBs *via* the error-prone non-homologous end joining (NHEJ) pathway, which leads to the formation of small insertions or deletions (indels) in the target sequences (**Figure 4A**). Alternatively, if a repair template is supplied with homology to the target site, the host's repair machinery activates homology-directed repair (HDR), resulting in error-free replacement of the target DNA (**Figure 4A**). However, HDR is typically characterized by lower efficiency than NHEJ-mediated repair. Furthermore, as it is not active in post-mitotic cells, HDR has a very limited ability to introduce such specific changes in the brain. Also, the DSBs needed to trigger efficient HDR increase the possibility of off-target effects, and even on-target HDR can have negative effects on cells (Haapaniemi et al., 2018; Ihry et al., 2018). This limitation motivated the development of single-base-pair editing and prime-editing technologies to enable precision genome editing in

post-mitotic tissues such as the brain (discussed in detail below and reviewed in Komor et al., 2018; Anzalone et al., 2019).

Lastly, the ability of Cas9 to sequence-specifically bind DNA is of immense value in and of itself, independent of its catalytic activity. Indeed, for many theoretical applications, Cas9 endonuclease activity would be detrimental. To address this, mutations were identified in the RuvC (D10A) and HNH (H840A) nuclease domains which destroy the catalytic activity of Cas9 while maintaining its RNA-guided DNA-targeting capacity (Jinek et al., 2012; Qi et al., 2013). Cas9 is thus transformed from a targeted nuclease to a site-specific DNA recognition module. This exceptional modularity has motivated many groups to repurpose catalytically dead Cas9 (dCas9) for control over gene expression, by tethering dCas9 to a diverse range of transcriptional and epigenetic effectors (see **Figures 4–6**; also reviewed in Thakore et al., 2016).

Base Editing Technology

The most common genetic variants associated with human disease in the CNS are point mutations and functional single-nucleotide polymorphisms (SNPs; Nussbaum, 2018). As such, a gene-editing system with the capability to safely, efficiently, and accurately convert single nucleobases has the potential to completely correct many genes implicated in neurodegenerative disease. The creation of a cytosine base-editor (CBE) in David Liu's lab was the first major advancement towards the development of such tools (**Figure 4B**). Komor et al. (2016) fused catalytically deficient, or "dead," Cas9 (dCas9) with rat APOBEC1, a cytosine deaminase enzyme. The resulting complex catalyzes the conversion of all cytosines (Cs) within a 5–6 nucleotide window to uracils (Us); this window ranges from approximately 12–18 nucleotides upstream of the 5' end of the dCas9's PAM. The uracil is then read as thymine during replication, completing the C-to-T conversion. However, this intermediate formation of uracil can trigger cellular uracil DNA glycosylase to perform base excision repair, reverting the uracil to cytosine and limiting the base editor's ability. To combat this problem, a second tool (base editor 2; BE2), was created. It additionally includes the fusion of a uracil glycosylase inhibitor onto dCas9, blocking base excision repair, and significantly increasing the base editor's efficiency. To further improve BE2, dCas9 was replaced with a Cas9 nickase which cuts only the non-edited strand. Nicking the non-edited strand induces mismatch repair, where the cell preferentially cleaves away the nicked strand and repairs it based on the intact (in this case, edited) strand. This new construct (BE3) was tested in a variety of human cell lines, resulting in the permanent correction of 15–75% of genomic DNA targets. The creation of a single-stranded break did increase the possibility of indel formation from less than 0.1% to

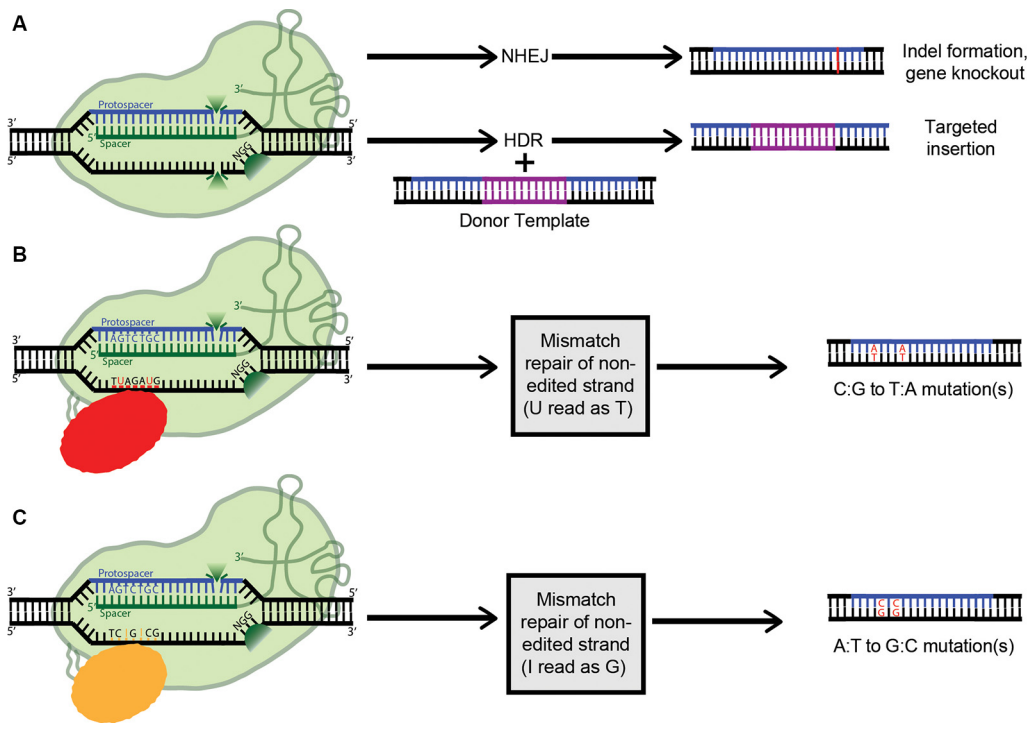

FIGURE 4 | Applications of clustered regularly interspaced short palindromic repeats (CRISPR) technology **(A)** Active Cas9 introduces a double-stranded DNA break, which is repaired *via* non-homologous end joining (NHEJ), creating indels. Alternatively, if a dsDNA donor template is provided, the dsDNA break can be repaired by homologous recombination, resulting in a targeted insertion. **(B)** Cytosine Base Editors catalyze the conversion of all cytosines within a 5–6 nucleotide window to uracils. Uracil is then read as thymine during replication, completing the C:G to T:A conversion. **(C)** Similarly, Adenosine Base Editors (ABEs) catalyze the conversion of all adenosines within a 5–6 nucleotide window to inosines. Inosine is then read as guanine during replication, completing the A:T to G:C conversion.

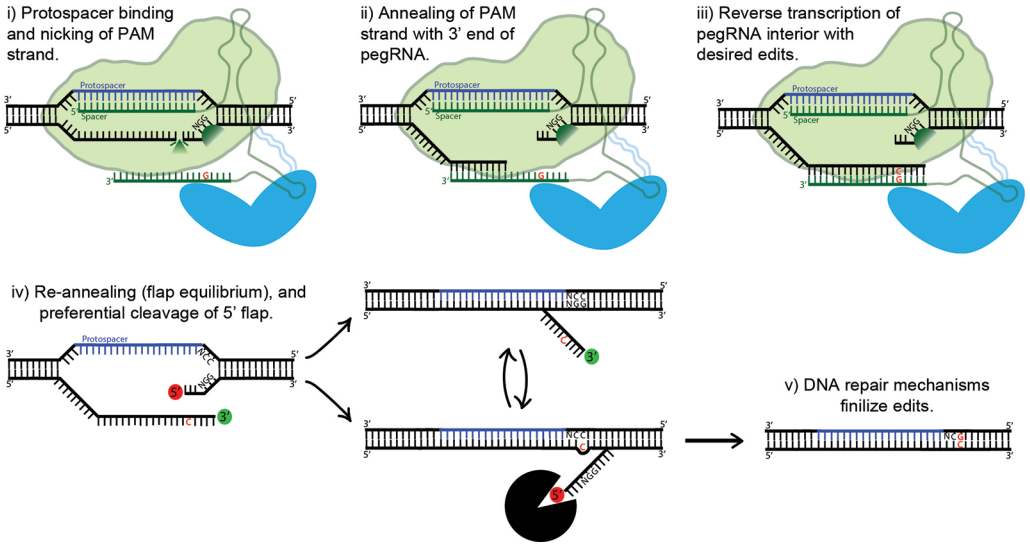

FIGURE 5 | Proposed mechanism of prime editing. First, the 5' end of the pegRNA binds to the protospacer of the target DNA and the protospacer-adjacent motif (PAM) strand is nicked (i). The nicked PAM strand then hybridizes with the primer binding site (PBS) at the far 3' end of the pegRNA (ii). The interior of the pegRNA then serves as a template for reverse transcription, which extends from the free 3'-OH of the PAM strand (iii). The prime editing complex then disengages, leaving the target site with two redundant PAM strands, or "flaps" (iv). The unedited 5' flap is preferentially degraded by cellular endonucleases, allowing the edited 3' flap to hybridize with the non-PAM strand. Finally, DNA repair mechanisms transfer the desired edits to the non-PAM strand (v).

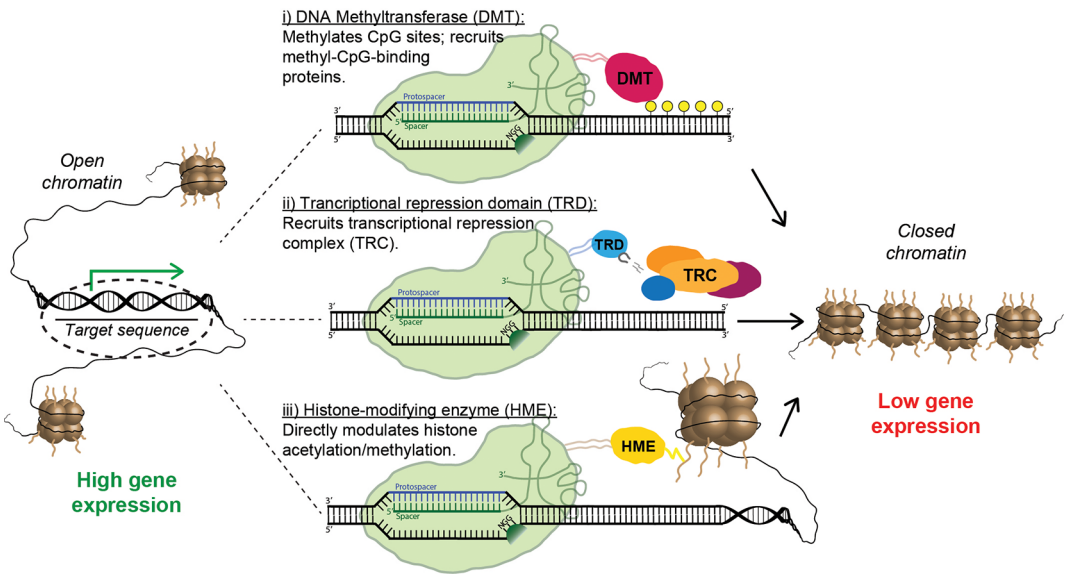

FIGURE 6 | Strategies for epigenetic repression of risk-factor genes using Cas9 fusion proteins. Fusions containing the catalytic domain of a DNA methyltransferase cause targeted methylation of CpG sites and the recruitment of inhibitory methyl-CpG-binding proteins (i). Alternatively, a transcriptional repression domain (TRD) can be fused to Cas9, leading to the direct recruitment of transcriptional repression complexes (ii). Finally, multiple forms of inhibitory histone-modifying enzymes can be fused to Cas9, altering histone acetylation/methylation patterns and causing the formation of closed chromatin (iii).

approximately 1%; however, this is still a remarkably low rate (Komor et al., 2016).

Since then, the base editing system has been further enhanced. A second copy of the uracil glycosylase inhibitor and a bacteriophage protein called Gam was fused to the nCas9. Gam functions by binding to the free ends of DSBs, thus preventing NHEJ-mediated repair and reducing indel formation. These changes resulted in BE4-Gam, which is characterized by higher base editing efficiency and decreased indel frequency (Komor et al., 2017). However, Gam binding may lead to cell death

rather than NHEJ repair, which is unlikely to be appropriate for therapeutic applications. Separately, Koblan et al. (2018) added two Nuclear Localization Signals (NLS) to nCas9 and performed codon-optimization and ancestral sequence reconstruction on APOBEC, yielding BE4max, and ancBE4max. BE4max was then used to efficiently edit two previously challenging to modify disease-relevant SNPs; MPDU1 in human patient-derived fibroblasts and SCN9a intron 6a in mouse neuroblastomas (Koblan et al., 2018). Other researchers have focused on limiting or expanding the cytosine deaminase activity window, and these new constructs allow for C-to-T conversions within a window as short as 3 or as long as 12 nucleotides (Rees and Liu, 2018).

By definition, cytosine base editors catalyze only C-to-T conversions, greatly limiting the range of correctable disease-causing mutations. As such, the creation of an adenosine base editor (ABE), which causes A-to-G conversions, vastly broadens the applicability of base editing (**Figure 4C**). The first ABE was created by Gaudelli et al. (2017) who fused nickase Cas9 with deoxyadenosine deaminase, which catalyzes the conversion of adenosine to inosine. Similarly to the two-step cytosine editing mechanism, the inosine is then read as guanine during replication, completing the A-to-G conversion. Notably, deoxyadenosine deaminase is not a naturally occurring enzyme and had to be forcefully evolved from the adenosine deaminase TadA, which only recognizes RNA substrates (Gaudelli et al., 2017). This multistep artificial selection process resulted in ABE7, which displayed an average editing efficiency of 53% in HEK293T cells, with an indel formation rate of less than 0.1%. However, a major downside of ABE7 in comparison with its CBE counterparts is incompatibility with Cas9 of any origin other than *Streptococcus pyogenes* (SpCas9). This incompatibility is due to the low DNA-bound residence time of non-SpCas9, coupled with the slow enzymatic rate of deoxyadenosine deaminase. To address this problem, Richter et al. (2020) used phage-assisted-continuous evolution (PACE) and phage-assisted non-continuous evolution (PANCE) methods to enhance the catalytic rate of the deoxyadenosine deaminase enzyme 590-fold, creating ABE8e. ABE8e also displays increased processivity, which is especially beneficial for multiplexed approaches. However, the downside to the new system is an expected increase in Cas9-dependent off-target editing. Similarly, using a modified version of the artificial selection system they established during the creation of ABE7, Gaudelli and colleagues created an array of new 8th generation ABEs, which are characterized by increased activity and editing efficiency, and a broader window of editing. It will be interesting to see whether any 8th gen ABEs or ABE8e can outperform ABE7 *in vivo*, and to what extent. ABE7 has already shown success in an adult mouse model of Duchenne muscular dystrophy, able to correct the *DMB* gene in 17% of myofibers, with no indels or off-targets detected. The 17% rate of cells corrected is highly significant, considering only 4% expression is needed to improve muscle function (Ryu et al., 2018).

Prime-Editing Technology

Base editing's profound capabilities are unfortunately limited to C-to-T/G-to-A (CBE) and A-to-G/T-to-C (ABE) base substitutions. This shortcoming inspired David Liu's lab to develop an ingenious approach to gene editing called prime editing (Anzalone et al., 2019). The protein complex is composed of nCas9 fused with an engineered reverse transcriptase. Importantly, the prime editing guide RNA (pegRNA) differs significantly from regular sgRNAs and plays a major role in the system's function. The pegRNA acts as both a guide for the nickase Cas9 domain and a template for the fused reverse transcriptase domain (see **Figure 5**). First, the 5' end of the pegRNA binds to its DNA target, exposing the noncomplementary strand. The unbound DNA of the "PAM strand" (termed as such because it contains the downstream PAM motif) is then nicked. The very 3' end of the pegRNA then acts as a PBS, hybridizing with the recently nicked PAM strand. The exposed 3'-OH group on the nicked PAM strand is then extended by reverse transcriptase, using the interior of the pegRNA as its template. The result is two redundant PAM strands, or "flaps": the edited 3' flap that was just reverse transcribed from the pegRNA and the unedited 5' flap. Which of these two flaps hybridizes with the non-PAM strand is theoretically an equilibrium process; in fact, the unedited 5' flap is thermodynamically favored to hybridize over the edited flap. However, 5' flaps are also preferentially degraded by cellular endonucleases, which are abundant because of their function in lagging strand synthesis. Thus, the 5' flap is usually degraded, and the 3' flap inserted and ligated (see **Figure 5**). The outcome of this step is a DNA heteroduplex with one edited strand and the other nonedited. The introduction of a nick in the nonedited strand can be accomplished by providing a separate (traditional) sgRNA which guides the prime-editing complex to the unedited strand. The edited strand is thus preferentially used as a template for DNA repair. The addition of the sgRNA represents the latest advancement in the prime-editing system, dubbed PE3. Ideally, the sgRNA should be designed such that it matches the edited strand and not the original, forcing unedited strand nicking to only occur post-edit. This ensures that two nicks are never present at one time, greatly reducing indel formation. This optimal use-case (which is not always possible, due to PAM sequence constraints) also confers a new label: PE3b. PE3b has been shown to support targeted insertions of up to 44 bps, deletions of up to 80 bps, and all 12 types of point mutations, without requiring double-strand breaks or a donor DNA template. Its efficiency in HEK293T cells ranges from approximately 20–50% with 1–10% indel formation. Furthermore, PE3b supports simultaneous combinational edits ranging from 3 bps upstream to 29 bps downstream of the Cas9 PAM motif (Anzalone et al., 2019). In sum, the advantages of prime editing over base editing are numerous: no window of activity removes the possibility of "bystander" mutations, there are less stringent PAM requirements due to the varied length of the RT template, and pegRNA has an approximately 4.4-fold lower off-target editing rate vs. sgRNA. The low off-target rate is due to the need for complementation at Cas9 binding, PBS binding, and RT product complementation for flap resolution. That being said, at the moment base editing offers higher efficiency and lower indel formation, and thus should be used over prime editing whenever possible. Prime editing is still in it is infancy, and it is *in vivo* efficacy is yet to be determined.

However, the potential impact on gene editing is enormous, underscored by theoretically being able to correct 89% of known pathogenic mutations and disease-associated genetic variants (Anzalone et al., 2019).

EPIGENETIC REGULATION BY CRISPR/dCas SYSTEMS

First, a note on the definition of the term "epigenetics." In its strictest sense, epigenetics refers to heritable, information-bearing DNA modifications apart from the nucleotide sequence itself (Adli, 2018). The two main types of these epigenetic marks (DNA methylation and histone modifications) will be discussed below. However, we will be using the term "epigenetics" in its more colloquial sense, which more loosely refers to any regulation of gene expression (i.e., transcription) not taking place at the primary sequence level. In the past half-decade, the fusion of catalytically dead Cas9 with various regulatory domains has given researchers unprecedented control over gene expression *in vitro* and *in vivo*, allowing for the therapeutic reprogramming of cell and tissue behavior. Here we will review the current state of dCas9-based epigenetic controllers.

DNA Methylation

The C^5 position of DNA-incorporated cytosine can be methylated by DNA methyltransferase enzymes (DNMTs); in mammals, this modification occurs *only* when the cytosine is part of the specific two-base sequence CpG. Cytosine methylation is highly mutagenic; spontaneous deamination of 5-methylcytosine produces thymine, thus converting the CG dinucleotide to TG. Over evolutionary time, the CpG sites which were constitutively methylated have been eliminated from the human genome by precisely this mechanism. The remaining sites, referred to as "CpG islands," are enriched in the promotor regions of genes, where their methylation causes stable, heritable transcriptional repression (Egger et al., 2004). Furthermore, dysregulation of DNA methylation is the cause of multiple neurodevelopmental disorders, including Fragile X syndrome, in which the expansion of a CGG repeat in the *FMR1* promotor leads to *de novo* DNA methylation and silencing of gene expression (Jin and Warren, 2000), and Rett syndrome, which is caused by mutations in the transcriptional inhibitor methyl-CpG-binding protein 2 (MeCP2), which specifically binds methylated DNA (Amir et al., 1999).

Multiple groups have reported efficient, targeted DNA methylation and gene silencing by fusing dCas9 to the *de novo* DNA methyltransferase enzyme DNMT3A (Liu et al., 2016; McDonald et al., 2016; Vojta et al., 2016). Furthermore, the dCas9-DNMT3A activity can be significantly increased by the additional fusion of the DNMT3A heterodimerization partner DNMT3L (Saunderson et al., 2017; Stepper et al., 2017). The use of DNMT3A has also been combined with the "SunTag" signal amplification system (Huang et al., 2017). In this system, dCas9 is conjugated to a repeating peptide epitope, which then recruits multiple copies of an antibody-effector fusion protein to the desired genomic location. Importantly, Pflueger and colleagues reported that the use of SunTag-DNMT3A resulted in a substantial decrease in off-target DNA methylation compared to a direct dCas-DNMT3A fusion strategy (Pflueger et al., 2018). DNA methyltransferase domains other than DNMT3A have also been fused to dCas9 with similar results, including the prokaryotic DNMT MQ1 (Lei et al., 2017).

Conversely, efficient DNA demethylation has been achieved using dCas9 fusions with the catalytic domain of the methylcytosine dioxygenase TET1 (Choudhury et al., 2016a; Liu et al., 2016). Liu and colleagues evaluated the therapeutic potential of this system by targeting the CGG expansion which causes fragile X syndrome. They found that dCas9-TET1 reduced methylation of the FMR1 promotor and reversed the fragile X-associated loss of the *FMR1* gene product FMRP (Liu et al., 2018). Importantly, the restored expression of FMRP was maintained following the engraftment of *ex vivo* edited cells into mouse brains (Liu et al., 2018). Notably, TET1 has also been employed in conjunction with the SunTag system (Morita et al., 2016).

Histone Modifications

In nature, DNA does not exist as free strands, but is wrapped around nucleosomes—octamers of histone proteins—like "beads on a string"; the other notable epigenetic marks are applied to these histones rather than DNA itself. Lysine residues in the N-terminal tails of DNA-bound histones are subject to two distinct forms of chemical modification: acetylation and methylation. Histone acetylation, which occurs at multiple lysines resides across histones, neutralizes the lysine's positive charge, weakening the association of the nucleosome subunits. Generally, this leads to an increase in DNA accessibility and transcriptional activation (Egger et al., 2004). More complex histone methylation generally occurs upstream of acetylation. In contrast to acetylation, methylation of different lysine residues produces profoundly varied, often opposing effects on transcription. Further complicating the process, lysine residues can be mono-, di-, or tri-methylated, which also lead to different downstream effects. The combination of these two factors (and the presence of less common histone modifications) results in what is termed the "histone code." Briefly, methylation of stimulatory lysines, such as histone 3-lysine 4 (H3K4), causes the recruitment of transcriptional activation complexes, histone acetylation, and an increase in transcription. Conversely, methylation of inhibitory lysines such as H3K9 and H3K27 causes the recruitment of nucleosome-binding proteins, leading to the formation of higher-order chromatin structures and transcriptional silencing. Further complexities of the histone code are beyond the scope of this discussion, but have been excellently reviewed elsewhere (Bannister and Kouzarides, 2011).

CRISPR-based tools have been developed for bidirectional manipulation of both acetylation and methylation. Hilton et al. (2015) showed that a fusion of dCas9 and the catalytic domain of the p300 histone acetyltransferase caused robust, target-specific histone acetylation and gene activation. Conversely, Kwon et al. (2017) showed that a dCas9-histone deacetylase 3 (HDAC3) fusion protein reliably produced target-specific histone deacetylation, although this effect curiously led to

opposing transcriptional effects in two different cells lines. To affect methylation, a variety of histone methyltransferase domains have also been fused to dCas. Interestingly, direct methylation of H3K4 by a dCas-PRDM9 fusion was sufficient to cause reactivation of silenced genes (Cano-Rodriguez et al., 2016), but direct methylation of H3K27 (by one of three methyltransferase fusion constructs) was not sufficient for *de novo* gene silencing (O'Geen et al., 2017). Lastly, Kearns et al. (2015) employed a fusion of dCas9 and the histone demethylase LSD1. They found that dCas9-LSD1 is capable of causing targeted loss of H3K4 methylation, which notably caused gene repression only when targeted to enhancer (but not promotor) regions (Kearns et al., 2015).

Transcriptional Regulators

Remarkably, CRISPR-mediated transcriptional modulation can be achieved while using only catalytically inactive Cas9 and sgRNA. Multiple groups have shown that the mere binding of dCas9 to promoters and other regulatory regions can repress transcription by sterically hindering the RNA polymerase machinery (Gilbert et al., 2013; Larson et al., 2013; Qi et al., 2013); this effect has been dubbed "CRISPR interference" (CRISPRi). Nevertheless, the repressive capacity of the system is vastly improved when dCas9 is linked to a transcriptional repressor domain (TRD). The most commonly used is the Krüppel-associated box (KRAB), a small domain found in ∼400 human zinc-finger transcription factors; recruitment of KRAB is associated with methylation of H3K9 and gene silencing (Huntley et al., 2006). Multiple groups have shown that transcriptional inhibition using a dCas9-KRAB fusion protein is vastly superior to CRISPRi using dCas9 alone (Gilbert et al., 2013; Thakore et al., 2015). Furthermore, Yeo et al. (2018) recently demonstrated that dCas9 fused to a bipartite repressor consisting of KRAB and MeCP2 was even more effective than dCas9-KRAB. Interestingly, a homo-dimerizing dCas9 construct delivered with multiple sgRNAs, which causes the direct formation of artificial DNA loops, also had an inhibitory effect on transcription, presumably by promoting assembly of higher-order chromatin structures (Hao et al., 2017). Unsurprisingly, epigenetic activation can also be achieved using CRISPR-derived tools, most often by directly fusing dCas9 to a transcriptional activation domain such as VP64 (Maeder et al., 2013; Perez-Pinera et al., 2013) or a tripartite activation construct such as VPR or VPH (Chavez et al., 2015; Weltner et al., 2018). However, inhibition of toxic risk-factor genes—rather than stimulating expression of a loss-of-function gene—is the primary strategy for the treatment of NDDs (**Figure 6**). Thus, we will leave further details of CRISPR activation to other capable reviewers (Pickar-Oliver and Gersbach, 2019).

In vivo Applications and Size Constraints

Despite the impressive and rapidly diversifying array of CRISPR/Cas-derived tools, an uncomfortable fact remains. The vast majority of the genome- and epigenome-editing constructs described in the previous sections have only been used *in vitro*. Efficient delivery *in vivo* is a significantly more difficult problem. It must further be noted that all the genome editing tools are relatively large and are currently unable to be packaged into single AAVs. To overcome the significant restraints imposed by AAV's ∼4.7 kb functional packaging capacity, researchers have adopted a clever strategy. A large or multi-component transgene is physically split into two pieces, which are packaged into separate AAV vectors. The resulting AAVs are then co-delivered, and the complete protein is reassembled *in situ* by a split intein—a pair of domains which "splice themselves out," thus joining two peptide chains end-to-end (Chew et al., 2016; Moreno et al., 2018). Integrase-deficient lentiviral vectors are another appealing option, as they are easily capable of packaging either base-editing tool along with the associated sgRNA and all other required/beneficial transcriptional elements. Furthermore, the large packaging capacity of Lentiviruses may prove critical for the delivery of prime-editors, as the complete PE3 system with all the included elements would not even fit in a dual-AAV system. In their original publication describing prime editing, Anzalone et al. (2019) delivered PE3 along with a reporter construct *via* a dual-Lentivirus system, equivalent to the process described with AAV. Incidentally, our lab has expertise efficiently packaging 10 kb inserts (measured LTR-to-LTR) into Lentiviral vectors. With this in mind, the packaging of the PE3 system in all-in-one lentivirus is theoretically possible, even though with likely lower efficiency. Similarly, an LV vector could easily be configured to package PE2, which confers lower efficiency but also lower indels than PE3, all-in-one. Size restrictions are also critical when working with dCas9-effector complexes, although to a more flexible extent. One common solution when using CRISPR in mice is to simply use a transgenic line stably expressing dCas9 fused to a domain from one of several protein-protein or RNA-protein interaction systems. The complementary domain can then be fused to an epigenetic effector of choice and delivered along with the targeting sgRNA, all of which will fit in a single AAV (Liao et al., 2017; Wangensteen et al., 2018; Zhou et al., 2018). It should be noted that a similar method could be used when using the aforementioned genome editing tools in mice. Lastly, the previously discussed split-intein dual-AAV method has also been used to deliver dCas9-based epigenetic modulators in animal models (Chew et al., 2016; Moreno et al., 2018).

Despite its successes, it must be emphasized that a dual-vector delivery platform has significant caveats. Preps have to be made separately and then combined, meaning twice the viral load must be injected for an equivalent effect compared to an all-in-one system. Furthermore, each target cell must be co-transduced by each vector, or the system fails. However, *in vivo* delivery of epigenetic CRISPR tools in a single AAV is tantalizingly close at hand. In a recent study by Chew et al. (2016), *in vivo* delivery of SaCas9 (which is substantially shorter than the more commonly used SpCas9) fused to the KRAB repressor domain (which contains a mere 45 amino acids) required a second AAV only for delivery of the guide RNA (Thakore et al., 2018); for comparison, the longer SpCas9-KRAB construct (along with sgRNA) easily fits in a single lentiviral vector (Zheng et al., 2018). In a parallel effort to create very small epigenetic modulators, some groups have taken inspiration from the CRISPR system but jettisoned the use of CRISPR itself. Remarkably, Rauch et al. (2019) were able to rationally assemble an active, guide

RNA-directed endonuclease out of pre-existing catalytic and RNA-binding domains. This system, dubbed CIRTS, is less than 1/3 the size of SpCas9, and easily able to fit in a single AAV. Although such creative approaches are potentially of great value, CRISPR currently has no competition as the gene-manipulation platform of choice; CIRTS only targets mRNA, and its efficiency pales in comparison to equivalent CRISPR-derived tools. In the coming years, the development of robust CRISPR-based gene editing tools which are capable of being packaged in a single AAV vector will be of the utmost importance. Fortunately, given the amount of scientific talent invested in the advancement of CRISPR/Cas, we do not doubt that single-AAV delivery will soon become commonplace.

OVERVIEW OF CRISPR/CAS SYSTEMS AND THEIR USE FOR THE TREATMENT OF NDDs

NDDs are defined as any disease that causes the progressive deterioration of nerve cells in the central or peripheral nervous system, a category which naturally encompasses a variety of conditions. However, of the ∼7.4 million Americans with an NDD, the vast majority suffer from one of only two: Alzheimer's disease (AD; 5.4 million) and Parkinson's disease (PD; 1.5 million; Pal, 2012). As these conditions will be covered in-depth, we must, unfortunately, omit any detailed discussion of other NDDs, the most prominent being multiple sclerosis, which currently affects approximately 400,000 Americans.

AD is a debilitating neurodegenerative disorder characterized by cognitive decline, the risk for which increases significantly with age (reviewed in Gottschalk et al., 2016; Alzheimer's Dementia, 2020). Phenotypically, AD is characterized by the formation of extracellular plaques of β-amyloid protein (Aβ) and intracellular tangles consisting of the tau protein. To date, the only gene consistently found to be associated with the common, sporadic form of AD (late-onset AD; LOAD) is apolipoprotein E (*APOE*; 2020). APOE was discovered nearly five decades ago (Shore and Shore, 1974), though it took more than two decades to find that APOE has a vital function in the brain (Pitas et al., 1987). Humans have multiple variants of the *APOE* gene (McIntosh et al., 2012), the two most important of which are APOEε3 and APOEε4 (Castellano et al., 2011), while all other animals have only a single APOE isoform (resembling human APOEε3). Only a single amino acid difference exists between APOEε3 (Cys112) and APOEε4 (Arg112). Nevertheless, carrying the APOEε4 variant significantly increases lifetime risk for LOAD, and the presence of two copies is associated with further increased risk (Friedmann, 1976; Alzheimer's Dementia, 2020) and earlier disease onset (Moskvina et al., 2013; Nussbaum, 2013). We and other groups have suggested that alterations in the expression of APOE in general, and the ϵ4 isoforms in particular, maybe an important mechanism in the etiology of LOAD (Gottschalk et al., 2016). Therefore, the development of CRISPR/Cas-based therapies targeting APOE and/or APOEε4 expression would offer a valuable epigenetics-based approach for the treatment of LOAD. Below, we will describe current progress and future efforts towards targeting APOE.

Also, we aim for this review to provide a perspective on the etiopathogenesis of PD, which may provide an alternative avenue of research and treatment for the disease. The presence of alpha-synuclein (α-syn) aggregates defines a spectrum of disorders collectively termed synucleinopathies, of which PD is arguably the most well-characterized. Aggregated α-syn is the primary component of Lewy bodies, the defining pathological feature of PD, and point mutations or multiplications in the *SNCA* gene (which expresses α-syn) result in familial PD. The tight link between α-syn expression and PD has led to the hypothesis that α-syn accumulation may produce toxicity through a gain-of-function mechanism. Indeed, misfolding of α-syn leads to the formation of toxic oligomers and beta-pleated sheets, which are thought to impair the proper function of the mitochondria, proteasome, and lysosome-dependent degradation pathways (Poewe et al., 2017). These contribute to neuronal death, mostly within dopaminergic neurons of the substantia nigra pars compacta. This in turn leads to dopamine deficiency in the striatum, which is responsible for the overt symptoms of PD (Poewe et al., 2017).

As elevated levels of α-syn have been implicated in the pathogenesis of PD, targeting SNCA expression levels is an attractive neuroprotective strategy, and manipulations of SNCA expression have demonstrated beneficial effects (reviewed in Tagliafierro and Chiba-Falek, 2016). Several studies have attempted to reduce the expression of α-syn and rescue PD-related phenotypes by directly targeting SNCA mRNA. Flierl et al. (2014) showed that a lentivirus expressing a short hairpin RNA (shRNA) targeting SNCA was capable of rescuing multiple phenotypic abnormalities in SNCA-Tri (triplicated) human neuroprogenitor cells (NPCs), including viability, growth, energy metabolism, and stress resistance (Flierl et al., 2014). Efficient knockdown of SNCA was also reported in a study utilizing small interfering RNA (siRNA), which was injected directly into the monkey substantia nigra (McCormack et al., 2010). A siRNA-based approach also achieved a significant improvement in motor function in a fly model of PD (Takahashi et al., 2015). Notwithstanding these successes, the RNAi approach bears two significant caveats. First, RNAi can affect the expression of genes other than the intended targets, as shown by whole-genome expression profiling after siRNA transfection (Jackson et al., 2003). Second, RNAi does not support the fine resolution of knockdown severity, where tight regulation is needed to achieve a physiological level of SNCA expression (Tagliafierro and Chiba-Falek, 2016). For example, an AAV-siRNA system targeting SNCA caused significant toxicity and a massive loss of nigrostriatal dopaminergic neurons in rat models, inadvertently showing that a *complete* loss of α-syn can cause neurodegeneration (Gorbatyuk et al., 2010).

These examples demonstrate the need for novel therapeutic strategies targeting the regulatory mechanisms controlling SNCA expression, rather than directly targeting the mRNA or the protein, such that precise regulation of α-synuclein levels can be achieved. To this end, our group recently developed a system, comprising an all-in-one lentivirus, for targeted DNA

methylation (i.e., epigenome editing) within a regulatory region in SNCA intron 1. This system (dCas9 fused with the catalytic domain of DNMT3A methyltransferase, and associated sgRNA), when delivered to hiPSC-derived dopaminergic neurons from PD patients with SNCA triplications, yielded fine-tuned downregulation of SNCA mRNA and protein levels (Kantor et al., 2018). Furthermore, this effect rescued PD-related cellular phenotypes in these cells, including mitochondrial ROS production and cellular viability (Kantor et al., 2018). These results provide a proof-of-concept validation that DNA hypermethylation at SNCA intron 1 is an effective means of SNCA repression, confirming this general approach as a novel epigenetics-based therapeutic strategy for PD.

While most cases of PD and AD are sporadic, a small subset of both AD and PD cases result from single, causative mutations, which are inherited in a classic Mendelian fashion. These familial forms of AD/PD present earlier in life and are generally very severe. Specifically, early-onset AD is caused mostly by mutations in *APP, PSEN1*, and *PSEN2* (Masters et al., 2015). The pathological beta-amyloid peptide discussed above is a cleavage product of *APP*. Mutations in any of these three genes result in increased AB42/AB40 ratios, and the increase in aggregation-prone AB42 leads to early plaque formation and symptom onset (Masters et al., 2015). In addition to the previously mentioned mutations/multiplications in the *SNCA* gene, autosomal-dominant forms of PD are caused by mutations in leucine repeat kinase 2 (*LRRK2*), and autosomal-recessive PD is caused by mutations in parkin, PTEN-induced putative kinase 1 (*PINK1*), and Daisuke-Junko-1 (*DJ-1*, Scott et al., 2017). These and other genes involved in the etiology of PD, including FBX07, ATP13A2, DNAJC1, PLA2G634, SYNJ1, VPS35, eiF4G1, and CHCHD2, are reviewed elsewhere (Scott et al., 2017).

While the devil is always in the details, for these patients the overall therapeutic strategy is obvious: simply correct the causative genetic mutation, using base editing if it is a valid target, or prime editing if not. This strategy is very similar to that which would be appropriate for any other CNS disease caused by a single, correctable genetic mutation. The first proof-of-concept study validating a base-editing approach *in vivo* on post-mitotic sensory cells came from David Liu's lab (Yeh et al., 2018). The authors used base editing to install an S33F mutation in the β-catenin gene, successfully upregulating Wnt signaling (which is involved in mitosis of cochlear supporting cells and cellular reprogramming). In contrast, delivery of nuclease-active Cas9 to install the S33F mutation *via* HDR did not produce a measurable induction of Wnt signaling (Yeh et al., 2018). Two years earlier, the same lab validated the base-editing system *in vitro* by converting APOEε4 into APOEε3 in immortalized mouse astrocytes, in which the endogenous APOE gene was replaced by human APOEε4. In this study, Komor et al. (2016) transfected the CBE system and an appropriate sgRNA placing the target cytosine at position 5 relative to a downstream PAM, resulting in a conversion rate of up to 10%. Indeed, the generation of APOEε3/4 iPSC lines *via* base-pair editing has become a routine task for many labs and is now offered as a service from biotech companies. As an example of this technique, BE4max was used to generate base-edited isogenic hiPSC lines using a transient reporter for editing enrichment (BIG-TREE). Relevantly, the researchers efficiently generated multiple clonal lines bearing different APOE genotypes, with an astonishing 90% of isolated clones being edited (Brookhouser et al., 2020).

Base-editing technology has become available only very recently. Interestingly, an older editing technology—the zinc-finger nuclease (ZFN) system—was recently applied to generate isogenic APOEε3 and ε4 iPSC lines, by Wang and coworkers (Wang et al., 2018). Using human neurons derived from the isogenic iPSCs, they showed that APOEε3-expressing neurons had higher levels of tau phosphorylation, unrelated to their increased production of Aβ peptides. Further, they displayed GABAergic neuron degeneration. Gene editing to APOEε3 rescued these phenotypes, indicating a specific effect of APOEε3. Crucially, the authors also reported that APOE knockout neurons behave similarly to those expressing APOEε3, and that re-introduction of APOEε3 restored the pathological phenotypes associated with AD; these results suggest that APOEε4 has a toxic gain-of-function effect.

As mentioned above, to best of our knowledge, base/prime editing systems have not yet been applied to animal models of familial AD or PD, but recent days have seen groundbreaking results targeting other CNS diseases. For example, Levy et al. (2020) recently applied a dual-AAV9 system to deliver SpCas9-CBE to the brain of a mouse model of Niemann-Pick disease type C. They successfully edited approximately 48% of cortical cells (mixed cell types from unsorted tissue), and 0.3% of cerebellar cells, with minimal indel formation, off-targets, or bystander mutations. The result was an increase in surviving Purkinje neurons and an increase in lifespan of about 10%. Even more recently, Li et al. (2020) used an ABE derived from the very short *Campylobacter jejuni* Cas9 (CjCas9) to correct an oncogene-activating mutation in the *TERT* gene promotor, which occurs in glioblastoma and many other cancer types. Impressively, localized intracranial injection of a pair of AAVs expressing CjCas9-ABE (and its associated sgRNA, respectively) was capable of arresting the growth of *TERT* mutation-driven gliomas (Li et al., 2020). Notably, compared to many "editable" diseases (particularly developmental disorders) treating familial NDDs with base/prime editing would have at least one major advantage. Unlike inherited developmental disorders, the symptoms of familial NDDs present (relatively) late in life. Thus, viral gene therapy could plausibly be administered until adolescence/adulthood, as opposed to requiring delivery during infancy or earlier.

All that being said, the vast majority of both AD and PD cases are not of the early-onset type. Unlike familial cases, the etiologies of late-onset AD/PD are quite complex, being driven by an intricate web of generally low-impact genetic and environmental risk factors. Unfortunately, attempts to identify unifying pathogenic mechanisms based on the genetics of the familial forms have had mixed results, at best. One of the strongest pieces of evidence for the "amyloid hypothesis" of Alzheimer's pathogenesis is the existence of familial AD caused by mutations in APP, PSEN1, and PSEN2. Regardless, several AD therapies based on this hypothesis have recently suffered devastating failures in clinical trials for late-onset AD

(Mullard, 2019). Importantly, many of these therapeutics very effectively cleared beta-amyloid plaques from the brain, yet patients saw no improvements, nor delays in disease progression. If the mechanisms underlying these diseases can be fully elucidated, the potential for prevention or reversal of progression and pathophysiology will increase considerably (Gottschalk et al., 2016; Tagliafierro and Chiba-Falek, 2016; Kampmann, 2017; Lutz et al., 2020).

As far as therapeutic options are concerned, safe, permanent "knockdowns" can be achieved using base editing. For example, a mouse model of ALS was treated by using BE3 to introduce a nonsense mutation in SOD1, resulting in prolonged survival and slowed disease progression, even in adult mice (Lim et al., 2020). However, the use of dCas9-based epigenetic effectors similar to those developed in our laboratory (Tagliafierro and Chiba-Falek, 2016) provides an additional, complementary approach to gene repression.

A major concern over viral vectors is lingering uncertainty over their safety profile. Although progress has been made to reduce the toxicity of viruses by directed evolution and engineering, most viruses that infect cells and deliver genes will inevitably integrate their genetic elements into the host genome. These elements can pose long-term safety risks. Furthermore, viral vectors possess a significant risk associated with their ability to activate deleterious immune responses. Last but not least, a drawback of viruses is that their production is labor-intensive, and clinical applications are expensive because each step of clinical-grade viral vector manufacturing must strictly comply with good manufacturing practices (GMP).

While we focus here on factors most relevant to viral vector design and production, we would be remiss if we did not note other novel delivery technologies being developed in parallel. Recently, Park et al. (2019) used a "traditional CRISPR" strategy (i.e., creation of disruptive indels by nuclease-active Cas9) to successfully alleviate behavioral deficits in two mouse models of familial AD. These constructs were delivered not by a viral vector, but *via* nano complexes composed of a synthetic, amphiphilic peptide (Arg_7-Leu_{10}). This is a highly innovative approach—reminiscent of traditional chemical transfection—which hopefully has therapeutic potential as well.

A few final points are worthy of discussion before concluding. First, future gene therapies for early- and late-onset AD/PD may be quite different. Second, and critically, the small size of many of the epigenetic repressor modules would make packaging into a single all-in-one AAV delivery vector relatively straightforward. Lastly, both AAV-based delivery and the epigenetic strategy itself carry significant advantages. Epigenetic approaches have the benefit of never physically modifying the DNA target, ruling out an entire class of potential off-target effects. And as mentioned above, AAV vectors have an unparalleled safety profile. Indeed, AAVs are the only delivery vehicle approved to administer CRISPR-based therapeutics to humans (Wang et al., 2020). As such, while developing AAV-compatible epigenetic therapies for late-onset AD and PD is sure to be a challenge, it is a worthy one, with the promise of lasting clinical reward.

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JR and MM wrote the review, with guidance and edits from OC-F and BK.

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Gene Therapy Vector Encoding Neuropeptide Y and Its Receptor Y2 for Future Treatment of Epilepsy: Preclinical Data in Rats

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Gene therapy to treat pharmacoresistant temporal lobe epilepsy in humans is now being developed using an AAV vector (CG01) that encodes the combination of neuropeptide Y and its antiepileptic receptor Y2. With this in mind, the present study aimed to provide important preclinical data on the effects of CG01 on the duration of transgene expression, cellular tropism, and potential side effects on body weight and cognitive function. The CG01 vector was administered unilaterally into the dorsal and ventral hippocampus of adult male rats and expression of both transgenes was found to remain elevated without a sign of decline at 6 months post-injection. CG01 appeared to mediate expression selectively in hippocampal neurons, without expression in astrocytes or oligodendrocytes. No effects were seen on body weight as well as on short- or long-term memory as revealed by testing in the Y-maze or Morris water maze tests. Thus these data show that unilateral CG01 vector treatment as future gene therapy in pharmacoresistant temporal lobe epilepsy patients should result in stable and long-term expression predominantly in neurons and be well tolerated without side effects on body weight and cognitive function.

Keywords: NPY, Y2, learning and memory, AAV viral vector, hippocampus, gene therapy

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INTRODUCTION

Epilepsy is the fourth most common disorder of the central nervous system, affecting up to 1% of the world population (Fiest et al., 2017). Since up to 1/3 of epilepsy patients remain resistant to currently available anti-epileptic therapeutics (Picot et al., 2008; Brodie et al., 2012), there is a great unmet need to explore novel treatment avenues. In recent years, gene therapy with viral vectors has emerged as an attractive alternative treatment strategy, particularly for focal epilepsies that also account for the greatest proportion of epilepsies (Wykes and Lignani, 2018). Several different targets have been suggested for therapeutic gene regulation, including neuropeptides [e.g., neuropeptide Y (NPY), galanin, dynorphin], potassium ion channels, and designer receptors exclusively activated by designer drugs (DREADDs; Wykes et al., 2012; Simonato, 2014; Agostinho et al., 2019; Weston et al., 2019). The most frequent type of pharmacoresistant epilepsy is mesial temporal lobe epilepsy

(mTLE) with hippocampal sclerosis (Blümcke et al., 2012). Many studies have established the seizure-suppressant effects of NPY against seizures in the hippocampus both in rodents (Woldbye et al., 1996, 1997, 2005; Vezzani et al., 1999; Klemp and Woldbye, 2001) and hippocampal slices from pharmacoresistant human epilepsy patients (Patrylo et al., 1999; Ledri et al., 2015; Wickham et al., 2019). NPY elicits its biological actions in the brain mainly by binding to Y1, Y2, and Y5 receptors, members of a G-protein coupled receptor superfamily (Berglund et al., 2003). In the hippocampus, the seizure-suppressant effects of NPY appear to be mediated primarily *via* activation of Y2 receptors (El Bahh et al., 2005) while Y5 receptors may also play a role particularly outside the hippocampus (Woldbye et al., 1997, 2005; Marsh et al., 1999). In contrast, Y1 receptors appear to act in an opposite manner (Benmaamar et al., 2003; Lin et al., 2006; Olesen et al., 2012).

Using adeno-associated viral (AAV) vectors, it has been shown that hippocampal overexpression of NPY (Richichi et al., 2004; Noè et al., 2008; Noe et al., 2010; Gøtzsche et al., 2012) and/or its antiepileptic receptor Y2 (Woldbye et al., 2010; Ledri et al., 2016) has antiepileptic effects *in vivo* in rodents. A similar seizure-suppressant effect has been reported after AAV-mediated overexpression of NPY or the Y2 agonist NPY13–36 in the piriform cortex (Foti et al., 2007). Combined overexpression of NPY and Y2 in the hippocampus exerted a superior seizure-suppressant effect compared to single transgene expression (Woldbye et al., 2010). To test NPY/Y2 combination gene therapy for human patients with mTLE, we recently provided proof-of-concept with a single vector (CG01) that mediates simultaneous hippocampal overexpression of NPY and Y2 using a translational chronic epilepsy model (Melin et al., 2019). In this model, AAV injection was applied successfully into the hippocampal seizure focus *after* spontaneous recurrent seizures were established. In the present study, we further conducted a series of preclinical experiments in rats to provide important knowledge of the expression and potential side effects of CG01 before future clinical testing, including duration of transgene expression, cells types targeted (i.e., cellular tropism), as well as effects on body weight and cognitive function.

MATERIALS AND METHODS

Animals

All procedures were performed following the Danish Animal Experiments Inspectorate and approved by the local Ethical Committee for Laboratory Animal Research. A total of 52 adult male Wistar rats (Charles River; 200–220 g on arrival) were housed in standard plastic cages on a 12 h light/dark cycle with *ad libitum* access to food and water and adapted for 7 days before experiments.

Viral Vectors

Two recombinant serotype-1 AAV vectors kindly provided by CombiGene AB (Lund, Sweden) were used in the study: CG01 which encodes human pre-pro-neuropeptide Y (NPY) and its receptor Y2 (AAV1-CAG promotor-pre-proNPY-IRES-hY2-WPRE-BGHpA) and CG07 which is an empty control vector (AAV1-CAG promotor-EMPTY-WPRE-BGHpA; Melin et al., 2019). Both vectors were driven by a synthetic CAG promoter (chicken beta-actin promoter hybridized with the CMV immediate early enhancer sequence). An internal ribosome entry site (IRES) located between the two transgenes assured translation of both.

AAV Vector Surgery

Two separate experiments were performed. The experimental design is shown in **Figure 1**. In Experiment-1, 14 rats were injected unilaterally in the hippocampus with CG01 vector and subsequently sacrificed at 1, 2, 3, 4, 8, 13, and 26 weeks post-injection (n = 2). Also, two treatment-naïve rats were sacrificed at 1 and 26 weeks, respectively. In Experiment-2, 36 rats were randomly allocated to three groups (n = 12 per group). The rats in the first group were left untreated (naïve), the second and third groups of rats were injected unilaterally in the hippocampus with CG07 and CG01 vectors, respectively. Animals were subjected to two behavior tests starting 3 weeks after the vector administration.

Before the intracerebral injection, rats were weighed and anesthetized using a 4% isoflurane-oxygen mixture. Anesthesia was maintained with a 1–2.5% isoflurane-oxygen mixture. Each rat received 1 μl/g s.c. injection of temgesic (0.3 mg/ml) and, in the scalp region, 1 μl/g s.c. injection of a mixture of lidocaine (10 mg/ml) and mepivacaine (10 mg/ml). Rats were injected in the right hippocampus in two areas using the following coordinates: dorsal hippocampus (AP −3.3 mm, ML +1.8 mm, DV −2.6 mm relative to dura); ventral hippocampus (AP −4.8 mm, ML +5.2 mm, DV −6.4 mm and −3.8 mm relative to dura) using a stereotaxic frame (Kopf, Sweden). Injections were performed with a 5-μl Hamilton syringe mounted with a glass pipette. The vector of choice was injected at each site in a volume of 1 μl with a speed of 0.1 μl/min and a final concentration of 1×10^{12} genomic particles/ml. The syringe was allowed to remain at the site for 10 min to prevent the backflow of the injected solution. After the surgery and every 24 h for the next 2 days, each rat received 1 μl/g s.c. injection of analgesic carprofen (5 mg/ml). Recovery of the rats was observed closely by the experimenter for 48 h post-surgery.

Quantitative PCR (qPCR)

At the end of Experiment-1, the rats were anesthetized, brains quickly removed from the skull, and left and right hippocampi were snap-frozen and kept at −80°C until processing. The right (CG01-injected) and left (non-injected) hippocampi were homogenized with QIAzol lysis reagent (Qiagen, Copenhagen, Denmark) and total RNA was extracted using RNeasy Lipid Tissue Kit (Qiagen) following the manufacturer's protocol. On-column DNAse I treatment was performed using RNase-Free DNase Set (Qiagen). RNA purity (260 nm/280 nm ratio) and concentration of the samples were measured on NanoDrop (Thermo Fischer Scientific) and 0.5 μg of RNA was used for cDNA synthesis following High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems). QPCR was run on a LightCycler 480 (Roche) using SYBR Green I Master kit (Roche). The cycling conditions were: 5 min at 95°C followed by

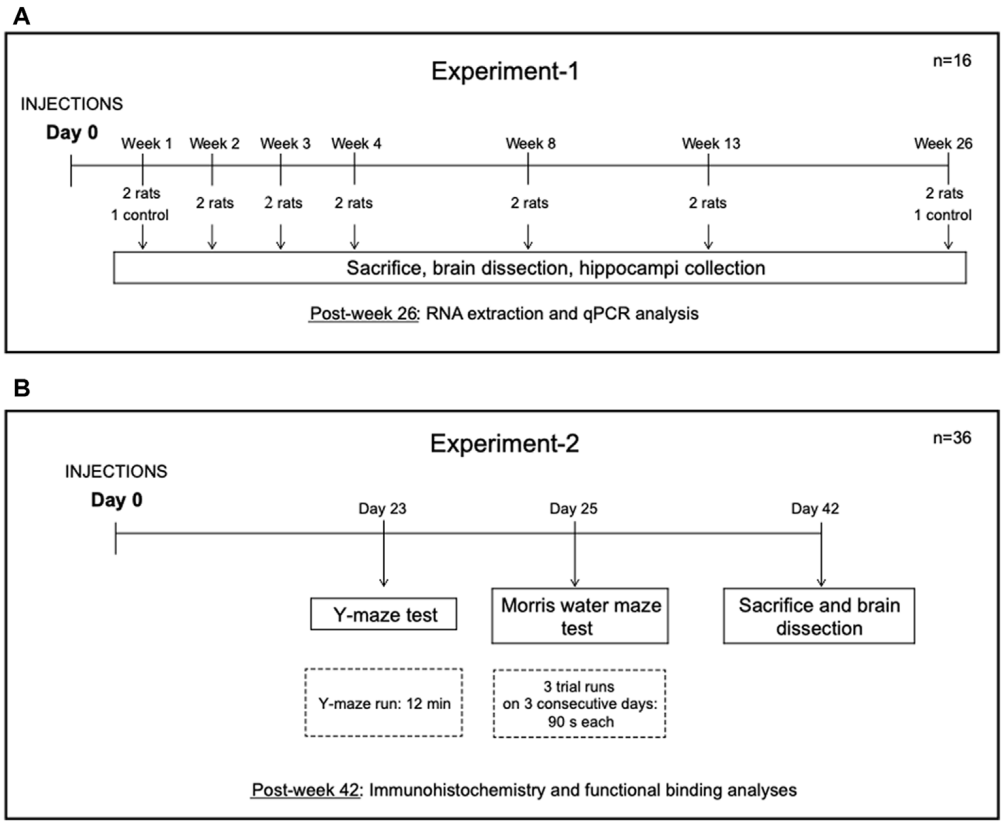

FIGURE 1 | Design of Experiment-1 **(A)** and Experiment-2 **(B)**.

45 cycles of 10 s at 95°C, 15 s at 60°C and 10 s at 72°C; one cycle of 5 s at 95°C, 1 min at 65°C, and then continuous acquisition mode at 97°C for 5 acquisitions per 1°C; one cooling cycle for 10 s with ramp rate 2.0°C/s.

The expression levels of human *NPY* and *Y2* were normalized to the *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) expression level, the gene with the least variable expression among several tested reference genes, i.e., *beta-actin* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *ribosomal protein L13a* (RPLI3A), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide* (YWHAZ; see **Supplementary Figure 1**) and thus used as a reference gene in this study. Primers were acquired from Eurofins Genomics and sequences are shown in **Table 1**. Data are presented as increases in the cycle threshold (Ct) values for *NPY* or *Y2* expression levels in the CG01-injected side minus the non-injected side normalized to *HPRT* and subsequently inverted (multiplied with −1) to visualize increases as positive values.

TABLE 1 | Primers used for quantitative PCR (qPCR) experiment.

Gene name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
NPY	GGAGGACATGGCCAGATACT	ATCTCTGCCTGGTGATGAGG
Y2	GGCCATCTTCCGGGAGTATT	GCCAGGCCACTTTTCAGTAC
ACTB	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA
GAPDH	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA
HPRT	GCAGACTTTGCTTTCCTTGG	CGAGAGGTCCTTTTCACCAG
RPLI3A	ACAAGAAAAAGCGGATGGTG	TTCCGGTAATGGATCTTTGC
YWHAS	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA

Cognitive Testing

Only rats from Experiment-2 were subjected to behavioral testing starting on day 21 post-surgery.

Y Maze Test

The Y maze, also called spontaneous alternation (SA) test, uses the nature of the rodents to explore the unrestricted areas and their tendency to enter the new area rather than the one previously visited (Momeni et al., 2015). The rats were handled for 2 days before the test and habituated to the test room for 1 h on the test day. The Y maze was composed of three opaque arms distributed 120° apart from each other. The arms were marked A, B, and C, where B was the introductory arm. The maze was surrounded by black curtains with four cues allocated around the maze. On the trial day, each rat was placed in arm B, facing the closed end of the arm, and allowed to freely explore the arms for 12 min. The sequence of the arm entries was recorded. Between testing of each rat, the maze was cleaned with water to reduce olfactory cues. To assess spatial working memory, the percentage of SA was calculated as follows: SA% = [number of alternations/(total number of entries – 2)] × 100. A lower

alternation percentage indicates lower spatial working memory (Momeni et al., 2015).

Morris Water Maze Test

The Morris water maze was conducted as described previously (Soud et al., 2019). Briefly, a water maze pool (160 cm in diameter, 60 cm high) was filled with 21°C (±1°C) warm water. The pool was virtually divided into four quadrants, and the escape platform (10 cm in diameter) was placed in one of the quadrants, submerged 1.5 cm under the water surface. The area of the water maze was surrounded by black curtains with four visual orientation cues glued on the inner side. Before the test, the rats were handled for 2 days. In the reference memory training, each rat was subjected to three trials for three consecutive days. On each of the trial days, the rat was removed from its home cage and carefully placed on the introductory line in the pool. On each trial the rat was allowed to freely explore the pool until the platform was found, but for a maximum of 90 s. The rats that failed to find the platform during this period were gently guided to the platform. The rats were allowed 20 s orientation time on the platform before being removed from it. Probe tests were performed on 7 and 14 days after the last trial day. During the probe test, the rats performed two trials. During the first trial, the platform was not present in the pool and the rat was allowed to explore the pool for 60 s. Then the platform was gently reintroduced to its usual position in the pool and the rat was guided to the platform where it was allowed to spend 20 s. A second trial was performed like previous trials on training days where the rat was allowed to explore the pool for a maximum of 90 s with the platform in its usual position and allowed to stay on the platform for 20 s.

Using the SMART 3.0 Video Tracking System (Panlab, Harvard, UK) the learning/short-term memory abilities in the test animals were assessed as calculated by the mean latency to reach the platform for each training day. To estimate more long-term spatial memory, the time spent in the platform quadrant on the probe test days was calculated. Swim speed of the animals during the training days was also measured to determine potential effects on motor activity.

NPY Immunohistochemistry

Only rats from Experiment-2 were used for this analysis. Animals were anesthetized by isoflurane mixture, the brains were quickly removed from the skull, snap-frozen in powdered dry ice, and kept in a −80°C freezer. Rat brains were cut into 14-μm coronal sections on Cryostat CM3050S (Leica), and sections covering the dorsal and ventral hippocampus were collected on SuperFrost PLUS slides and kept on −80°C until use. The slides were defrosted and fixed in 4% paraformaldehyde for 20 min and subsequently washed three times in potassium PBS (KPBS) for 10 min. The sections were then blocked in 10% normal goat serum (NGS), 0.25% Triton X-100 in KPBS for 1 h. Slides were incubated overnight with rabbit anti-NPY antibody (1:500, Sigma–Aldrich, #N9528) diluted in 5% NGS, 0.25% Triton X-100 in KPBS. After washing in KPBS, slides were incubated with secondary goat anti-rabbit Alexa555Plus antibody (1:500, Invitrogen, #A32732), diluted in the same buffer as the primary antibody for 2 h. The slides were washed in T-KPBS for 10 min and two times in KPBS for 10 min. The sections were cover-slipped with the anti-fade mounting medium DABCO (Sigma–Aldrich). Images were acquired on a fluorescence microscope (Olympus BX61 microscope) using the CellSens software. Histological evaluation of the levels of NPY-immunoreactivity was performed using ImageJ 1.49 by densitometric measurements of optical densities in the dentate gyrus, CA3, and CA1 areas of the dorsal and ventral hippocampus by an experimenter blinded to vector treatment of the animals.

For co-staining experiments, sections were additionally blocked in the same blocking solution and incubated with either mouse anti-NeuN (1:100, Merch Millipore; #MAP377) or mouse-anti GFAP (1:500, Sigma–Aldrich; #G3895) overnight followed by Alexa488-conjugated donkey anti-mouse (1:200, Thermo Fisher Scientific; #A21202) or with rabbit-anti Olig2 antibody conjugated with Alexa488 (1:100, Abcam; Ab225099) for 2 h.

Y2 Functional Binding

Functional binding was performed as previously described (Woldbye et al., 2010). Sections were defrosted and air-dried for 30 min at room temperature (RT), rehydrated in assay buffer A (50 mM Tris-HCl, 3 mM MgCl, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) for 10 min at RT and then preincubated in assay buffer B, composed of assay buffer A supplemented with 0.2 mM dithiothreitol, 1 μM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, Sigma–Aldrich; #C101), 0.5% w/v BSA, and 2 mM guanosine-5′-diphosphate (GDP; Sigma-Aldrich, DK) for 20 min at RT. Further, the slides were incubated in assay buffer B supplemented with 40 pM [^{35}S]-GTPγS (1,250 Ci/mmol; NEG030H250UC; PerkinElmer, DK) for 1 h at 25°C in the presence of NPY peptide (Schafer-N, Copenhagen, DK) at 10^{-6} M to which the Y1 receptor antagonist BIBP3226 (10^{-6} M; Bachem AG, Switzerland; #4034548) and the Y5 receptor antagonist L-152,804 (10^{-5} M; Tocris Cookson, UK; #1382) were added to specifically visualize functional Y2 binding only. To confirm the specificity of this binding assay, the Y2 receptor antagonist BIIE0246 (10^{-6} M; Tocris Cookson, UK; #1700) was added to NPY together with BIBP3226 and L-152,804 at concentrations as above to block Y2 receptor functional binding. Basal binding was determined by incubation in assay buffer B supplemented with 40 pM [^{35}S]-GTPγS (1,250 Ci/mmol) without NPY receptor ligands. Since all used NPY receptor antagonists were dissolved in DMSO, DMSO was also added to other incubation buffers (0.1% as final concentration). Incubation was terminated by 2 × 5 min washing in ice-cold 50 mM Tris-HCl buffer (pH 7.4). Sections were dried and exposed to Kodak BioMax MR films together with ^{14}C-microscales (Amersham Life Sciences) for 5 days at −20°C. Films were developed in Kodak GBX developer. Y2 receptor functional binding levels were measured in the dorsal/ventral hippocampus by an experimenter blinded to vector treatment of the animals as previously described (Christensen et al., 2006).

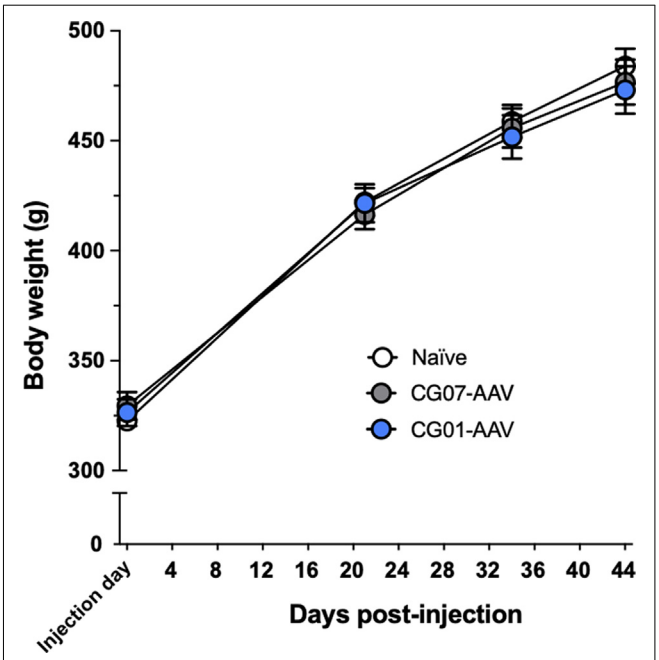

FIGURE 2 | Body weight did not differ between the three groups during Experiment-2 (n = 12 per group).

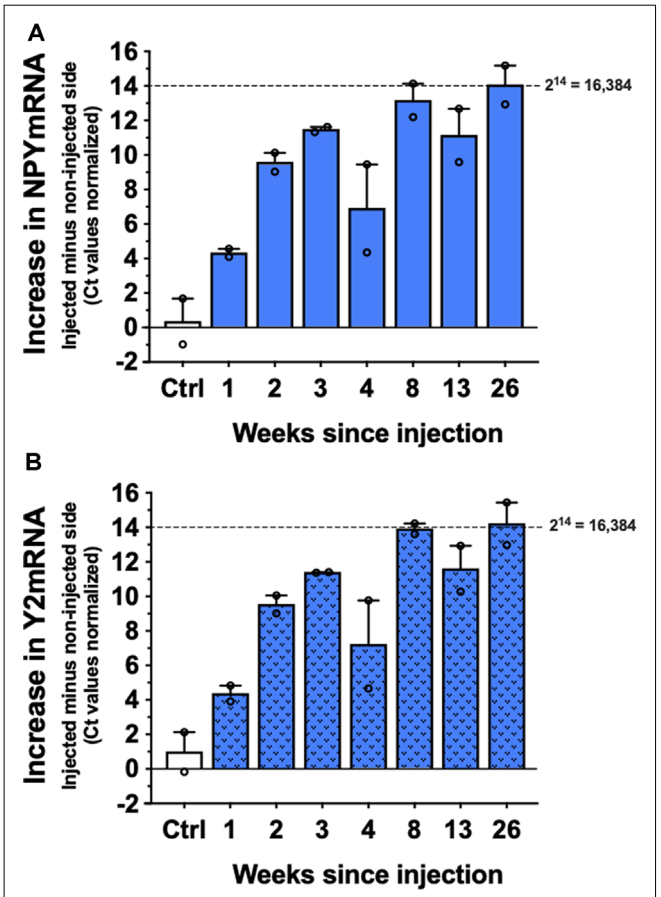

FIGURE 3 | Levels of NPYmRNA **(A)** and Y2mRNA **(B)** at different time points after right intrahippocampal CG01 injection or in non-injected controls (Ctrl) normalized to Hypoxanthine-Guanine Phosphoribosyltransferase (HPRT) levels (n = 2). The graphs show the increases in normalized Ct values in the injected side subtracted the non-injected side. Columns are means with SEM values as error bars. Individual values are depicted as small circles.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism v8.4.3. Non-parametric Kruskal–Wallis ANOVA test followed by Wilcoxon matched-pairs signed rank test was used for immunohistochemistry and functional binding data while parametric one-way ANOVA or two-way repeated measures mixed model ANOVA were used for body weight and behavioral data. Potential correlation between CG01-mediated overexpression of NPY/Y2 (ratios of ipsilateral vs. contralateral sides) and behavioral performance was analyzed using Spearman's correlation. $P < 0.05$ was considered significant.

RESULTS

No Effect of the Vector Treatment on Body Weight

No signs of suffering or discomfort was observed in the animals before and after the vector injections during the whole course of the experiments. NPY is a known orexigenic agent in the hypothalamus, causing prominent increase in food intake and body weight (Loh et al., 2015) and, consequently, the animals were observed for potential weight gain (**Figure 2**). No significant differences were found between the three groups with regard to body weight (non-significant treatment effect in repeated measures two-way ANOVA: $F_{(2,33)} = 0.073$, $P = 0.929$).

Long-Term Expression of NPY and Y2 Transgenes

As evident from **Figure 3**, the expression of both CG01 vector-encoding genes, NPY and Y2, were upregulated at week 1 and appeared to reach close to maximum after 3 weeks. Both transgene expressions showed no sign of decay as long as 26 weeks after CG01.

CG01-Mediated Overexpression of NPY and Y2 as Revealed by NPY Immunohistochemistry and Functional Y2 Binding

To confirm proper CG01-mediated overexpression of NPY and Y2 transgenes, the brains of the rats undergoing memory testing were processed for NPY immunohistochemistry and Y2 functional binding. As expected, NPY immunohistochemical examination of the rat brains revealed increased NPY-immunoreactivity ipsilateral to the CG01-injection in the hippocampal regions dentate gyrus, CA3, and CA1 both at dorsal and ventral levels compared to the contralateral non-injected side (**Figures 4A,H,I**) as confirmed with densitometric measurements (**Figure 4L**). In contrast, NPY-immunoreactivity was modest and without side differences in CG07-injected and naïve rats (**Figures 4F,G,J,K**). High-magnification images (**Figures 4B–E**) showed that increased NPY-immunoreactivity

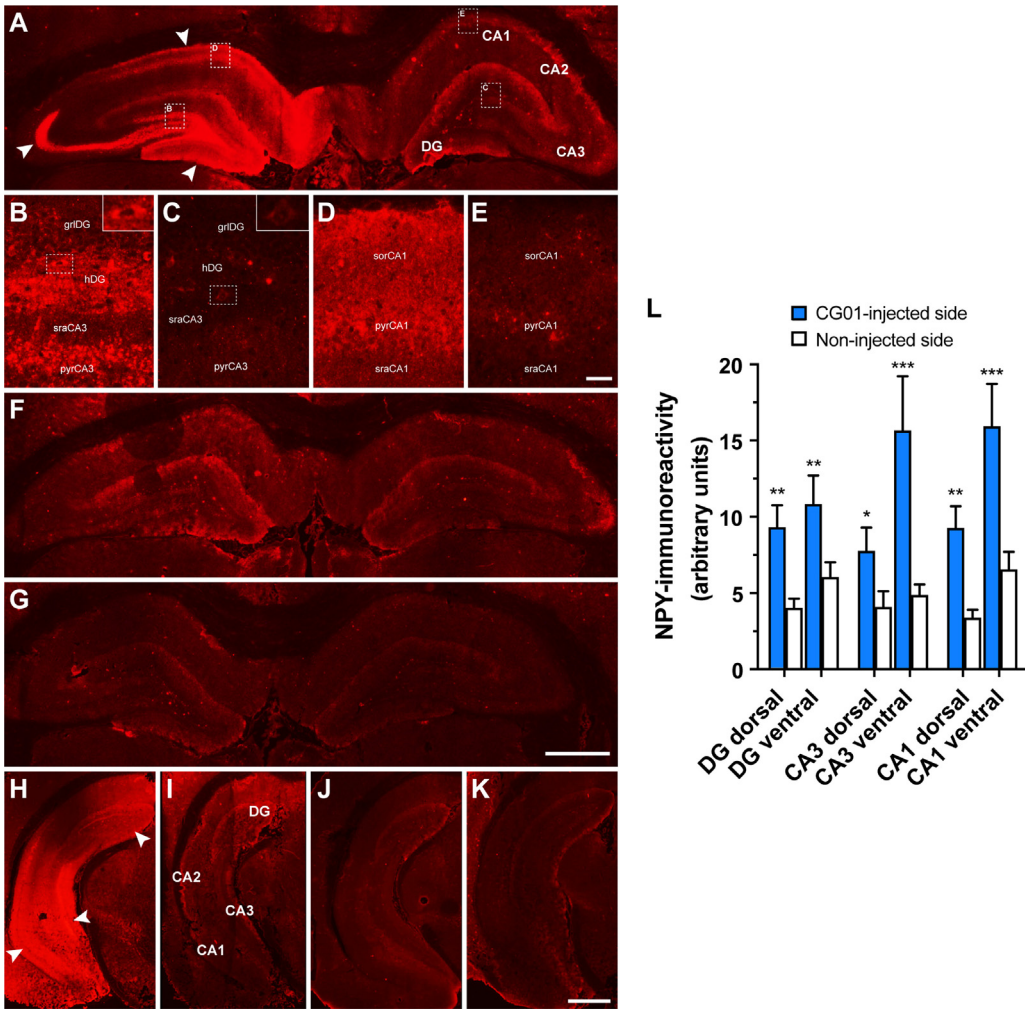

FIGURE 4 | Increased neuropeptide Y (NPY)-immunoreactivity shown in the right dorsal hippocampal dentate gyrus (DG), CA3, and CA1 of a CG01-injected rat (white arrows) compared to the contralateral non-injected side **(A)**. High-magnification images of hatched areas in panel **(A)**, showing increased NPY-immunoreactivity in the granular layer (grlDG) and hilus (hDG) of the DG and adjoining CA3 stratum radiatum (sraCA3) and pyramidal layer (pyrCA3) ipsilaterally **(B)** as compared to contralaterally **(C)** to the injection, as well as ipsilateral CA1 stratum oriens (sorCA1), pyramidal layer (pyrCA1), and stratum radiatum (sraCA1) **(D)** as compared to contralateral non-injected side **(E)**. Inserts in right top corners of **(B)** and **(C)** show increased NPY-immunoreactivity in dentate hilar interneurons in ipsilateral compared to contralateral side. NPY-immunoreactivity shown in a CG07-injected rat **(F)** and in a naïve rat **(G)**. Similarly, increased NPY-immunoreactivity shown in the DG, CA3, and CA1 in the ventral part of the hippocampus of a CG01-injected rat (white arrows; **H**) compared to non-injected hippocampus of the same rat **(I)**. NPY-immunoreactivity in the ventral hippocampus of CG07-injected rat **(J)** and in naïve rat **(K)**. Magnification bars = 1 mm in panels **(A,F,G)**, 50 μm in panels **(B–E)**, and 1.5 mm in panels **(H–K)**. Densitometric measurements confirmed that CG01 increased NPY-immunoreactivity in dorsal and ventral parts of hippocampus compared to the contralateral non-injected side **(L)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-injected side, Kruskal–Wallis ANOVA followed by Wilcoxon matched-pairs signed rank tests. Data are means ± SEM (n = 10–12).

in the DG was mainly observed in mossy fibers and interneurons in the hilus (inserts of **Figures 4B,C**), but some labeling was also seen in cell bodies in the granular layer (**Figures 4B,C**) and in the molecular layer (**Figure 4A**). In the dorsal CA3, increased NPY-immunoreactivity was particularly strong in the stratum lucidum (**Figure 4A**), but labeling was also observed in some cell bodies of the CA3 pyramidal layer, particularly in the CA3c region (**Figures 4B,C**). At ventral levels of CA3, labeling was higher (**Figure 4L**), also in the pyramidal layer of ventral CA3 (**Figures 4H,I**). In the dorsal CA1, elevated NPY-immunoreactivity was observed in the pyramidal layer (**Figures 4D,E**) and stratum oriens, but less so in the stratum radiatum where CA3 pyramidal projections terminate. As for the hippocampal CA2, more than two thirds of the CG01-injected animals were also observed to display increased NPY-immunoreactivity ipsilaterally vs. contralaterally in the dorsal (not shown) and ventral (**Figures 4H,I**) parts.

As evidence that the [^{35}S]-GTPγS Y2 functional binding assay was working, there was a clear increase in Y2-stimulated (NPY + Y1 antagonist + Y5 antagonist) binding (**Figures 5A,D,F,H,J,K**) compared to basal binding (**Figures 5B,E,G,L**) in all treatment

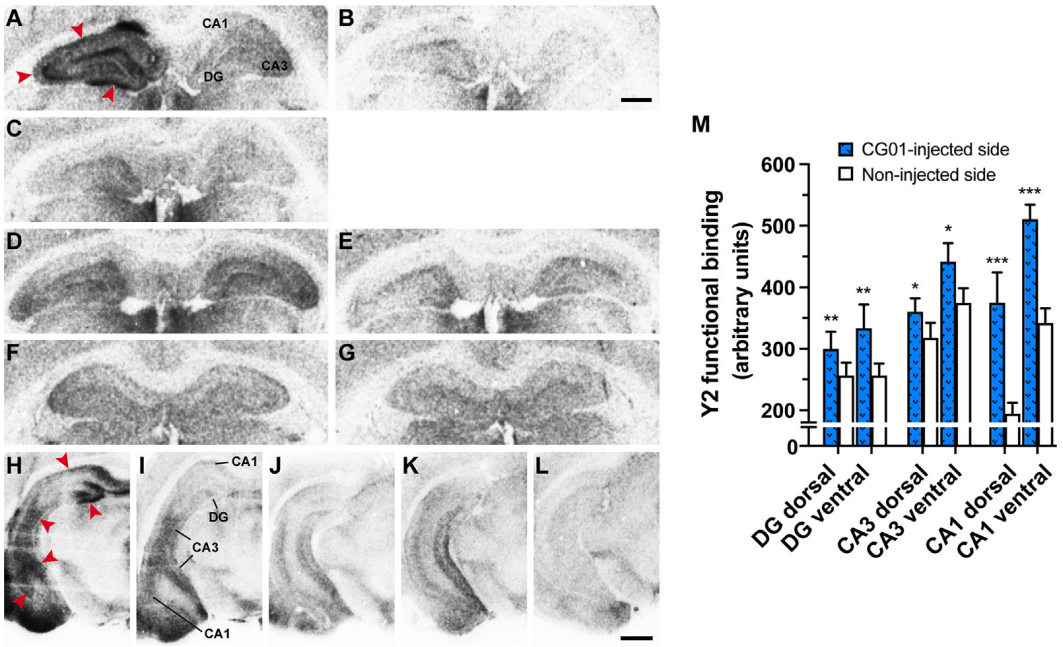

FIGURE 5 | Unilateral intrahippocampal administration of CG01 increased Y2 transgene expression as seen by increased Y2 functional binding (NPY + Y1 antagonist + Y5 antagonist) in the right dorsal hippocampal DG, CA3, and CA1 of a CG01-injected rat (red arrows) compared to the contralateral non-injected side **(A)**. Basal binding (no addition of NPY; **B**) and blocking of Y2 binding (NPY + Y1 antagonist + Y5 antagonist + Y2 antagonist; **C**) are shown in CG01-injected rat. Y2 functional binding is shown in CG07-injected **(D)** and in naïve rat **(F)** with corresponding basal binding (**E** and **G**, respectively). Increased Y2 functional binding was also seen in DG, CA3, and CA1 in ventral part of the CG01-injected hippocampus (red arrows; **H**) compared to non-injected hippocampus **(I)**. Y2 functional binding displayed in ventral hippocampus of CG07-injected rat **(J)** and in naïve rat **(K)** while **(L)** shows basal binding in CG01-injected ventral hippocampus. Magnification bars = 1 mm in panels **(A–G)** and 1.5 mm in panels **(H–L)**. Densitometric measurements confirmed that CG01 increased Y2 functional binding in dorsal and ventral parts of hippocampus after unilateral intrahippocampal administration compared to the contralateral non-injected side **(M)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-injected side, Kruskal–Wallis ANOVA followed by Wilcoxon matched-pairs signed rank tests. Data are means ± SEM (n = 10–12).

groups. Consistent with the NPY-immunoreactivity results, Y2 functional binding was also increased in CG01-injected DG, CA3, and CA1 regions ipsilaterally compared to the noninjected side both at dorsal and ventral levels (**Figures 5A,H,I**), and as confirmed by densitometric measurements (**Figure 5M**). As confirmation that increased labeling was due to Y2 functional binding, the signal was blocked after addition of Y2 antagonist (**Figure 5C**). Thus the assays confirmed that both NPY and Y2 transgenes were well expressed in all the studied hippocampal regions on the injected side. In general, NPY-immunoreactivity and/or functional Y2 binding were confined to the DG and hippocampus proper, but in some animals, particularly at ventral levels, some vector-mediated expression was also observed to spread into the subiculum (**Figure 5A**) and adjoining areas, including the entorhinal cortex, amygdalopiriform transition area, and cortical amygdaloid nuclei (**Figures 4H,I**, **5H,I**).

NPY Expression in Hippocampal Cells (Tropism)

Further, we performed immunohistochemical co-staining to investigate the cell populations expressing NPY after CG01-treatment in the dorsal dentate gyrus and CA3. Co-staining for NPY and the neuronal marker NeuN showed extensive overlap of immunoreactivity in neuronal fibers particularly from dentate granule neurons passing through the dentate hilus to terminate in the CA3 stratum lucidum (**Figures 6A–F**) and interneurons in the dentate hilus (**Figure 6E**) while cells expressing GFAP- or Olig2-immunoreactivity did not appear to co-express NPY (**Figures 7A–J**). This suggests that CG01-mediated hippocampal NPY overexpression predominantly targets neurons.

No Effects of CG01 On Short- or Long-Term Memory

In order to check whether the injection of CG01 could affect cognitive function of the animals, two memory tests were carried out. Using the Y-maze SA test, which is a simple test evaluating spatial memory (Gøtzsche and Woldbye, 2016), no significant effect was detected between CG01 vector treated compared to the control groups, as revealed by percentage triads conducted during the test (**Figure 8A**; one-way ANOVA: $F_{(2,32)} = 0.98$, $P = 0.39$). Similarly, using the Morris water maze test, which is a more complex test used to evaluate learning as well as short- and long-term memory (Morris et al., 1986; Vorhees and Williams, 2006), no overall significant effect of CG01 was revealed during the three training days (**Figure 8B**; non-significant treatment effect in repeated measures two-way mixed effects model: $F_{(2,33)} = 3.24$, $P = 0.052$) nor during the

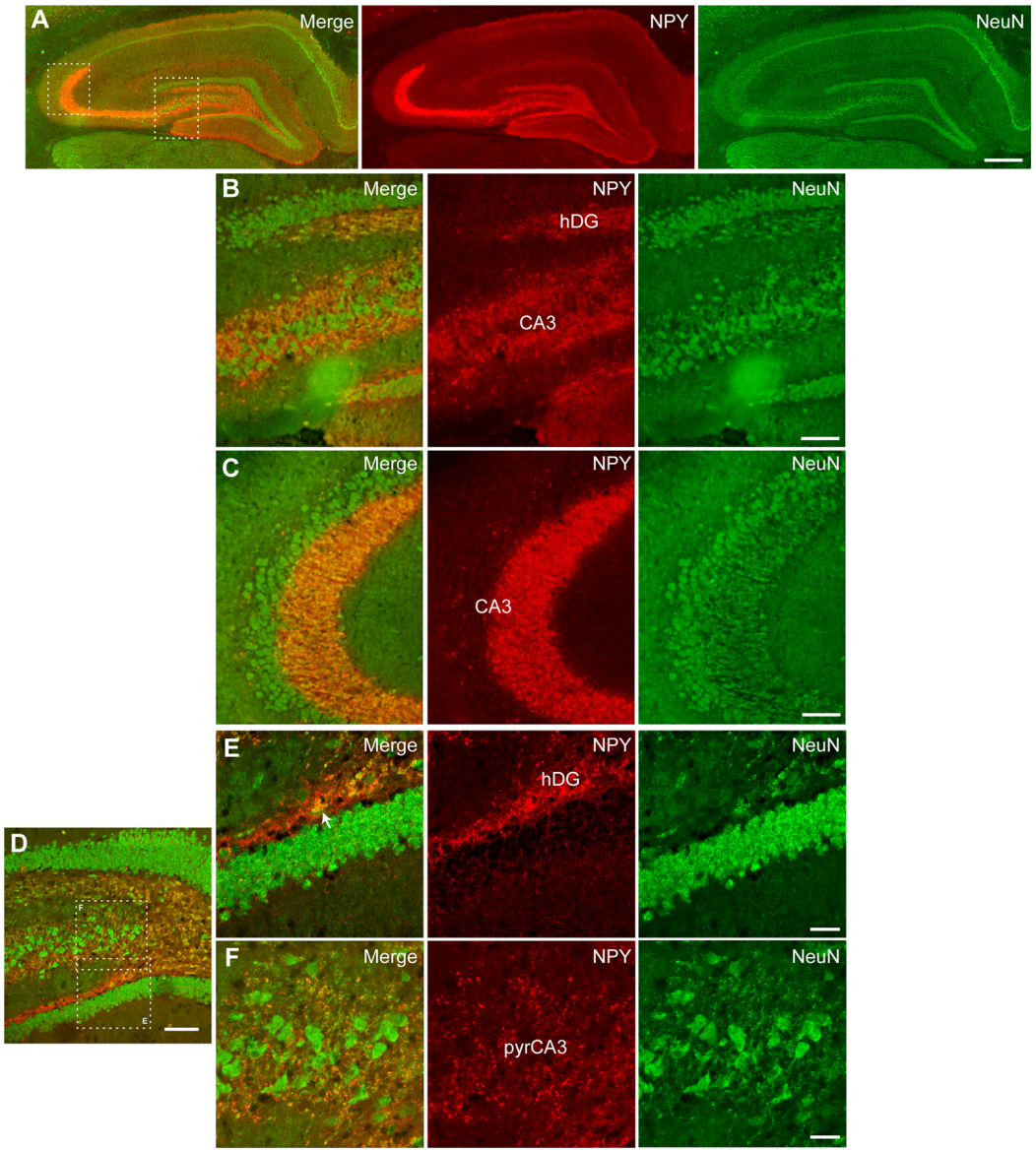

FIGURE 6 | Neuronal tropism in the dorsal hippocampal DG and CA3 of CG01-treated rat as evidenced by NPY-immunoreactivity being co-expressed with NeuN-immunoreactivity as revealed by fluorescent **(A–C)** and confocal microscopy **(D–F)**. **(A)** Fluorescent microscopy overview images of NPY/NeuN co-staining (merge), NPY- and NeuN-immunoreactivity, with high-magnification images of hatched areas in panel **(A)**, showing hilus of the DG (hDG; **B**) and CA3 **(B,C)**. **(D)** Confocal microscopy overview image of NPY/NeuN co-staining, with high-magnification images of hatched areas in panel **(D)**, showing co-labeling in fibers and interneuron (white arrow) in hDG **(E)** and fibers in pyramidal layer of CA3 (pyrCA3; **F**). Scale bars: 500 μm **(A)**, 100 μm **(B–D)**, and 30 μm **(E,F)**.

probe tests (**Figure 8C**; $F_{(2,65)} = 0.28$, $P = 0.76$). However, during the training days, there was a clear effect of time ($F_{(1.96,63.7)} = 42.69$, $P < 0.0001$) and no evidence of interaction ($F_{(4,65)} = 1.21$, $P = 0.32$), indicating respectively that all groups learned the memory task of finding the escape platform and did this equally well. As confirmation that the animals of all groups remembered the location of the platform, all groups displayed a mean time spent in the test quadrant clearly above the theoretical 25%, i.e., 15 s out of the total 60 s probe test periods (**Figure 8C**, dashed line). Swim speed during the learning sessions also did not differ significantly between the groups (**Figure 8D**; $F_{(2,33)} = 0.54$, $P = 0.59$), indicating that treatment with the CG01 vector also had no locomotor side effects that could have influenced the memory responses measured in this test. Performance in the memory tests after CG01-treatment was not correlated in any of the measured hippocampal regions to levels of NPY-immunoreactivity (Spearman r performed on ratios of ipsilateral vs. contralateral non-injected sides: Y-maze: $P = 0.09$–0.81; Morris water maze, days 1–3, probe tests: $P = 0.11$–0.96) or Y2 functional binding (Spearman r: Y-maze: $P = 0.22$–0.86; Morris water maze, days 1–3, probe tests: $P = 0.12$–0.97).

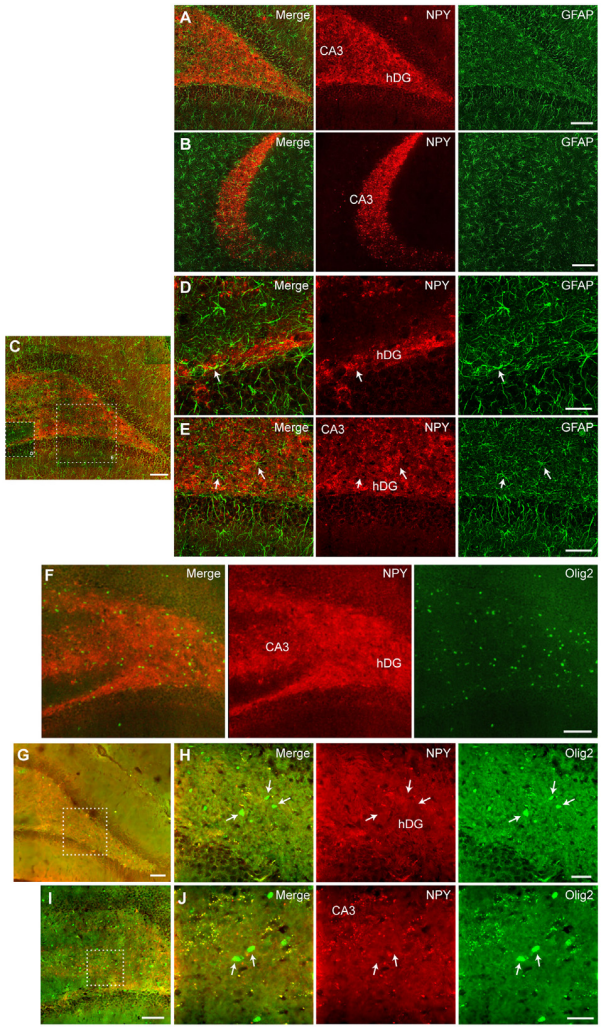

FIGURE 7 | Fluorescent **(A,B,F)** and confocal microscopy **(C–E,G–J)** revealed no co-staining between NPY and GFAP **(A–E)** or Olig2 **(F–J)** in the dorsal hippocampus after CG01 treatment, suggesting that CG01 does not express in astrocytes or oligodendrocytes. Fluorescent microscopy images of NPY/GFAP co-staining (merge), NPY- and GFAP-immunoreactivity in the dentate hilus (hDG; **A**) and CA3 **(A,B)** showing no overlap between NPY- and GFAP-immunoreactivity. **(C)** Similarly, confocal microscopy overview image shows no overlap between NPY- and GFAP-immunoreactivity in the DG and CA3, with high-magnification images of hatched areas in panel **(C)**, showing hDG **(D)** and CA3 (**D,E**; white arrows indicate astrocytes). **(F)** Fluorescent microscopy overview images of NPY/Olig2 co-staining (merge), NPY- and Olig2-immunoreactivity showing no overlap in the hDG and CA3 of the dorsal hippocampus. **(G,I)** Confocal microscopy overview images of NPY/Olig2 co-staining, with high-magnification images of hatched areas in panels (**G** and **I**), showing no overlap in the dentate hilus **(H)** and CA3 (**J**; white arrows indicate oligodendrocytes). Scale bars: 100 μm **(A–C,F,G,I)**, 30 μm **(D,H,J)**, and 60 μm **(E)**.

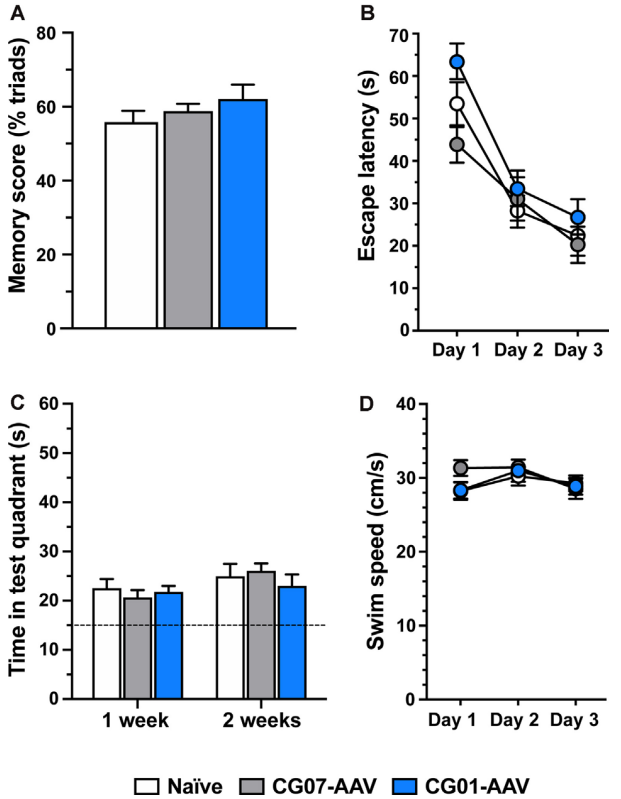

FIGURE 8 | CG01 unilateral intrahippocampal administration did not significantly affect learning and memory function (% triads) compared to CG07-AAV control vector treatment or compared to treatment naïve control rats as revealed by testing in the Y-maze test (**A**; n = 11–12 rats per group). Similar result was obtained in the Morris water maze test where escape latencies during learning sessions **(B)** and during probe tests 1 and 2 weeks after **(C)** did not differ significantly between the groups (n = 11–12 rats per group). Likewise, swim speed did not differ between the groups during the learning trials **(D)**.

DISCUSSION

Currently, gene therapy is being developed for pharmacoresistant temporal lobe epilepsy using unilateral intrahippocampal gene therapy with CG01, an AAV vector mediating overexpression of NPY and Y2 (Drew, 2018). Our groups have explored the effects of unilateral NPY/Y2 gene therapy and shown that hippocampal overexpression of both transgenes induces significant seizure-suppressant effect in a chronic rat temporal lobe epilepsy model (Ledri et al., 2016; Melin et al., 2019). Potential side effects should be fewer or less pronounced after unilateral hippocampal gene therapy. Consistent with this view, unilateral surgical excision of a large part of hippocampus and amygdala of the epileptic focus is considered acceptable with regards to cognitive side effects (Sheikh et al., 2019). Thus to pave the way for future clinical testing, the present study provides important knowledge of the effects of CG01 in adult rat hippocampus on the duration of transgene expression, cellular tropism, and potential side effects on body weight and cognitive function in rats.

As for the duration of CG01-mediated transgene overexpression, the present study shows that there are prominent increases in both NPY and Y2 mRNA levels in the injected hippocampus compared to the non-injected side, reaching close to maximum after 3 weeks and remaining at this level or at even higher levels as long as 26 weeks after CG01 vector injection. Twenty-six weeks may correspond

to around 13–18 years of lifetime in humans (Andreollo et al., 2012; Agoston, 2017) and suggests that CG01-mediated expression of NPY and Y2 transgenes could last for long duration in humans. Consistent with this view, AAV vector-mediated overexpression has been detected as long as 10 years post-surgery in Parkinson's disease patients (Chu et al., 2020) and remained unchanged for 15 years in non-human primates (Sehara et al., 2017).

Having established that NPY-immunoreactivity was clearly upregulated by CG01 treatment, the cellular distribution of transgene expression (i.e., tropism of the AAV viral vector) was explored using immunohistochemical co-staining between NPY and specific cell markers in hippocampal dorsal dentate gyrus and CA3. NPY-immunoreactivity was found to co-localize in NeuN-positive neuronal fibers and cells in the dentate hilus and mostly fibers in the CA3 region. In contrast, no co-staining was seen between NPY- and GFAP- or Olig2-immunoreactivity, indicating that, in the hippocampus, CG01 predominantly, if not exclusively, mediates transgene expression in neurons and not in astrocytes or oligodendrocytes. Cellular tropism is driven by the serotype and promotor (Watakabe et al., 2015; Hudry and Vandenberghe, 2019). *In vitro*, the AAV1 serotype has been shown to transduce preferentially pyramidal neurons in CA3/CA1 of primary hippocampal cultures, but also to a minor degree astrocytes when using the hCMV promoter (Royo et al., 2008). Nonetheless, consistent with the present study, *in vivo*, the AAV1 vector transduced neurons in the dentate granular layer and pyramidal neurons of the CA3/CA1 (Burger et al., 2004), and another study using AAV1 to induce hippocampal NPY expression also reported predominantly neuronal expression (Noe et al., 2010). Further consistent with the present findings with CG01, when targeting the hippocampus of adult rats with an AAV1 vector utilizing the CAG promoter, selective transduction of neurons was observed without transduction of astrocytes or microglia (Jeon et al., 2015). Previous data suggest a minor transduction of oligodendrocytes when injecting an AAV1 into mice (Wang et al., 2003), but in contrast to these findings we did not observe expression of NPY in oligodendrocytes in rats.

NPY has powerful feeding stimulatory effects by acting in the hypothalamus (Loh et al., 2015). Since AAV-mediated bilateral overexpression of NPY in limbic rodent brain regions, including the hypothalamus (Tiesjema et al., 2007) and amygdala (Christiansen et al., 2014), has been associated with increased body weight, we also measured body weight in the present study. No significant effects on body weight were observed after unilateral hippocampal CG01 administration compared to both CG07 control vector administration and naïve rats. This is consistent with several previous studies indicating that targeting hippocampus with vectors mediating overexpression of NPY is not associated with weight gain (Richichi et al., 2004; Woldbye et al., 2010; Christiansen et al., 2014; Soud et al., 2019).

Central administration of NPY is known to have both inhibitory and stimulatory effects on memory, depending on the brain region, dose, memory test, and application time point in the learning process (for review see Gøtzsche and Woldbye, 2016). Experiments with Y2 receptor knockout mice and Y2 receptor antagonist indicate that Y2 receptors play an important role in mediating hippocampal memory-related effects of NPY (Redrobe et al., 2004; Gonçalves et al., 2012; Hörmer et al., 2018). Studies with AAV vectors encoding NPY have shown that hippocampal NPY overexpression may attenuate the memory-related synaptic phenomenon long-term potentiation (LTP) *in vitro* (Sørensen et al., 2008a,b). Similarly, direct NPY application inhibits hippocampal LTP (Whittaker et al., 1999; Sørensen et al., 2008b). However, in electrically kindled animals (a chronic epileptic condition), LTP was not further decreased after NPY-AAV treatment compared to control vector (Sørensen et al., 2009). This suggests that although naïve rodents may display reduced LTP after NPY-AAV treatment, this is not evident in epileptic animals. This is consistent with the finding in TLE patients that they may experience memory impairment (Elger et al., 2004), and suggests that future treatment with NPY gene therapy may not additionally impair their memory. Nonetheless, *in vivo*, bilateral AAV-mediated hippocampal NPY overexpression has been shown to transiently inhibit memory and learning in a two-platform spatial discrimination water maze test in rats (Sørensen et al., 2008a). Thus, on days 3 and 4 of training, the rats appeared to remember the location of the correct platform less well than control vector-treated rats, but on the last days of training (i.e., days 5–7), NPY-AAV treated rats were equally good as controls. In two other studies, no significant effect was found after bilateral NPY-AAV treatment in seizure-naïve rats in the same model (Noè et al., 2008; Noe et al., 2010). The reason for this discrepancy is not clear, it may be relevant that the latter studies used a CAG promotor (as opposed to neuron-specific enolase promotor; Sørensen et al., 2008a), serotype 1 (as opposed to mixed serotype 1/2) and injected the vector in both the septal temporal parts of hippocampus (as opposed to only septal). Consistent with the lack of effect on hippocampal mediated memory, NPY-AAV treatment also did not influence memory in a passive avoidance test (Noe et al., 2010). Similarly, our group recently showed that bilateral septal/temporal hippocampal overexpression of NPY under a synapsin promotor and with mixed serotype 2/8 also did not influence memory in a Morris water maze test (Soud et al., 2019).

No previous studies have examined potential effects on memory after vector-mediated Y2 overexpression in normal rodents. However, re-expression of Y2 receptors in the dorsal hippocampus of Y2 knockout mice decreased spatial memory in mice (Hörmer et al., 2018). The present study using an AAV vector (CG01) with a CAG promotor to induce overexpression of both Y2 and NPY in septal and temporal hippocampus was not associated with a significant effect on short-term or long-term memory using the hippocampus-dependent Morris water maze test. Consistent with this finding, the Y maze SA test also showed no significant difference in task performance between CG01 and the two control groups, indicating no differences in working memory. Finally, no effect was seen on locomotor activity in the water maze, suggesting that locomotor effects were also not influenced by CG01 treatment. Noe et al. (2010) also did not find significant effects on locomotion after NPY-AAV treatment. Taken together, these data indicate that CG01-mediated overexpression of NPY and Y2 does not significantly influence memory in the tested animal models.

In conclusion, the present study showed that unilateral CG01-mediated overexpression of NPY and Y2 transgenes in the dorsal and ventral parts of the hippocampus was not associated with significant effects on learning and memory as revealed by the Y-maze and Morris water maze memory tests in adult male rats. No effects of CG01 administration were seen on body weight either. CG01-mediated levels of NPY/Y2 transgene expression were long-lasting, remaining at maximum levels all the way to the last time point of the study, 26 weeks, after intrahippocampal injection. CG01 appeared to selectively induce transgene expression in hippocampal neurons. These data suggest that treatment with CG01 in future clinical trials for pharmacoresistant temporal lobe epilepsy patients should not have significant side effects on body weight or memory.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Danish Animal Experiments Inspectorate.

AUTHOR CONTRIBUTIONS

JS, KD, EM, SR, and DW conducted the experimental work and analyzed the data. AE, KA, EM, MK, and DW conceived and designed the study. JS, SP, KD, MK, and DW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.603409/full#supplementary-material.

SUPPLEMENTARY FIGURE 1 | Different reference genes were tested and HPRT was found to be the least regulated and was consequently used for determining NPY and Y2 mRNA levels. Data are pooled averaged values for both sides of the hippocampus (L + R) at different time points after CG01 injection (n = 1–2 rats).

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REVIEW
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AAV Targeting of Glial Cell Types in the Central and Peripheral Nervous System and Relevance to Human Gene Therapy

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Different glial cell types are found throughout the central (CNS) and peripheral nervous system (PNS), where they have important functions. These cell types are also involved in nervous system pathology, playing roles in neurodegenerative disease and following trauma in the brain and spinal cord (astrocytes, microglia, oligodendrocytes), nerve degeneration and development of pain in peripheral nerves (Schwann cells, satellite cells), retinal diseases (Müller glia) and gut dysbiosis (enteric glia). These cell type have all been proposed as potential targets for treating these conditions. One approach to target these cell types is the use of gene therapy to modify gene expression. Adeno-associated virus (AAV) vectors have been shown to be safe and effective in targeting cells in the nervous system and have been used in a number of clinical trials. To date, a number of studies have tested the use of different AAV serotypes and cell-specific promoters to increase glial cell tropism and expression. However, true glial-cell specific targeting for a particular glial cell type remains elusive. This review provides an overview of research into developing glial specific gene therapy and discusses some of the issues that still need to be addressed to make glial cell gene therapy a clinical reality.

Keywords: gene therapy, AAV, glia, astrocyte, oligodendrocyte, microglia, peripheral nerve, Müller glia cell

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INTRODUCTION

The term glia relates to types of non-neuronal cells in the central nervous system (CNS) and peripheral nervous system (PNS) that maintain homeostasis and are active regulators of numerous physiological functions. The glial cells of the CNS include astrocytes, which support the blood-brain barrier (BBB), provide nutrients to neurons and play a crucial role in maintaining extracellular ion balance and neurotransmitter levels in the CNS. Microglia play roles relating to both the immune response and homeostasis (Kierdorf and Prinz, 2017) and oligodendrocytes primary function is to myelinate axons and provide metabolic support (Bradl and Lassmann, 2010). The retina, which is considered part of the CNS, contains Müller glia, which like astrocytes play a role in regulating blood flow, uptake of neurotransmitters, regulation of ion levels and energy storage (Bringmann et al., 2006).

Several glial cell types play similar roles within the PNS. The gut contains enteric glia, which share many similarities with CNS glia (Grubisic and Gulbransen, 2017) and are crucial for

the survival of enteric neurons. Moreover, they play a key role in homeostasis, metabolism and neurotransmission as well as gut epithelial integrity, and regulate gut motility (Ruhl et al., 2004). Schwann cells are the myelinating cells of the PNS and are involved in maintaining ionic balance and providing support to axons (Kidd et al., 2013). Satellite cells are associated with neurons in peripheral ganglia and have similar functions to astrocytes in the CNS (Hanani, 2005, 2010). As well as their role in normal physiological functions of the nervous system, glia are activated under pathological conditions and contribute significantly to disease pathology in many neurodegenerative diseases, neurotrauma, peripheral neuropathies and gut inflammation. Glial cells are therefore a potential cell target for several therapeutic approaches to treat diseases of the nervous system (Ahmed et al., 2017; Spear and Mawe, 2019; Eastlake et al., 2020).

One such approach is the use of gene therapy which employs viral vectors to deliver genetic material with therapeutic potential into a cell. Different viral vector systems have been developed to mediate gene delivery to different organ systems, including the CNS and PNS (Kantor et al., 2014). The use of viral vector gene therapy for the nervous system is appealing as many drugs cannot cross the BBB efficiently and it can overcome the need for repeated delivery of often short-acting drugs into the brain, spinal cord, retina and cochlea by allowing for a single, long-lasting intervention.

One of the most well-characterized vectors for gene therapy is derived from adeno-associated virus (AAV). These are considered the ideal for human gene therapy approaches as they are small and non-replicative, can transduce dividing and non-dividing cells, are non-pathogenic to humans and can provide long-lasting changes in gene expression (Ingusci et al., 2019). AAVs have been used to target a number of different tissue and cell types successfully within the CNS and PNS including neurons, astrocytes, oligodendrocytes, microglia, Müller glia, Schwann cells, and satellite cells (Berns and Giraud, 1996; Rabinowitz and Samulski, 1998; Xiang et al., 2018; Sargiannidou et al., 2020). A large number of clinical trials using AAV have demonstrated the relative safety of AAV gene therapy (Mastakov et al., 2002; Penaud-Budloo et al., 2018). However, to date, these trials have targeted neuronal cell types and retinal pigment epithelium in the retina. An AAV gene therapy approach has real potential for targeting of glial cells and in preclinical studies targeting of different glial cell types has been achieved (Howard et al., 2008; Hammond et al., 2017).

AAV Vectors

Wild type AAVs are small, 4.7 kb, linear, single-stranded DNA (ssDNA) viruses in the Parvovirus family. They are composed of an icosahedral protein capsid of three types of subunit (VP1, VP2, and VP3), totaling 60 copies in a ratio of 1:1:10 (VP1:VP2:VP3). The genome consists of a *rep* gene, encoding four proteins necessary for viral replication; a *cap* gene that encodes the three capsid subunits through alternative splicing and translation from different start codons; and a third gene that encodes an assembly activating protein (AAP) which promotes virion assembly. These are flanked by inverted terminal repeats (ITRs) which are needed to direct genome replication and packaging (Samulski and Muzyczka, 2014). For therapeutic use, the *rep* and *cap* genes are removed and replaced by an expression cassette containing the therapeutic transgene under the control of a promoter and flanked by the AAV ITRs, forming a recombinant AAV (rAAV) (During et al., 2003). There are hundreds of variants of AAV, including the 11 natural serotypes; AAVs 1–11. The natural serotypes are defined by antigenically distinct viral capsids and although most were first isolated in humans, later serotypes were identified in non-human primate species, including rhesus and cynomolgus macaques (Gao et al., 2004; Mori et al., 2004).

AAV Tropism

In the CNS, while most AAV vectors have a preference for targeting neurons, both naturally-occurring and engineered serotypes have been shown to transduce glia (**Figure 1**). The tropism of an AAV for a particular cell type is dependent on the interaction of the capsid with cell surface receptors (Lisowski et al., 2015). The vector initially attaches to a cell surface glycan, which acts as a primary receptor. For efficient entry to the cell, the virus must then interact with a co-receptor. Twenty-three different glycan receptors have been identified, although the primary receptor for some serotypes has not yet been determined, whilst a number of co-receptors have also been identified (reviewed in Lisowski et al., 2015; Srivastava, 2016). AAV capsids can be modified, changing their ability to interact with specific receptors and therefore the cell types they will transduce, and this has been used successfully to change AAV tropism for a particular cell or tissue and to improve transduction efficiency.

Different strategies can be used to alter the tropism of AAV capsids (reviewed in Castle et al., 2016; Deverman et al., 2018). Chemical modification of the virus capsid can lead to improved transduction efficiency and mask native receptors allowing the vector to target alternate receptors (Bartlett et al., 1999; Ponnazhagan et al., 2002; Le et al., 2005; Carlisle et al., 2008; Horowitz et al., 2011), but these have had limited use *in vivo*. Hybrid capsids that combine the advantageous properties of specific selected AAV serotypes have been developed that lead to improved transgene expression and tropism (Koprich et al., 2010). Short peptides can also be inserted into the capsids, and their presence can allow for interaction with a specific target cell receptor (Chen et al., 2009).

Approaches can involve rational design, which is underpinned by an understanding of the function of capsid protein residues such as key residues involved in receptor binding. Mutation of these residues can lead to unique cellular tropism (Murlidharan et al., 2015), and insertion of specific peptide sequences can change cell tropism and modify the ability of the AAV vector to cross the BBB (Adachi et al., 2014; Albright et al., 2018). Another approach used to develop novel capsids is directed evolution. This involves generating highly diverse capsid libraries and using iterative rounds of selection either *in vitro* or *in vivo* to enrich for the most potent AAV variant with the desired tropism. This diversity can be created using capsid-shuffling, which involves the nuclease digestion of different AAV serotype *cap* genes that are then randomly reassembled to form chimeric

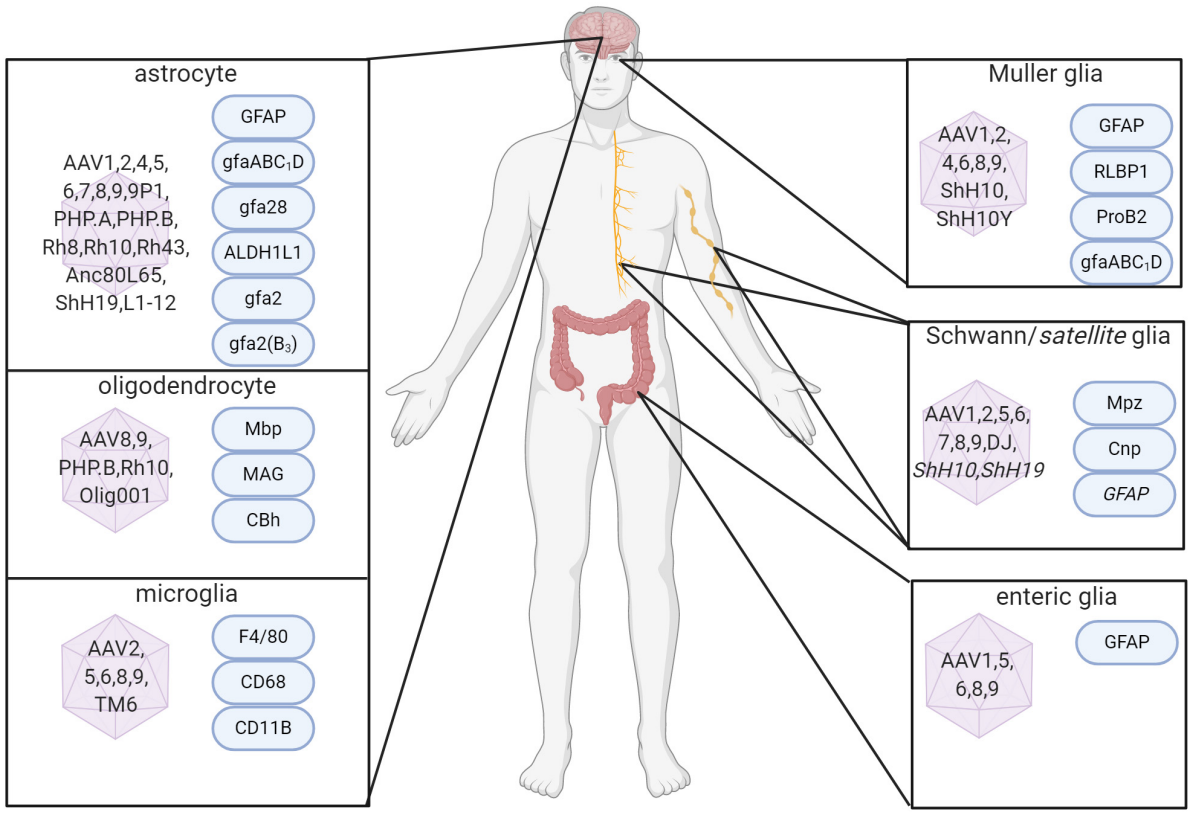

FIGURE 1 | Capsid serotypes and promoters for glial targeting of AAV. Overview depicting naturally-occurring and engineered AAV viral vectors with known glial cell tropism in the CNS and PNS and relevant cell-specific promoters. References used for this figure are detailed and cited in the text. Created with BioRender.com.

genes (Koerber et al., 2009); peptide insertion, where every virus particle is engineered to display a random peptide at the capsid surface (Muller et al., 2003); or error prone PCR, which involves amplifying AAV *cap* genes in error-prone PCR reaction, with the resulting PCR products cloned to generate a diverse AAV plasmid library (Koerber et al., 2006). A more recent approach called CREATE (Cre-recombination-based AAV targeted evolution) uses Cre/lox technology to generate novel capsids and involves delivering capsid genomes containing loxP sites to animals with Cre expression in a defined cell population and then selective amplification and recovery of *Cap* sequences that transduced the target population (Deverman et al., 2016). A recent approach called BRAVE (barcoded rational AAV vector evolution), allows for large scale selection of capsids using only a single *in vivo* round of screening, unlike previous methods that require multiple rounds of enrichment (Davidsson et al., 2019). The directed evolution approach has had the greatest success in shifting AAV tropism toward certain glial cell types, and examples of this are described in the appropriate sections below.

ASTROCYTES

Astrocytes play a role in several homeostatic functions within the brain and spinal cord, including controlling uptake and release of neurotransmitters, modulating synaptic activity and the supply of metabolites to neurons. Astrocytes are also components of the blood-brain barrier (BBB), where they play a crucial role in BBB integrity and function (Sweeney et al., 2019). As well as these supportive roles in normal CNS function they are well known to respond in a number of CNS disorders including Alzheimer's disease and other aging-related dementias (Dzamba et al., 2016; Garwood et al., 2017), Parkinson's disease (Booth et al., 2017), Huntington's disease (Palpagama et al., 2019), Amyloid Lateral Sclerosis (ALS) (Yamanaka and Komine, 2018) and traumatic conditions such as ischemia (Rossi, 2015), spinal cord injury (Gaudet and Fonken, 2018; Okada et al., 2018), and traumatic brain injury (Burda et al., 2016).

Astrocytes respond to CNS insult by transforming their phenotype via a process called reactive gliosis. Once stimulated by injury or inflammation, several cell pathways are activated that can be either damaging or protective, many of which could be targeted as a treatment. As a result of insult or neurodegeneration, astrocytes produce molecules such as inflammatory cytokines, which activate microglia and infiltration of peripheral immune cells leading to chronic inflammation (Stephenson et al., 2018). Following traumatic injuries of the CNS, activated astrocytes migrate to the lesion where they eventually form a glial scar that produces axonal growth

inhibitors, preventing axonal regeneration (Burda et al., 2016; Okada et al., 2018). Astrocyte activation leads to the loss of proper synaptic and plasticity regulation. The control of glutamatergic transmission by astrocytes is adversely affected by oxidative stress and increased production of pro-inflammatory factors (Liu et al., 2006; Santello et al., 2011). Another key role of astrocytes is in the regulation of ion flux, and disruptions to this interfere with neurotransmitter uptake by astrocytes (Djukic et al., 2007; Kucheryavykh et al., 2007). Changes in astrocytic modulation of synaptic function have been demonstrated in a model of ALS (Benkler et al., 2013). Expression of the potassium Kir4.1 channel is lost in the SOD1 mouse (Kaiser et al., 2006) and high levels of endothelin-1, which leads to activation of the AMPA receptor, is produced by activated astrocytes in this model and leads to motor neuron cell death (Ranno et al., 2014). Astrocytes produce a number of growth factors, including nerve growth factor (NGF), brain-derived growth factor (BDNF), and fibroblast growth factor (FGF) which all play an essential role in neuronal function (Miyazaki and Asanuma, 2017). Reduced growth factor levels have been associated with neurodegenerative disease. A decrease in serum BDNF levels is associated with cognitive impairment in dementias and changes in BDNF levels in the hippocampus may be linked with emotional symptoms relating to Alzheimer's disease (Budni et al., 2015).

As well as the negative effect of reactive gliosis, astrocytes can have protective effects. For example, activating the TGF-β signaling pathway in astrocytes limits the degree of inflammation following stroke (Cekanaviciute et al., 2014). As a reaction to oxidative stress, expression of Toll-like receptor-3 is increased in astrocytes, which upregulates anti-inflammatory cytokines whilst reducing the levels of pro-inflammatory cytokines (Bsibsi et al., 2006). The interferon pathway in astrocytes is also protective. Interferon regulatory factor 3 suppresses astrocyte inflammatory cytokine gene expression following inflammatory insult (Tarassishin et al., 2011). Interferon-1 production by astrocytes is known to regulate immune responses of brain endothelial cells via anti-inflammatory effects (Rothhammer et al., 2016).

Mutations in key astrocyte genes have been associated with neurodegenerative disease. Mutations of Fyn tyrosine kinase are associated with increased inflammatory responses in Alzheimer's disease (Lee et al., 2016, 2017). Mutations in the gene encoding TGF-β have been associated with AD risk (Caraci et al., 2012, 2018). Mutations in the astrocyte protein apolipoprotein E4 can impair amyloid-beta (Aβ) clearance (Liu et al., 2013) and may be linked to oxidative stress and inflammation (Liu et al., 2015). PARK7, a regulator of astrocyte metabolism has been found to be mutated in cases of familial Parkinson's disease (Bandopadhyay et al., 2004), is important for astrocyte mitochondrial function and its loss leads to oxidative stress (Kumaran et al., 2007; Larsen et al., 2011). Therefore, astrocytes are a potential cell target for AAV-mediated gene therapy strategies that modulate either the inflammatory or protective effects of reactive gliosis or through potentially expressing normal copies of mutated genes expressed in astrocytes.

AAV-Based Approaches for Targeting Transgene Expression to Astrocytes

A number of studies have looked at the tropism of AAV vectors for astrocytes. Most AAV serotypes demonstrate broad tropism without absolute specificity, but some differ in their absolute levels of transgene delivery to specific tissues. This depends on the experimental model as cell receptors for AAVs are likely expressed differently *in vitro* and *in vivo* (Royo et al., 2008). This is reflected in the variation of AAV tropism observed in each study as shown in **Table 1**.

In primary cultures of rat CNS cells, AAV5 appears to demonstrate the strongest glial tropism under the control of a constitutively active CAG or CMV promoter (Harding et al., 2006; Howard et al., 2008). Further, AAVs 1, 2, 6, 7, 8, and especially 9 can transduce both neurons and astrocytes (Howard et al., 2008; Royo et al., 2008; Schober et al., 2016). Newer, more novel serotypes have expanded the repertoire and potential for glial transduction, but many of these have yet to be compared with the naturally-occurring serotypes (Cearley et al., 2008).

In vivo animal studies have demonstrated some astroglial transduction with AAVs 1, 2, 5, 6, and 8 (Davidson et al., 2000; Wang et al., 2003; Harding et al., 2006; Klein et al., 2008; Hutson et al., 2012; Schober et al., 2016; Hammond et al., 2017), but AAVrh43 has been shown to have more specific astrocyte targeting when compared to AAV8 (Lawlor et al., 2009). In a separate study, AAV4 has demonstrated strong transduction of astrocytes when injected into the brain parenchyma (Liu et al., 2005). However, this has not been replicated in side-by-side comparisons with other serotypes. AAVs rh8 and rh10, in addition to rh43 and 9 can penetrate the BBB and transduce both neurons and glial cells (Foust et al., 2009; Gray et al., 2011; Yang et al., 2014). Glial transduction was more robust in adult animals, while transduction in neonatal animals was primarily neuronal (Foust et al., 2009; Gray et al., 2011; Yang et al., 2014). This may be an example of differential receptor expression causing altered tropism, in this case between neonatal and mature adult mice. Despite the existence of certain trends, many of the studies only compare a limited number of AAV serotypes and other variables are not controlled for between studies. Even AAV purification methods have led to differences in tropism (Klein et al., 2008). This indicates that for new experiments, it is worth comparing as many serotypes as possible to ensure the best choice for a specific set of experimental conditions.

Several synthetic AAVs that have been developed to improve transduction of the CNS have demonstrated improved ability to target astrocytes. AAV9P1 is a synthetic AAV9 variant that produces selective and robust astrocyte transduction *in vitro* (Kunze et al., 2018). This vector was identified from a screen of 30 artificial AAV variants, generated by introducing specific peptides into the AAV capsid sequence of AAV1, 2, 6, 8, and 9. While this variant was shown to have relatively good astrocyte specificity *in vitro* (the transduction rate for primary human neurons was around 10%), to date no data is available on whether this specificity is still seen *in vivo*. The CREATE approach led to the discovery of a variant AAV-PHP.B, which can transduce the CNS much more efficiently than AAV9, and is able to transduce

TABLE 1 | Astrocyte transduction of AAV serotypes with pan-cellular promoters.

Study	Hammond et al., 2017	Harding et al., 2006 (mouse)	Harding et al., 2006 (glioma)	Schober et al., 2016	Klein et al., 2008	Wang et al., 2003	Howard et al., 2008	Royo et al., 2008	Hutson et al., 2012	Lawlor et al., 2009	Davidson et al., 2000	Liu et al., 2005
Model	*In vivo*	*In vivo*	Xenograft	*In vivo/vitro*	*In vivo*	*In vivo*	*In vitro*	*In vitro*	*In vivo*	*In vivo*	*In vivo*	*In vivo*
Promoter	CBA	CAG	CAG	CMV	CMV	CMV	CMV	CMV	CMV	CAG	RSV	RSV
1/AAV2 ITR	++			++		++	−^	+	++			
2/2		+	++	++		+	−	++	+		+	
3/2									−			
4/2									−		−	++
5/2		++	++	++			++		++		++	
6/2		+	++	+++			−^		++			
7/2		+	++				−^	+				
8/2	++*	+	++		++		−^	++	+	+		
9/2	++*				−		−	++				
rh43/2										+++		

**, wild-type virus; ^, had transduced astrocytes 2 days after treatment.*
AAV, adeno-associated virus; CBA, CMV-Enhancer/Chicken β-Actin Promoter; CMV, Human Cytomegalovirus Immediate/Early Gene Promoter and Enhancer; CAG, Chicken β-Actin/Cytomegalovirus Hybrid Promoter; RSV, Rous Sarcoma Virus Long Terminal Repeat Promoter.

the majority of astrocytes (> 75%) in multiple CNS regions of the mouse brain (Deverman et al., 2016). However, this does not have selectivity for astrocytes as it can effectively transduce neurons and oligodendrocytes. Another variant AAV-PHP.A improved the selective targeting to more than 80% of transduced cells being ALDH1L1 + ve. However, when AAV-PHP.A and AAV-PHP.B were used to transduce human iPSC-derived cortical spheroids, only around 15% of AAV-PHP.A transduced cells were glial fibrillary acidic protein (GFAP)-positive, compared with 40% for AAV-PHP.B. There did not appear to be any difference in selectivity for astrocytes between the two variants (Deverman et al., 2016). The AAV capsid Anc80L65, developed using *in silico* reconstruction of the viral evolutionary lineage transduces astrocytes with around four times the efficacy of AAV9 (Hudry et al., 2018). A study that utilized molecular evolution to engineer novel AAV variants using directed evolution and a panel of 4 distinct AAV libraries found variants that had increased astrocyte transduction. Two AAV mutants, ShH19 and L1–12, transduced astrocytes 5.5- and 3.3-fold, respectively, compared to the parent AAV2. However, the percentage of astrocytes showing expression from these vectors was very low; 15% for ShH19 and 9% for L1–12 (Koerber et al., 2009). While these vectors described above are not astrocyte-specific, the use of these with astrocyte-specific promoters could have potential.

Most of the work to date developing astrocyte-specific promoters for gene therapy has focused on the use of the promoter for GFAP. GFAP is an intermediate filament protein that is expressed almost exclusively by astrocytes (Yang and Wang, 2015). This fact has led to its promoter being used to direct transgene activity to astrocytes, and there is a large amount of literature that shows this can be achieved. Many studies have tested the incorporation of GFAP promoters to drive astrocytic-specific expression using viral vectors. Transgene expression under the transcriptional control of the 2.2 kb human *GFAP* promoter, gfa2, has been shown to be expressed in astrocytes throughout the brain (Lee et al., 2008). However, as with many cell-type-specific promoters, the large size of this promoter has severe limitations when used with AAV vectors, due to it occupying a considerable amount of the vector genome. Different strategies have been employed to shorten the *GFAP* promoter to make it more suitable for use in AAV vectors. An AAV vector containing a truncated 448 base-pair gfa28 promoter (Lee et al., 2006, 2008), was able to drive gene expression much more strongly than the full-length promoter when tested *in vitro*. However, when this promoter was used *in vivo* in mice, the level of transgene expression driven by this promoter was comparable to the full-length gfa2 promoter, expression was restricted to certain CNS regions, and neuronal expression was observed as well as in astrocytes. Based on this finding Lee et al. (2008) created transgenic mice with promoters containing different enhancer fragments to determine which were required to silence neuronal signaling, and to restrict expression to specific brain regions. This work led to the discovery of a 681 bp *GFAP* promoter, gfaABC$_1$D, which exhibited mostly the same expression pattern in the brain as the full-length 2,210 bp gfa2 promoter but had a twofold greater expression that was largely restricted to astrocytes (Lee et al., 2008). Similarly a 681 bp gfaABC$_1$(mC$_{1.1}$)D variant had expression limited to astrocytes in the dorsal and caudal cortex, hippocampus and caudal vermis of the cerebellum. This study demonstrates that it may be possible to further limit gene expression to specific glial populations by modifying cell-specific promoters (Lee et al., 2008). de Leeuw et al. inserted additional copies of the *GFAP* enhancer regions to determine if these would increase its transcriptional activity. Injection of an adenoviral construct containing the gfa2 promoter engineered to contain three copies of the B enhancer region [gfa2(B$_3$)] resulted in greater gene expression in astrocyte cell cultures and expression that was limited to GFAP-positive cells when injected into the basal ganglia of mice (de Leeuw et al., 2006). However, again due to its size, there are issues in using this in the context of AAV vectors.

A number of studies have used AAV vectors containing the 681 bp gfaABC$_1$D with the goal of obtaining astrocyte specificity (Xie et al., 2010; Theofilas et al., 2011; Dirren et al., 2014;

Dvorzhak et al., 2016; Vagner et al., 2016; Taschenberger et al., 2017; Griffin et al., 2019, 2020; Testen et al., 2020). While several studies show good evidence of astrocyte specificity (Xie et al., 2010; Theofilas et al., 2011), other studies report transduction of other cell types (Taschenberger et al., 2017; Griffin et al., 2019). For instance, we observed transgene expression in lower motor neurons but not in neurons of the dorsal horn following vector infusion in the adult rat spinal cord (Griffin et al., 2019). Very high levels of transgene expression in lower motor neurons was also reported with the full-length GFAP promoter (Peel and Klein, 2000). One approach that has been used to overcome the issue of lack of astrocyte specificity is the incorporation of cell-specific microRNAs (miRNAs) to suppress off-target transgene expression in particular cell types (Brown et al., 2006). Endogenous expression of miR124, which is specific to neurons is able to repress gene expression in neuronal cells (Colin et al., 2009) and addition of miRNA recognition sequences to viral constructs can suppress leaky gene expression from AAV vectors (Shimizu et al., 2014). When target sequences for miR124 were included in the 3′ UTR of an AAV expression plasmid containing a transgene under the control of the gfaABC$_1$D promoter, neuronal transgene expression in the rat striatum was completely absent compared to around 10% neuronal expression with the gfaABC$_1$D promoter alone (Taschenberger et al., 2017). However, the presence of the miR sequence strongly reduces the number of astrocytes expressing the transgene to around 10% of that seen with the gfaABC1D promoter alone, calling into question the usefulness of this approach for improving astrocyte specificity.

One issue with the use of a GFAP promoter is that the levels of GFAP expression can be variable in different parts of the CNS and relatively low in some brain regions (Hajos and Kalman, 1989; Kalman and Hajos, 1989). Therefore using this promoter may not always be appropriate. Aldehyde dehydrogenase family 1, member L1 (ALDH1L1) has been characterized as a pan-astrocytic marker that is found more homogeneously throughout the brain than GFAP (Cahoy et al., 2008). Mudannayake et al. (2016) tested several different AAV serotypes under the control of a putative rat *Aldh1l1* promoter for astrocyte selectivity in the rat substantia nigra pars compacta (SNpc) brain region and found transgene expression was exclusively expressed in neurons and independent of AAV serotype used. Neuronal-specific transgene expression was also found following intrahippocampal vector infusion, but expression was found in both neurons and astrocytes in the striatum following intrastriatal vector infusion. In a later study by Koh et al. (2017) using a human *ALDH1L1* promoter, an AAV-hALDH1L1-Cre vector was injected into several brain regions of the Ail4 (RCL-tdTomato) mouse, found tdTomato gene expression was also seen to be predominantly neuronal in most brain regions analyzed. Interestingly, in the thalamus, this expression pattern was reversed, with the majority of tdTomato expression found in astrocytes (92%) with minimal neuronal expression (2%). Therefore, the use of the ALDH1L1 promoter may have the potential for targeting astrocyte expression in the thalamus, especially as GFAP expression in this region appears to be very low (Kalman and Hajos, 1989).

Other potential astrocyte-specific gene promoters have also been suggested (Kery et al., 2020) including *Slc1a3*, which codes for the glutamate transporter SLC1A3 (also known as GLAST or EAAT1) (Sery et al., 2015). A 636 bp region 5′ upstream of the gene can drive strong gene expression, and so this relatively small promoter might have potential use in AAV vectors (Hagiwara et al., 1996). Another potential promoter is *Gjb6*, which codes for the gap junction protein Connexin30. Connexin30 is only expressed in gray matter astrocytes and so this promoter could be used to specifically target these populations (Nagy et al., 1999; Sohl et al., 2004).

OLIGODENDROCYTES

Oligodendrocytes are the myelin-producing cells of the CNS. This myelin forms an insulating membrane that wraps tightly around axons that allows for rapid signal conduction and is crucial for normal CNS function (Kuhn et al., 2019). Oligodendrocytes and the myelin sheath also provide trophic support for axons, such as the production of neurotrophic factors (Bradl and Lassmann, 2010) and lactate that is passed to axons to partake in the metabolic pathways involved in producing ATP (Bercury and Macklin, 2015). Oligodendrocytes are particularly sensitive to excitotoxic and cytotoxic factors and damage of the CNS. The high metabolic rate required for myelination and the presence of high levels of iron, which is required as a co-factor for this process, can lead to high levels of reactive oxygen species, free radical formation and lipid peroxidation. This combined with the presence of low levels of the antioxidant enzyme glutathione in oligodendrocytes makes this cell type particularly sensitive (Bradl and Lassmann, 2010). Oligodendrocyte pathology is, therefore, present in a range of CNS disorders (Fern et al., 2014). This includes leukodystrophies, which are a group of inherited disorders that lead to white matter degeneration (Vanderver et al., 2015), multiple sclerosis (Procaccini et al., 2015), Alzheimer's disease (Nasrabady et al., 2018), Parkinson's disease (Bohnen and Albin, 2011), Fragile X syndrome (Filley, 2016), ischemic stroke (Wang et al., 2016), spinal cord, and traumatic brain injury (Fern et al., 2014; Hassannejad et al., 2019; Pukos et al., 2019), as well as in conditions such as schizophrenia and depression (Wang et al., 2014; Najjar and Pearlman, 2015). Gene therapy approaches have the potential to protect against toxicity or to promote remyelination.

AAV-Based Approaches for Targeting Transgene Expression in Oligodendrocytes

No natural AAV capsid that exhibits primary oligodendrocyte tropism has been described. While a small number of serotypes can transduce this cell type when combined with pan-cellular markers, the overall transduction efficiency is low (Lawlor et al., 2009). These include AAV8 and 9 when paired with a cytomegalovirus (*CMV*) promoter (Hutson et al., 2012; Bucher et al., 2014) and AAV8 with a chicken β-actin (*CBA*) promoter (Gray et al., 2010). In a preclinical study of metachromatic leukodystrophy, AAVrh10 has been found to transduce

oligodendrocytes when driven by a cytomegalovirus/β-actin hybrid (*CAG*/*cu*) promoter (Piguet et al., 2012). AAV-PHP.B's widespread transduction of the mouse CNS includes oligodendrocytes when using a *CAG* promoter (Deverman et al., 2016). DNA shuffling and directed evolution approaches have also produced a chimeric capsid that transduced both neurons and oligodendrocytes (Gray et al., 2010), with a novel AAV capsid shown to have excellent oligodendrocyte preference (Powell et al., 2016). The Olig001 vector, which contains a chimeric mixture of AAVs 1, 2, 6, 8, and 9 had a > 95% specificity for oligodendrocytes (as assessed by GFP expression) following striatal vector infusion into rats even though transgene expression was under the control of the CBA promoter. The other 5% of cells transduced were neurons, and no astrocyte or microglial expression was seen.

In order to specifically target transgene expression to oligodendrocytes, a number of cell-specific promoters have been trialed. Initial studies used the promoter from the gene for myelin basic protein (MBP), which is a major constituent of the myelin sheath of both oligodendrocytes and Schwann cells. A 1.9 kb *Mbp* promoter was able to drive GFP expression in the MOCH-1 transformed oligodendrocyte cell line and primary rat oligodendrocyte cultures (Chen et al., 1998). In primary cultures, GFP expression was almost exclusively in oligodendrocytes, although some expression was observed in astrocyte-like cells. When AAV vector was injected into the cerebral hemisphere of mice, GFP expression appeared to be only in oligodendrocytes and was not seen in astrocytes, microglia or neuronal filaments. Building on this work, the authors then investigated cell and tissue specificity and the duration of transgene expression following injection of the vector into different regions of the mouse brain (Chen et al., 1999). High-levels of GFP expression were almost exclusively seen in white matter areas of the brain with very limited expression in areas of gray matter. When cell-specificity was determined based on morphology, anatomic location, and cell-type specific immunohistochemistry, GFP expression was found to be almost exclusively in oligodendrocytes with no expression seen in neurons, astrocytes or microglia.

While this data suggests that cell-specific promoters such as those for MBP can be used to target oligodendrocytes specifically, it is also important to determine how the stage of development would impact this. This is essential to understand when using somatic gene transfer approaches for glia in the developing brain to treat genetic conditions such as leukodystrophies. von Jonquieres et al. (2013) tested this by injecting a chimeric AAV 1/2 vector expressing GFP under the control of the *Mbp* promoter into the striatum of mice at postnatal day 0 (P0) (neonates), P10 and P90 (adults). While in the P10 and P90 animals, the majority of GFP staining was localized to oligodendrocytes, for the P0 animals only around 25% of GFP + ve cells were oligodendrocytes, with the majority (56%) being astrocytes and the number of oligodendrocytes transduced was very low at only 3%. While for the P10 and P90 animals the majority of transduced cells were oligodendrocytes, there was a different pattern in the degree of transduction of other cell types. In the P90 animals, around 20% of transduced cells were neurons, and no GFP was detected in astrocytes. However, in the P10 animals, no GFP was detected in neurons, but it was seen in astrocytes, although in small numbers (3.6% of GFP + ve cells). Perhaps most interestingly at P10, transduction was almost exclusively in oligodendrocytes (96%), compared with around 75% in the adult mice. The authors suggested that this vector could allow for treatment of developmental gliopathies as brain development in the P10 mouse corresponds to that seen at the beginning of the last trimester in human pregnancy (Clancy et al., 2001). When the vector was injected into the brains of P10 homozygous ASPA$^{lacZ/lacZ}$ mice, which are a model of the early onset leukodystrophy Canavan disease, a similar pattern of oligodendrocyte specificity was seen as in WT mice (von Jonquieres et al., 2013). While this is promising for the potential to treat developmental gliopathies such as leukodystrophies, due to the marked difference in cell expression patterns seen between P0 and P10 animals, much more work would be required to understand changes in expression during human development before it could be used for such a purpose. In a subsequent paper these authors looked at transduction of non-chimeric AAV variants rh20, rh39, and cy5 and their ability to drive expression under the control of the *Mbp* promoter, in the striatum of adult mice. They showed that oligodendrocyte specificity was greater for rh39 (91%) and cy5 (87%) when compared to AAV 1/2 (78%) (von Jonquieres et al., 2016).

While the *Mbp* promoter seems to have potential for oligodendrocyte targeting, its relatively large size and the poor oligodendrocyte specificity following early neonatal vector delivery (von Jonquieres et al., 2013) would limit its usefulness. However, MBP-driven transgene expression from an AAV vector has been shown to be effective in a model of oligodendrocyte disease. The Cx32/Cx47 double-knockout mouse is a well-characterized model of hypomyelinating leukodystrophy-2. An AAV vector containing the *Gjc2/Cx47* gene under the *Mbp* promoter was delivered to the internal capsule of P10 animals. This resulted in greater survival and significant motor improvement, improved myelination and reduced oligodendrocyte apoptosis, inflammation and astrogliosis (Georgiou et al., 2017).

The myelin-associated glycoprotein (MAG) has been tested for its ability to drive oligodendrocyte-specific expression using AAV (von Jonquieres et al., 2016). MAG is a protein responsible for recognition of axons and maintenance of myelin. Based on an *in silico* analysis of the MAG promoter, the authors generated AAV plasmids expressing GFP under the control of either a 2.2 kb *MAG* promoter or truncated 1.5 and 0.3 kb fragments. All three AAV constructs were packaged into cy5 vectors and injected into the striatum of adult mice. All three constructs showed very good oligodendrocyte specificity, with GFP expression almost exclusively confined to oligodendrocytes, with between 98.4% for the 2.2 kb to 90.7% for the 0.3 kb promoter. The percentage of oligodendrocytes transduced was 65, 82, and 57% for the 2.2, 1.5, and 0.3 kb promoters, respectively. When the vector containing the 2.2 kb promoter was injected into the brains of P0 pups, the specificity for oligodendrocytes was seen to be around 80% in comparison to the *Mbp* promoter where only around 25% specificity for oligodendrocytes was seen, suggesting use of the *MAG* promoter may be better suited to treatment of developmental gliopathies. However, the authors did not

test the smaller promoters. The specificity and percentage of oligodendrocytes transduced appeared to be less for the smaller promoters. It would have been interesting to look at their profile in P0 pups, especially as the 0.3 kb promoter is likely to be the most useful for use with AAV vectors due to its small size.

A recent report by Powell et al. (2020) demonstrated that modification of constitutive promoters can shift gene expression from neurons to oligodendrocytes. Infusion of an AAV9 vector with a full-length *CBA* promoter into the rat striatum led to predominantly neuronal (88.4%) transgene expression. However, the use of a truncated CBA promoter (*CBh*) showed only 46% of neurons but 38% of oligodendrocytes were labeled. When an AAV2 vector was used, expression was predominantly neuronal for both promoters. This suggests that certain AAV capsids can influence promoter activity between different cell types. When six glutamate residues were inserted into the VP2 region of the AAV9 capsid, this shifted the transgene expression profile from the full-length promoter from neurons to oligodendrocytes (80%). However, when these amino acids were changed in AAV2, no change in expression was seen. This ability for capsid sequence to influence gene expression could be used to target particular cell types.

MICROGLIA

Studies have tested various AAV serotypes for their ability to transduce microglia, and the level of transduction is generally low (Maes et al., 2019). In early studies, Bartlett et al. (1998) found that while AAV2 was able to transduce microglia, as determined using Cy3-labeled virions, no transgene expression was observed. Similarly, no transgene expression was observed in primary microglia cultures following application of AAV1–9 or rh10 vectors (Rosario et al., 2016) although others showed 80% of the cells expressed GFP when AAV2-CMV-eGFP was applied to primary neonatal and adult microglia (Su et al., 2016). When different serotypes (AAV2, AAV5, AAV6, AAV8, and AAV9) were applied to cultured neonatal microglia, AAV6-CMV-eGFP produced an 80-fold increase in transgene expression compared to AAV2-CMV-eGFP while AAV8 resulted in a 25-fold increase in transgene expression. However, when the expression of microglial M1 and M2 activation markers was assessed to determine the effect of viral vector-mediated transduction and/or transgene expression, AAV6 and AAV9 elevated expression of the scavenger receptor MARCO, while the M2 phenotype marker YM1 was down-regulated in cells treated with AAV5 and AAV8. AAV2 did not produce any significant change in any activation markers assessed or influence the phagocytic activity of the microglia (Su et al., 2016). Therefore, development of an AAV to target microglia *in vivo* will require an understanding of the effect a serotype will have on the activation state of microglia, to prevent any unwanted, potentially detrimental inflammatory side-effects of the treatment.

Several macrophage-specific promoter sequences, human *CD11B* and *CD68*, and murine *F4/80*, have been assessed for their ability to provide specific microglial targeting both *in vitro* and *in vivo*. When AAV2 and AAV5 vectors expressing transgenes under control of these promoters were applied to rat primary microglia cultures, transduction efficiencies of around 25% for *F4/80*, 10% for *CD68*, and only one cell per field-of-view for *CD11B* were reported for both vectors. While these transduction efficiencies are low, transgene expression did appear to be microglia-specific as no expression was detected following transduction of rat primary neuronal cultures (Cucchiarini et al., 2003). When AAV5 vector containing the *F4/80* promoter was injected in to brains of adult rats, transgene expression appeared largely localized to F4/80-labeled cells.

Modified AAV capsids have also shown some success in improving transduction of microglia. Rosario et al. modified the AAV6 capsid (AAV6) through site-directed mutagenesis of two tyrosine residues to phenylalanine and a threonine to valine (Y731F/Y705F/T492V), which has been shown to increase transduction efficiency in monocyte-derived dendritic cells (Pandya et al., 2014). The combination of this modified AAV capsid and either the *F4/80* or *CD68* promoter resulted in > 95% transduction of microglia and no neuronal or astrocytic expression in mixed glial or primary microglial cultures. When tested *in vivo*, microglial specificity was shown to be around 75% for F4/80 and 20% for CD68 following injection into P0 rat pups and the adult rat hippocampus, although the total numbers of microglia showing expression appeared to be low (Rosario et al., 2016).

An important consideration is the impact that the activation state of microglia will have on expression from specific vectors used. For example, levels of CD68 are greater in pro-inflammatory microglia, which could explain the low level of expression seen in a healthy brain. This expression pattern could potentially be of use when targeting activated microglia, where it is likely that gene expression would be highest in this population of cells.

MÜLLER CELLS

Müller glial cells are a major cellular component of the retina and engage in numerous roles vital to retinal function, such as structural, nutritional, homeostatic, osmotic, metabolic, and growth factor support to retinal neurons (Bringmann et al., 2006; Reichenbach and Bringmann, 2013). These glial cells also interact with blood vessels and are involved in the function of the blood–retina barrier and regulating retinal blood flow (Newman, 2015). In response to retinal injury, stress, or degeneration, Müller glial cells undergo active gliosis (Graca et al., 2018). A number of diseases including diabetes, macular edema, and ischemia lead to hypertrophy and hyperplasia of the Müller glia that contributes to a chronic inflammatory retinal environment and ultimately cell death (Coughlin et al., 2017). It has been demonstrated that Müller cells have a neuroprotective phenotype. For example, they respond to disease or injury-mediated photoreceptor stress by upregulating secretion of neurotrophic factors such as basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) (Greenberg et al., 2007). Mutations in several genes expressed in Müller glial cells have been linked to retinal dystrophies (Maw et al., 1997;

Zhao et al., 2015). Therefore Müller glial cells have been proposed as targets for therapeutic approaches to retinal diseases such as gene replacement therapy or boosting neurotrophin secretion to enhance their neuroprotective properties (Dorrell et al., 2009; Pellissier et al., 2015). As these cells transverse the entire thickness of the retina, they would have the ability to facilitate expression of factors throughout all layers of the retina (Reichenbach and Bringmann, 2013).

A number of different AAVs have been shown to have tropism for Müller cells. When AAV 1, 2, and 5 were delivered to the retinas of E13 mice via subretinal injection, only AAV1 and 2 transduced Müller cells (Surace et al., 2003). Intravitreal injection of AAV 1, 5, 8, and 9 showed occasional Müller cell transduction in 2 month-old C57BL/6 mice (Lebherz et al., 2008). Subretinal injection of AAV8 and 9 also shows Müller cell transduction in 4 week-old mice, but transduction was not seen with AAV5 (Allocca et al., 2007). When retinal transduction by AAV2 was compared following intravitreal injection into P0, P14 and adult mice, transduction was seen in Müller cells at all ages with highest levels of transduction observed in adults, where around 10% of AAV transgene expression was observed (Harvey et al., 2002). AAV4 and 6 also have tropism for Müller cells (Hellstrom et al., 2009). In all these cases the number of Müller cells transduced was low, compared with other retinal cell populations. However, Pellissier et al. (2014) reported that use of AAV9 vectors led to transduction of around 30% of Müller cells along with photoreceptor cells and retinal pigment epithelia. These authors used AAV9 under the control of a CMV promoter to deliver the Crumbs-homolog-2 (CRB2) protein, which is expressed in Müller cells and photoreceptors, to the retinas of *Crb2* and *Crb1Crb2*$^{F/+}$conditional knock-out mice, which are both models of severe, progressive retinal degeneration (Pellissier et al., 2015). This led to improved retinal function and morphology compared to untreated animals, demonstrating the potential for AAV to target and modify Müller cell-related gene mutations.

As Müller cells express GFAP, this has been used to target transgene expression to these cells. AAV-mediated delivery of neurotrophic factors, under control of the *GFAP* promoter has been shown to be protective in animal models of retinal disease. A mouse model that has a defective gene for the VLDL receptor (VLDLR) shows excessive retinal neo-vascularization (NV), which is associated with common causes of vision loss (Klein et al., 2004). The retinal phenotype of these animals is similar to that seen in human patients with certain retinal NV diseases, including glial abnormalities as evidenced by increased Müller cell activation associated with the area of NV. In order to target activated Müller cells as a potential therapy, Dorrell et al. (2009) tested an AAV2 vector containing GFP under the control of a minimal 0.35 kb human *GFAP* promoter consisting of the A/B and D sequences (Besnard et al., 1991). When the vector was delivered by intravitreal injection to wild-type mice, limited GFP expression was noted in Müller cells while in *Vldlr*$^{-/-}$ mice, a greater number of Müller cells expressed GFP, and this was also associated with transgene expression in processes adjacent to areas of NV. GFP expression appeared to be Müller cell-specific, unlike non-specific expression mainly localized to ganglion cells that was found using a non-selective *CAG* promoter. The authors then used this system to deliver neurotrophin-4 (NT-4), which is known to protect neurons in models of retinal degeneration. Using an AAV-GFAP-NT4 vector, a similar expression pattern of NT-4 as found for GFP was observed, and this was associated with protection of the retina from neuronal degeneration.

A number of AAV variants have shown enhanced targeting for Müller cells. Based on work which determined novel AAV capsids that more efficiently transduced both primary human astrocytes *in vitro* and rat astrocytes *in vivo* (Koerber et al., 2009) and the shared properties between astrocytes and Müller cells, Klimczak et al. (2009) explored the effectiveness of a number of AAV variants for their potential for intravitreal transduction of Müller cells. Compared to very low transduction from the parent AAV6, one novel variant named ShH10 demonstrated a striking increase in both transduction efficiency and specificity for Müller cells. ShH10 was able to produce diffuse expression throughout the retina with approximately 94% of transduced cells being Müller cells, with limited transduction in interneurons (2%), and retinal ganglion cells (4%) compared with approximately 76% of transduced cells being Müller cells, 3% interneurons, and 21% retinal ganglion cells for the related AAV2 virus. ShH10 was also more efficient at transducing Müller cells, transducing 22 vs. 14% of total Müller cells compared with AAV2. These authors then extended to testing the ability of the ShH10 vector to deliver GDNF to the retina via Müller cells, and its ability to modulate retinal degeneration in the S334-4ter rat model of retinitis pigmentosa (Dalkara et al., 2011). To optimize GDNF production by improving the transduction capability of the vector, they modified ShH10 via a tyrosine to phenylalanine amino acid change to create ShH10.Y445F. Mutations in conserved tyrosine residues in AAV capsids have been shown to enhance vector transduction including in the retina (Zhong et al., 2008; Petrs-Silva et al., 2009). This vector led to transduction of approximately 50% of Müller cells in the retina of TgS334 rats. This was over 100% greater transduction than that seen in WT rats, demonstrating that this mutation allows for a greater transduction efficiency, although the authors also acknowledge that the degenerating retina may be a more permissive environment for AAV transduction. When this vector was used to deliver a GDNF transgene in the diseased retina, they were able to demonstrate long-term expression of therapeutic and safe levels of GDNF for up to 5 months, and this was accompanied by a slowdown in retinal degeneration, as assessed by electroretinogram and histology. Interestingly, when the ShH10Y vector was used to specifically target Müller cells in the *Crb2* and *Crb1Crb2*$^{F/+}$ models described above, no improvement in retinal function was seen (Pellissier et al., 2015), as opposed to AAV9, which targeted Müller cells and photoreceptor cells and led to functional improvement. This demonstrates that depending in the disease, specific targeting of just one cell type with an AAV may not be appropriate. These vectors combined with the use of cell-specific promoters have been used in an attempt to further improve Müller cell specificity (Pellissier et al., 2014). Pellissier et al. (2014) looked at the transduction profile of AAV6 and the two AAV6-derived variants ShH10 and ShH10Y via injection into the vitreous of *Crb1*$^{-/-}$ mice, a model of retinal dystrophy. The ShH10Y variant had an enhanced ability to

transduce Müller cells, with 2–3 times the transduction efficiency of the other vectors. This supports the idea that the increase in transduction seen by Dalkara et al. (2011) is due to the vector modification and not the diseased environment. The authors tested ShH10Y vectors with GFP expression under the control of a full-length (1.8 kb) or minimal (0.4 kb) *Cd44* promoter or the 2.8 kb *RLBP1* promoter (Pellissier et al., 2014). However, the *Cd44* promoters showed only low levels of expression, that was not restricted to Müller cells. Conversely, the *RLBP1* promoter caused high levels of expression that was restricted to Müller cells. However, due to its size, a shortened promoter would likely be required for it to be of practical use for expression of most genes. More recently Cao et al. (2020) found using an AAV2/6 mutant rAAV2/6-S663L led to increased tropism for Müller cells when injected intravitreally into 5 week-old mice and when gene expression as driven by the *GFAP* promoter that gene expression was specifically seen in Müller cells.

Due to the fact that natural "cell-specific" promoters often target expression to more than one cell type, attempts have been made to screen a library of promoters driving transgene expression in regions of interest. Jüttner et al. developed a library of 230 AAVs, each with a different synthetic promoter and tested their transduction in the retina of mice, non-human primates and humans (Juttner et al., 2019). This screen found a number of synthetic promoters that targeted Müller cells with 100% specificity and one in particular (labeled ProB2) transduced 45% of Müller cells.

Attempts have been made to create vectors that will restrict gene expression to an injured retina. Hypoxia in the eye is known to be a causative factor in a number of retinal diseases, such as diabetic retinopathy and age-related macular degeneration (Campochiaro, 2015). When an AAV2 vector containing a hybrid promoter consisting of the $GfaABC_1D$ promoter and hypoxia-responsive and aerobically silenced elements (HRSE) (Wenger, 2002) was injected into a mouse model of oxygen-induced retinopathy, high levels of gene expression was seen in Müller cells of damaged eyes but was completely absent in mice exposed to normoxia (Prentice et al., 2011). This approach would allow for gene expression only in regions of hypoxia, which could be beneficial in reducing off-target effects of gene expression in healthy tissue.

PERIPHERAL NERVE

Schwann Cells

Schwann cells are the major glia of the PNS. Two types of Schwann cells are found; myelinating, which form a myelin sheath around peripheral axons and non-myelinating, which are involved in maintaining ionic balance and providing support to axons (Kidd et al., 2013). Many neuropathies are related to mutations or inflammation of immune cells (Martini and Willison, 2016). Charcot-Marie-Tooth (CMT) disease, or non-syndromic inherited peripheral neuropathy, is one of the most common neurogenic disorders. Clinical characteristics include distal weakness, sensory loss and deformities of the feet. It is a genetically heterogeneous set of disorders with over 100 different genes implicated in disease causation. Mutations can occur in myelinating Schwann cells, and these cause demyelinating forms of neuropathy (Laura et al., 2019; Sargiannidou et al., 2020). A number of gene therapy approaches using lentiviral vectors for gene replacement strategies in models of CMT have been trialed and demonstrated promise (Eggers et al., 2013; Allodi et al., 2014; Sargiannidou et al., 2015; Kagiava et al., 2016). To date, only a few studies have looked at the feasibility of using AAV vectors to target Schwann cells in CMT.

Several AAV vectors demonstrate tropism for Schwann cells. Hoyng et al. (2015) tested AAV 1–9 for their ability to transduce cultured primary rat and human Schwann cells and rat and human nerve segments. This study showed a few differences in tropism between the species and between cells and nerve segments. AAV1 was the most efficient at transducing rat Schwann cells, with twice the number of transgene-expressing cells compared to any other AAV tested. In human Schwann cells, AAV2 and AAV6 were seen to perform equally well. However, a different transduction pattern was seen in nerve segments. AAV 1, 5, 7, and 9 were all equally successful in transducing rat nerve segments, whereas AAV2 was superior in human nerve segments. More recently, AAV1, AAV2, AAV6, and AAV-DJ were found to efficiently transduce primary human Schwann cells, with levels of transgene expression for AAV6 and AAV-DJ being 2–3 times that seen in AAV1 or 2 (Bai et al., 2019). A study by Homs et al. (2011) to determine whether AAV vectors could specifically target Schwann cells found that sciatic nerve injection of AAV8 led to specific Schwann cell expression with limited ($< 1\%$) neuronal gene expression, unlike the CNS where significant neuronal tropism is seen. This could be explained by differences in the expression of receptors between the CNS and PNS (Homs et al., 2011).

Due to the presence of common transcription binding elements, a number of oligodendrocyte promoters are also able to drive gene expression in Schwann cells. However, the size of these promoters precludes their use in AAV vectors. A full-length *Mbp* promoter is capable of driving expression in Schwann cells but the shorter 1.3 or 1.9 kb fragments shown to drive expression in oligodendrocytes do not contain the enhancer elements required for Schwann cell expression (Mathis et al., 2000; Forghani et al., 2001). The 2,3-cyclic nucleotide (*Cnp*) and proteolipid protein (*Plp*) promoters have been shown to be expressed in Schwann cells, and a *Cnp* promoter has been used to drive expression in oligodendrocytes using lentivirus, but are again too big to use with AAV (Kagiava et al., 2014; Schiza et al., 2015). The 2.2, 1.5, and 0.3 kb fragments of the *MAG* promoter should drive expression in Schwann cells, but this has not been tested. These promoter fragments contain an RNF10 site which has been suggested would allow transgene expression in Schwann cells (von Jonquieres et al., 2016). The myelin-specific myelin protein zero (*Mpz*) promoter has been shown to have high selectivity for Schwann cells. This along with its relatively short length (1.1 kb) (Messing et al., 1992) and its successful use in targeting Schwann cells using lentivirus (Sargiannidou et al., 2020), suggests it is likely to be a good candidate for use with AAV vectors.

Satellite Cells

Another glial cell type found in peripheral nerves are satellite cells. These are associated with neurons in the sensory, sympathetic and parasympathetic ganglia and are thought to play similar functions to astrocytes in the CNS (Hanani, 2005, 2010). Following nerve injury, satellite cells become activated, leading to chemokine/cytokine release (Ohara et al., 2009; Souza et al., 2013). This activation is an important component of pain signaling, and dysregulation can lead to chronic pain (Gosselin et al., 2010; Ji et al., 2013). Modulation of satellite cells can alter the pain responses after nerve injury, and genetic manipulation of satellite cells has been proposed as a potential treatment for pain control (Jasmin et al., 2010). A recent study tested different AAV vectors for their ability to transduce satellite cells. AAV6 as well as AAV shH10, and AAV shH19, which have been shown to have a preference for transduction of Müller glia in the retina were used and injected into the dorsal root ganglion (DRG) of adult rats. Strong expression that was restricted to neurons was observed when transgene expression was under the control of a *CMV* promoter. Conversely, when expression was driven by a *GFAP* promoter, expression was almost exclusively in satellite cells (Xiang et al., 2018). Interestingly the AAV shH10 vector, the novel capsid variant of AAV6 that demonstrates almost exclusive glial tropism in the retina, was almost exclusively neuronal on the DRG. This suggests that cellular receptors for different AAVs can vary between different glial populations in the CNS and PNS.

ENTERIC GLIA

The gut contains its own nervous system, termed the enteric nervous system (ENS) which regulates gastrointestinal functions such as motility, local blood flow, transport of molecules across the mucosa and modulates endocrine and immune functions (Costa et al., 2000). It consists of two interconnected ganglionated plexuses that surround the digestive tract. As well as enteric neurons, the ENS contains enteric glia, which are present in numbers up to 6 times higher than the number of neurons. These glia express astrocyte markers such as GFAP and S100 and are typically thought of as astrocytes of the gut. The role of enteric glia in gut function is not completely understood, but they are involved in regulating motility via interactions with enteric neurons (McClain et al., 2014). Furthermore, they also play a role in the maintenance of gut epithelial integrity and loss of enteric glia has been shown to lead to intestinal inflammation (Aube et al., 2006). It has also been suggested that enteric glia may influence barrier integrity through interaction with immune cells (Ibiza et al., 2016). The ability of enteric glia to mediate immune responses could be a possible underlying mechanism for Crohn's disease (Pochard et al., 2018) and they are known to contribute to the inflammation that occurs with conditions of the gut including irritable bowel disease, enterocolitis, and gut infections (von Boyen et al., 2004; Linan-Rico et al., 2016; Lilli et al., 2018). An age-related decrease in the number of enteric glia may play a role in chronic, low-grade inflammation that is associated with age-related gut motility disorders (Franceschi et al., 2007). Treatments for Crohn's disease are limited and can have serious side-effects (Seyedian et al., 2019). Therefore, gene therapy targeting of enteric glia with neuroprotective strategies in the early stage of such diseases, may be a useful approach to help treat these disorders.

Several studies have looked at the delivery of AAV to the gastrointestinal tract. Oral or enema delivery of AAV serotypes 1–10 have shown transduction of GI tissue including the lamina propria and endothelial cells, but transduction of the ENS was not determined (Shao et al., 2006). When AAV vectors were injected directly into the descending colon, neuronal and enteric glia transduction was observed in the myenteric and submucosal plexuses (Benskey et al., 2015). Of the serotypes tested, AAV serotypes 1, 5, 6, 8, and AAV8-double Y-F + T-V showed both neuronal and glial transduction. However, the authors note that glial transduction was rare for most serotypes except for AAV6, where the level of transduction was roughly equal between neurons and glia. Systemic delivery of AAV serotypes has demonstrated tropism for enteric glia, although few studies have attempted to target glia directly. Both intravenous and intrathecal delivery of AAV9 to juvenile mice leads to transduction of enteric ganglia, but no glial transduction was reported (Schuster et al., 2014). Gombash et al. (2014) showed that intravenous injection of scAAV8 and scAAV9 (which have known tropism for astrocytes) containing a GFP reporter into neonatal and juvenile mice led to GFP expression that was found exclusively in myenteric neurons. GFP expression was occasionally detected in S100 positive glia in neonatal animals for scAAV8. To determine if tropism could be directed toward glia, the authors engineered an AAV9 vector with GFP expression under the control of the *GFAP* promoter, as GFAP expression is present in enteric glia, similar to astrocytes in the CNS. Following intravenous injection of an AAV9-GFAP-GFP vector into neonates, GFP expression was found principally in enteric glia of the myenteric ganglia. However, it should be noted that the number of glial cells transduced was less than 5%. This study also trialed AAV 6, which has been shown to cross the BBB (Zhang et al., 2011) but no transgene expression was detected. This is likely due to differences in vasculature between the ENS and CNS that impact on the virus's ability to cross the endothelial barrier. In another study (Buckinx et al., 2016) using AAV8 and AAV9 to transduce myenteric and submucosal neurons, about 25–30% of neurons were found to be expressing eGFP. All subtypes of neurons expressed GFP, but no expression was seen in glia (assessed using S100 and GFAP). While it appears that AAV-mediated transgene expression can be somewhat tailored toward enteric glia, further work is required to make this a viable approach to target these cells. One issue is the use of a GFAP promoter and systemic delivery of AAV9, as this would make specific targeting of enteric glia, without potential off-target effects on other GFAP-expressing cells, impossible. Currently, an enteric-glia-specific promoter has not been identified. However, transcriptional profiling of enteric glia suggests that they are developmentally closer to oligodendrocytes and Schwann cells than astrocytes and can adopt some Schwann cell markers (Rao and Gershon, 2018). However, the gut microenvironment shifts these cells toward an astrocyte-like phenotype as shown by expression of GFAP, which is not seen in Schwann cells (Gulbransen and Christofi, 2018).

Therefore, enteric glia are considered a novel type of glia, and that an enteric glia-specific promoter may need to be developed. This different phenotype may also explain the low transduction efficiency of enteric glia compared with other glial types. Further testing of novel AAVs would likely be needed to make this a viable approach to therapy.

CONCLUSION

Through the use of specific serotypes and cell-specific promoters, glial cell targeting is possible and has shown some promise in experimental models, particularly in the retina. However, there are still several hurdles that need to be overcome for truly glial-specific AAV tropism to be achieved for use in human gene therapy. While several AAV serotypes have increased tropism toward glia, they are not glia-specific, and so any gene therapy transgene will be present in other cell types. Furthermore, the percentage of glial cells that are transduced is also often low, raising the question of whether transgene expression levels would be enough to have a therapeutic effect. The development of promoters that are highly specific for a particular glial cell type would be beneficial to avoid the possibility of off-target effects which might be likely with systemic delivery of an AAV vector expressing transgenes under the control of the GFAP promoter for example, due to it being expressed in a number of glial cell types throughout the body. An issue with the use of cell-specific promoters is that they are often large, which can preclude their use in an AAV vector context. However, a number of studies have demonstrated the use of shortened promoter sequences for driving glial cell-specific transgene expression. The development of new methods for synthetic promoter design holds real promise (Juttner et al., 2019). This study used a number of approaches to design synthetic promoters based on conserved upstream sequences of highly cell-specific genes and cis-regulatory regions active in cell types of interest. This approach led to the discovery of promoters that provide 100% specific gene expression in different retinal cell types, including the 500 bp ProB2 promoter, which shows 100% specificity for Müller cells. Applying this approach to other cell types could lead to the discovery of highly cell-type-specific promoters of appropriate size for use in AAV vectors.

The AAV literature also reports differences in transduction results depending on the model used and the method of delivery. Observations from *in vitro* studies often do not translate to the *in vivo* situation, and differences can be seen depending on the animal species or strain used or the method of delivery. For example, the ability to systemically deliver an AAV vector with a particular cellular tropism or cellular promoter would be beneficial. The variants AAV9-PHP.B and PHP.eB have been reported to allow for significant transduction of the CNS following intravenous infusion but depending on the animal model or method of delivery used, different results are seen (Hordeaux et al., 2018; Simpson et al., 2019). While enhanced CNS tropism has been shown in a number of mouse strains including C57BL/6J mouse where it was first demonstrated, no such increase in transduction efficiency is seen in the BALB/cJ mouse strain (Hordeaux et al., 2018; Matsuzaki et al., 2019). These results are explained by lower levels of the receptor for AAV9-PHP.B, LY6A, in BALB/cJ mice (Huang et al., 2019; Batista et al., 2020). When this virus was delivered to cats, sheep and non-human primates the efficiency of transduction of the CNS was low (Hordeaux et al., 2018; Matsuzaki et al., 2018; Liguore et al., 2019; Batista et al., 2020; Liu et al., 2020) and these species, as well as humans, have no known ortholog for *Ly6a* (Batista et al., 2020). This demonstrates that the use of non-human species to select novel AAV capsid variants could inadvertently limit the usefulness of candidate capsids that are isolated. These differences can also be more subtle. Using the cell-type-specific markers GFAP and Olig-2, He et al. (2019) assessed the cell-specific tropism of AAV serotypes 2, 5, 7, 8, and 9 in C57 and FVB mice. In the case of oligodendrocyte transduction, AAV8 resulted in 23% of oligodendrocytes being EGFP positive in C57 mice, significantly more than was seen measured in FVB mice at only 4.4%. In FVB mice, AAV7 vectors transduced a significantly larger number of oligodendrocytes than AAV8 vectors. Therefore, the choice of model can have a significant impact on the results that are obtained, and it will be important to understand the species and cell-type specific localization of AAV receptors to ensure accurate targeting of AAVs.

In a review by Maes et al. (2019) on the use of gene therapy to target microglia, the authors proposed guidelines for the reporting of viral transduction of this cell type. A similar set of guidelines for design and reporting of glial-targeting gene therapy studies could be of benefit for moving these approaches to clinically useful interventions. In order to confirm the results observed in animals, experiments using human model systems are crucial. While a number of non-human primate studies of AAV tropism have been carried out, these are not practical or financially viable for many research groups to undertake. Studies could be carried out in primary cells derived from the human brain; however, these may not express the same AAV receptors and lack the differentiation of the *in vivo* environment. In order to better understand the tropism and glial cell-specific transduction of AAV vectors, studies using primary human cells as close to their *in vivo* context are needed (Lisowski et al., 2015). One example that has been proposed is the use of humanized mouse models containing chimeric tissues, such as the FRG mouse model that allows animals to be generated with chimeric mouse-human livers (Azuma et al., 2007). While such an approach does not apply to the nervous system, the use of induced pluripotent stem cells (iPSCs) and human brain organoids have potential to understand tropism and expression of transgenes in human cells. Recent studies have used this approach to study AAV transduction and expression in the retina (Mookherjee et al., 2018; Quinn et al., 2019; Tornabene et al., 2019; Garita-Hernandez et al., 2020; Lane et al., 2020). The study by Quinn et al. (2019) specifically looked at the expression in Müller cells within the organoids. Using AAV and ShH10Y445F vectors with either the CMV or RLBP1 promoter showed good Müller cell transduction, with the authors reporting a ShH10Y445F-RLBP1-GFP vector being specific for transduction in Müller cells. A similar tropism and expression potency was seen in cultured adult human retinal explants. This is an important

observation, as it demonstrates findings seen in organoids may recapitulate what would be seen in adult human tissue. Studies have also used human iPSC-derived cerebral organoids to study AAV transduction (Latour et al., 2019; Depla et al., 2020). However, these studies only looked at neuronal transgene expression achieved from the AAV vectors used. While organoids are likely useful for testing AAV for treating developmental disorders, using optimal differentiation conditions enables the creation of cerebral organoids containing mature neurons and astrocytes (Yakoub, 2019) that could be used to study AAV glial tropism. Models of human intestinal organoids that contain an ENS (including glia) could potentially be used for this purpose (Schlieve et al., 2017).

AUTHOR CONTRIBUTIONS

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frontiers in Molecular Neuroscience

MINI REVIEW
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NPY and Gene Therapy for Epilepsy: How, When,... and Y

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Neuropeptide Y (NPY) is a neuropeptide abundantly expressed in the mammalian central and peripheral nervous system. NPY is a pleiotropic molecule, which influences cell proliferation, cardiovascular and metabolic function, pain and neuronal excitability. In the central nervous system, NPY acts as a neuromodulator, affecting pathways that range from cellular (excitability, neurogenesis) to circuit level (food intake, stress response, pain perception). NPY has a broad repertoire of receptor subtypes, each activating specific signaling pathways in different tissues and cellular sub-regions. In the context of epilepsy, NPY is thought to act as an endogenous anticonvulsant that performs its action through Y2 and Y5 receptors. In fact, its overexpression in the brain with the aid of viral vectors can suppress seizures in animal models of epilepsy. Therefore, NPY-based gene therapy may represent a novel approach for the treatment of epilepsy patients, particularly for pharmaco-resistant and genetic forms of the disease. Nonetheless, considering all the aforementioned aspects of NPY signaling, the study of possible NPY applications as a therapeutic molecule is not devoid of critical aspects. The present review will summarize data related to NPY biology, focusing on its anti-epileptic effects, with a critical appraisal of key elements that could be exploited to improve the already existing NPY-based gene therapy approaches for epilepsy.

Keywords: viral vectors, epilepsy, gene therapy, Y2 receptor, NPY

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NPY DISCOVERY, EVOLUTION, AND FUNCTION

Described in 1982, neuropeptide Y (NPY) is a 36-aminoacid peptide that shares high homology with its family members pancreatic peptide (PP) and peptide YY (PYY). The NPY ancestral gene appeared in vertebrates, evolving from an ortholog NPY-like system that regulates energy homeostasis in invertebrates acting on growth and reproduction (De Jong-Brink et al., 2001; Kooijman and Troost, 2007; Gershkovich et al., 2019). The family of Y peptides probably originated through a chromosome quadruplication event that took place during jawed vertebrate emergence (Larhammar and Salaneck, 2004).

NPY has a widespread expression throughout the central (CNS) and peripheral nervous system (PNS) and it is typically co-released with other neurotransmitters. An unusually broad repertoire of receptor subtypes mediate its actions, each activating specific signaling pathways in different tissues and cellular sub-regions (Leblanc et al., 1987; Keast, 1991; Dumont et al., 1992; Elfvin et al., 1997; Cerdá-Reverter and Larhammar, 2000; Wai et al., 2004).

During evolution, the NPY-like system has increased the complexity of its actions, with effects that in humans range from cell proliferation to the control of energy metabolism, pain and neuronal activity (Kuo et al., 2007; Tilan and Kitlinska, 2016). NPY is involved in cardiovascular and metabolic diseases, as well as in respiratory and neurologic disorders (Pedrazzini et al., 2003; Vezzani and Sperk, 2004; Atanasova and Reznikov, 2018), acting as a paracrine hormone in the periphery and behaving like a neuromodulator in the CNS.

In the CNS, NPY exerts its modulatory action both at cellular (excitability, neurogenesis) and at circuit level (food intake, stress response, and pain perception). It is expressed in different areas of the brain, from the neocortex to the posterior root of spinal nerves, usually in GABAergic interneurons, but also in long projecting catecholaminergic neurons; e.g., in the brainstem and in certain hypothalamic nuclei (Chronwall et al., 1985; de Quidt and Emson, 1986; Silva et al., 2005a; Benarroch, 2009). In the mesial temporal lobe, NPY is widely expressed in different subnuclei of the amygdala, where it is thought to exert a potent anxiolytic effect (Tasan et al., 2010; Wood et al., 2016), and in the hippocampus, where it displays an inhibitory action on excitatory synaptic transmission, mostly by reducing glutamate release (Colmers et al., 1985; Klapstein and Colmers, 1992; Greber et al., 1994; Mcquiston and Colmers, 1996). It is worth noting that, coherently with its homeostatic role, NPY projecting neurons are also close to circumventricular organs and sensory/secretory blood-brain interfaces (Wagner et al., 2015).

GENE STRUCTURE

The human NPY gene (∼8 kb) is located on chromosome 7p15 (genomic coordinates (GRCh38): 7:24,284,189-24,291,861). Regulatory elements have been found within 530 bases from the transcription start site and further regulatory sequences enhancing transcription and mRNA stability may be present up/downstream that region or even inside introns (Waldbieser et al., 1992; Waschek, 1995; Zhou et al., 2008). Single nucleotide polymorphisms (SNPs) in the coding region may increase NPY synthesis (Mitchell et al., 2008). The full length mRNA is 551 bp long (Minth et al., 1984). After translation in the endoplasmic reticulum, upon signal peptide truncation, NPY is directed to the secretory pathway.

PEPTIDE TRAFFICKING, PROCESSING AND RELEASE

While trafficking inside dense core vesicles (DCVs), the full coding sequence of NPY, prepro-NPY, is sequentially split into three fragments (**Figure 1A**): (1) an N-terminus 28-amino acid (aa) signaling peptide, (2) the mature 36 aa, 4.2 kDa, peptide (NPY_{1-36}), and (3) a 30-aa C-terminal flanking peptide of neuropeptide-Y (CPON). A glycine-lysine-arginine (G-K-R) site in proximity of the C-terminus of the mature 36 aa peptide is crucial for CPON cleavage by pro-hormone convertases and for the amidation of the mature NPY, performed by carboxypeptidase E and peptidyl-glycin-α-amidating monooxygenase. The CPON structure is highly conserved during evolution (Cerdá-Reverter and Larhammar, 2000). It has been suggested that it may play a role in epilepsy control, but current data do not confirm this hypothesis (Soud et al., 2019).

NPY and CPON containing DCVs are released upon calcium influx. The need of a long, high frequency firing rate for NPY release (Lundberg et al., 1986; van den Pol, 2012) has been questioned by evidence that NPY is released by hippocampal neurons even during physiological synaptic activity (Li et al., 2017).

METABOLISM

Once released in the extracellular space, mature NPY can bind to its receptors and activate signal transduction (Walther et al., 2011) or be metabolized, either close or far away from its release site, in the cerebrospinal fluid or in the blood. Proteolytic processing can alter the NPY signaling at either the N-terminal or C-terminal portion of the peptide and usually results in a modification of receptor binding affinity or inactivation followed by complete degradation, depending on a number of peptidases with compartment-dependent concentration and activity (Allen et al., 1987; Wagner et al., 2015).

The most common pathway of NPY metabolism is N-terminal cleavage by dipeptidyl peptidase IV (DP4) which is responsible for the formation of NPY_{3-36}, followed by C-terminal processing by enzymes like kallikrein, cathepsins or angiotensin-converting enzyme (ACE) that in turn yield inactive NPY fragments. Aminopeptidase (AmP) instead produces NPY_{2-36}, catalyzing a less efficient cleavage within the N-terminal region compared to DP4, which results in a lower relative concentration of this metabolite (Abid et al., 2009). Both NPY_{3-36} and NPY_{2-36} display a decreased affinity for Y1 receptors, therefore preferentially binding to other (Y2 and Y5) receptor subtypes (Grandt et al., 1996; Hubers et al., 2018; Yang et al., 2018).

After inactivation, other plasmatic peptidases catalyze the metabolism of smaller fragments, with the kidney playing a major role in residual NPY metabolism (Satoh et al., 1999). The estimated plasma half-life in human and animal studies is between 5 and 20 min (Pernow et al., 1986; Potter, 1987).

NPY RECEPTORS

The NPY system is not only multi-ligand, as described above, but also multi-receptor, and this makes it a complex target for therapeutic applications.

In fact, five different NPY receptors are expressed in mammals: Y1, Y2, Y4, Y5, and y6. While Y1, Y2, Y4, and Y5 are functional in all mammals, y6 is a pseudogene in humans and other primates and is missing also in the rat genome (Larhammar and Salaneck, 2004). NPY displays an especially high affinity for the Y1, Y2, and Y5 receptor subtypes: even if structurally different, these three receptors can respond to the same ligands. Y1 and Y4 form a receptor superfamily, while Y2 and Y5 have

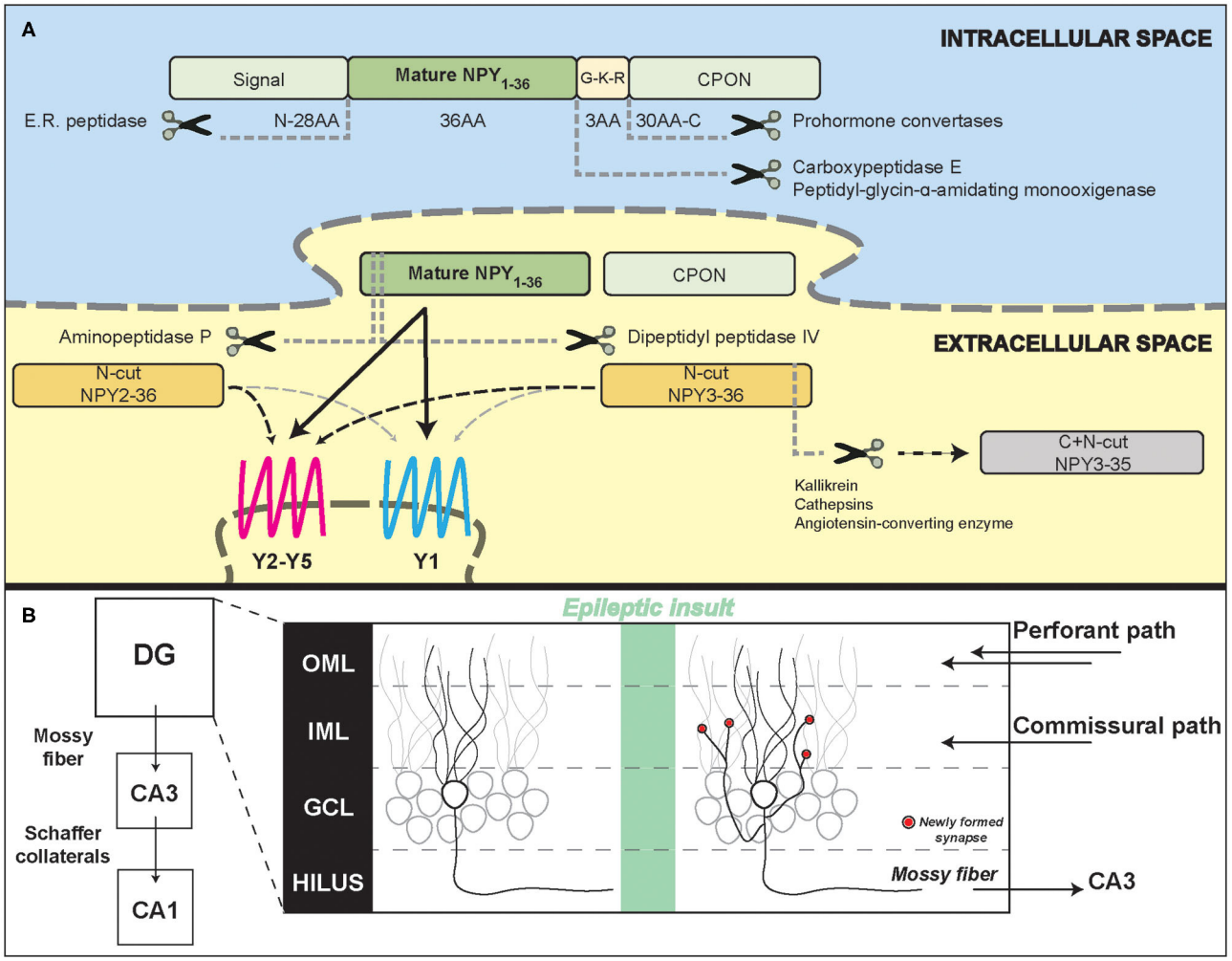

FIGURE 1 | Neuropeptide Y processing and its potential role in the epileptic hippocampal network. **(A)** Schematic representation of NPY intracellular processing and extracellular metabolism. **(B)** Illustration of hippocampal formation rearrangements after an epileptic insult. Red dots represent synapses newly formed by the mossy fiber sprouting in the inner molecular layer that contain NPY and pre-synaptic Y2 receptors. DG, dentate gyrus; CA3/CA1, Cornu Ammonis; OML, outer molecular layer; IML, inner molecular layer; GCL, granule cell layer.

distinct, individual features (Larhammar and Salaneck, 2004). NPY receptors (YRs) have different affinities for the Y family hormone ligands, with Y4Rs binding preferably PP and Y2Rs binding NPY and N-terminally truncated peptides with similar affinity (Lindner et al., 2008). The genes encoding for NPY receptors are located on human chromosome 4 and probably arose by a duplication event from an ancestral NPY/PYY-binding receptor. All NPY receptors are widely expressed in the mammal brain, Y2 being the most abundant (Dumont et al., 1998). High levels of NPY binding can be revealed in the cortex, hippocampus, amygdala, striatum and cerebellum (Dumont et al., 1993).

Specific binding to Y1 receptors can be visualized in different layers of the cortex, in the CA1 and CA3 stratum radiatum, oriens, in the dentate gyrus of the hippocampus in the amygdala, striatum, cerebellum and, at lower levels, in some thalamic, hypothalamic and brainstem nuclei (Dumont et al., 1990, 1993; Aicher et al., 1991; Cabrele and Beck-Sickinger, 2000; Kopp et al., 2002). Outside the CNS, Y1Rs are also found in the adipose tissue and in vascular smooth muscle cells (Castan et al., 1993; Lindner et al., 2008). Y1Rs are mainly localized post-synaptically in neurons of the hippocampus (especially in CA3, CA1 and dentate gyrus), striatum and cortex (Wahlestedt et al., 1986; Caberlotto, 1997; Kopp et al., 2002), with a prominent somatic and dendritic localization (Kopp et al., 2002). However, some studies also suggest a pre-synaptic localization (Colmers et al., 1987, 1988; Flood and Morley, 1989; Pickel et al., 1998; Brumovsky et al., 2002; Glass et al., 2002; Kopp et al., 2002; Stanić et al., 2006; Li et al., 2017). Albeit NPY and Y1R scarcely co-localize (Stanić et al., 2011), the presence of Y1R on the cell soma of NPY-containing hilar interneurons and cultured hippocampal neurons is suggestive of a possible role

of these receptors in an autoinhibitory feedback (St-Pierre et al., 2000; Paredes et al., 2003).

Together with Y5Rs, Y1Rs play an important role in regulating feeding behavior and energy homeostasis (Baldock et al., 2007; Nguyen et al., 2012). Y1R-mediated antidepressant and anxiolytic effects have been described in rodents (Wahlestedt et al., 1993; Verma et al., 2012), while the role in epilepsy remains controversial (see below). The anxiolytic effect of NPY in the basolateral amygdala has been attributed to the activation of Y1Rs (Sajdyk et al., 2004; Giesbrecht et al., 2010).

Y2Rs are expressed in many brain regions, including the hippocampus, thalamus, hypothalamus and cortex; in the peripheral nervous system, Y2Rs are found in parasympathetic, sympathetic and sensory neurons; finally, they are also present in the intestine and in certain blood vessels (Wahlestedt et al., 1986; Stjernquist and Owman, 1990; Gehlert et al., 1992; Dumont et al., 1993; Rettenbacher and Reubi, 2001). In the hippocampus, Y2 receptors are particularly enriched in the CA1 and CA3 areas, respectively in the stratum radiatum and in the pyramidal cell layer (Colmers et al., 1987, 1988, 1991; Monnet et al., 1992). Expression of Y1 and Y2 receptors is often complementary. For example, high levels of Y2Rs are detectable in the stratum oriens and radiatum of CA1-CA3, where Y1 receptor levels are relatively low, while the opposite is true in the dentate gyrus molecular layer (Stanić et al., 2011). Y2Rs are highly expressed in the terminal regions of mossy fibers and Schaffer collaterals (Jacques et al., 1997), where they act pre-synaptically by inhibiting calcium-mediated neurotransmitter release (Klapstein and Colmers, 1993). While NPY and a Y2R selective agonist inhibit evoked EPSPs on CA1 pyramidal cells, a Y2R selective antagonist is able to block the inhibitory action of NPY on glutamate release (El Bahh et al., 2002).

Y2Rs are expressed by both GABAergic and glutamatergic terminals (Stanić et al., 2006, 2011) and may therefore inhibit the release of both neurotransmitters, in particular under chronic epileptic conditions (Martire et al., 1993; Greber et al., 1994; Klapstein and Colmers, 1997; Vezzani and Sperk, 2004; Silva et al., 2005b). This makes Y2Rs an interesting target in epilepsy (Vezzani and Sperk, 2004). Y2Rs can also be localized along the course of axons in fiber tracts (in Schaffer collaterals, the fimbria and the stria terminalis (Dum et al., 2017)). These receptors are functionally coupled with G-protein signaling and show high affinity for their ligand (Dum et al., 2017), leaving open the possibility of a modulation through NPY volume transmission.

Y5Rs are mainly found in the hypothalamus and in the hippocampus (in the pyramidal cell layer of the CA2 region, with lower concentrations in the hilar region of the dentate gyrus and in the CA3 subregion), where they participate in the modulation of hippocampal excitability (Gerald et al., 1996; Dumont et al., 1998; Guo et al., 2002). Together with Y1Rs, Y5Rs contribute to the regulation of food intake and energy homeostasis, but they also display anticonvulsant effects (Woldbye et al., 1997; Criscione et al., 1998; Nanobashvili et al., 2004). Y5R KO mice display a reduced NPY-mediated inhibition of glutamatergic synaptic transmission and are therefore more susceptible to kainate-induced seizure mortality (Marsh et al., 1999; Baraban, 2004).

NPY receptors are G protein-coupled receptors (GPCRs) with seven transmembrane domains, acting preferentially *via* hetero-trimeric Gi/o proteins (Michel et al., 1998). They can trigger a variety of intracellular responses, including inhibition of adenylyl cyclase, regulation of potassium and calcium channels and activation of the mitogen-activated protein kinase (MAPK) cascade in some cell types (Howell et al., 2005; Lu et al., 2010; Thiriet et al., 2011; Shimada et al., 2012). Binding of the ligand to the receptor stabilizes an active receptor conformation, essential for inducing intracellular signal transduction. NPY binding modes vary with individual receptors, with different amino acids impacting anchoring, affinity and binding (Beck-Sickinger et al., 1994; Merten et al., 2007; Walther et al., 2012; Pedragosa-Badia et al., 2013; Kaiser et al., 2015; Yang et al., 2018). NPY peptides reach the receptors by lateral diffusion, after being pre-associated with the membrane through their C-terminal domain (Lerch et al., 2004; Thomas et al., 2005) that is also essential for the binding of NPY to specific receptors, in particular Y2 (Beck-Sickinger et al., 1994).

NPY receptors are predominantly expressed at the cell surface and sequence motifs essential for endoplasmic reticulum export and delivery to the membrane have been identified, particularly in the C-terminal portion of the protein (Walther et al., 2011, 2012). Y2Rs display desensitization (Ziffert et al., 2020a) but can undergo arrestin beta3-dependent and independent internalization only when exposed to high concentrations of agonist (Lundell et al., 2011; Walther et al., 2011). The low rate of Y2R internalization may depend on the presence of a N-terminal extracellular domain rich in acidic/anionic residues (Parker et al., 2001; Gicquiaux et al., 2002).

NPY AND EPILEPSY

A consistent amount of data demonstrates the functional involvement of the NPY system in epilepsy. This statement is supported by two lines of evidence: (1) the epileptogenic process and epilepsy itself modify the expression pattern of the genes encoding NPY and its receptors; (2) acting as neuromodulators, NPY peptides control network excitability and homeostasis.

NPY expression is increased both in rodent and human hippocampal sections from temporal lobe epilepsy (TLE) surgical samples (Sperk et al., 1992; Furtinger et al., 2001), despite the strong loss of hilar GABAergic interneurons that physiologically express NPY. This is because the excitatory granule cells, which in epilepsy give rise to mossy fiber sprouting (MFS), have been demonstrated to ectopically produce and release NPY (Mathern et al., 1995; McCarthy et al., 1998). MFS, the aberrant sprouting of granular axons that recurrently innervate granule cell dendrites in the molecular layer generating an auto-excitatory loop (**Figure 1B**), is a marker of TLE, even if its pathophysiological role is still controversial (Cavarsan et al., 2018).

In patients with hippocampal sclerosis, another common pathological trait of TLE, a shift toward higher Y2 receptor density is observed in the CA1, CA3, in the hilar region and in the inner molecular layer of the hippocampus (Furtinger et al., 2001). This receptor up-regulation may support a persistent Y2R

signaling, because it has been recently shown that Y1, but not Y2, receptors are rapidly internalized and recycled after binding to their ligand (Ziffert et al., 2020a,b). As noted above, increased Y2Rs signaling may imply an anti-epileptic effect (El Bahh et al., 2005). In fact, Y2R knockout mice are totally insensitive to the anti-epileptic actions of NPY, both *in vitro* and *in vivo* (Woldbye et al., 2005).

As opposed to Y2 receptor up-regulation in the epileptic hippocampus, it has been shown that Y1 receptor mRNA and binding actually decrease in kindled rats (Gobbi et al., 1998) and in intra-hippocampal kainate-treated mice (O'Loughlin et al., 2014). A reduced density of Y1Rs has been also demonstrated in human patients with hippocampal sclerosis, indicating a reduced expression of the receptor or a loss of Y1R-expressing neurons (Kofler et al., 1997; Furtinger et al., 2001). In addition, as mentioned above, Y1Rs are rapidly internalized after binding to NPY (Ziffert et al., 2020a,b). Y1R may be responsible of unfavorable effects in epilepsy, because administration of Y1R antagonists produces antiepileptic effects in animal models (Gariboldi et al., 1998; Vezzani et al., 2000) and Y1 KO mice display reduced mortality rate upon NPY administration (Lin et al., 2006). Thus, their reduced density and signaling may be interpreted as an antiepileptic adaptive mechanism. It cannot be excluded, however, that this adaptive downregulation could be linked to epilepsy-induced depressive or anxious behavior, described in patients and in animal models (Yilmazer-Hanke et al., 2016; Vrinda et al., 2017; Zanirati et al., 2018).

Similarly, the decreased density of Y5R in epilepsy models (Bregola et al., 2000) may represent a maladaptive alteration because the pharmacological activation of Y5Rs has been reported to exert antiseizure effects (Woldbye et al., 1997).

Expression levels of NPY-related genes may strongly vary across species, with rats having higher expression of both NPY and Y2 compared to mice (Nadler et al., 2007; Károly et al., 2015). Discrepancy between rodents and humans have been also found at the electrophysiological level. In human slices, prepared from surgically resected hippocampi of drug-resistant patients, NPY application reduces both lateral perforant path-evoked excitatory response in granule cells (Patrylo et al., 1999) and currents evoked by medial perforant path stimulation (Ledri et al., 2015). Conversely, experiments on hippocampal slices from an animal model of epilepsy (pilocarpine-treated rats) show that NPY does not affect the response of granule cells to perforant path stimulation but reversibly inhibits recurrent synaptic transmission of mossy fibers on granule cells themselves (Tu et al., 2005).

Even if the precise mechanism of action of the NPY system on the epileptic network has not been completely clarified, a clear effect of the neuropeptide in inhibiting epileptiform activity on human hippocampal sections challenged with [0] Mg^{2+}/4-amino-piridine has been demonstrated (Wickham et al., 2019), further corroborating the idea that the anti-epileptic effect is predominantly mediated by Y2. It has been shown indeed that the effect of NPY administration can be abolished by treatment with a specific Y2 receptor antagonist (Tu et al., 2005; Ledri et al., 2015; Wickham et al., 2019).

An epileptic insult in the brain can result in a synchronous activation of granule cells that fail to inhibit the propagation of excitation from the entorhinal cortex to the hippocampus. Subsequent compensation mechanisms might arise, and it is tempting to speculate that granule cells, with the death of their target inhibitory neurons, sprout their axons to the molecular layer, increasing excitability but, at the same time, producing synapses containing both NPY and Y2R at the presynaptic level. Within this view, NPY would act as a compensatory negative feedback, activated upon high frequency stimulation, where NPY is released from granular axons and reduce the overall hyperactivity of the local neuronal network. This hypothesis is also in line with the discrepancies that have been observed between mice and rats, with the latter showing higher recurrent mossy fiber sprouting and displaying higher levels of NPY and Y2 immunoreactivity coupled with a stronger inhibitory effect upon NPY application (Tu et al., 2005).

Taken together, these data suggest a significant involvement of NPY in the epileptogenic process, supporting the idea that both pharmacological and genetic approaches targeting the NPY system may represent effective strategies for the treatment of epilepsy. In the frame of this article, we will focus on the latter (gene therapies).

EXPLOITING NPY IN GENE THERAPY

In the last two decades, a great effort has been devoted to the development of gene therapy products for life-changing treatments in epilepsy. In that context, one of the most prominent strategies has been the direct infusion in epileptogenic areas of recombinant adeno-associated vectors (rAAVs) designed to modulate the NPY system (**Table 1**).

Early attempts in this direction explored the anti-seizure potential of NPY overexpression mediated by rAAV serotype 2 (rAAV2) vector injection in the hippocampus (Richichi et al., 2004) or piriform cortex (Foti et al., 2007) in the rat kainate model of epilepsy. Importantly, Richichi et al. (2004) compared the effects of serotypes AAV2 and chimeric AAV1/2, both vectors with the human NPY gene driven by the neuron-specific enolase promoter (pNSE). A long-term transgene expression, confined in hilar interneurons, was observed with AAV2, while more widespread expression in diverse subtypes of neurons was observed with the AAV1/2 serotype, that also conferred a more robust protection from epileptogenesis and chronic seizures. Y1 or Y2 double knockout mice, contrary to the wild type, did not display any protection from seizure activity upon NPY gene therapy, indicating that activation of one (most likely Y2) or both of these receptor subtypes was essential for the NPY effect (Lin et al., 2006). More recently, the AAV1/2 expressing-NPY vector was infused into the thalamus or somatosensory cortex in a rat model of genetic generalized epilepsy (GAERS, Genetic Absence Epileptic Rats from Strasbourg), resulting in a reduced seizure activity, in particular when injected in the thalamus (Powell et al., 2018).

Some concerns on the potential for translatability to human application were raised by Sørensen et al. (2008). These

TABLE 1 | Comparison of different gene therapy strategies designed to modulate the NPY system, based on the use of recombinant adeno-associated vectors.

First author (year)	Species	Model of epilepsy	Vector	Time of vector delivery	Transgene
Richichi et al. (2004)	WT rats	Intrahippocampal and intracerebroventricular kainic acid; Kindling	rAAV2_NSE-NPY; rAAV1/2-NSE-NPY	Before seizure onset	Human pre-pro-NPY
Lin et al. (2006)	WT mice; Y1 -/- and Y2 -/- mice	Systemic kainic acid	rAAV1/2_NSE-NPY	Before seizure onset	Human NPY cDNA
Foti et al. (2007)	WT rats	Intraperitoneal kainic acid	rAAV2_CBA-NPY; rAAV2_CBA-NPY13-36	Before seizure onset	Full length and NPY13-36 (Species not specified)
Sørensen et al. (2008)	WT rats	None	rAAV1/2_NSE-NPY	N/A	Human pre-pro-NPY
Noè et al. (2008)	WT rats	Electrically induced status epilepticus	rAAV1/2_CBA-NPY	After seizure onset	Human pre-pro-NPY
Sørensen et al. (2009)	WT rats	Kindling	rAAV1/2_NSE-NPY	Before seizure onset	Human pre-pro-NPY
Noè et al. (2010)	WT rats	Intrahippocampal kainic acid	rAAV1_CBA-NPY; rAAV1/2_CBA-NPY	After seizure onset	Human pre-pro-NPY
Woldbye et al. (2010)	WT rats	Kindling; Subcutaneous kainic acid	rAAV1/2_NSE-NPY; rAAV1/2_NSE-Y2	After seizure onset	Human pre-pro-NPY Full length mouse Y2 receptor
Gøtzsche et al. (2012)	WT rats	Subcutanous kainic acid	rAAV1/2_NSE-Y5; rAAV1/2_NSE-NPY	Before seizure onset	Human pre-pro-NPY Full length mouse Y5 receptor
Olesen et al. (2012b)	WT mice	Subcutaneous kainic acid	rAAV1/2_NSE-Y1	After seizure onset	Full length mouse Y1 receptor
Olesen et al. (2012a)	WT mice	Subcutaneous kainic acid	rAAV1/2_NSE-Y5	After seizure onset	Full length mouse Y5 receptor
Dong et al. (2013)	WT rats	Intrahippocampal kainic acid	rAAV1/2_CMV-NPY	Before seizure onset	Full length NPY (species not specified)
Zhang et al. (2013)	WT rats	Intracerebroventricular kainic acid	rAAV1/2_NPY (unknown promoter)	Before seizure onset	Not specified
Nikitidou Ledri et al. (2016)	WT rats	Intrahippocampal kainic acid	rAAV1/2_NSE-NPY; rAAV1/2_NSE-Y2	Before seizure onset	Human pre-pro-NPY Full length mouse Y2 receptor
Powell et al. (2018)	GAERS (Genetic Absence Epilepsy Rats)	None	rAAV1/2_NSE-NPY	N/A	Human pre-pro-NPY
Melin et al. (2019)	WT rats	Intrahippocampal kainic acid	rAAV1_CAG-NPY/Y2	Before seizure onset	Human pre-pro-NPY Human Y2 receptor

authors claimed an impairment of synaptic plasticity and the attenuation of long-term potentiation of Schaffer collateral-CA1 synapses in naive rats upon unilateral vector injection in the hippocampus, with consequent deficits of hippocampal-based spatial discrimination learning (Sørensen et al., 2008). These unexpected findings were contrasted by the authors themselves in a following study that showed seizure protection with no impact on working memory performance tasks in kindled rats injected in both hippocampi with the AAV1/2-pNSE-NPY vector (Sørensen et al., 2009).

In any event, the initial attempts of NPY gene therapy had limited relevance for clinical translation: they were all carried out before epilepsy onset, in a scenario that is obviously non-reproducible in real patients and that did not take into account the aberrant changes occurring during epileptogenesis, which may significantly affect treatment effectiveness. In order to overcome this limitation, Noè et al. (2008) tested the effect of hippocampal injection of an AAV1/2 vector expressing NPY after the establishment of epilepsy in rats and found a decrease in seizure activity. Interestingly, this study also demonstrated preserved levels of Y2R into the AAV-injected hippocampus, with functional transport and high levels of release of the recombinant NPY to nerve terminals upon induction of neuronal depolarization. In a following report, the same authors delivered NPY using rAAV1, and observed a widespread transgene expression pattern throughout the injected hippocampi and a potent effect on seizure reduction, with no detectable evidence of immune response or cognitive impairment (Noè et al., 2010).

NPY is directly involved in the regulation of brain excitability by regulation of intracellular calcium and glutamate release, mainly through binding to and activation of Y1, Y2, and Y5

receptors (Berglund et al., 2003). As described above, whereas converging evidence supports an anti-epileptic role of Y2 (and to a lesser extent of Y5) receptors, the involvement of Y1Rs remains debated, with some evidence of pro-epileptic effects. Therefore, a simple increase in NPY levels may become a double-edged sword.

These considerations prompted alternative gene therapy strategies, oriented not only at increasing NPY secretion into the epileptic focus but, also, at re-shaping the NPY ligand-receptor system by the delivery of genes encoding for the different NPY receptors. To date, the only study performed to evaluate the effects of a brain overexpression of Y1 in an animal model of epilepsy indicates an increased susceptibility to kainate-induced seizures (Olesen et al., 2012b), consistent with the mentioned evidence of Y1R-mediated pro-epileptic effects (Gariboldi et al., 1998; Benmaamar et al., 2003). One study proved seizure reduction through the delivery in the rat hippocampus of an AAV pool of vectors for the concomitant expression of both Y5 and NPY (Gøtzsche et al., 2012), but no protective effect was observed with the AAV-Y5 vector alone (Gøtzsche et al., 2012; Olesen et al., 2012a). More robust and promising data have been obtained by overexpressing Y2 receptors, i.e., by seconding the adaptive up-regulation of these receptors observed in the epileptic tissue. Y2Rs proved to be sufficient to suppress acute seizures even when overexpressed alone, although the therapeutic outcome significantly increased in the case of concurrent treatment with an NPY expressing vector (Woldbye et al., 2010).

Attempts of combinatorial gene delivery have been accomplished by using two separate rAAV vectors (Nikitidou Ledri et al., 2016). This procedure, however, faces some limitations, such as an unknown transduction efficiency of the different vectors upon brain infusion or the potential obstacles that a heterogeneous viral pool could face in case of clinical application. In order to solve such issues, Melin et al. (2019) used an AAV1-based vector specially designed for the concurrent expression of both NPY and Y2 from a single viral construct, injected into both dorsal and ventral hippocampus to target the epileptogenic focus. This dual-gene vector delivery led to a detectable overexpression of both NPY and Y2R within the injected hippocampi, particularly pronounced into the dorsal CA1 and CA3 regions, and resulted in a remarkable decrease of EEG seizure frequency and duration in the kainic acid model of TLE (Melin et al., 2019).

PROBLEMS AND OPPORTUNITIES

As described above, both NPY and its receptors display a high degree of complexity, from synthesis, processing and compartmentalized delivery or regulated secretion, to an intricated variety of biological effects, both at local and global circuit level. These elements have profound implications for gene therapy.

The majority of data reported in the literature derive from experiments performed with viral vectors constructed to express pre-pro-NPY (**Table 1**). In this context, the use of the full length NPY sequence may be advantageous, since it allows using the endogenous cellular machinery to process and pack the pro-peptide into vesicles, where the mature NPY is formed and then stored. In this way stimulus-dependent release of the peptide (e.g., at the onset of a seizure) can be preserved. Biosynthesis and stimulus-dependent release of mature NPY have been indeed shown *ex vivo* (Noè et al., 2008). While all this may occur in cells that physiologically express NPY, NPY gene delivery alone may not be sufficient for regulated release of the mature peptide in cells lacking/under-expressing one or more of the regulatory elements (e.g., processing enzymes, trafficking proteins) needed in such a complex multi-step system. One option to circumvent this problem could be linking the NPY gene sequence to the sequence of the laminar protein fibronectin (FIB), which induces a constitutive secretion as opposed to a regulated secretion (Foti et al., 2007). Finally, even after release, the effects of peptidases should be taken into account to understand and modulate NPY signaling.

The modulation of YRs expression requires an even more finely regulated sequence of events. Functional specificity of the NPY system depends largely on receptors. In this context, the processes of anterograde transport, internalization, recycling or degradation have been thoroughly characterized for only a few NPY receptors. These considerations lead to the suggestion that, if no specific cell targeting strategy is employed, gene therapy-induced overexpression of NPY or NPY receptors may be more efficient in (or even restricted to) cells that physiologically or pathophysiologically express them. The levels of released NPY and the coupling between ligand and receptor are also crucial for inducing the desired effect in the right cell target. It may be possible to obtain a certain degree of receptor selectivity by using, for example, N-terminally truncated forms of NPY (like NPY_{3-36} or NPY_{13-36} (Beck-Sickinger and Jung, 1995; Sajdyk et al., 2002; Foti et al., 2007; Pedragosa-Badia et al., 2013) that could favor Y2Rs dependent signaling.

OUTLOOK FOR HUMAN STUDIES USING VIRAL VECTOR-BASED STRATEGIES

Despite this complexity, several anti-epileptic gene therapy strategies proved successful in modulating the inhibitory/excitatory balance within animal brain regions involved in seizure onset by focal overexpression of NPY alone or in combination with Y2 or Y5 receptors. Even if extended long-time studies to exclude side effects or neuropathological changes due to application of viral vectors still need to be performed (optimally in non-human primates), these compelling preclinical data may concretely prompt the design of a first-in-human gene therapy trial in drug-resistant epileptic patients. As an example, patients deemed suitable for surgical resection of a clearly mapped epileptogenic region may be enrolled in a first putative human study. This would allow to design a confined (and presumably more effective) transgene expression within the epileptogenic lesion only, while preserving the unaffected brain tissue and thereby lowering the risk of unpredictable side effects. In addition, should the treatment not prove to be effective or well-tolerated, patients would undergo resective surgery as originally planned.

Several issues should be taken into account in the study design. For example, hippocampal sclerosis, if extensive, may reduce vector diffusion and transduction efficacy, imposing

a personalization of the dose. The choice of vector would largely depend on the strategy employed to regulate the expression of the therapeutic gene. As described in this review, all studies on gene therapy-mediated overexpression of NPY and/or its receptors in epilepsy models have been performed by using AAV vectors. However, the limited cargo capacity of AAVs may hinder their adaptability for clinical translation, in particular when complex regulatory mechanisms must be set in place. In fact, it would be desirable to regulate the levels of transgene expression in a patient-tailored manner, in response to endogenous and/or exogenous clues. While an endogenous control of the transgene expression system that responds to physiological stimuli (for example, glutamate accumulation) would be preferable, the time needed for the biosynthesis and delivery of the therapeutic protein(s) would be too long to arrest an ongoing seizure. Therefore, a more concrete alternative, although not applicable to the response to individual seizures, but rather on a general control of seizure threshold, may rely on the administration of external factors (i.e., specific molecules). These elements could selectively activate or inhibit transgene expression, by acting on specific regulatory sequences delivered along with the therapeutic gene cassette, in the same viral vector. This option, however, would require the exploitation of neurotropic vectors capable to host much larger exogenous DNA cargos, for example HSV derived vectors (Ingusci et al., 2019a,b).

Once the remaining gaps in knowledge and hurdles for gene therapy will be overcome, we may finally be able to treat epilepsy by acting on endogenous systems of neuromodulation. In a way, this is something that we may have already done, unconsciously and much less finely, with certain anti-epileptic drugs (Brill et al., 2006).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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In vivo Genome Editing Therapeutic Approaches for Neurological Disorders: Where Are We in the Translational Pipeline?

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In vivo genome editing tools, such as those based on CRISPR, have been increasingly utilized in both basic and translational neuroscience research. There are currently nine *in vivo* non-CNS genome editing therapies in clinical trials, and the pre-clinical pipeline of major biotechnology companies demonstrate that this number will continue to grow. Several biotechnology companies commercializing *in vivo* genome editing and modification technologies are developing therapies for CNS disorders with accompanying large partnering deals. In this review, the authors discuss the current genome editing and modification therapy pipeline and those in development to treat CNS disorders. The authors also discuss the technical and commercial limitations to translation of these same therapies and potential avenues to overcome these hurdles.

Keywords: genome editing, neurological disease, CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR associated protein 9)-mediated genome editing, biotech companies, translational pipeline

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INTRODUCTION

Genome Editing and Modification in the CNS

The possibility to introduce any desired modification in specific sites of the genome of cells, genome editing, is a longstanding ambition in biotechnology and molecular medicine and is now making precision medicine a real possibility for the treatment of genetic diseases.

A big step forward in the generation of new genome editing tools was the observation that the introduction of a double-strand-break (DSB) in the desired genomic site can strongly enhance the integration of a desired donor DNA sequence (Rouet et al., 1994). The discovery of zinc finger proteins (ZFP) dramatically changed the genome editing scenario as they are eukaryotic zinc ion-regulated small protein motifs able to bind DNA in a sequence-specific manner (Klug and Rhodes, 1987; Kim et al., 1996). When fused to transcriptional activator or repressors (ZFP-TFs), they modulate the expression of endogenous genes (Rebar et al., 2002). The next advance, the transcription activator-like effector (TALE) proteins from *Xanthomonas* bacteria, specifically recognize one single base instead of three bases (Boch et al., 2009; Moscou and Bogdanove, 2009), and can work as programmable nuclease, called TALEN (Li et al., 2011; Miller et al., 2011; Zhang et al., 2011). However, the cloning and protein engineering work for ZFNs and TALENs is complex. It requires two different effectors to cut each DNA strand as *Fok*I works as a dimer and only laboratories with extensive expertise in molecular biology could take advantage of those techniques, thus not broadly adopted by the scientific community.

Conversely, the latest CRISPR tools are much simpler and more flexible to use and require minimal molecular skills to exploit them successfully in multiple genome editing strategies (Anzalone et al., 2020). The main simplification is that DNA target specificity is ensured by short nucleic acid sequences (short guide RNA, sgRNA) rather than protein modules and their cloning is thus faster and cheaper. Beside the classic Cas9 which induce genomic DSBs favoring gene inactivation or gene correction, the nickase Cas9 is the basic platform for the base editor tools that make direct C to T or A to G conversion at the target site (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017). In addition, nuclease defective Cas9 (dCas9) can become a scaffold to which different effectors can be attached to deliver specific protein functions to genomic sites, such as transcriptional activators (CRISPRa), inhibitors (CRISPRi), epigenetic factors and histone modifiers (Shi et al., 2004; Mali et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013; Chavez et al., 2015; Hilton et al., 2015; Konermann et al., 2015; Thakore et al., 2015; Amabile et al., 2016; Liu et al., 2016; McDonald et al., 2016; Morita et al., 2016; Vojta et al., 2016; Xu et al., 2016; Xiong et al., 2017; Matharu and Ahituv, 2020). The more recent fusion of the dCas9 to a modified reverse transcriptase makes possible to rewrite new genetic information into a specified DNA site; in this case the prime editing exploits a guide RNA (prime editing guide RNA, pegRNA) that provides specificity and encodes the edit to be introduced at the same time (Anzalone et al., 2019).

Advances in genome editing strategies encouraged researchers to exploit those tools for preclinical studies even in the CNS, that has always represented a major challenge. The main reason is that neurons are post-mitotic cells and HDR is mainly restricted to cycling cells, specifically in S and G2, when homologous recombination between sister chromatids normally occurs (Lin et al., 2014). However, homology-independent targeted integration (HITI) and other similar systems have been recently described as improved NHEJ-based homology-independent strategy for targeted transgene integration, still based on CRISPR/Cas9, but also efficient in post-mitotic cells (Suzuki et al., 2016).

Preclinical Studies Using Genome Editing to Correct Neurological Diseases

ZFN and TALEN- based therapies have already been used in preclinical studies for several pathologies (Li et al., 2020). However, the technical limitations described above make these technologies challenging to be brought forward for treating CNS pathologies.

CRISPR-based genome editing to rescue neurological diseases has been recently tested in animal and *in vitro* human models. Several neurodevelopmental and neurodegenerative diseases have been tackled including Epilepsy, Autism Spectrum Disorder (ASD), Frontotemporal Dementia (FTD), Alzheimer's, Huntington's and Parkinson's diseases (Yang et al., 2017; Kantor et al., 2018; Krishnan et al., 2020; Stepanichev, 2020; Turner et al., 2020; Vermilyea et al., 2020; Zhou et al., 2020). These approaches are based on either genome editing, silencing, or regulation, and they have been employed to overcome the limitations of classical gene therapy approaches.

Gene silencing and deletion of pathogenic repeats have been tested in animal and human models of Angelman Syndrome (AS), Fragile X syndrome (FXS), FTD and Alzheimer's (Park et al., 2015, 2019; Xie et al., 2016; Gyorgy et al., 2018; Krishnan et al., 2020; Wolter et al., 2020). Although the results of these studies are promising showing rescue of the pathologies *in vitro* and *in vivo*, there are still preclinical tests to be performed in order to translate these approaches to the clinic. Some examples are the downstream effects of silencing a gene in a fully developed mature brain (Shitik et al., 2020), or the potential side effects of AAV integration in the DSBs (Wolter et al., 2020). Single hit mediated gene silencing of a pathogenic allele, as well as deletion of aberrant repeats, could have less impact on the immunological system. The disadvantages to these approaches are two-fold: the potential CRISPR-mediated off-target effects resulting in permanent changes to the genome and the delivery of these tools to patients. There is a massive ongoing effort to find better bioinformatic tools to predict off-target effects and in developing new delivery strategies to widely target CNS (Cota-Coronado et al., 2019).

CRISPRa, for example, has been already tested in *in vivo* animal models of neurodevelopmental and acquired epilepsies, and obesity (Matharu et al., 2019; Colasante et al., 2020a,b; Yamagata et al., 2020). These studies showed, for the first time, a long-lasting effect of endogenous gene upregulation either rescuing haploinsufficiency or modifying neuronal properties to treat pathological symptoms. Although there is great potential for effectively treating several CNS pathologies with CRISPRa, some hurdles for using this technology in humans still has to be addressed. These include the potential immunological response of the brain to long-term expression of dCAS9 (Crudele and Chamberlain, 2018) and a more efficient delivery (e.g., using smaller dCAS9).

On the other hand, the possibility of using genome editing to correct the pathological mutations is still an attractive prerogative of the CRISPR systems. Although the post-mitotic neuronal genome is difficult to modify, some recent techniques (Suzuki et al., 2016; Nishiyama et al., 2017; Yeh et al., 2019) allow gene modification in mature brain cells. In these studies, a successful insertion of new DNA in the genome of neurons has been shown to mildly rescue pathological conditions. Indeed, the main limitation is the low efficiency of the modifications that need to be addressed and improved before moving toward the clinic.

Furthermore, CRISPR base editors and CRISPR prime editing hold the potential to further improve the treatments for neurological diseases (Gaudelli et al., 2017; Anzalone et al., 2019; Duarte and Deglon, 2020). They are still behind in the preclinical pipeline due to the difficulties in the delivery of these constructs and the validation of the off-target effects. However,

their ability to correct single mutations (Base editor) or longer DNA sequences (Prime) with high efficiency, without indels, is promising for future translational treatments. Recently, it has been shown that CRISPR base editing can be successfully employed *in vivo* to treat Amyotrophic Lateral Sclerosis (ALS) (Lim et al., 2020), splitting the base editors with an intein-mediated *trans*-splicing system, but the efficiency is still low.

Overall, all these different CRISPR-based technologies have been tested either in animal or *in vitro* human models, revealing an unprecedented potential for translation. The next steps are the refinement of the tools, in terms of delivery, efficiency and off-target effects in order to enable the development of an extensive commercial pipeline.

DISCUSSION

The Current Therapeutic Pipeline to in Human Genome Editing

Despite the achievements in preclinical studies, therapeutic use of genome editing in the CNS is still in its infancy. Even though there are nine active clinical trials using *in vivo* genome editing[1] (Hirakawa et al., 2020) none of them are to treat a CNS indication. Yet the potential of these technologies to treat CNS disorders is of great interest to pharmaceutical companies as seen from their pre-clinical pipelines (**Table 1**).

Among the several biotech companies involved in genome editing and regulation, Sangamo Therapeutics (Sangamo), Editas Medicine and Beam Therapeutics are the only ones that have publicly stated their pipelines on *in vivo* genome editing therapies for the CNS. Interestingly, Beam Therapeutics, which uses CRISPR/Cas9-based base editing, has an undisclosed CNS project.

Sangamo and Biogen are co-developing up to another ten therapeutic candidates targeting a neurological indication using ZFP-TF, with one of the assets targeting a neuromuscular indication, whereas Editas Medicine and Asklepios BioPharmaceutical (AskBio) are developing a therapy utilizing AAV-CRISPR-Cas9. AskBio was acquired by Bayer in October 2020, positioning this large pharmaceutical company in the gene therapy and genome editing space[2]. Sangamo has disclosed that its pipeline includes therapies for tauopathies, synucleinopathies, Huntington's disease, neurodevelopmental disorders, prion disease and ALS/FTD[3].

On the other hand, other genome editing companies such as CRISPR Therapeutics, Intellia Therapeutics and Precision Biosciences have not entered the CNS space or have not yet disclosed their candidates.

Although there is great potential of prime editing it is too early for this technology to be added to commercial pipelines. Indeed, there are currently no publicized therapy assets using prime editing. To be noted, Beam Therapeutics licensed the IP for prime editing from Prime Medicine[4].

[1] www.clinicaltrials.gov 2020 Search 13 November 2020

[2] https://media.bayer.com/baynews/baynews.nsf/id/Bayer-acquires-Asklepios-BioPharmaceutical-to-broaden-innovation-base-in-cell-and-gene-therapy

[3] https://www.sangamo.com/pipeline

[4] https://www.businesswire.com/news/home/20191031005255/en/Beam-Therapeutics-Announces-Collaboration-and-Exclusive-License-Agreement-with-Prime-Medicine-for-Prime-Editing-Technology

TABLE 1 | Companies with *in vivo* genome editing and regulation assets at preclinical stage [search on November 10, 2020].

Company	Genome editing system	Approach	Affected Tissue/Organ/ Therapeutic Area	Indication	Delivery	Target or Gene Delivered
Sangamo Therapeutics/Biogen	ZFP-TF	Gene downregulation	CNS	Tauopathies	AAV	Tau
Sangamo Therapeutics/Biogen	ZFP-TF	Gene downregulation	CNS	Synucleinopathies (Inc., Parkinson's Disease)	AAV	Alpha-synuclein
Sangamo Therapeutics/Biogen	ZFP-TF	Gene downregulation	PNS and/or CNS	Neurological (Inc., a neuromuscular indication)	AAV	Unknown
Sangamo Therapeutics/Pfizer	ZFP-TF	Gene downregulation	CNS	ALS/FTD	AAV	Mutant C9ORF72
Sangamo Therapeutics/Takeda	ZFP-TF	Gene downregulation	CNS	Huntington's Disease	AAV	Mutant HTT
Sangamo Therapeutics	ZFP-TF	Gene downregulation	CNS	Prion	AAV	Unknown
Sangamo Therapeutics/Novartis	ZFP-TF	Gene downregulation	CNS	Neurodevelopmental Disorders (Inc., Autism Spectrum Disorder)	AAV	Unknown
Editas Medicine/Asklepios Biopharmaceutical	CRISPR/Cas9	Unknown	PNS and/or CNS	Neurological	AAV	Unknown
Beam Therapeutics	CRISPR/dCas (base editor)	Correction or Silencing	CNS	Unknown	AAV	Unknown

Public pre-clinical pipelines of biopharmaceutical companies using in vivo genome editing to treat CNS disorders. Queried the pipelines of genome editing companies and used search engines to find companies with publicly available information on its pipelines. Companies using genome editing and regulation technologies that do not publicize their pipelines are not shown.

Why are there still only few in vivo genome editing therapeutic programmes for the CNS? This is due to technical and commercial limitations. Biotechnology companies seek the indications with the largest patient population that are not adequately treated by current therapies. In this equation, companies also compute the risk of failure at a technical level. Delivering *in vivo* genome editing therapies to the CNS is technically harder than to other organ systems, which increases the risk of failure. In addition, CNS indications often have a more complex etiology than oncology or monogenic disorders in other organs. This can incentivize companies to invest in therapies that can target indications that have better defined genotype-phenotype relationships, such as oncology or monogenic disorders in the retina or liver.

The potential of off-target effects also plays an important role in the risk-aversion to the investment in CNS *in vivo* genome editing therapeutics. A permanent off-target change to the DNA could lead to material consequences for the patient. It is possible that biotechnology companies are waiting for increased specificity of CRISPR and other tools before targeting the CNS. In fact, seven out of nine disclosed *in vivo* genome editing therapies treating CNS indications (**Table 1**) are using tools acting on transcriptional regulation which leads to transient changes in neuronal gene expression, rather than genome modifications.

In summary, overcoming some technical limitations that are specific for CNS, such as temporal and spatial control of tool expression, delivery and targetability (Wang et al., 2020); as well as accuracy and efficacy (Zhang et al., 2015) could increase the interest of biotechnology companies toward *in vivo* genome editing for CNS disorders, and therefore also increase investments and number of therapies in the clinic.

Partnerships

Biopharmaceutical companies developing *in vivo* genome editing therapies and advanced therapeutics are partnering with other biotechnology companies in order to make progress on some of those key limitations. For example, the partnership with AskBio will enable Editas Medicine to leverage its knowledge and IP on capsid development and its AAV delivery system in order to overcome the aforementioned bottlenecks of *in vivo* genome editing in the CNS[5]. In the transient gene therapy space, Roche and Spark Therapeutics partnered with Dyno Therapeutics in order to use Dyno Therapeutics' CapsidMap™ platform to develop optimized AAV vectors for gene therapies targeting CNS and liver[6]. Those novel AAVs will have optimized tissue targeting and "immune-evading" properties.

Some CNS indications, however, have already an attractive commercial proposition. In fact, there are indications such as Huntington's and ALS, for which there is a large therapeutic unmet need and the etiology is clear and are therefore suitable indications to be treated with *in vivo* genome editing. For this reason, large biopharmaceutical companies have partnered with genome editing companies to treat CNS disorders (**Table 2**).

Sangamo has positioned itself as the leader in *in vivo* genome editing for CNS disorders with its ZFP-TF technology. With four large collaborations with Pfizer, Takeda Pharmaceutical Company (Takeda), Biogen and Novartis (**Table 2**), it has managed, at least publicly, to become the biopharmaceutical company with the largest amount of genome editing therapeutic assets for CNS indications.

All disclosed CNS *in vivo* genome editing therapeutics are in early stages, but their potential is reflected in the large partnering and licensing deals (**Table 2**).

Sangamo signed two collaboration agreements with Pfizer and Takeda for the development of therapies for ALS/FTLD and Huntington's, respectively. Under the collaboration with Pfizer, Sangamo will receive a $12m upfront payment from Pfizer[7]. In this agreement, Sangamo will be responsible for developing ZFP-TF candidates and Pfizer responsible for research, development, manufacturing and commercialization for the ZFP-TF program. Sangamo is eligible to receive development and commercial milestones of up to $150m, as well as tiered royalties on net sales.

More recently, Sangamo announced a global collaboration with Biogen to develop gene regulation therapies for tauopathies including Alzheimer's disease, for synucleinopathies including Parkinson's disease, a third undisclosed neuromuscular disease target, and up to nine additional undisclosed neurological disease targets. Sangamo will use its ZFP-TF platform to develop these assets. Biogen paid $350m upfront with up to $2.37b in development, regulatory, and commercial milestone payments[8]. In July 2020, Sangamo and Novartis announced a global collaboration to develop and commercialize gene regulation therapies to address three neurodevelopmental diseases, including autism spectrum disorder. The target genes are undisclosed. Novartis will pay $75m to Sangamo as an upfront license fee payment with a potential $720m in other development and commercial milestone payments. The agreement also stipulates that Sangamo is eligible to receive a high single-digit to sub-teen double-digit royalties on net commercial sales arising from the collaboration[9].

Patenting and Licensing

The commercialization route for biologics and advanced therapeutics, including genome editing therapeutics, is different

[5]https://www.askbio.com/editas-medicine-and-askbio-enter-strategic-research-collaboration-to-explore-in~vivo-delivery-of-genome-editing-medicines-to-treat-neurological-diseases/

[6]https://www.dynotx.com/news/press-releases/dyno-therapeutics-enters-collaboration-and-license-agreement-with-roche-to-develop-next-generation-aav-gene-therapy-vectors-for-cns-diseases-and-liver-directed-therapies/

[7]https://www.pfizer.com/news/press-release/press-release-detail/sangamo_and_pfizer_announce_collaboration_for_development_of_zinc_finger_protein_gene_therapy_for_als

[8]https://investors.biogen.com/news-releases/news-release-details/biogen-and-sangamo-announce-global-collaboration-develop-gene

[9]https://investor.sangamo.com/news-releases/news-release-details/sangamo-announces-global-collaboration-novartis-develop-genomic

TABLE 2 | Licensing deals from co-developed *in vivo* genome editing and regulation CNS assets (excluding AskBio/Editas) https://investor.sangamo.com/news-releases/news-release-details/sangamo-announces-global-collaboration-novartis-develop-genomic.

Licensee	Licensor	Phase	Indication	Upfront ($m)	Milestone Payments (Up to $m)	Year
Pfizer	Sangamo Therapeutics	Pre-clinical	ALS/FTLD	12	150	2018
Takeda Pharmaceutical Company	Sangamo Therapeutics	Pre-clinical	Huntington's	Unknown	Unknown	2019
Biogen	Sangamo Therapeutics	Pre-clinical	Tauopathies, Synucleinopathies (Inc., Parkinson's disease), a neuromuscular target and up to nine other undisclosed neurological indication	350	2,370	2020
Novartis	Sangamo Therapeutics	Pre-clinical	Neurodevelopmental Disorders (Inc., Autism Spectrum Disorder)	75	720	2020

FIGURE 1 | Number of patents filing that mention both CRISPR and CNS. Google patents search November 10 2020.

from that of small molecules. Small molecule developers usually do not require a license for a critical technology (such as genome editing tools) in order to commercialize a therapy. In the case of advanced therapeutics, such as the use of CRISPR, any academic or commercial institution would require a license to key IP in order to have "freedom-to-operate" and to commercialize its CRISPR-based therapeutic. This is a major barrier to entry since developing a *de novo* genome editing tool in order to avoid expensive CRISPR licenses requires years of fundamental research (Brinegar et al., 2017).

However, there are still 52,603 CRISPR patents filed globally (Google patents search 10/11/2020), of which 5,447 mention the CNS. The total number of patent filings that mention both CRISPR and CNS have been increasing since 2016 (**Figure 1**), demonstrating both the academic and institutional interest in the use of genome editing in the CNS.

For each CRISPR patent filed, there can be multiple licenses. For example, the Broad Institute licensed its key patents, non-exclusively, to The Monsanto Company (part of Bayer) for use in agriculture (StatNews, 2016[10]), but licensed it exclusively to Editas Medicine for human therapeutic use (Editas Medicine, 2014[11]).

Conclusion and Future Perspectives

Although, as aforementioned, CRISPR/Cas9 tools can be designed and implemented much more easily than ZFPs, most of the preclinical studies that companies are running are based on ZFPs. This might be partially due to the more recent advent of CRISPR and the associated off-target effects, which have to be further tested. We now have several genome editing tools in our hands to really change the course of neurological disease treatment. Preclinical studies are promising and there are extensive efforts in the scientific community to find approaches to overcome the current barriers to developing a first in human genome editing therapeutic for CNS diseases. We envision that the next 5–10 years will be fundamental to understand whether we can completely eradicate some severe intractable neurological diseases using genome editing. The road to clinic is still full of hurdles but the speed of development in the field is one of the fastest ever seen in science.

AUTHOR CONTRIBUTIONS

PL, GC, and GL conceived the review and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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[10]https://www.statnews.com/2016/09/22/monsanto-licenses-crispr/

[11]https://ir.editasmedicine.com/news-releases/news-release-details/editas-medicine-licenses-genome-editing-technology-broad?ID=2125239&c=254265&p=irol-newsArticle

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REVIEW
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Current Approaches for Glioma Gene Therapy and Virotherapy

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Glioblastoma (GBM) is the most common and aggressive primary brain tumor in the adult population and it carries a dismal prognosis. Inefficient drug delivery across the blood brain barrier (BBB), an immunosuppressive tumor microenvironment (TME) and development of drug resistance are key barriers to successful glioma treatment. Since gliomas occur through sequential acquisition of genetic alterations, gene therapy, which enables to modification of the genetic make-up of target cells, appears to be a promising approach to overcome the obstacles encountered by current therapeutic strategies. Gene therapy is a rapidly evolving field with the ultimate goal of achieving specific delivery of therapeutic molecules using either viral or non-viral delivery vehicles. Gene therapy can also be used to enhance immune responses to tumor antigens, reprogram the TME aiming at blocking glioma-mediated immunosuppression and normalize angiogenesis. Nano-particles-mediated gene therapy is currently being developed to overcome the BBB for glioma treatment. Another approach to enhance the anti-glioma efficacy is the implementation of viro-immunotherapy using oncolytic viruses, which are immunogenic. Oncolytic viruses kill tumor cells due to cancer cell-specific viral replication, and can also initiate an anti-tumor immunity. However, concerns still remain related to off target effects, and therapeutic and transduction efficiency. In this review, we describe the rationale and strategies as well as advantages and disadvantages of current gene therapy approaches against gliomas in clinical and preclinical studies. This includes different delivery systems comprising of viral, and non-viral delivery platforms along with suicide/prodrug, oncolytic, cytokine, and tumor suppressor-mediated gene therapy approaches. In addition, advances in glioma treatment through BBB-disruptive gene therapy and anti-EGFRvIII/VEGFR gene therapy are also discussed. Finally, we discuss the results of gene therapy-mediated human clinical trials for gliomas. In summary, we highlight the progress, prospects and remaining challenges of gene therapies aiming at broadening our understanding and highlighting the therapeutic arsenal for GBM.

Keywords: gene therapy, glioma, viral vectors, non-viral vectors, HSV1-TK, mutant IDH1 3, immunotherapy, FMS-like tyrosine kinase 3 ligand

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INTRODUCTION

Molecular Alterations in Gliomas

Gliomas are group of heterogeneous primary brain neoplasms which differ in grade of malignancy, histology and genomic alterations. They may arise from neural stem cells (NSCs), NSC-derived astrocyte or oligodendrocyte precursor cells (Zong et al., 2012, 2015) and represent almost 30% of the central nervous system (CNS) tumors, and 80% of malignant CNS tumors (Ostrom et al., 2015, 2018). Most gliomas are diffuse and have been traditionally classified based either on histologic type: astrocytoma, oligodendroglioma, oligoastrocytoma (a rare mixed glioma) (Perry and Wesseling, 2016) or on their malignancy grade: World Health Organization (WHO), grades I-IV (Louis et al., 2007, 2016; Reifenberger et al., 2017). Recently, the WHO classification was refined (Louis et al., 2016). The presence and distribution of genetic alterations in brain tumors is now a criterion to differentiate glioma subtypes (Louis et al., 2016; Masui et al., 2016; Wesseling and Capper, 2018) that can be related with a particular WHO grade and tumor histology (Parsons et al., 2008; Sturm et al., 2012; Brennan et al., 2013; Cancer Genome Atlas Research et al., 2015; Ceccarelli et al., 2016) (**Table 1**).

A recurrent point mutation in isocitrate dehydrogenase 1 (IDH1), usually at arginine 132 (R132H), is detected with high frequency in adult diffuse-gliomas, being particularly high in diffuse low-grade gliomas (LGGs; WHO grade II) (Cancer Genome Atlas Research et al., 2015; Ceccarelli et al., 2016; Delgado-Lopez et al., 2017; Ostrom et al., 2018). This mutation is also found in anaplastic astrocytomas (WHO grade III), and in a smaller proportion of glioblastomas originated from LGGs (secondary glioblastomas; WHO grade IV) (Cancer Genome Atlas Research, 2008; Bai et al., 2016; Ceccarelli et al., 2016; Louis et al., 2016). Mutant IDH1 gliomas are sub-classified, according with the loss of 1p/19q chromosomal segments, in mutant IDH1-1p/19q-codel and mutant IDH1-noncodel. Mutant IDH1 1p/19q-codel gliomas frequently harbor *TERT* promoter (*TERT*p) and *CIC* mutations, and are associated with oligodendrogliomas; whereas mutant IDH1-non-codel harbor mutations in alpha-thalassemia X-linked mental retardation (*ATRX*) and *TP53*, and associated with astrocytoma and oligoastrocytoma (Cancer Genome Atlas Research et al., 2015; Bai et al., 2016; Ceccarelli et al., 2016; Louis et al., 2016; Venteicher et al., 2017).

In adults, wild-type IDH1 (wt-IDH1) glioma patients retain ATRX function, and typically present *TP53* and *TERT*p mutations, and alterations in regulators of the receptor tyrosine kinase (RTK)-RAS-PI3K signaling cascade, including EGFR amplification and *PTEN* mutation or loss (Brennan et al., 2013; Louis et al., 2016; Masui et al., 2016; Reifenberger et al., 2017). Pediatric gliomas are mostly wt-IDH1, and they also can harbor *TP53* and *ATRX* inactivating mutations, additionally mutations in *H3F3A*, *HIST1H3B*, *HIST1H3C*, and *BRAF* (Rapidly Accelerated Fibrosarcoma type B) are frequent in pediatric high-grade gliomas (HGGs) (Bjerke et al., 2013; Venteicher et al., 2017). Based on these alterations, four pediatric HGG subtypes can be distinguished: H3.3-K27M; H3.1-K27M,

TABLE 1 | List of recurrent genomic alterations in glioma.

Glioma subtype	mIDH1 LGG codel	mIDH1 LGG non-codel	mIDH1 secondary glioblastoma	wt-IDH1 adult glioblastoma	Diffuse midline glioma	Pediatric HGGs	Pediatric LGGs
Grade	Diffuse glioma, WHO grade II/III	Diffuse glioma, WHO grade II/III	WHO grade IV	WHO grade IV	WHO grade IV	WHO grade IV	WHO grade I-III
Recurrent genetics alterations	IDH1, TERT, CIC, FUBP1, TCF12 Mutations; CDKN2A deletion	IDH1 or IDH2, TP53, ATRX mutations	IDH1 or IDH2, TP53, ATRX mutation; CDKN2A homozygous deletion	TERT, PTEN, TP53, PIK3CA, PIK3R1, NF1 mutation; PDGFRA amplification.	H3F3A-K27M or HIST1H3B/C-K27M, TP53, PPMD1, ACVR1, FGFR1 mutation, PDGFRA, MYC, MYCN, CDK4, CDK6, CCND1-3, ID2, MET amplification	H3F3A-G34, ATRX, TP53, DAXX mutation; EGFR amplification	BRAF-V600E mutation and KIAA1549:BRAF fusion, BRAF, RAF1, NTRK2 gene fusions; BRAF-V600E, NF1, KRAS, FGFR1 alterations
DNA/histone methylation	Histone and DNA Hyper-methylation; G-CIMP	Histone and DNA Hyper-methylation; G-CIMP	Histone and DNA Hyper-methylation; G-CIMP		Loss of histone-H3-lysine tri-methylation	Decreased H3K36me3 DNA hypo-methylation	
Chromosomal alterations	1p/19q co-deletion	loss of heterozygosity in 17p; 7q gain	loss of heterozygosity in 17p; 7q gain; 10q deletion	7q gain;			
Age	Young adults	Young adults	Adults	Adults	Children	Children	Children

characteristic of high grade midline gliomas, including diffuse intrinsic pontine glioma (DIPG); H3.3G34-R/V; and BRAF-V600E (Jones et al., 2017). BRAF alterations are also found in pediatric LGGs (Packer et al., 2017).

In addition, DNA methylation in CpG islands describes the CpG-island methylator phenotype (G-CIMP) which is associated with better prognosis and tightly related with IDH1 mutation (Noushmehr et al., 2010; Wiestler et al., 2014). Recently, a study performed over more than 1,000 diffuse glioma (TCGA) patients, identified glioma DNA methylation clusters (LGm1–LGm6) which are linked to molecular glioma subtypes (Ceccarelli et al., 2016). Also, the methylation of CpG islands in the O6-methylguanine-DNA methyltransferase (MGMT) promoter has been identified as a molecular marker of better response to treatment with DNA alkylating agents (Wick et al., 2014).

The genetic lesions described in gliomas impact tumor biology and signaling pathways. Important signaling pathways altered in gliomas include the growth factor receptor tyrosine kinase (RTK) signaling pathways, partly as a result of PDGF and EGFR overexpression (Verhaak et al., 2010; Nazarenko et al., 2012). RAS, PI3K/PTEN/AKT, RB/CDK N2A-p16INK4a, and TP53/MDM2/MDM 4/CDKN2A-p14ARF pathways are commonly activated in gliomas and has been involved in cancer cells proliferation (Nakada et al., 2011; Crespo et al., 2015). In addition, NOTCH signaling activity has been reported in WHO grade IV gliomas, and can be associated with hypoxia, PI3K/AKT/mTOR and ERK/MAPK molecular pathways, increase malignant features of gliomas (Gersey et al., 2019).

In pediatric gliomas the MAPK pathway or its downstream effectors, which contribute to tumorigenesis and growth of many types of cancers, can be activated as a consequence of *NF1* and *BRAF* gene mutations (Truong and Nicolaides, 2015; Mackay et al., 2017) In addition, BMP signaling, is also active in pediatric HGG tumor cells (Mendez et al., 2020). Approximately 25% of childhood brainstem gliomas harbor somatic mutations in Activin A receptor type I (*ACVR1*) (Fontebasso et al., 2014) which encode the type I BMP receptor ALK2, inducing BMP pathway activation (Olsen et al., 2014). Signaling pathway alterations, resulting from specific genetic lesions in gliomas, represent a valuable target to develop novel targeted gene therapies.

Barriers to Drug and Gene Delivery

One of the most challenging aspects in developing effective therapies for gliomas is the ability of the therapeutic agents to reach the tumor site at sufficient therapeutic concentrations (Shergalis et al., 2018). This is due to the presence of the Blood Brain Barrier (BBB), composed of a monolayer of endothelial cells held together by restrictive tight junctions (Vorbrodt and Dobrogowska, 2003). Pericytes, astrocytes, nerve terminals and central nervous system-border associated macrophages (BAMs), a specific myeloid subpopulation are closely associated with the endothelium and play critical roles in BBB development, maintenance and function (Abbott et al., 2010; Rajan et al., 2020). The BBB is a neuroprotective barrier that can block the passage of noxious agents but also the delivery of anti-tumor drugs including gene therapy delivery vehicles (Karim et al., 2016). Different strategies have emerged to offset these protective effects of the BBB, such as direct delivery of chemotherapeutics to the brain as well as the passive targeting based on the increased permeability and retention (EPR) effects (Yu et al., 2016). However, the passive targeting strategy is not sufficient to target invasive tumor cells, as the EPR effects tend to be weak near the infiltrating cancer cell tumor region (Juillerat-Jeanneret, 2008; Kim et al., 2015a). The blood-brain tumor barrier (BBTB), is also known to prevent drugs from accessing the tumor bulk, contributing to chemo-resistance and tumor recurrence. New strategies for actively targeting the BBB have been developed, such as disruptions in tight junctions (Karim et al., 2016), efflux transporter inhibition (Hoosain et al., 2015; Parrish et al., 2015) receptor-mediated transcytosis and/or endocytosis (Wei et al., 2014; Lajoie and Shusta, 2015).

Another consideration is the presence of P-glycoprotein efflux pumps that can actively transport lipophilic drugs out of the brain capillary endothelial cells that form the BBB. Although the BBB is altered at the tumor site, the dense endothelial cells' layer is not compromised and therefore the BBB remains effective at preventing drugs from reaching the tumor cells (Sarkaria et al., 2018). These issues need to be addressed during the preclinical phase, before bringing therapeutic candidates into clinical trials for brain cancer (Shergalis et al., 2018).

Due to the inefficient drug delivery across the BBB and development of drug resistance, gene therapy was envisioned as a promising strategy to overcome limitations of conventional therapies. Gene therapy for cancer treatment conventionally includes the introduction of growth regulating or tumor suppressor genes, RNA interference (RNAi) to inhibit the activity of oncogenes. It can also involve the delivery of suicide genes which can convert non-toxic prodrugs into active anti-cancer compounds. Other approaches include oncolytic and immunomodulatory gene therapy approaches (Candolfi et al., 2009; Puntel et al., 2010; Foreman et al., 2017; Mendez et al., 2020).

Delivery vectors such as viral vectors, non-polymeric nanoparticles (NPs) and polymeric NPs have been used to deliver the therapeutic payload in GBM and LGG (Caffery et al., 2019). To elicit therapeutic effects in the brain, nucleic acids used as therapeutic moieties need to surmount several barriers. Once they enter the blood circulation, they will encounter nuclease degradation, systemic elimination, reticuloendothelial system (RES) uptake before they can successfully cross the BBB, which is impermeable to hydrophilic macromolecules. After sufficient diffusion throughout the brain and into the tumor mass, the therapeutic gene needs to be endocytosed into targeted cells followed by endosome escape to avoid lysosomal degradation and eventually reach the cytoplasm for siRNA or further transport into the nucleus for plasmid DNA (Lu and Jiang, 2017). Viral vectors are attractive delivery vehicles, but they have not yet been clinically approved due in part to manufacturing challenges, high-cost, immunogenic and inflammatory responses, oncogenic mutations and limited loading capacity (Bergen et al., 2008; Rogers and Rush, 2012; Gomes et al., 2015). Non-viral delivery strategies offer alternative approaches that can be developed used to overcome the

barriers of gene delivery. Many non-viral vectors, including polymeric and non-polymeric are non-immunogenic and can be functionalized with targeting moieties to increase receptor-mediated uptake of vectors into tumor tissue.

Immune Responses in Glioma

Immunotherapy has proven successful against a growing number of tumors, unfortunately ongoing attempts to develop new immunotherapies for GBM have not yet demonstrated any significant improvement in glioma patients' survival. In Phase-III clinical trials, immune-checkpoint blockade immunotherapies, which looked highly promising in other solid cancers such as melanoma and lung cancer, were ineffective in GBM (Havel et al., 2019; Zhang et al., 2020). GBM exploits numerous strategies contributing to the evolution of an immunologically suppressive TME that eventually promotes systemic immunosuppression and antagonizes anti-GBM immune responses. GBM mediated immunosuppression is achieved by the production of cytokines and chemokines in the TME and subsequent recruitment of immunosuppressive cells, blocking intra-tumoral T-cell migration and activation (Perng and Lim, 2015). Systemic immunosuppression has been demonstrated by compromised adaptive immunity in murine GBM models and human subjects (Bloch et al., 2013; Chae et al., 2015). TGF-b and IL-10 play a central in maintaining the immunosuppressive TME, these cytokines are not only produced by GBM-infiltrating Tregs, but also by the GBM cells themselves (Perng and Lim, 2015). Another anti-inflammatory cytokine, IL-10 suppresses the activation and effector functions of DCs, macrophages and T cells, and inhibits the MHC-II expression in monocytes (Moore et al., 2001; Perng and Lim, 2015). Additionally, IL-10 promotes the expansion of myeloid-derived suppressor cells (MDSCs), Tregs (Tanikawa et al., 2012) and augments PD-L1 expression in monocytes and tumor-associated macrophages (TAMs) (Bloch et al., 2013). TGF-b is preferentially expressed in GBM cells, and involved in the blockade of T cell proliferation and activation in murine GBM models and human GBM patients (Bodmer et al., 1989). Higher TGF-b expression levels are correlated with poor prognosis and higher glioma grades (Zhang et al., 2014).

GBMs also produce large amounts of indolamine 2,3-dioxygenase (IDO) that triggers the recruitment of Tregs and suppresses effector T cells' activity by depleting tryptophan from the TME (Wainwright et al., 2012). GBM produces other immunosuppressive factors including colony stimulating factor 1 (CSF-1), NO, PGE2, Arg I, Gal-1, and VEGF (Nduom et al., 2015). PGE2 stimulates anti-inflammatory Th2 cytokines such as IL-4, Il-6, and IL-10 and suppress the production of Th1 cytokines. CSF-1 has been demonstrated to polarize the macrophages to M2 phenotype which enhances the glioma progression (Pyonteck et al., 2013). VEGF inhibits the DCs maturation and promotes angiogenesis (Gabrilovich et al., 1996). GBM derived chemokines CCL22 and CCL2 recruits Tregs which expresses CCR4 into the TME and blockade of these chemokines could improve antitumor immunity (Galvao and Zong, 2013).

Progression of GBM is dependent on the genetic lesions encountered within the tumor cells and also epigenetic alterations resulting in an immunosuppressive glioma microenvironment. Immunosuppressive cells abundant within the glioma microenvironment include of MDSCs (Kamran et al., 2017; Guo et al., 2018), TAMs (Wei et al., 2020), and Tregs (Chang et al., 2016). MDSCs have been shown to promote tumor angiogenesis *via* secretion of VEGF as well as MMP-9, and also augment the expression of checkpoint receptor ligand PD-L1 (Mirghorbani et al., 2013). We have recently demonstrated that depletion of MDSCs in glioma-bearing mice prominently augments the efficacy of our immune stimulatory gene therapy (Kamran et al., 2017). Immunotherapeutic strategies currently being investigated to treat GBM include passive immunotherapy with antibodies (Kamran et al., 2016), chimeric antigen receptor (CAR) T-cell therapy (Pituch et al., 2018; Choi et al., 2019) autologous activated lymphocytes therapy (Walker et al., 2019; Lee-Chang et al., 2021), immune-mediated gene therapy (Ali et al., 2005; Curtin et al., 2009; Mineharu et al., 2012; Kamran et al., 2017), oncolytic viral therapy (Mooney et al., 2019; Chastkofsky et al., 2020), or active immunotherapy with tumor cell based vaccines, peptides, or dendritic cells (Hdeib and Sloan, 2015; Polivka et al., 2017).

T-cell Exhaustion, TAMs, MDSCs, Tregs

In glioma, most of the macrophages found within the tumor microenvironment have immune suppressive functionality and support tumor progression (Hambardzumyan et al., 2016). This population of tumor associated macrophages, TAMs, can constitute up to one-third the total mass of the tumor (Roesch et al., 2018). Brain-resident microglial cells and bone marrow derived macrophages (BMDMs) are distinct myeloid cell populations with many shared features including their immunoregulatory abilities and many surface markers (Roesch et al., 2018). Distinguishing features of these populations are that naïve-mature microglia expresses $CD45^{lo/int}$ and BMDMs express $CD45^{hi}$ (Roesch et al., 2018).

MDSCs play a major immune suppressive role in the TME and are correlated with glioma progression and therapeutic resistance (Kamran et al., 2018b; Ostrand-Rosenberg and Fenselau, 2018). MDSCs are divided into two main subpopulations polymorphonuclear-MDSCs (PMN-MDSCs) and monocytic-MDSCs (M-MDSCs) (Kamran et al., 2018b). They are characterized by different sets of surface markers (Kamran et al., 2018b). MDSCs in GBM have also been found to express high levels of the T-cell exhaustion promoting molecule PD-L1 (Kumar et al., 2016). Study revealed that immune suppressive cells work, in part, by inducing T cell exhaustion. Many T cells within the TME of GBM exhibit an exhausted T cell phenotype, with lower secretion of IFN-γ, IL-2, and TNF-α (Woroniecka and Fecci, 2018; Woroniecka et al., 2018). In addition, exhausted T cells may highly express multiple "inhibitory" receptors, including PD-1, 2B4 (CD244), BTLA, CTLA-4, CD160, LAG-3, and Tim-3 (Wherry and Kurachi, 2015; Osuch et al., 2020). Currently, therapies targeting the classical immune checkpoint pathways responsible for inducing the exhausted T cell phenotype, PD-1 to PD-L1 and CD80/CD86 to CTLA4, are being used to reverse the dysfunctional state and enhance anti-tumor immune response (Kamran et al., 2017; Woroniecka and Fecci, 2018; Woroniecka et al., 2018). Although

blocking the immune checkpoints with anti-PD-1, anti-PD-L1 and anti-CTLA4 has shown promising results and is an effective strategy for many other types of cancers, their ability to bolster the immune response is limited in the case of GBM (Woroniecka and Fecci, 2018; Woroniecka et al., 2018). High frequencies of Tregs are found in gliomas and this occurrence has also been associated with tumor progression and immune evasion (Mu et al., 2017). Tumor cells recruit Tregs by the CCL22/CCR4 and CCL28/CCR10 signaling axes in GBM. IDO expression on GBM tumor cells has also been shown to stimulate Treg recruitment (Mu et al., 2017).

GENE THERAPY AND VIROTHERAPY IN GLIOMA

Gene therapy is a therapeutic approach that consists in utilizing genetic elements in order to treat or prevent disease. Whole genes, regulatory elements or oligonucleotides may be delivered to the target cells in glioma patients either by mechanical methods or using delivery vehicles. In order to achieve high therapeutic efficacy, gene therapy vectors must be chosen with caution, taking into consideration therapeutic transgene expression levels, distribution of gene expression within the TME, immunogenicity and biosafety (Castro et al., 2014; Asad et al., 2017; Kamran et al., 2018a). Gene therapy viral and non-viral vectors have shown efficacy in many pre-clinical studies since their first development in the 90s (Okura et al., 2014), but their clinical implementation still presents many challenges (Lowenstein et al., 2009), which we will highlight below. One of the advantages of gene therapy is that its local administration may overcome the challenges posed by the BBB for systemic delivery approaches. Virotherapy is also an attractive therapeutic approach for glioma; it entails the use of genetically engineered viruses, which are no longer virulent and thus, cannot cause disease, but have the capacity of replicating within tumor cells, causing tumor cell death and release of oncolytic viral particles which can continue to infect and kill neighboring tumor cells.

Viral Vectors for Gene Therapy

Adenoviral Vectors

Adenoviruses are non-enveloped, double stranded DNA viruses that exhibit many advantages i.e., feasibility for genetic manipulation, high titers, low biosafety risks, and excellent safety profile after delivery into the brain. They are able to transduce dividing and non-dividing cells, while their genome remains episomal, thus reducing the risk of insertional mutagenesis. Adenoviral vectors (AdV) genome consists of ∼35 kbp. They possess high cell tropism, since AdV are able to bind to the target cells *via* the interaction between their knob domain and the coxsackie and adenovirus receptor (Castro et al., 2014). AdV can also enter cells by endocytosis after interacting with cell surface integrins (Castro et al., 2014).

First-generation AdV were initially produced by deletion of E1 and E3 regions, which makes them non-replicative (Castro et al., 2014) and are replaced by the expression cassette, which could be up to 8 kbp. Several strategies have been developed for GBM using AdVs. AdVs encoding for the conditionally cytotoxic enzyme Herpes Simplex Virus thymidine kinase (HSV-TK) has exhibited very promising results in clinical trials in GBM. Expression of HSV-TK in glioma cells confers sensitivity to ganciclovir (GCV), as explained below (van Putten et al., 2010). An interesting immunotherapeutic strategy involved the development of a dendritic cell (DC)-specific AdV that targets DEC205, a DC surface receptor, expressing human glioma-specific antigen (CMV-IE) (Kim et al., 2018). This approach showed prolonged survival in a GBM model and, when rechallenged, brain tumor cells were completely rejected (Kim et al., 2018). Since AdV are not completely devoid of viral genes, they are immunogenic, which leads to transient transgene expression (Barcia et al., 2007).

Adeno-associated viruses (AAV) are small replication-defective non-enveloped single stranded DNA viruses from the non-pathogenic parvovirus family (Asad et al., 2017). AAV require a helper virus for its replication inside the host cell, such as adenovirus or HSV (van Putten et al., 2010). AAV have many advantages (Santiago-Ortiz and Schaffer, 2016) and among them AAV have a genome of 4.7 kbp, allows them to rapidly penetrate solid tumors, such as gliomas (Enger et al., 2002). It was reported that a single intracranial injection of AAV encoding human interferon (IFN)-β in human and murine GBM models increases tumor cell death and promotes long-term survival (GuhaSarkar et al., 2017). Many researchers have developed high-efficiency AAV for GBM cells, by selection in culture of a chimeric AAV capsid library generated by DNA shuffling of different *cap* genes, with several different AAV serotypes (Maguire et al., 2010; Zolotukhin et al., 2013). Despite of the many advantages of this vector, at the moment they are not being evaluated in clinical trials; this should be expected soon.

Retroviral Vectors

Retroviruses are single stranded positive sense RNA viruses, whose RNA genome is reverse transcribed into DNA that integrates into the genome of the host cell (Murphy and Rabkin, 2013). They have a cloning capacity of ∼8 kbp, with stable expression of the therapeutic transgene, and can only infect dividing cells (Murphy and Rabkin, 2013). Retroviral vectors (RV) encoding HSV-TK were the first viral vectors to be evaluated in clinical trials for glioma (NCT00001328). This study showed anti-tumor activity, but only in smaller tumors (Caffery et al., 2019). A tumor-selective non-lytic replicating RV, Toca 511, and an extended-release formulation of 5-fluorocytosine (5-FC), Toca FC, enables highly efficient transduction of glioma cells with cytosine deaminase (CD), an enzyme that activates the conversion of 5-FC into the anticancer drug 5-fluorouracil (5-FU) directly within the infected cells (Takahashi et al., 2014). Researchers showed that this treatment also sensitizes GBM cells to radiotherapy (Takahashi et al., 2014). A previous study also revealed tumor eradication and prolonged survival in immunocompetent mice (Ostertag et al., 2012).

Lentiviral Vectors

Lentiviruses are single stranded positive sense RNA viruses that have been widely evaluated for the treatment of GBM (Del

Vecchio et al., 2019). They are similar to RV but exhibit several advantages, mostly because lentiviral vectors (LV) integrate into the host genome but are less prone to insertional mutagenesis. The best-known lentivirus is the human immunodeficiency virus type (HIV)-1, which in 1994 was first seen to transduce lymphocytes (Parolin et al., 1994) and non-dividing cells (Naldini et al., 1996). Third generation HIV-based vectors have been developed with higher transduction efficiency and safety. These vectors may be modified in order to achieve tissue tropism by pseudotyping and exhibit low immunogenicity due to the lack of viral protein expression (Del Vecchio et al., 2019). Lymphocytic choriomeningitis virus-pseudotyped LV were developed to achieve higher transduction efficiency in GBM cells, including glioma stem cells, in relation to normal brain cells (Miletic et al., 2004; Huszthy et al., 2009). LV are the vectors of choice to express silencing RNA (Luan et al., 2015) or for engineering T cells so that they express chimeric antigen receptors specific for GBM antigens (Yu et al., 2017). Researchers developed a LV with a p2A peptide-enabled dual expression system allowing the expression of tumor suppressor proteins growth arrest specific (GAS)-1 and PTEN under the control of a CMV promoter (Sanchez-Hernandez et al., 2018). This vector inhibited the growth of human GBM cells *in vitro* and elicited inhibition of glioma progression in a human GBM xenograft model (Sanchez-Hernandez et al., 2018). A LV encoding a shRNA specific for TLX, an orphan nuclear receptor (NR2E1), essential for neural-stem cell renewal, inhibited human glioma stem cell tumorigenicity in mice, and induced the expression of DNA hydroxylase ten eleven translocation 3 (TET3), a potent tumor suppressor downstream of TLX (Cui et al., 2016). LVs have also been used to encode the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) 9 system. Using this system, it was reported that TEA domain transcription factor 1 (TEAD1) ablation inhibited human GBM cell migration and altered the migratory and epithelial mesenchymal transition (EMT) transcriptome signatures (Tome-Garcia et al., 2018).

Non-viral Vectors for Gene Therapy

Non-viral vectors are emerging as attractive platforms for gene therapy approaches for GBM. Recent studies discussed below, have demonstrated the potential of these delivery technologies.

Non-polymeric Delivery System

Liposomes

Liposomes are artificial, lipid-based microvesicles that are considered as a possible valuable system to achieve therapeutic efficacy in glioma. On this backdrop, a liposomal vector was devised in early 2000s to carry a plasmid coding for HSV-TK which was given to patients with recurrent GBM in a Phase I/II trial *via* intratumoral infusion, followed by administration of the prodrug ganciclovir (Reszka et al., 2005). This therapy was well-tolerated without major side effects. Also, they observed >50% reduction of tumor volume in patients. Although this was a small Phase I trial and thus, it was not powered to determine therapeutic efficacy. Moreover, Kato et al. demonstrated that siRNA-based downregulation of MGMT could enhance the chemosensitivity of malignant gliomas against TMZ using novel liposome, LipoTrust EX Oligo. Such liposome transduced glioma cells are found to be sensitized to TMZ both *in vivo* and *in vitro* models (Kato et al., 2010). A dual targeting with T7 and A7R peptides was developed to target vascular endothelial growth factor receptors 2 (VEGFR2) (Zhang et al., 2017). PEG-conjugated liposomes modified with the Transferrin receptor (TfR) monoclonal antibody (OX26) and chlorotoxin (CTX) significantly promoted cell transfection, increased the transport of plasmid DNA bearing hTERTC27 gene across the BBB and efficiently targeted brain glioma cells both *in vitro* and *in vivo*. This dual targeting therapeutic strategy of OX26/CTX-pL/pC27 against glioma exhibits significant therapeutic efficacy leading to diminished tumor volume and extended survival of glioma bearing rats (Yue et al., 2014). Other liposomal formulations with modified surface and core include magnetite-core cationic liposomes that can be used to activate a heat-shock sensitive promoter in the DNA carried by the liposome, thus regulating expression of the therapeutic gene such as TNFα in glioma cells (Ito et al., 2000).

Nanoparticles

NU-0129 is a spherical nucleic acid gold nanoparticle containing siRNAs targeting Bcl-2-like protein 12 (Bcl2L12) is now in early phase I clinical trials (NCT03020017) for patients with recurrent glioblastoma. It can cross BBB in xenograft GBM mice after systemic administration which results in increased apoptosis of glioma cells and reduced tumor progression (Jensen et al., 2013). RNA nanoparticles are also used to deliver anti-miR-21 in xenograft GBM mice, resulting in tumor regression and increased survival (Lee et al., 2017). Intravenously-administered chlorotoxin (CTX) coupled stable nucleic acid lipid particle (SNALP) formulated anti-miR21 oligo preferentially accumulates within the brain tumor and efficiently silence miR21 expression. This results in increased mRNA and protein levels of RhoB, leading to reduced tumor load and proliferation without inducing any systemic immunogenicity (Costa et al., 2015). Moreover, combined treatment of both nanoparticles formulated anti-miR21 oligo and tyrosine kinase inhibitor Sunitinib exerts enhanced apoptosis and improved survival in mice (Costa et al., 2015).

Development of a library with PBAE based nanoparticles carrying herpes simplex virus type I thymidine kinase (HSV-TK) DNA, resulted in apoptosis of transfected glioma cells. This led to increased median survival of glioma bearing animals when delivered intracranially (Choi et al., 2020). Furthermore, when HSV-TK DNA loaded nanoparticles are delivered in combination with the prodrug ganciclovir (GCV) to glioma cells *in vivo*, they elicited induction of apoptosis and reduction of tumor load in glioma bearing rats (Mangraviti et al., 2015). Another important type of anti-GBM treatment in gene therapy uses different types of RNA such as dsRNA, siRNA or miR101 associated to nanoparticulate systems resulting in enhanced apoptosis of GBM cells. Also inhibition of growth and migration of these cells can be induced through targeting miR34 or proteins like SOX9 and Ras with the same nanoparticulate systems (Shu et al., 2014; Kim et al., 2015b; Alphandery, 2020).

We have recently demonstrated that local treatment of glioma with sHDL (synthetic High-density lipoprotein) mimicking nanodiscs containing ApoAI mimetic peptide, phospholipids, immunogenic cell death inducing chemotherapeutics (ICD) docetaxel and adjuvant CpG oligodeoxynucleotide, effectively elicit anti-glioma T-cell activity and induce immunological memory response against tumor relapse (Kadiyala et al., 2019). We also engineered an albumin based NPs equipped with cell-penetrating iRGD peptide, containing siRNA against Signal Transducer and Activation of Transcription 3 factor (STAT3*i*) and demonstrated that when administered in combination with ionizing radiation, these NPs activate anti-GBM immunologic memory which results in tumor regression and long term survival of GBM bearing mice (Gregory et al., 2020). Other peptide modifications on nano-platforms have been explored to minimize off-target accumulation and facilitate active tumor targeting or mediate BBB transport. For example, IL-13Rα2 is overexpressed on glioma cells, therefore it is an attractive target for peptide-modified nanotherapies (Madhankumar et al., 2006). A study revealed that IL-13-conjugated nanoplatform enhanced therapeutic efficacy in a subcutaneous mouse model of glioma (Madhankumar et al., 2006). Moreover, transferrin receptor (TfR) has been extensively researched as a target for gliomas, because TfR is over-expressed on glioma cells (Kang et al., 2015). Despite exploiting the use of TfR as a target for decades, translation of systems leveraging these finding have been limited (Johnsen et al., 2019). On this backdrop, a seven amino acid peptide (sequence: HAIYPRH, T7), which has a greater affinity for TfR has been used for glioma targeting to deliver siRNA (Wei et al., 2016), coupled with other targeting ligands to demonstrate increased transport across the BBB and greater tumor penetration (Zong et al., 2014).

Oncolytic Viruses

Several oncolytic viruses have been evaluated in preclinical studies or clinical trials for the treatment of GBM. Specificity must be seriously evaluated, taking into consideration the infection capacity of the vector. Oncolytic viruses (OVs) are designed to recognize tumor receptors or to replicate under oncogene promoters in order to improve their tropism and avoid non-neoplastic cells. It was observed that the immunosuppression present in the tumor microenvironment promotes the OV infection capacity and improves the oncolysis (Tobias et al., 2013; Davola and Mossman, 2019). Once infected, the dying tumor cells start the presentation of tumor epitopes, triggering a viral-specific and tumor-specific T cell-mediated immune response, critical for the efficiency of the oncolytic virotherapy (Li et al., 2017). When tumor cells are lysed, tumor-associated antigens (TAA) are released into the tumor microenvironment and recognized by the immune system, which stimulates the recruitment of activated immune cells which overcome the tumor-mediated immunosuppression and activate a systemic response (**Figure 1**) (Marelli et al., 2018). When using antitumor viral gene therapy, the administration and distribution of the vectors must be evaluated, taking into consideration their ability to overcome antiviral immune responses and to cross the BBB.

A genetically engineered third generation oncolytic HSV, G47Δ that is armed with IL-12 showed increased survival in a syngeneic murine GBM stem cell model (Cheema et al., 2013). G47Δ was evaluated in a phase II clinical trial in patients with GBM, who received repeated intratumoral stereotactic injections, in addition to TMZ (Todo, 2019).

Newcastle disease virus (NDV) based vectors have a natural tropism for tumor cells, together with oncolytic potential and immuno-stimulatory properties (Schirrmacher et al., 2019). It was shown that the complementary treatment with LaSota strain of the naturally oncolytic NDV induces increased apoptosis in glioma cells, comparing with TMZ alone (Bai et al., 2018). The combination treatment also significantly extended survival in a rat xenograft tumor model (Bai et al., 2018).

Finally, *in vivo* immunovirotherapy with measles virus (MV) strains in combination with anti-PD-L1 blockade synergistically increased the survival of a murine syngeneic GBM model, together with the enhanced infiltration of activated $CD8^+$ T cells (Hardcastle et al., 2017). MV has already been evaluated in a dose-escalating phase I clinical trial in recurrent GBM in which no dose limiting toxicities were observed (NCT00390299) (**Table 3**).

APPROACHES TO GENE THERAPY

This section was structured taking into account the gene therapeutic approaches against glioma (**Table 2**) that are currently under Phase-I/II/III clinical trials (**Table 3**). We will discuss advantages and limitations of the proposed approaches. We have included the clinical trials that were listed, in clinicaltrials.gov using the key words: "Condition or disease: glioma;" "Study type: interventional studies (clinical trials)"; "Status: Recruiting, not recruiting, not yet recruiting, and active;" "Phase: Phase 1, Phase 2, Phase 3." For "other terms" we used the following words: gene therapy, virus, and antibody. **Table 3** was updated in October 2020 and includes all the clinical trials found under those key words. Trials were organized depending upon 10 major viral vectors currently used in clinical trials.

Suicide Gene Therapy: Conditional Cytotoxic Therapy

Suicide gene therapy is the most studied gene therapy approach for the treatment of glioma. This strategy is based on genes encoding for an enzyme that converts a non-toxic prodrug into a cytotoxic drug. Gene therapy vectors allow restricting enzyme expression to the transduced brain tumor cells, without altering the normal brain parenchyma. In addition, this strategy is toxic for cells that are replicating, and thus, specifically targets dividing tumor cells.

Genetically engineered neural or mesenchymal stem cells (NSC, MSC) may be used as vectors for suicide gene therapy, given their ability to migrate toward tumor cells. Recently, Tamura and colleagues evaluated the efficacy of a LV encoding HSV-TK under the control of a tet-inducible system for the treatment of GBM using neural stem/progenitor cells (NS/PCs) derived from induced pluripotent stem cells (hiPSCs). Results

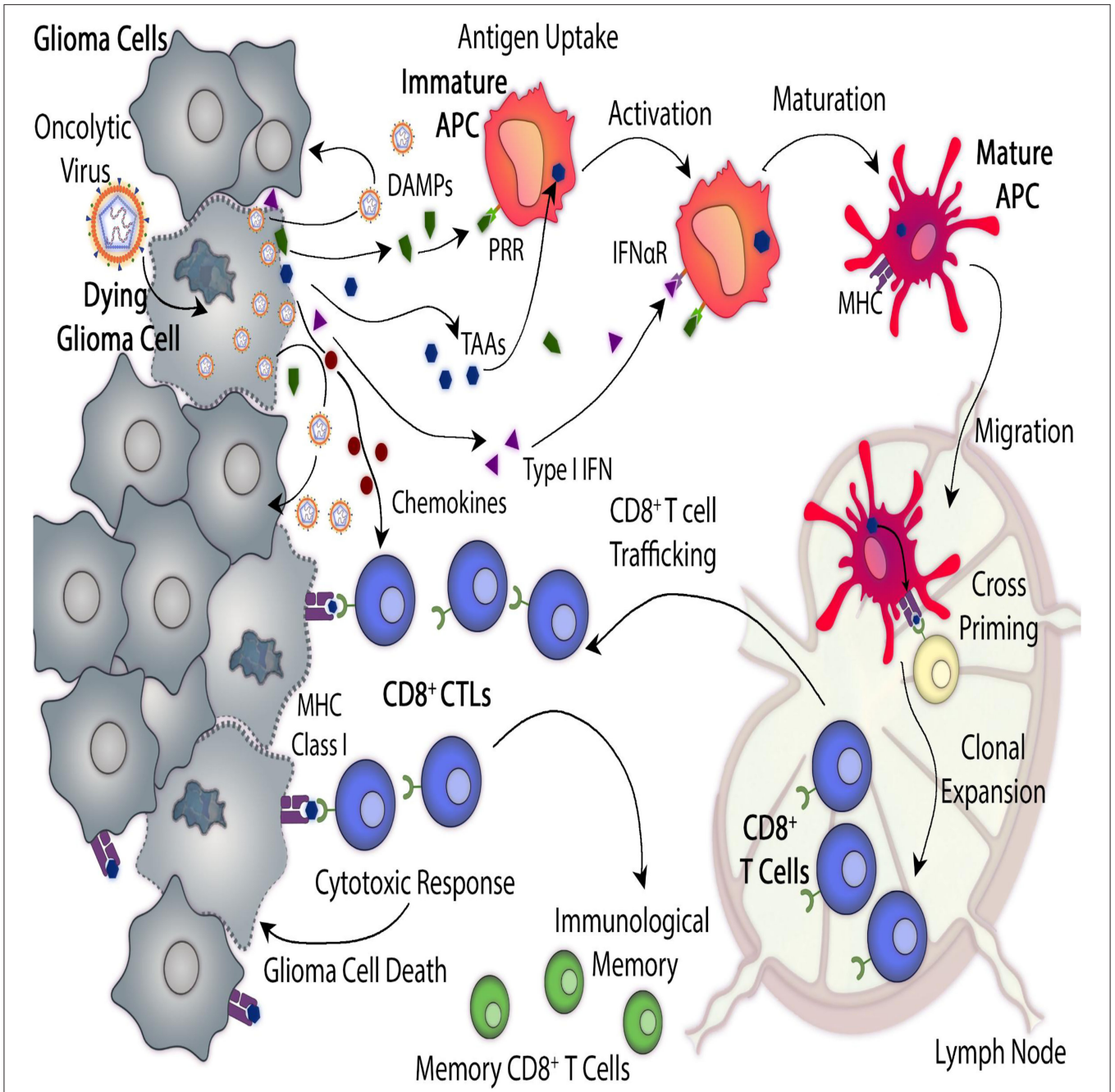

FIGURE 1 | Antitumor mechanisms mediated by oncolytic virus-mediated therapy. Oncolytic viruses (OVs) induce glioma cell death by infecting cells and replicating within them. In addition, OVs trigger immunogenic cell death (ICD) which leads to anti-glioma immunity. Direct virus-mediated cell lysis induces the release of additional virus particles which can infect neighboring glioma cells and continue their replicative cycle. ICD produces immune stimulatory molecules such as tumor cells-derived damage-associated molecular patterns (DAMPs), chemokines and type I interferons (Type I IFN) and they also induce the release of tumor-associated antigens (TAAs). These molecules recruit antigen-presenting cells (APCs) to the site of viral infection, where they get activated, they engulf TAAs and recognize DAMPs which interact with their pattern recognition receptors (PRRs). Mature APCs migrate to the regional lymph node where they prime anti-tumor cytotoxic $CD8^+$ T lymphocytes (CTLs) which leads to anti-glioma immunity. Viral-mediated release of type I IFN and chemokine elicits the recruitment of tumor-specific CTLs to the tumor site. As glioma cells express TAAs, presented by their major histocompatibility complex (MHC) class I, they are recognized, and therefore killed, by $CD8^+$ T cells.

showed the directional migration of these NS/PCs and the consequent inhibition in tumor growth in a human GBM xenograft model (Tamura et al., 2020). Currently, there are around 20 Phase-I/II clinical trials testing the effectiveness of AdV in different types of glioma (**Table 3**). The great majorities of these are studying either the effect of HSV-tk

TABLE 2 | List of Viral vectors used in glioma gene therapy.

Vectors	Genome	Advantages	Disadvantages
Adenovirus (AdV)	Non-enveloped dsDNA Cloning capacity: 8 kbp	Production in high titers. Transduction of diving and non-dividing cells. Non-integrative, avoids insertional mutagenesis. Replicative or oncolytic. Transduce wide varieties of cells. Feasibility for being safely manipulated. Robust expression.	Highly Immunogenic. Existence of anti-AdV immunity in the population, leading to the clearance of the vector.
Adeno-associated virus (AAV)	Non-enveloped dsDNA Cloning capacity: 8 kbp Parvovirus family	Helper virus-dependent replication. They remain episomal in the absence of helper virus. Transduce dividing and non-dividing cells. Lack of immunogenicity Its small size allows the penetration into solid tumors. Transduce wide varieties of cells. Long-term expression.	Possibility of insertional mutagenesis. Low transduction in certain cell types. Existence of anti-AdV immunity in the population, leading to the clearance of the vector. Small cloning capacity.
Retrovirus (RV)	Enveloped ss(+)DNA Cloning capacity: 8 kbp	Stable transgene expression. Feasibility to be modified to achieve higher tropism.	Possibility of inserional mutagenesis. Infection of dividing cells only. Production in low titers Their manipulation requires more biosafety.
Lentivirus (LV)	Enveloped ss(+)DNA Cloning capacity: 8 kbp	Transduce dividing and non-dividing cells. Stable transgene expression. Their integration is less prone to insertional mutagenesis than RV. Transduce hematopoietic cells. Feasibility for being engineered to avoid integration, increasing biosafety. Feasibility to be modified to achieve higher tropism	Possibility of insertional mutagenesis. Engineered non-integrative LV have less stable transgene expression. Production in low titers. Their manipulation requires more biosafety.
Baculovirus (BV)	Enveloped dsDNA Cloning capacity: 38 kbp	Non-integrative, avoids insertional mutagenesis. They do not replicate in human cells, which makes them very safe. There is no pre-existent anti-BV immunity in the population. Transduction of wide varieties of cells. Feasibility to be modified to achieve higher tropism. Large cloning capacity.	They have not been evaluated in clinical trials. Unstable long-term storage.
Herpes simplex virus (HSV)	Enveloped dsDNA Cloning capacity: 20 kbp	Non-integrative, avoids insertional mutagenesis. Replicative or oncolytic. Feasibility to be modified to achieve higher tropism. Large cloning capacity.	Pathogen to humans, so they must be engineered. Immunogenicity. Existence of anti-HSV immunity in the population, leading to the clearance of the vector. Production in low titers.
Newcastle disease virus (NDV)	Enveloped ss(-)RNA Paramyxoviridae family	Non-pathogen to humans. High tropism Oncolytic Selective replication in tumor cells	Limited gene insertion. Limited manipulation.
Measles virus (MV)	Enveloped ss(-)RNA Paramyxoviridae family	High tropism. Feasibility to be modified for being retargeted. Oncolytic Selective replication in tumor cells.	Pathogen to humans, so attenuated strains must be used. Limited gene insertion.

gene therapy or studying the effect of oncolytic AdVs in combination with valacyclovir/ganciclovir or standard of care (SOC) therapies (Chiocca et al., 2011) (**Table 3**). However, encouraging results from a multi-institutional Phase-II study (NCT00589875) (Wheeler et al., 2016) contrasted with negative results from a Phase-III randomized open-label trial using a similar approach (NCT00870181) (Ji et al., 2016). A Phase-I trial is currently evaluating the intratumoral delivery of Ad-TK and oral administration of the prodrug valacyclovir coupled with SOC and the checkpoint inhibitor Nivolumab in newly diagnosed patients with HGG (NCT03576612). Also, a phase III clinical trial revealed that adjuvant therapy with HSV-tk and ganciclovir through retroviral gene therapy delivered to the surgical resection cavity in combination with radiation in adults with previously untreated GBM failed to improve the overall survival of (Rainov, 2000). Although the feasibility and good biosafety profile of this gene therapy strategy were supported in this study. The failure of this specific protocol may be due mainly to the presumably poor rate of delivery of the HSV-tk gene to the tumor cells. In addition, the current mode of manual injection of vector-producing cells

TABLE 3 | List of Viral vectors under clinical trials for glioma.

Sr. no.	Viral vector	Gene therapy	Combination therapy	Condition	Phase	Clinical trial	Status	ID
1	AdV	HSV-TK	Valacyclovir + Std treatment	GBM, Anaplastic astrocytoma	I-II	HSV-tk and XRT and chemotherapy for newly diagnosed GBM	Recruiting	NCT03603405
2		HSV-TK	Valacyclovir + radiation	Recurrent GBM, Astrocytoma grade III	I-II	HSV-tk + Valacyclovir + SBRT + chemotherapy for recurrent GBM	Recruiting	NCT03596086
3		AdV-TK	Ganciclovir + chemotherapy	High grade glioma	II	ADV-TK Improves outcome of recurrent high-grade glioma	Completed	NCT00870181
4		HSV-TK	Valacyclovir + radiation	Malignant glioma, GBM, Anaplastic astrocytoma	Ib	Phase 1b study of AdV-tk + Valacyclovir combined with radiation therapy for malignant gliomas	Completed	NCT00751270
5		HSV-TK	Valacyclovir + Std treatment	Malignant glioma, GBM, Anaplastic astrocytoma	IIa	Phase 2a study of AdV-tk with standard radiation therapy for malignant glioma (BrTK02)	Completed	NCT00589875
6		HSV-TK + Flt3L		Malignant glioma, GBM	I	Combined cytotoxic and immune-stimulatory therapy for glioma	Active	NCT01811992
7		HSV-TK	Ganciclovir + Chemotherapy	Brain and CNS tumors	I	Gene therapy in treating patients with primary brain tumors	Completed	NCT00002824
8		p53		Brain and CNS tumors	I	Gene therapy in treating patients with recurrent malignant gliomas	Completed	NCT00004041
9		p53		Brain and CNS tumors	I	Gene therapy in treating patients with recurrent or progressive brain tumors	Completed	NCT00004080
10		AdV-TK	Valacyclovir + Radiation	Pedriatic brain tumors including GBM, anaplastic astrocytoma, recurrent ependymomas	I	A phase I study of AdV-tk + prodrug therapy in combination with radiation therapy for pediatric brain tumors	Active	NCT00634231
11		Delta-24-RGD (oncolytic AdV)		Recurrent GBM	I-II	Safety study of replication-competent adenovirus (delta-24-rgd) in patients with recurrent glioblastoma	Completed	NCT01582516
12		DNX-2440 (oncolytic AdV)		Recurrent GBM	I	DNX-2440 oncolytic adenovirus for recurrent glioblastoma	Active	NCT03714334
13		DNX-2401 (conditionally replicative and oncolytic AdV)	IFN-γ	Recurrent GBM or gliosarcoma	Ib	DNX-2401 with interferon gamma (IFN-γ) for recurrent glioblastoma or gliosarcoma brain tumors (TARGET-I)	Completed	NCT02197169
14		DNX-2401 (conditionally replicative and oncolytic AdV)	TMZ	Recurrent GBM	I	Virus DNX2401 and temozolomide in recurrent glioblastoma	Completed	NCT01956734

(Continued)

TABLE 3 | Continued

Sr. no.	Viral vector	Gene therapy	Combination therapy	Condition	Phase	Clinical trial	Status	ID
15		AdV-TK	Ganciclovir + Chemotherapy	Recurrent high grade gliomas	II	ADV-TK improves outcome of recurrent high-grade glioma	Completed	NCT00870181
16		DNX-2401 (conditionally replicative and oncolytic AdV)	Pembrolizumab	GBM or gliosarcoma	II	Combination Adenovirus + pembrolizumab to trigger immune virus effects (CAPTIVE)	Active	NCT02798406
17		DNX-2401 (conditionally replicative and oncolytic AdV)		Recurrent high grade gliomas	I	Oncolytic Adenovirus DNX-2401 in treating patients with recurrent high-grade glioma	Recruiting	NCT03896568
18		Ad-RTS-hIL12*	Veledimex	GBM or anaplastic oligoastrocytoma	I	A study of Ad-RTS-hIL-12 with veledimex in subjects with glioblastoma or malignant glioma	Active	NCT02026271
19		Neural stem cells loaded with + oncolytic AdV	Radio and Chemotherapy	Malignant glioma	I	Neural stem cell based virotherapy of newly diagnosed malignant glioma	Completed	NCT03072134
20		Ad-RTS-hIL12	Veledimex	Pedriatic brain tumors or diffuse intrinsic pontine glioma	I	A study of Ad-RTS-hIL-12 + Veledimex in pediatric subjects with brain tumors or DIPG	Active	NCT03330197
21	HSV-1	M032-HSV* + IL-12		Recurrent GBM, progressive GBM, anaplastic astrocytoma or gliosarcoma	I	Genetically engineered HSV-1 phase 1 study for the treatment of recurrent malignant glioma (M032-HSV-1)	Recruiting	NCT02062827
22		C134-HSV* + IRS-1		GBM, anaplastic astrocytoma, gliosarcoma	I	Trial of C134 in patients with recurrent GBM (C134-HSV-1)	Recruiting	NCT03657576
23		G207 (oncolytic HSV-1)		Recurrent brain cancer	I-II	Safety and effectiveness study of G207, a tumor-killing virus, in patients with recurrent brain cancer	Completed	NCT00028158
24		G207 (oncolytic HSV-1)	Radiotherapy	Pediatric recurrent or refractory cerebellar brain tumors	I	HSV G207 in children with recurrent or refractory cerebellar brain tumors	Recruiting	NCT03911388
25		G207 (oncolytic HSV-1)	Radiotherapy	Pediatric progressive or recurrent supratentorial brain tumors	I	HSV G207 alone or with a single radiation dose in children with progressive or recurrent supratentorial brain tumors	Recruiting	NCT02457845
26		G47delta (oncolytic HSV-1)	TMZ	GBM	II	Results of a phase II clinical trial of oncolytic herpes virus G47Δ in patients with glioblastoma	Completed	Todo, 2019

(Continued)

TABLE 3 | Continued

Sr. no.	Viral vector	Gene therapy	Combination therapy	Condition	Phase	Clinical trial	Status	ID
27	LV	Temferon*		GBM with unmethylated MGMT promoter	I-IIa	A Phase I/IIa study evaluating temferon in patients with glioblastoma and unmethylated MGMT (TEM-GBM)	Recruiting	NCT03866109
28		NK-92/5.28.z*		GBM	I	Intracranial injection of NK-92/5.28.z cells in patients with recurrent HER2-positive glioblastoma (CAR2BRAIN)	Recruiting	NCT03383978
29		Modified γδ T cells, resistant to chemotherapy (DRI*)	TMZ	GBM	I	Novel gamma-delta (γδ)T cell therapy for treatment of patients with newly diagnosed Glioblastoma (DRI)	Recruiting	NCT04165941
30		CAR T cells with a chlorotoxin tumor targeting domain		Recurrent or progressive $MPP2^+$ GBM, recurrent grade III glioma, recurrent grade II glioma	I	Chimeric antigen receptor (CAR) T cells with a chlorotoxin tumor-targeting domain for the treatment of $MPP2^+$ recurrent or progressive glioblastoma	Recruiting	NCT04214392
31		IL13Rα2-specific hinge optimized 41BB-co-stimulatory CAR truncated $CD19^+$ autologous T cells*		Recurrent or refractory GBM	I	Genetically modified T-cells in treating patients with recurrent or refractory malignant glioma	Recruiting	NCT02208362
32		HER2(EQ)BBzeta/$CD19t^+$ T cells*		Recurrent or refractory GBM	I	Memory-enriched T cells in treating patients with recurrent or refractory grade III-IV glioma	Recruiting	NCT03389230
33		Autologous $CD8^+$ T cells that express IL13ζ CAR and HSV-TK	Ganciclovir	Recurrent or refractory high-grade malignant glioma	I	Cellular adoptive immunotherapy using genetically modified T-lymphocytes in treating patients with recurrent or refractory high-grade malignant glioma	Completed	NCT00730613
34	MV	Carcinoembryonic Antigen (CEA)		Recurrent GBM	I	Viral therapy in treating patients with recurrent glioblastoma multiforme	Completed	NCT00390299
35	NDV	NDV-HUJ		Recurrent GBM	I-II	Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme	Completed	Freeman et al., 2006
36	RV	Autologous T cells expressing receptors anti-mutated neoantigens		GBM, non-small cell lung cancer, ovarian cancer, breast cancer, gastrointestinal cancer, genitourinary cancer	II	Administration of autologous T-cells genetically engineered to express T-cell receptors reactive against mutated neoantigens in people with metastatic cancer	Recruiting	NCT03412877

(Continued)

TABLE 3 | Continued

Sr. no.	Viral vector	Gene therapy	Combination therapy	Condition	Phase	Clinical trial	Status	ID
37		Toca 511	Toca FC	Recurrent GBM, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma	I	A study of a retroviral replicating vector combined with a prodrug administered to patients with recurrent malignant glioma	Completed	NCT01156584
38		Leukocytes expressing anti-EGFRvIII CAR*	Aldesleukin, Fludarabine, Cyclophosphamide	Malignant glioma, GBM, brain cancer, gliosarcoma	I-II	CAR T cell receptor immunotherapy targeting EGFRvIII for patients with malignant gliomas expressing EGFRvIII	Completed	NCT01454596
39		Autologous HER2-CD28 CMV-T cells		GBM	I	CMV-specific cytotoxic T lymphocytes expressing CAR TARGETING HER2 in patients with GBM (HERT-GBM)	Completed	NCT01109095
4		$CD34^{+}$ cells are transduced with a fibronectin assisted RV expressing MGMT	Filgrastim, iomustine, procarbazine hydrochloride, vincristine sulfate	Bone marrow suppression, brain and CNS tumors	I	Combination chemotherapy plus gene therapy in treating patients with CNS tumors	Completed	NCT00005796
41		Neural stem cells that express cytosine deaminase	5-fluorocytosine	Recurrent high-grade gliomas	Pilot	A pilot feasibility study of oral 5-fluorocytosine and genetically-modified neural stem cells expressing *E. coli* cytosine deaminase for treatment of recurrent high grade gliomas	Completed	NCT01172964
42		Toca 511	Toca FC ± Iomustine, bevacizumab	Recurrent GBM, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma	I	Study of a retroviral replicating vector combined with a prodrug to treat patients undergoing surgery for a recurrent malignant brain tumor	Completed	NCT01470794
43		Toca 511	Toca FC	Recurrent GBM, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma	I	Study of a retroviral replicating vector given intravenously to patients undergoing surgery for recurrent brain tumor	Completed	NCT01985256
44		Chemoprotected autologous stem cells	Radiation, carmustine, O6-benzylguanine	GBM or gliosarcoma	I-II	O6-benzylguanine-mediated tumor sensitization with chemoprotected autologous stem cell in treating patients with malignant gliomas	Active	NCT00669669

(Continued)

TABLE 3 | Continued

Sr. no.	Viral vector	Gene therapy	Combination therapy	Condition	Phase	Clinical trial	Status	ID
45		Allogenic $CD8^+$ T cells expressing IL13-ζ and HSV-TK	Aldesleukin	Recurrent or refractory malignant glioma	I	Phase I study of cellular immunotherapy for recurrent/refractory malignant glioma using intratumoral infusions of GRm13Z40-2, an allogeneic $CD8^+$ cytolitic T-cell line genetically modified to express the IL 13-zetakine and HyTK and to be resistant to glucocorticoids, in combination with interleukin-2	Completed	NCT01082926
46		HSV-TK	Ganciclovir and radiotherapy	GBM	III	A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme	Completed	Rainov, 2000
47	RV vs. AdV	HSV-TK	Ganciclovir	Malignant glioma		Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses	Completed	Sandmair et al., 2000
48	VACV	TG6002 (oncolytic VACV) + FCU1*	5-FC	Recurrent GBM	I	Safety and efficacy of the oncolytic virus armed for local chemotherapy, TG6002/5-FC in recurrent GBM patients (ONCOVIRAC)	Recruiting	NCT03294486
49	PVS	PVSRIPO*		Recurrent GBM	I	PVSRIPO for recurrent GBM	Active	NCT01491893
50	H-1PV	ParvOryx		Progressive primary or recurrent GBM	I-II	Parvovirus H-1 (ParvOrxy) in patients with progressive primary or recurrent GBM. (ParvOryx01)	Completed	NCT01301430

**(18) IL-12 under the transcriptional control of the RheoSwith Therapeutic System (RTS). (21) M032 is an oncolytic HSV that only infects and kills tumor cells. (22) C134 is an oncolytic HSV that safely replicate and kill glioma cells. (27) Temferon: autologous $CD34^+$-enriched hematopoietic stem and progenitor cells exposed to transduction with a lentiviral vector driving myeloid specific interferon-alpha2 expression. (28) The NK-92/5.28.z cell line (also referred to as HER2.taNK) represents a stable, lentiviral-transduced clone of ErbB2 (HER2)-specific, second-generation CAR-expressing derivative of clinically applicable NK-92 cells. 29) DRI, Drug resistant immunotherapy. (31) A preparation of ex vivo expanded, genetically modified autologous central memory-enriched T-cells (Tcm) transduced with a replication-incompetent, self-inactivating (SIN) lentiviral vector expressing a hinge-optimized, chimeric antigen receptor (CAR) specific for interleukin-13 receptor alpha 2 (IL13Ra2), and containing the cluster of differentiation 137 (CD137; 4-1BB) co-stimulatory signaling domain fused to the signaling domain of the T cell antigen receptor complex zeta chain (CD3-ζ), and a truncated form of human cluster of differentiation 19 (CD19t). (32) A preparation of genetically modified autologous central memory (Tcm) enriched T cells transduced with a lentiviral vector expressing a chimeric antigen receptor (CAR) consisting of an anti-human epidermal growth factor 2 (HER2) single chain variable fragment (scFv) derived from trastuzumab, with a 4-1BB (CD137) costimulatory domain that is linked to the signaling domain of the T-cell antigen receptor complex zeta chain (CD3-zeta) (BBz), and truncated CD19 (CD19t). (38) CAR, chimeric antigen receptor. (48) FCU1 encodes a bifunctional fusion protein that converts 5-FC into 5-FU. (49) PVSRIPO is an attenuated chimera that restricts the virus to infect CNS cells but not spinal cord motor neurons.*

with a non-migratory fibroblast phenotype limits the distribution of these cells and the released replication-deficient RV to the immediate vicinity of the needle track. Further evaluation of the RV-mediated gene therapy strategy must incorporate refinements such as improved delivery of vectors and transgenes to the tumor cells and improved delivery of the prodrug across the BBB and blood-tumor barrier to the transduced tumor cells (Rainov, 2000).

Other clinical trials for recurrent glioblastoma or gliosarcoma evaluated directly injected, genetically modified, conditionally replicative and oncolytic human-derived adenovirus, DNX-2401 in combination with IFNγ (NCT02197169). This trial established an active infection with the virus replicating in, and killing neighboring glioma cells. Similarly, patients with recurrent GBM were also treated with DNX-2401 which was delivered into brain tumor followed by up to two 28-day cycles of oral temozolomide (TMZ) using a schedule of 7 days on/7 days off to evaluate the efficacy of this combination (NCT01956734). Both these clinical trials showed encouraging results with respect to survival outcome. In another Phase-I/II trial (NCT01582516) recurrent GBM patients were treated with replication competent adenovirus i.e., Delta-24-RGD through convection-enhanced delivery (CED), showing similar results. Although these trials offered good safety data and indications of anti-glioma activity, one must await results of Phase 3 clinical trials in order to assess therapeutic benefits.

Another example of a conditional cytotoxic approach involves the expression of the yeast or bacterial enzyme CD in cancer cells, activates the conversion of the prodrug 5-FC into the anticancer drug 5-FU (Takahashi et al., 2014). CD is virtually absent in mammalian cells, which makes 5-FC non-toxic to human cells under normal conditions (Okura et al., 2014). Toca 511, is a replication competent RV encoding CD that has demonstrated to promote tumor eradication in mouse glioma models (Ostertag et al., 2012), together with durable antitumor immune responses (Mitchell et al., 2017). Vocimagene amiretrorepvec (Toca 511) or Toca 511 with flucytosine (Toca FC) have been evaluated in Phase I clinical trials which demonstrated safety and good tolerability, with tumor regression at the site of infusion and durable responses in patients with recurrent high-grade glioma (Cloughesy et al., 2018). However, a recent phase III clinical trial (NCT02414165) revealed that among 403 randomized patients who underwent tumor resection for first or second recurrence of GBM or anaplastic astrocytoma, administration of Toca511 or Toca FC compared with standard of care did not improve overall survival or other efficacy end points (Cloughesy et al., 2020).

Novel conditionally cytotoxic enzymes have been recently developed i.e., a novel isocytosine deaminase (ICD) named Vcz converts the prodrug 5-fluoroisocytosine (5-FIC) into 5-FU (Kazlauskas et al., 2019) and the purine nucleoside phosphorylase (PNP), which converts the prodrug fludarabine phosphate (F-araAMP) to diffusible toxic fludarabine (2-F-araA; 2-FA), these have yet to reach testing in the clinical arena.

Targeted Toxins

Toxins have been evaluated in several anti-glioma studies targeting IL13Rα2, the urokinase-type plasminogen activator (uPA) receptor, growth factor receptors and transferrin receptors, due to their differential expression status in glioma cells when compared to normal brain cells (Candolfi et al., 2011; Castro et al., 2011). The natural ligands of these receptors are fused to the catalytic and fusion domains of cytotoxic bacterial products such as *Pseudomonas* and *Diphtheria* exotoxins, which are then internalized and cause apoptosis within glioma cells.

Our group developed a regulatable AdV encoding a mutated human IL-13 fused to *Pseudomonas* exotoxin (PE), under the control of the tet-inducible promoter system that specifically binds to IL13Rα2, expressed by GBM cells that differs from the physiological IL4R/IL13R receptor (Candolfi et al., 2010). When comparing this AdV with the hIL-13-PE protein formulation used in clinical trials (Cintredekin Besudotox) and a second-generation mhIL-13-PE, we found that even though both proteins exhibited severe neurotoxicity Ad-mediated delivery of IL-13-PE, in the presence of Doxycycline, led to tumor regression and long-term survival in over 70% of the animals without apparent neurotoxicity (Candolfi et al., 2010).

Tumor Suppressor Gene Therapy

The aim of tumor suppressor gene therapy is to restore the function of tumor suppressor genes which are commonly inactivated in glioma cells. These genes can regulate diverse cellular functions including cell-cycle regulation, regulation of cellular proliferation and death and DNA damage repair system.

TP53 Gene

p53 is well-documented tumor suppressor gene located on chromosome 17p. Inactivation of p53 is one of the most commonly mutated tumor suppressors in glioma which accounts for ∼50% in grade II and III glioma, 25–30% in primary and 60–70% in secondary GBM (England et al., 2013). Tumor suppressor gene therapy using p53 as a target was first tested by delivering through replication-deficient adenovirus (Kwiatkowska et al., 2013). The most commonly used adenoviral vector for p53 is the type 5 adenovirus in which the E1 region is replaced with the cDNA of the wild type p53 gene and is driven under the control of a CMV promoter (Ad5CMV-p53) (Cirielli et al., 1999; Lang et al., 1999; Li et al., 1999; Shono et al., 2002). There was a marked inhibition of growth in implanted gliomas and significant prolongation of survival of animals following the delivery of wild type p53 gene (Badie et al., 1998; Cirielli et al., 1999; Li et al., 1999). Delivery of wild type p53 also suppressed angiogenesis in GBM (Van Meir et al., 1994). SGT-53 is a transferring receptor-targeted liposomal vector encapsulating wild-type p53 plasmid DNA that can cross the BBB and target GBM cells. This resulted in a reduction of MGMT and induction of apoptosis in GBM xenografts mice (Kim et al., 2014). Ad5CMV-p53 can be most effective when used in combination with radiation and chemotherapy (Biroccio et al., 1999; Shono et al., 2002). Similarly, combined treatment of p53 transfection with FasL, GM-CSF, and B7-1 gene enhances apoptosis and inhibits cell growth (Shinoura et al., 2000; Pan et al., 2010). All these results led to phase-I trials of Ad5CMV-p53 gene therapy in recurrent malignant glioma (NCT00004041, NCT00004080). In another study, combination of baculovirus mediated delivery of p53 gene

with sodium butyrate, a histone deacetylase inhibitor markedly reduced the growth of glioma cells and enhanced the survival of glioma bearing animals (Guo et al., 2011).

Systemic delivery of a nano-platform encapsulating wild type p53 (scL)-p53 also sensitizes cancer stem cells (CSCs) and bulk tumor cells to TMZ and increase apoptosis (Kim et al., 2014). In another study, Misra et al. generated a p53-EGFP-C3 fusion construct which expressed GFP to allow an estimation of p53 mediated anti-glioma activity and delivered them to glioma cells through a cationic cholesterol based nanocarrier prepared by mixing cationic cholesterol Gemini (ChoL-5L) with natural lipid DOPE in a molar 1:4 ratio. Introduction of wild type p53 cDNA through this nanocarrier induced apoptosis and significantly reduced the tumor volume in mice (Misra et al., 2014). Similarly, a nanoplatform assembled by coupling β-cyclodextrin and the cationic polymer polyethyleneimine to a hydrophobic polymer pullulan (PPEICD) was used to codeliver the antitumor drug mitoxantrone and wild type p53 cDNA to glioma cells. Herein β-cyclodextrin serves as a nanocontainer for mitoxantrone while the cationic part can condense p53 cDNA. Delivery of this nanocomplex induced cell death in glioma cells (Mitha and Rekha, 2014).

p16 Gene

P16/CDK4/Rb/E2F is the most commonly altered pathway in gliomas. Therefore, over-expression of p16 gene through recombinant replication-deficient adenovirus significantly reduced the invasion of glioma by suppressing the activity of MMP2 (Chintala et al., 1997). Moreover, data from a previous study revealed that retroviral delivery of $p16^{INK4A}$ gene could effectively inhibit the progression of glioma but only when endogenous pRb is intact (Xande et al., 2020). Similarly, intratumor injection of pCL retrovirus encoding full-length human p16 cDNA resulted in 95% reduction of gliomas *in situ* through necrosis and cell-cycle arrest (Hung et al., 2000). Another study also revealed that adenoviral delivery of p16 gene enhanced radiation induced cell killing possibly by a non-apoptotic mechanism with abnormal nucleation in glioma cells (Hama et al., 2003). Moreover, restoration of the wt-p16 activity into p16-null SNB19 glioma cells significantly inhibited tumor cell invasion (Chintala et al., 1997). Similarly, down-regulation of integrin α(v)β(3) expression and integrin-mediated signaling in glioma cells by adenoviral transfer of antisense urokinase-type plasminogen activator receptor and wild type p16 cDNA resulted in decrease adhesion, migration, proliferation and enhanced survival (Adachi et al., 2001, 2002). However, sometimes cell-cycle arrest following transfer of p16 gene to glioma cells resulted in the development of chemoresistance to some cytotoxic drugs such as cisplatin, paclitaxel, topotecan and ACNU (Fueyo et al., 1998; Hama et al., 1998).

Deregulation of E2F transcription factor, specifically E2F-1 is a critical target of any alteration of the p16/Rb/E2F pathway in glioma. E2F-1 positively regulates the transcription of S-phase genes and drives the cell-cycle progression through G1 checkpoint. Study revealed that transfer of E2F-1 along with p53 to gliomas induced apoptosis and appeared to be more effective than wild type p53 as it can induce apoptosis even in p53 resistant glioma cells (Fueyo et al., 1998). In fact, vectors expressing p16 and p21 were more effective than wild type p53 at improving survival (Wang et al., 2001). Thus, Adenovirus mediated transfer of E2F-1 alone or in combination with wild type p53 to glioma cells should propel the development of clinical trials for glioma treatment. Deregulated p16 expression also plays a crucial role in angiogenesis in glioma. Therefore, transfer of p16 cDNA through recombinant replication-defective adenoviral vector to glioma cells markedly inhibited angiogenesis through suppressing vascular endothelial growth factor (VEGF) expression (Harada et al., 1999).

However, in a recent report, when $p16^{INK4A}$ was expressed under the control of Tet repressor system in glioma cells on a long-term basis, it decreased the expression of Rb, suggesting that this gene therapy approach involving $p16^{INK4A}$, could ultimately have led to the selection of Rb-deficient gliomas (Simon et al., 2002).

PTEN Gene

Phosphatase and Tensin Homolog on chromosome number 10 (PTEN) is a tumor suppressor gene which contains a central catalytic phosphatase core domain that negatively regulates PI3K by dephosphorylation of PIP_3 to PIP_2 and can act as an excellent target for gene therapy (Kanu et al., 2009). PTEN is inactivated in 33% of all gliomas resulting in aberrant activation of PI3K pathways (Dunn et al., 2012). Therefore, the transfer of chromosome 10 to glioma cells induced thrombospandin-1 and inhibited angiogenesis in glioma (Hsu et al., 1996). Restoration of PTEN activity in glioma cells led to suppression of their neoplastic phenotype (Cheney et al., 1998). Forced PTEN expression through AdV conferred sensitivity to temozolomide and/or ionizing radiation (Inaba et al., 2011). Adenoviral re-expression of PTEN in glioma cells inhibited Akt kinase activity, leading to tumor cell apoptosis (Davies et al., 1998). Additionally, adenoviral expression of PTEN demonstrated an anti-angiogenic response in glioma along with decreased proliferation and increased apoptosis in gliomas *in vivo* (Abe et al., 2003; Lu et al., 2004). Another study revealed that replication-defective adenoviral vector, i.e., MMCB mediated PTEN gene transfer to malignant glioma inhibited the growth and survival of the tumor cells, suppressing the tumorigenecity of malignant gliomas (Cheney et al., 1998). It has been found that, overexpression of EGFR and mutation/deletion of PTEN is one of the main genetic changes identified in gliomas. It was demonstrated that combined infection of glioma cells with antisense-hTERT and wt-PTEN bearing adenovirus significantly inhibited proliferation and reduced tumor load both *in vivo* and *in vitro* (You et al., 2007). Similarly, introduction of an expression plasmid carrying shRNA against hEGFR and wt-PTEN cDNA to glioma cells significantly suppressed the tumor cell proliferation, reduced the tumor invasion and promoted tumor cell apoptosis in gliomas (Han et al., 2010). TIMPs (the inhibitors of MMP2) and PTEN are known to be inhibitors of the invasive activities of malignant gliomas. Therefore, adenoviral delivery of TIMP2 and PTEN/MMAC1 cDNA to human glioma cells significantly inhibited invasive phenotype and growth of gliomas *in vivo* (Lu et al., 2004).

Gene Therapy Targeting Signaling Pathways

EGFR and EGFRvIII

EGFRvIII is the most common variant, leading to constitutively active EGFR signaling in glioma (Gan et al., 2013). EGFRvIII is often co-expressed with full-length EGFR in glioma cells. This complicates our understanding of its contribution to tumorigenesis (Shinojima et al., 2003; Fan et al., 2013). Delivery of both viral and non-viral vectors containing antisense-RNA to target EGFRvIII into intracranial glioma xenografts reduced tumor load significantly (Shir and Levitzki, 2002). Treatment with antisense-RNA or siRNA of U251 glioma expressing EGFRvIII also reduced tumor volume (Kang et al., 2006). This EGFR specific siRNA is directed against the TK-domain and were shown to cause 90% knockdown of EGFR mRNA (Kang et al., 2006). Thus, the overall median survival increased by almost 90% (Kang et al., 2006). Blocking the gene expression of both EGFR and β-catenin significantly inhibited the glioma invasive ability (Wang et al., 2013). It was shown that cyclodextrin-modified dendritic polyamine complexes (DexAMs) were effective at delivering EGFRvIII siRNA efficiently and selectively to glioblastoma with minimal toxicity (Kim et al., 2011). Furthermore, co-delivery of EGFRvIII siRNA and erlotinib in GBM was found to significantly inhibit cell proliferation and induce apoptosis in glioblastoma cells (Kim et al., 2011). Similarly, the use of an expression plasmid Pgenesil-1 vector viz. psiRNA-EGFR-PTEN on U251 glioma resulted in the suppression of cell proliferation, arrest of cell cycle, reduction of cell invasion and promotion of apoptosis both *in vitro* and *in vivo* (Han et al., 2010). Herein the vector expresses a small hairpin RNA-targeting EGFR and wild-type PTEN cDNA in glioma cells (Han et al., 2010). In addition, ribozyme targeting EGFRvIII inhibits ERM5-1 and U87MG GBM cells (Halatsch et al., 2000). Herein, anti-EGFRvIII hairpin ribozyme resulted in significant reduction in glioma proliferation (Halatsch et al., 2000). Moreover, treatment with anti-EGFRvIII hairpin ribozymes was shown to reduce EGFRvIII mRNA by 90% and inhibit anchorage-independent growth of U87MG glioma cells (Karpel-Massler et al., 2009). On the other hand, adjuvant miRNA-based therapies also showed potential for glioma treatment. miR-7 appears to be an effective inhibitor of the EGFR signaling in glioma by direct inhibition of the EGFR and down-regulation of Akt signaling, leading to decreased invasiveness of glioma. miR-7 treatment also helped to overcome the radio-resistance properties of glioma (Padfield et al., 2015). Taking into account both the preclinical and clinical experience of targeting the EGFR signaling pathway for GBM therapeutics, it can be concluded that as a monotherapy this approach is unlikely that it will work in the clinical arena, due in part to the heterogeneity of GBM and also the numerous alternative growth promoting pathways that are used by glioma cells. Nevertheless, targeting the EGFR pathway would be a valuable adjuvant strategy to be used in combination with other therapeutic approaches.

VEGF

The expression of VEGF is up-regulated in gliomas. Therefore, targeting VEGF could a promising approach for glioma management. It was shown that efficient delivery of anti-sense VEGF cDNA *via* an adenoviral Ad5CMV-αVEGF vector, into subcutaneous human glioma tumors established in nude mice, inhibited tumor growth (Im et al., 1999). Moreover, direct intra-tumoral injection of a VEGF siRNA-encoding plasmid complexed with linear PEI, efficiently reduced the vascularization of tumors in xenografts (Niola et al., 2006). Like VEGF, high-affinity VEGF receptor Flk-1/KDR (VEGFR-2) also plays a key role in tumor angiogenesis. Strategies to block VEGFR-2 signaling were successfully used to inhibit experimental tumor growth as this is the main signaling axis required for the proliferating tumor endothelium. It has been found that retroviral delivery of mutant-VEGFR1 that lacks the intracellular tyrosine kinase domain led to a strong reduction of glioma growth and angiogenesis in a xenografted C6 glioma model (Heidenreich et al., 2004). Also, the retroviral transfer of full-length VEGFR-1 cDNA caused a significant reduction of glioma growth. The inhibitory effects of the VEGFR-1 mutants and the full length VEGFR-1 were mediated through host tumor endothelial cells. The formation of heterodimers between VEGFR-2 and full length or truncated VEGFR-1 might contribute to the glioma inhibitory effect by modulating distinct signal transduction pathways (Heidenreich et al., 2004). Soluble vascular endothelial growth factor receptor (sFlt-1) also plays an important role in anti-glioma treatment. Co-delivery of sFlt-1 and angiostatin-endostatin fusion gene (Statin-AE) through non-viral sleeping-beauty (SB) transposons to glioma xenografts showed marked reduction in tumor vessel density and tumor load (Ohlfest et al., 2005). Similarly, co-infection of glioma cells with both anti-angiogenic gene therapy vectors Ad-Flk1-Fc, which expresses a soluble VEGF receptor and oncolytic virus dl922/947 whose replication and subsequent cytotoxicity are restricted to cancer cells, yielded significantly higher anti-glioma effect than monotherapy (Thorne et al., 2006). In another study, construction of an oncolytic adenovirus-based shRNA expression system i.e., Ad-DeltaB7-shVEGF revealed a marked reduction in glioma vasculature and tumor load *in vivo*. This study also demonstrated that the duration and magnitude of VEGF silencing by Ad-DeltaB7-shVEGF was greater than the efficacy elicited by the replication-incompetent adenovirus expressing sh-VEGF (Ad-DeltaE1-shVEGF) (Yoo et al., 2007). The delivery of a replication-incompetent adenovirus expressing, VEGF promoter-targeted transcriptional repressor Cys2-His2 zinc-finger proteins, F435-KOX namely Ad-DeltaE1-KOX significantly reduced angiogenesis and tumor load (Kang et al., 2008). Likewise, using the previously mentioned oncolytic adenovirus Ad-DeltaB7 expressing F435-KOX, namely Ad-DeltaB7-KOX, elicited similar anti-glioma efficacy in a human xenograft model (Kang et al., 2008). VEGF and high-affinity VEGF receptor Flk1/KDR (VEGFR2) are key regulators of glioma angiogenesis, thus, inhibition of VEGFR2 expression would inhibit the development of new blood vessels within the tumor microenvironment (TME) and inhibit glioma progression.

Data also revealed that delivery of genetic sequences of antisense RNAs to alter the splicing pattern and expression of the VEGFR2 transcript using pAAV-U7-smOPT vector markedly reduced glioma growth *in vivo* (Muralidharan et al., 2019).

Blood Brain Barrier Disruptive Gene Therapy

Treatment of gliomas could be improved markedly by the development of non-invasive therapeutic approaches that elicit robust, endothelial cell-selective gene expression in specific brain regions. Focused ultrasound (FUS) is one such targeted and non-invasive technique that can be used to activate gas filled microbubbles (MBs) to oscillate within the bloodstream. MBs expand and contract upon sonication by FUS producing cavitation. Stable cavitation is induced by relatively lower amplitude of FUS zzzzz. Generally, FUS elicits endothelial selective transfection without opening the BBB. Study found that magnetic resonance (MR)-guided MB enhanced low intensity pulsed FUS (LIFU) transiently open the BBB and delivers a liposome loaded MGMT inhibitor, O^6-(4-bromothenyl) guanine (O^6BTG) in mice bearing TMZ-resistant gliomas, thereby sensitizing murine and human gliomas to TMZ both *in vivo* and *in vitro* (Papachristodoulou et al., 2019). In another study, researchers developed a VEGFR2-targeted and cationic microbubble (VCMB) gene vector with FUS exposure to allow transient gene delivery. They delivered pHSV-TK/GCV with VCMB under FUS exposure for transgene expression and antitumor effect (Chang et al., 2017). It was also found that there was a significant increase in median survival following single treatment of FUS with doxorubicin in 9L gliosarcoma bearing rats (Treat et al., 2012). Another example is 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) which showed only a relatively limited effect against glioma. However, FUS-mediated delivery of BCNU to glioma-bearing rats greatly increased the intracellular retention and inhibition of tumor progression *in vivo* (Deng et al., 2019). Moreover, Fan et al. fabricated PEG-b-PMBSH-loaded MBs which are formed by boron-containing nanoparticles coupling with MBs for the treatment of GL-261 bearing mouse glioma model (Fan et al., 2019). Thus, FUS in conjunction with MBs has emerged as a unique non-invasive modality for MR image-guided gene delivery to the brain which involves transient disruption of BBB which may induce a sterile inflammatory response. It was found that activating circulating cationic plasmid bearing MBs with pulsed low pressure (i.e., 0.1 MPa) 1.1-MHz FUS facilitates sonoselective gene delivery to the endothelium selectivity varied inversely with the FUS pressure that means with high pressures i.e., 0.3 MPa and 0.4 MPa FUS consistently inducing BBB opening and extravascular transfection.

IMMUNE STIMULATORY GENE THERAPY

Cytokine Mediated Gene Therapy

Cytokine mediated gene therapy involves tumor-selective gene transfer and *in situ* expression of various cytokine genes such as IL2, IL4, IL12, and IFNβ/γ which can induce robust immune responses to glioma cells (Iwami et al., 2010; Tobias et al., 2013). Gliomas can effectively evade the host immune response (Natsume and Yoshida, 2008; Kwiatkowska et al., 2013). The unique characteristics of the CNS immune system in the context of an intracranial glioma, these include a paucity of antigen-presenting DCs, high levels of anti-inflammatory TGF-β and expression of immune checkpoint molecules by glioma cells and tumor infiltrating immunosuppressive cells. These mechanisms play important roles to protect the CNS from immunological attack. Therefore, it is challenging to stimulate the system to develop an effective anti-glioma response (Assi et al., 2012). The susceptibility of glioma stem cells to the cytotoxic effects of the immune system provides the basis for development of anti-glioma immune gene therapy.

Interferon β/γ

IFNβ is a pleiotropic cytokine with antitumoral activity. Therefore, when h-IFNβ expressing adenoviral vector viz. Ad.hIFNβ was introduced into human gliomas stereotactically, it induced increase amount of tumor cell apoptosis *in vivo* (Chiocca et al., 2008). Local administration of intracranial IFNβ gene delivery through adeno-associated viral vectors viz. AAV/P2-Int-mIFNβ also successfully treats orthotopic gliomas with concomitant activation of microglia surrounding the tumors. It is interesting to note that treatment with TMZ prior to AAV-IFNβ abrogated any benefits from the later, while the reverse order of treatment doubled the median survival compared to control population (GuhaSarkar et al., 2017). Moreover, cationic liposome mediated IFNβ gene transfer significantly changes antitumor immune responses and inhibits neovascularization. Many gliomas showed necrotic changes and increased infiltration of $CD8^+$ T-cells and macrophages within the tumor following administration of Ad.hIFNβ (Wakabayashi et al., 2008). A phase I/early phase II clinical trial demonstrated the safety and efficacy of this liposomal approach to deliver a plasmid coding for IFN-β in patients with recurrent malignant gliomas following resection of the tumor (Yoshida et al., 2004). This study revealed that there is upregulation of transgene expression and antitumor activity in most of the patients recruited for the study.

Direct injection of the IFNβ gene with a replication deficient adenovirus demonstrated tumor regression in human glioma xenograft, through the activation of NK cells. It also enhanced the generation of DC, T_H and macrophage cells and stimulated the generation of cytotoxic T-cells activity. Survival was significantly increased in glioma bearing mice (Qin et al., 2001).

Similarly another proinflammatory cytokine IFNγ, produced by NK, DC, and T-cells diminishes the invasive phenotype of glioma cells by inhibiting its interactions with extracellular matrix molecules (Schroder et al., 2004). Use of adenovirus expressing TNFα or IFNγ into tumors enhanced infiltration of $CD4^+$ and $CD8^+$ T cells along with increased expression of MHCI/II on the glioma cells *in vivo*. Intracranial administration of both these genes significantly increases the survival of glioma bearing animals (Ehtesham et al., 2002). In the ongoing trials, $CD34^+$-enriched hematopoietic stem and progenitor cells (HSPCs), NK-cells or different CAR T-cells are administered either with TMZ or with ganciclovir (**Table 3**). For instance, in a Phase-II trial, patients with GBM who have an unmethylated

MGMT promoter administered with single dose of autologous $CD34^+$-enriched HSPCs exposed to transduction with a 3rd generation LV driving myeloid-specific IFN-α2 expression (NCT03866109) (**Table 3**).

IL12

IL12 is one of the most potent anti-tumor cytokines, driving a Th1 response in tumor bearing animals (Tatsumi et al., 2003). Despite its therapeutic success in multiple animal models of cancer, the utility of systemically administered recombinant cytokine has been limited by its toxicity. This has encouraged the development of local IL12 delivery systems through gene transfer. Mice bearing GL-26 gliomas in the right corpus striatum when treated with direct intratumoral administration of replication-deficient adenoviral AdmIL-12 vector, it significantly prolonged the survival of glioma bearing animals with robust infiltration of $CD4^+$ and $CD8^+$ T-cells (Liu et al., 2002). Another study using vaccinia virus expressing IL12 resulted in effective inhibition of subcutaneous C6 glioma growth in mice (Chen et al., 2001). Moreover, combination therapy of glioma with recombinant vaccinia virus mediated IL2 expression, resulted in significant tumor inhibition with concomitant elevation of NK, Mac-1^+ and NKT cells in blood and IFNγ and TNFα expression in tumors (Chen et al., 2000, 2001). Neural stem cells (NSCs) isolated from hippocampi of human embryo were used for lipofectamine-mediated transfer of the IL12 gene to rat glioma cells (Yang et al., 2004). Several other groups have delivered IL12 using different non-adenoviral gene therapy vectors. Among them, γ34.5-deleted HSV-1 (oHSV) expressing mouse-IL12 was shown to exert its oncolytic activity and perform better than other IL12 bearing oHSVs in rodent models of GBM (Hellums et al., 2005). Similarly, Semliki forest virus (SVF) vectors were also used for the delivery of hIL-12 gene to RG2 rat glioma model (Roche et al., 2010). SVF carrying IL12 gene alone when administered through an implanted cannula to the brain, reduced the tumor load and prolonged the survival of RG2 glioma bearing animals not only through the oncolytic activity of SVF but also through activating an anti-tumor immune response (Roche et al., 2010). Despite this, the broad tropism of the SVF-based expression vector may limit its use as a glioma gene therapy vector unless this limitation can be overcome. Human umbilical cord blood-derived mesenchymal stem cell (UCB-MSC) have also been used as gene delivery vehicles, i.e., UCB-MSC-IL12M expressing IL12. It was shown that they inhibited GL26 intracranial tumor growth and prolonged survival when administered in the contralateral brain hemisphere (Ryu et al., 2011). Moreover, surviving mice generated memory response against tumor antigens (Ryu et al., 2011). Non-replicative AAV and replicative HSV have also been used to express IL12 in malignant glioma, resulting in significant inhibition of tumor growth and increased expression of IFNγ with microglial activation and recruitment of T and NK cells (Ahn et al., 2016; Barrett et al., 2018). These data demonstrated that cytokine gene therapy through viral vector mediated IL-12 gene expression may be a promising strategy for glioma treatment. Recently, two different Phase-1 dose-escalation trials (NCT02026271, NCT03330197) revealed that when the resection cavity walls were injected with a fixed dose of a regulatable ADV-hIL12 vector i.e., Ad-RTS (RheoSwitch Therapeutic System)-hIL12, together with an oral activator of IL12 expression, veledimex (VDX), the expression of IFNγ increased in peripheral blood in the enrolled patients. To minimize systemic toxicity, the ligand-inducible expression switch, RTS was developed to locally control the production of IL12 in the tumor microenvironment, during fixed periods of time. Also increased infiltration of PD-1^+ immune population was observed, following Ad-RTS-hIL12 therapy in some of the re-resected tumor samples. Since this was a Phase I trial, it was not powered to assess therapeutic efficacy (Chiocca et al., 2019). Administration of Ad-RTS-hIL12 to glioma patients also revealed pseudo-progression with increased frequencies of tumor infiltrating lymphocytes (TILs) producing IFNγ and expressing PD1 (Chiocca et al., 2019). These inflammatory infiltrates also support an immunological anti-glioma effect of h-IL12 (Barrett et al., 2018; Chiocca et al., 2019).

ONCOLYTIC VIROTHERAPY

Oncolytic virotherapy (OV) is based on genetically engineered viruses with the ability to infect and replicate within tumor cells and then lyse them, releasing new infectious viral particles that can infect neighboring cells leading to immunogenic cells death and immune stimulation (**Figure 1**). As such this approach cannot be considered as gene therapy, nevertheless, OV have been engineered to also harbor therapeutic transgenes, which we will discuss briefly below. In this case, they can be considered gene therapeutic platforms. In addition, OVs have been genetically engineered to express therapeutic transgenes to further boost antitumor immunity.

Among all the studied viruses, only one wild-type virus, an oncolytic double-stranded human RNA *orthoreovirus* (referred as reovirus) is under clinical trial as Reolysin in GBM patients (NCT00528684) (Samson et al., 2018). Reovirus is pathologically benign and it is tumor cytotoxic, making it an appealing OV for therapeutic development. Reovirus selectively targets transformed cells with activated Ras signaling pathways and can lyse cancer cells (Zhao et al., 2016). A dose escalation Phase-I clinical trial is currently evaluating the combination of intravenously administered Reolysin and subcutaneously administered GM-CSF in patients with recurrent HGG (NCT02444546).

The first attenuated mutant HSV serotype 1 TK deficient virus, called *dlsptk*, was incapable of replicating in non-dividing cells like neurons but could replicate in human brain tumor cells and kill them *in vitro* (Martuza et al., 1991). HSV1719 is a *first-generation* virus that is devoid of the γ34.5 (Δγ34.5) gene that suppresses PKR/eIF-2a signaling pathway and IFN-induced anti-viral mechanisms. This virus was evaluated in three successful Phase I trials in GBM patients (summarized in Ning and Wakimoto, 2014). The *second-generation* vector G207 also contains a gene-disrupting insertion of *lacZ* reporter sequence into U_L39, a gene encoding for the large subunit of the viral ribonucleotide reductase (ICP6), that is required for

replication in non-cycling cells (Aghi et al., 2008). Oncolytic selectivity is thought to occur because mutations in viral ICP6 and γ34.5 functions are respectively complemented by mammalian ribonucleotide reductase and GADD34, whose genes are expressed in cycling cells. Therefore, effective replication of OVs might be limited to a subpopulation of tumor cells, as the majority of tumor cells would not be cycling. This approach provides evidence that ICP6-negative OVs can replicate in quiescent tumor cells carrying specific oncogene deletions, independent of cell-cycle status. G207 successfully completed three trials in the USA, showing a well-tolerated antitumor response when the virus was inoculated after or before the tumor resection (Markert et al., 2000, 2009). Currently, there are two Phase I trials recruiting pediatric patients with recurrent or refractory cerebellar brain tumor (NCT03911388) or supratentioral brain tumors (NCT02457845) to determine the safety of G207 alone or combined with radiotherapy. On the other hand, the vector C134 is a chimeric hCMV/oHSV-1 which encodes the protein kinase R evasion gene IRS1 under the control of human CMV, which maintains the late viral proteins synthesis in malignant glioma cells improving amplification and prolonging survival in two different mouse models implanted intracranially with U87MG and U251MG glioma cells (Shah et al., 2007). A Phase I trial is recruiting GBM patients to evaluate this vector (NCT03657576) (**Table 3**). The interim analysis of a study using the genetically engineered oncolytic HSV, G47Δ showed that the 1-year survival rate of 13 patients was 92.3% which was significantly higher when compared to 15% survival rate in control population. A study also showed efficient induction of antitumor immunity and successful targeting of cancer stem cells. Another Phase-II trial (NCT00028158) with conditionally replicating oncolytic-HSV1 viz. G207 demonstrated anti-tumor activity and long-term presence of viral DNA in patients, without any serious adverse effects. No patients developed HSV-encephalitis (Markert et al., 2000). Other clinical trials with HSV are still recruiting (**Table 3**). Moreover, in a Phase-I/II trial, patients with recurrent-GBM were repeatedly administered with oncolytic HUJ, an attenuated lentogenic (nonvirulent) isolate of NDV revealed good tolerability with minimum adverse effects. This finding warrant continued evaluation of NDV-HUJ in GBM (Freeman et al., 2006).

The replication-competent adenovirus DNX-240, marketed as Tasadenoturev, was generated to restrict the viral replication to cells with retinoblastoma pathway deficiency (Fueyo et al., 2003). DNX-240 was first studied in a double-arm Phase-I trial to treat patients with recurrent glioblastoma (rGBM), reporting 20% of patients surviving more than 3 years and three complete responders (NCT00805376) (Lang et al., 2018). Another strategy involves the delivery of neural stem cells transduced with OV Ad5-DNX-2041 or NSC-CRAd-Survivin-pk7 in patients with rGBM and newly diagnosed malignant gliomas respectively (NCT03896568, NCT03072134). Moreover, a Phase II trial is still active, involving the delivery of genetically modified oncolytic adenovirus (DNX-2401) followed by intravenous immune checkpoint inhibitor pembrolizumab to evaluate the treatment efficacy (NCT02798406) (**Table 3**).

Several studies have shown the therapeutic potential of live attenuated oncolytic polio/rhinovirus recombinant (PVSPIRO) in patients with grade IV malignant glioma to evaluate the efficacy of this vector (NCT02986178) (**Table 3**). PVSPIRO has tropism toward CD155 that highly expressed in tumor cells, enables infected tumor cell cytotoxicity and stimulation of an inflammatory response (Brown et al., 2017). Finally, a third PVSRIPO-based therapy is ongoing for pediatric patients with rGBM (NCT03043391) (**Table 3**). Collectively, the successful accrual of these trials will demonstrate whether improved safety, tumor specificity, and efficacy of OVs alone or in combination with other therapies can be translated into the clinic arena.

COMBINATION THERAPIES

In an effort to overcome the shortcomings of monotherapies, combination therapies have been developed. Adenoviruses expressing a secretable angiostatin-like molecule (AdK3) in combination with 7.5 Gy radiation dosage in rat C6 gliomas appeared to be more cytotoxic than either treatment alone (Griscelli et al., 2000). Similarly, IL24 can also induce tumor cell death through various mechanisms including endoplasmic stress induced apoptosis, autophagy, anti-angiogenesis and immune activation (Emdad et al., 2009). In GBM models, the anti-tumor effects of Ad-bearing IL24 were also enhanced by radiation (Yacoub et al., 2003a,b). Histone deacetylase (HDAC) inhibitor was also shown to increase Ad-MDA-7/IL24 lethality through ER stress and activation of the extrinsic apoptotic pathways (Dent et al., 2010). Recently, a complex liposome was engineered to carry both a therapeutic gene TRAIL and a cytotoxic drug paclitaxel combined with re-targeting by inserting a peptide angiopep2 that facilitates BBB crossing. This preparation can effectively deliver TRAIL to glioma cells *in vitro* (Sun et al., 2012). Thus, these approaches constitute a valuable adjuvant therapeutic strategies for glioma. Moreover, combination of drugs with different phase specific cytotoxicities such as combination of p19 and p53 gene therapy, where p19 is important to inactivate p53 inhibitors and p53 itself triggers apoptosis, appear promising to target gliomas. Combination therapy with systemically administered liposomal p53 i.e., SGT-53 and TMZ enhanced antitumor efficacy compared to TMZ alone, demonstrating the ability of SGT-53 to improve chemo-sensitivity (Kim et al., 2014, 2015b).

We have pioneered the combination of Ad-Flt3L and Ad-TK. Combining both these two genes results in GCV phosphorylation which ultimately resulting in tumor cell death (Castro et al., 2014; Kamran et al., 2018a). This induces the release of tumor antigens into the tumor microenvironment and damage-associated molecular pattern molecules (DAMPs), which are molecules that when released into the TME or translocated to the cell membrane during cell death, they trigger an immune response against self-antigens (Kamran et al., 2018a; Altshuler et al., 2020). Our results indicate that release of DAMPs such as HMGB1 from Ad-TK infected tumors is required for the efficacy of Ad-TK+Ad-Flt3L mediated immunotherapy (Candolfi et al., 2009; Curtin et al., 2009). Flt3L increases the migration and

infiltration of DCs into the TME. This glioma infiltrating DCs are able to phagocytose antigens that are released during TK-induced glioma cell death (**Figure 2**) (Curtin et al., 2009; Candolfi et al., 2012). Moreover, HMGB1 activates DCs through TLR2 and then activated DCs transport the antigens to the draining lymph node, generating T-cell mediated cytotoxic immune response (Curtin et al., 2009). This combination therapy provides long-term survival and immunological memory in multiple glioma models. In addition, we have also combined Ad-mediated gene therapy with DC vaccination (Mineharu et al., 2011). We found that compared to either therapy alone, combination of intra-tumoral Ad-Flt3L/Ad-TK with DC vaccination resulted in long-term survival in 90% of glioma bearing animals. Our findings indicated that Ad-Flt3L/Ad-TK modifies the TME that enhances the efficacy of DC vaccination (**Figure 2**) (Mineharu et al., 2011). Work from our team has also recently shown the combining Ad-Flt3L/Ad-TK-mediated gene therapy together with immune-check point blockade, using CTLA4 or anti-PDL1, it significantly increased median survival when compared with either treatment used independently (Kamran et al., 2017). Similar results were obtained when we tested Flt3L/Ad-TK-mediated gene therapy in combination with depletion of immunosuppressive MDSCs (Kamran et al., 2017). Again, indicating that combination therapies are an attractive way forward to develop novel treatment for GBM.

In our first human Phase-I dose escalation trial (NCT01811992) using a combination of two adenoviral vectors expressing HSV1-tk and Flt3L for the treatment of newly diagnosed, resectable malignant gliomas we observed evidence of biological activity as evidenced by increased frequencies of DCs, CD4 and CD8 T cells within the TME (Lowenstein et al., 2019). Our results showed for the first time that reprogramming of the host's brain immune system to recognize gliomas could present an attractive approach for the treatment of malignant brain tumors (Lowenstein and Castro, 2018).

DISCUSSION AND CONCLUSIONS

Although innovative gene-mediated therapies and oncolytic virotherapies (OV) have been developed to treat gliomas, to date, they have failed in improving patients' outcomes compared to current standard of care treatment modalities, including surgery, radiotherapy and chemotherapy. Moreover, drug design and clinical trial implementation all come at a considerable economic cost that often limits the timely development of potentially promising treatments. Opportunities to address the lack of clinical benefit of genetic-based therapies and OV in the clinical arena may be provided by accurate preclinical *in vivo* models which recapitulate the disease processes. We envisage that testing gene therapies in more representative models would be essential to allow scientists to differentiate effective from ineffective therapies before their implementation in the clinical setting (Calinescu et al., 2015; Nunez et al., 2020). The extensive molecular characterization of gliomas has been instrumental in improving our understanding of glioma progression and their response to therapies. Genetic lesions in gliomas also play a critical role in modulating the TME. We believe that all these characteristics need to be closely modeled in preclinical models in order to offer a stronger footing on which to base the development and implementation of gene therapy mediated clinical trials.

As of yet, despite showing promise in the preclinical setting, different innovative therapies have been failed to show efficacy in Phase III clinical trials for GBM. The failure of these treatments can be attributed to tumor heterogeneity, tumor immune escape, and development of resistance to the therapy, the presence of the BBB, anatomical location, GBM invasiveness and immune suppressive TME. Gene therapy approaches that rely on the transduction of most of the tumor cells to be effective, can encounter unsurmountable challenges, due to the low transduction efficacy of currently available delivery platforms. This could be overcome by the use of convection enhanced delivery to achieved widespread transduction, manual delivery at multiple tumor locations and/or combination with immune stimulatory approaches which would rely of tumor antigen specific T cells to eradicate any remaining tumor cells.

In addition, the limitations of OV include limited replication capacity of OV after a few replicative cycles, the lack of widespread distribution of the oncology viruses throughout the tumor mass. Also the immune system of the host, may curtail replicative potential of the oncolytic viruses. As the idea of gene therapy gained hold, an ideal vector system quest began. Searching the database ClinicalTrials.gov for "gene therapy/transfer and the viral delivery system," adenovirus returns 69 studies, adeno-associated virus 41, herpes simplex virus 8, retro virus 61 and lentivirus 20 trials and plasmid delivery returns 19 studies. Currently, the use of AAV and lentiviral vectors is on the rise, while adenoviral vectors appear stable over time. Different viral vectors can be engineered to selectively replicate and kill tumor cells. In spite to the demonstrated safety of different viral and non-viral vector administration to glioma patients, gene therapy still needs to prove its potential as a valuable therapeutic tool for the treatment of gliomas. It has recently become apparent that there is a need for combinatorial treatments in order to elicit higher therapeutic efficacy and better outcomes in the clinical arena. Combinatorial immune-gene therapies offer promising approaches for improving patient survival in GBM. Considering the numerous therapeutic approaches developed, the several possible targets, the improved current SOC and alternative dosing regimens and delivery routes, the number of potential combinations has increased exponentially. Several combinatorial approaches are today under clinical trials.

In this respect, results from a Phase I clinical trial in which anti-PD-L1 was administered before and after GBM resection, demonstrated the importance of the selection of the starting point of the treatment. Moreover, as drug penetration in the brain is an issue for GBM treatment, different ways of administering these agents are being assessed and, so far, intracranial delivery, though invasive, has demonstrated to be the most efficient in several approaches. Nanoparticles have emerged as a new and safe method for the delivery of agents targeting brain tumors and preclinical results are

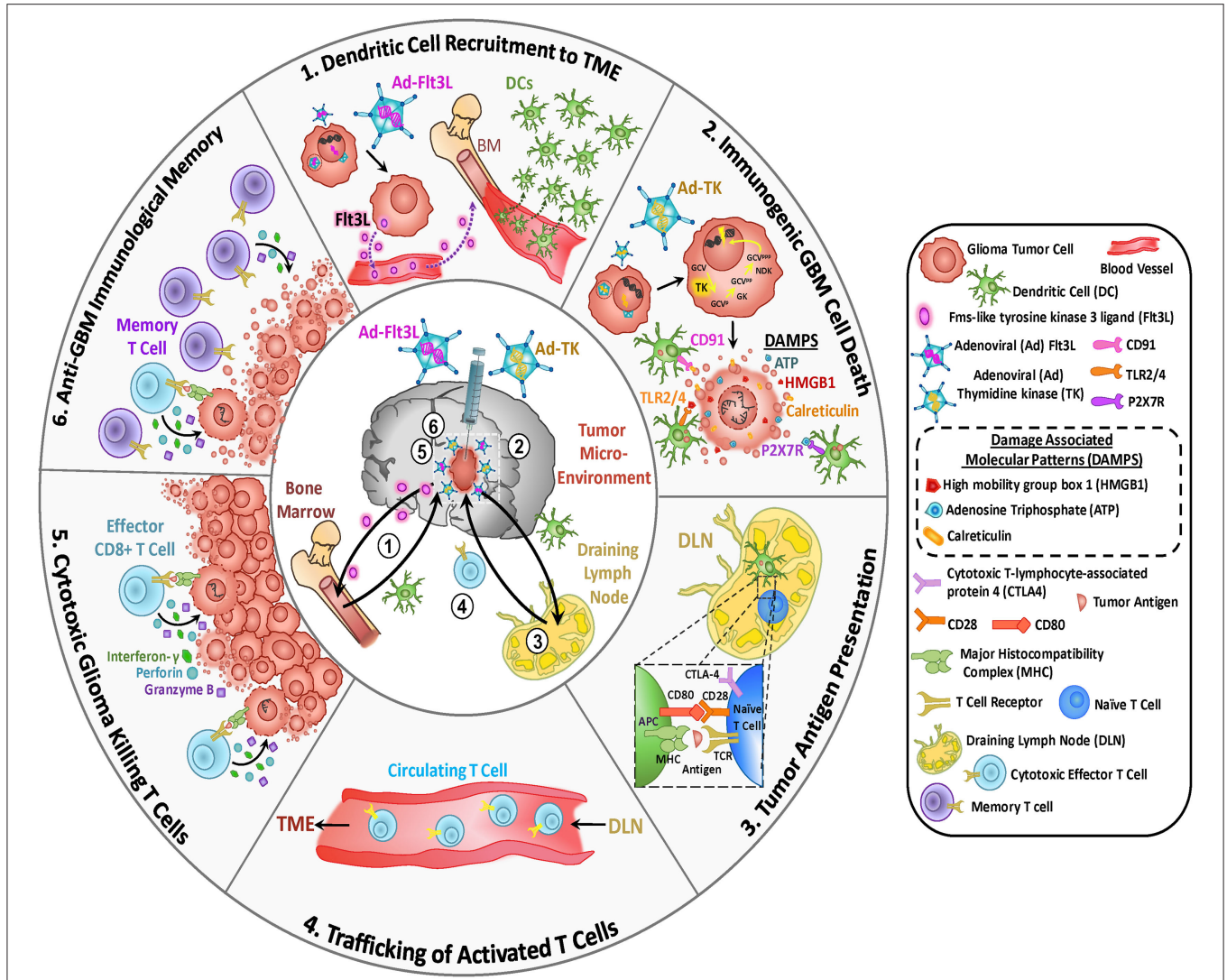

FIGURE 2 | Mechanism underlying the anti-glioma immune response following TK/Flt3L gene therapy. First generation adenoviral vectors (Ad) encoding HSV1-Thymidine Kinase (TK) and HSV1- FMS-like tyrosine kinase 3 ligand (Flt3L) are injected into the tumor cavity following surgical resection. (1) Dendritic Cell Recruitment to Tumor Microenvironment (TME): Tumor cells infected with Ad-Flt3L express Flt3L (pink circles) releasing it into the circulation. Flt3L in the bone marrow (BM) to induces dendritic cells (DCs) expansion, migration, and accumulation within the TME. (2) Immunogenic Glioblastoma (GBM) Cell Death: The prodrug ganciclovir (GCV) is administered systemically. Tumor cells infected with Ad-TK express TK protein which is capable of converting GCV to GCV-monophosphate (GCVp). This intermediate is further phosphorylated by cellular kinases: guanylate kinase (GK) and nucleoside diphosphokinase (NDK). GCV triphosphate (GCVppp) is a purine analog that selectively inhibits DNA replication in proliferating tumor cells leading to DNA breaks and apoptosis. The expression of TK in the presence of GCV mediates the release of damage associated molecular patterns (DAMPs), i.e., HMBG1, Calreticulin, and ATP from dying tumor cells. Expression of Flt3L recruits DCs into the tumor milieu where they take up brain tumor antigens released from the dying glioma cells. These DAMPs bind their corresponding receptors expressed on DCs. HMGB1 binds to TLR2/4, which promotes the production of cytokines and tumor antigen cross-presentation. The binding of extracellular ATP to purigenic receptor P2X7R further promotes the recruitment of DCs. Calreticulin binds to the CD91 receptor, which plays a major role in immunosurvillence. (3) Tumor Antigen Presentation: The DCs loaded with tumor antigens migrate to the cervical draining lymph node (DLN) where they present tumor antigens (Ag) to naïve T cells on MHC, priming tumor specific anti-glioma effector T cells. (4) Trafficking of Activated T cells: Primed $CD8^+$ effector T cells enter circulation from DLN and migrate toward the TME. (5) Cytotoxic Glioma Killing T Cells: The tumor specific effector T cells enter the TME and kill residual glioma cells *via* the production of granzyme B, perforin and effector cytokine IFN-γ. (6) Anti-GBM Immunological Memory: Continual exposure of T cells to tumor antigens promotes immunological memory. Memory T cells (CD103 and CD69) facilitate an anti-tumor response resulting in inhibition of tumor recurrence.

encouraging. It would be interesting to test the efficacy of these particles for the delivery of immune-stimulatory agents in the clinical setting. Finally, there is an urgent need for increased translational research and novel clinical trials to determine the potential efficacy of these novel therapies in glioma patients.

AUTHOR CONTRIBUTIONS

KB, FN, SH, BLM, SVF, SC, JY, MSA, AA, AC, MLV, MC, PL, and MGC wrote the manuscript with overall guidance, revisions, and edits from PRL and MGC. All authors read and approved the final version of the manuscript.

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Comparative Effectiveness of Intracerebroventricular, Intrathecal, and Intranasal Routes of AAV9 Vector Administration for Genetic Therapy of Neurologic Disease in Murine Mucopolysaccharidosis Type I

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Mucopolysaccharidosis type I (MPS I) is an inherited metabolic disorder caused by deficiency of the lysosomal enzyme alpha-L-iduronidase (IDUA). The two current treatments [hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT)], are insufficiently effective in addressing neurologic disease, in part due to the inability of lysosomal enzyme to cross the blood brain barrier. With a goal to more effectively treat neurologic disease, we have investigated the effectiveness of AAV-mediated IDUA gene delivery to the brain using several different routes of administration. Animals were treated by either direct intracerebroventricular (ICV) injection, by intrathecal (IT) infusion into the cerebrospinal fluid, or by intranasal (IN) instillation of AAV9-IDUA vector. AAV9-IDUA was administered to IDUA-deficient mice that were either immunosuppressed with cyclophosphamide (CP), or immunotolerized at birth by weekly injections of human iduronidase. In animals treated by ICV or IT administration, levels of IDUA enzyme ranged from 3- to 1000-fold that of wild type levels in all parts of the microdissected brain. In animals administered vector intranasally, enzyme levels were 100-fold that of wild type in the olfactory bulb, but enzyme expression was close to wild type levels in other parts of the brain. Glycosaminoglycan levels were reduced to normal in ICV and IT treated mice, and in IN treated mice they were normalized in the olfactory bulb, or reduced in other parts of the brain. Immunohistochemical analysis showed extensive IDUA expression in all parts of the brain of ICV treated mice, while IT treated animals showed transduction that was primarily restricted to the hind brain

with some sporadic labeling seen in the mid- and fore brain. At 6 months of age, animals were tested for spatial navigation, memory, and neurocognitive function in the Barnes maze; all treated animals were indistinguishable from normal heterozygous control animals, while untreated IDUA deficient animals exhibited significant learning and spatial navigation deficits. We conclude that IT and IN routes are acceptable and alternate routes of administration, respectively, of AAV vector delivery to the brain with effective IDUA expression, while all three routes of administration prevent the emergence of neurocognitive deficiency in a mouse MPS I model.

Keywords: MPS I, IDUA, AAV9, gene therapy, intracerebroventricular administration, intrathecal injection, intranasal infusion

INTRODUCTION

The mucopolysaccharidoses are a group of rare inherited lysosomal disorders caused by a deficiency in the activity of specific lysosomal enzymes, leading to aberrant glycosaminoglycan (GAG) catabolism (Muenzer, 2011; Wraith and Jones, 2014). This results in abnormal GAG accumulation in lysosomes and leads to progressive cellular damage in multiple organ systems. Mucopolysaccharidosis type I (MPS I) is caused by deficiency of the enzyme α-L-iduronidase (IDUA) and has a disease spectrum that ranges from mild to severe. The severe form of the disease (Hurler syndrome) is the most prevalent of MPS I, with an incidence of 1:100,000. Accumulation of heparan and dermatan sulfate leads to systemic disease including growth impairment, hepatosplenomegaly, cardiac disease, skeletal dysplasia, severe neurocognitive impairment, and if untreated generally death is observed by age 10. Current treatments include allogeneic hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). HSCT is effective in treatment of peripheral disease, with improvement and partial restoration of several symptoms such as growth, mobility, and hepatosplenomegaly. Although HSCT impedes neurological decline, post-transplant patients continue to exhibit below normal IQ and impaired cognitive ability (Krivit, 2004; Hess et al., 2004; Orchard et al., 2007). Recombinant enzyme is used in patients immediately upon diagnosis and is effective in the treatment of systemic disease (Rohrbach and Clarke, 2007). However, ERT has limited effect on neurologic disease due to inability of the enzyme to cross the blood brain barrier (Begley et al., 2008).

A primary goal of genetic therapy for MPS I is delivery of enzyme to the CNS, in order to address neurologic manifestations of the disease. AAV vectors, especially AAV serotype 9, are particularly effective in transducing a wide variety of tissues in the body, including tissues of the CNS. AAV9 has also been shown to cross the blood brain barrier, which makes it particularly useful for systemic delivery with access to the brain (Duque et al., 2009; Foust et al., 2009; Gray et al., 2011; Zhang et al., 2011). Different routes of administration can thus be used to access the CNS, including direct injection into the parenchyma of the brain (Desmaris et al., 2004; Ciron et al., 2009; Ellinwood et al., 2011), injection of vector into cerebroventricular space (Wolf et al., 2011; Janson et al., 2014; Hordeaux et al., 2018), IT administration into the cisterna magna or the lumbar area (Watson et al., 2006; Hinderer et al., 2014a, 2015; Hordeaux et al., 2019), intravenous injection (Hinderer et al., 2014b; Belur et al., 2020), and intranasal (IN) administration (Wolf et al., 2012; Belur et al., 2017). Site specific *in vivo* genome editing using engineered zinc finger nucleases delivered via AAV8 targeted to the liver, leads to prevention of neurobehavioral deficits in MPS I mice (Ou et al., 2019). Direct vector injection into the CNS is invasive, but it is also the most effective means of transducing large areas of the brain in comparison to other routes of administration, especially when administered intracerebroventricularly.

We previously reported the effectiveness of intracerebroventricular (ICV) IDUA-transducing AAV8 vector in the prevention of neurocognitive dysfunction in neonatally treated MPS I mice (Wolf et al., 2011). We also demonstrated the effectiveness of intranasally administered IDUA transducing AAV9 (Belur et al., 2017) and the high level of systemic IDUA achieved in adult MPS I mice intravenously administered IDUA-expressing AAV9 or AAVrh10 vector (Belur et al., 2020). Results from these studies show the potential for achieving high-level expression of IDUA and delivery to the CNS using a less invasive route of AAV vector administration.

In further pursuit of this goal, here we report a direct comparison of intracerebroventricular (ICV), intrathecal (IT), and intranasal (IN) routes of IDUA-transducing AAV9 vector in adult MPS I mice. Supraphysiological levels (1000 times higher than wt) of IDUA were widespread in different parts of the brain after ICV injection of IDUA-expressing AAV9. IDUA levels in the brain were comparatively reduced (about 10-fold) after IT administration as opposed to the ICV route, although relatively higher in the hindbrain than in the forebrain or the midbrain. Minimally invasive IN instillations restored wild-type or near wild-type levels of enzyme in all parts of the brain, with a much higher level of enzyme observed in the olfactory lobe. Despite the varying levels of enzyme found in different parts of the brain, all 3 routes of administration prevented neurocognitive deficit in treated animals as determined in the Barnes maze. We conclude that while ICV infusion of IDUA-transducing AAV9 achieves the highest level of IDUA expression in the CNS, the lower levels of IDUA observed after less invasive IT or IN infusion are nonetheless sufficient to ameliorate neurocognitive deficit in MPS I mice.

MATERIALS AND METHODS

Vector Construct

Generation of the miniCAGS regulated IDUA (AAV-MCI) expression cassette (pTR-MCI) has been described previously (Wolf et al., 2011). This vector was packaged into AAV9 virions at the University of Pennsylvania vector core, generating recombinant (r) AAV9-IDUA. Vector titer was 1×10^{13} genome copies/ml.

Animals and Immunomodulation

The MPS I mouse strain was generously provided by Dr. E. Neufeld and IDUA$^{-/-}$ offspring were generated from homozygous IDUA$^{-/-}$ and homozygous$^{-/-}$ by heterozygous$^{+/-}$ breeding pairs. Animals were maintained under specific pathogen-free conditions in AAALAC-accredited facilities. Animal work was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. In order to avoid immune responses, MPS I IDUA-deficient animals were immunotolerized starting at birth with an intravenous injection of 5.8 μg/g Aldurazyme (supplied by Dr. P. Orchard), followed by 5 subsequent weekly intraperitoneal injections. A second group of animals was immunosuppressed with cyclophosphamide (CP) at a dose of 120 mg/kg, administered weekly by intraperitoneal injection, starting at 1–3 days after vector infusions.

Vector Infusions

Immunotolerized and immunosuppressed MPS I animals were administered vector through the ICV, IT, and IN routes with AAV9-MCI vector at 3 months of age. For **ICV delivery**, (immunotolerized, $n = 9$; immunosuppressed, $n = 4$; no immunomodulation, $n = 4$) mice were anesthetized with 100 mg/kg ketamine and 16 mg/kg xylazine. The animal was secured in a Kopf stereotactic frame, and the lateral ventricle was targeted with a Hamilton syringe (AP, +0.4 mm anterior to bregma; ML, +0.8 mm right from midline; depth, 2.4 mm deeper from dura) using standard surgical techniques. Ten microliters (1×10^{11} vector genomes) of AAV9-MCI was infused into the right lateral ventricle by hand using a 10 μl Hamilton 701 N syringe (Hamilton Chromatography). Briefly, once the syringe was inserted to the designated coordinates, infusion of the AAV vector was begun after a 1 min break. One microliter of vector was infused per minute for a period of 10 min. After completion of the infusion, the syringe was left in place for two additional minutes before removal of the syringe and suturing of the scalp. The animals were returned to their cages on heating pads for recovery. For **IT injections** (immunotolerized, $n = 9$; immunosuppressed, $n = 5$; no immunomodulation, $n = 3$), 10 μl containing 1×10^{11} vector genomes was injected. The needle (30-gauge, 0.5-inch) was connected to a length of PE10 tubing, which was then connected to a second needle that was attached to a 50 μl Luer-hub Hamilton syringe. The injection was administered to conscious mice by gently gripping the iliac crest of the rodent and inserting the needle (bevel side up) at about a 45° angle centered at the level of the iliac crest. The injector positions the needle such that it slips between the vertebrae and makes contact with the dura mater. A reflexive flick of the tail indicated puncture of the dura mater. The injector depresses the Hamilton syringe and introduces the injectate into the CSF of the subarachnoid space (Hylden and Wilcox, 1980; Fairbanks, 2003). For **IN administration** (immunosuppressed, $n = 7$), mice were anesthetized and placed supine. Vector was administered by applying a series of four 3 μl drops with a micropipette to the nasal cavity of each mouse, alternating between right and left nostrils, at 1 min intervals between each nostril, for a total of 12 μl and a full dose of 1×10^{11} vector genomes.

IDUA Enzyme Assay

Animals were sacrificed at 3 months post-vector infusion, transcardiacally perfused with 50 ml PBS, and brains dissected into right and left hemispheres. Each hemisphere was microdissected on ice into olfactory bulb, cortex, striatum, hippocampus, cerebellum, thalamus, and brainstem. Tissues were frozen on dry ice and stored at −20°C until processed. Tissues were homogenized in 0.9% saline in a bullet bead blender, and homogenates were clarified by centrifugation. Tissue lysates were assayed for IDUA activity in a fluorometric assay using 4-MU iduronide as substrate (Glycosynth, England), as previously described (Garcia-Rivera et al., 2007). Emitted fluorescence was measured in a BioTek Synergy Mx plate reader. Protein was measured using the Pierce assay. Enzyme activity is expressed as nmol 4-methylumbelliferone released per mg protein per hour (nmol/mg/h).

GAG Assay

Tissue lysates were assayed using the Blyscan Sulfated Glycosaminoglycan Assay kit (Accurate Chemical, NY) based on the manufacturer's protocol. Tissue GAGs were normalized to protein and expressed as μg GAG/mg protein.

Quantitative Polymerase Chain Reaction

Genomic DNA was extracted from tissue homogenates using the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific). Reaction mixtures contained 200 ng of DNA, 2× IQ SYBR Green Supermix (Bio-Rad), and 200 nM each of forward and reverse primer. IDUA primers used were forward primer: 5′-AGGAGATACATCGGTACG-3′ and reverse primer: 5′-TGTCAAAGTCGTGGTGGT-3′. PCR conditions were: 95°C for 2 min, followed by 40 cycles of 95°C for 40 s, 58°C for 30 s, and 72°C for 1 min. The standard curve for IDUA consisted of serial dilutions of plasmid pTR-MCI.

Immunohistochemistry

At 3 months post-vector infusion, mice were deeply anesthetized and perfused via the heart with calcium-free Tyrode's solution (in mM: NaCl 116, KCl 5.4, $MgCl_2 \cdot 6H_20$ 1.6, $MgSO_4 \cdot 7H_2O$ 0.4, NaH_2PO_4 1.4, glucose 5.6, and $NaHCO_3$ 26) followed by fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9). Tissues were dissected and stored in PBS containing 10% sucrose and 0.05% sodium azide at 4°C for a minimum of 24 h before being frozen and sectioned

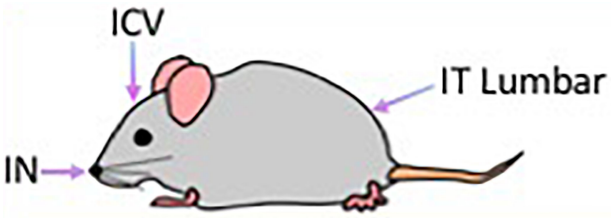

FIGURE 1 | Routes of AAV9-IDUA administration to access the brain. ICV, Intracerebroventricular; IT, Intrathecal; IN, Intranasal.

at 14 μm thickness using a cryostat. Sections were mounted onto gel-coated slides and stored at −20°C until further use. For immunohistochemical staining, sections were incubated in diluent (PBS containing 0.3% Triton-X100; 1% bovine serum albumin, 1% normal donkey serum) for 1 h at room temperature followed by incubation in primary antisera overnight at 4°C. Primary antisera included sheep anti-IDUA (specific for human IDUA; R & D Systems, Minneapolis, MN, 1:500), rabbit anti-Iba1 (Wako, 1:1,000), rabbit anti -LAMP1 (Abcam 1:500), rabbit anti-NeuN (Abcam, 1:500). For IDUA immunostaining, n = 3(ICV), n = 2 (IT), n = 1 (MPS I, Het). Sections were rinsed in PBS, incubated in species appropriate secondary antisera (Cy2 1:100, Cy3 1:300, Cy5 1:300; Jackson ImmunoResearch, West Grove, CA) for 1 h at room temperature, rinsed again using PBS, and coverslipped using glycerol and PBS containing p-phenylenediamine (Sigma). Images were collected using an Olympus Fluoview 1000 confocal microscope and adjusted for brightness and color using Adobe Photoshop software.

Barnes Maze

At 6 months of age (3 months post-vector infusion), mice (n = 9–10 animals per group) were analyzed for neurocognitive deficits and spatial navigation using the Barnes maze as described previously (Belur et al., 2017). Animals were administered 6 trials a day for 4 days. Latency to escape was recorded and analyzed.

Statistical Analyses

GraphPad Prism (GraphPad software) was used for all statistical analyses. For IDUA plasma activity and Barnes maze, data were compared to normal heterozygote levels and untreated MPS I mice, respectively, using two-way ANOVA, followed by Dunnett's multiple comparisons test. Tissue IDUA activity and GAG levels were compared to heterozygote levels using the Kruskal Wallis test. Significance cutoff of < 0.05 was used.

RESULTS

High Levels of Enzyme Activity in the Brain After CNS-Directed Delivery of IDUA Transducing AAV9

Intracerebroventricular (ICV), intrathecal (IT), and intranasal (IN) routes of AAV9 delivery to the CNS were comparatively evaluated for IDUA expression in an IDUA deficient mouse model of MPS I (**Figure 1**). Expression of human IDUA in C57BL/6 mice can be compromised by immune response, so in our studies animals were either immunotolerized with Aldurazyme or immunosuppressed with cyclophosphamide (CP) as described in Methods, with subsequent administration of AAV9-IDUA vector at 3 months of age. Experimental animals were euthanized at 12 months of age and tissues harvested for analysis of IDUA expression, storage material and vector biodistribution.

ICV administration of AAV9-IDUA into MPSI animals resulted in supraphysiological levels of IDUA in all areas of the micro-dissected brain (**Figure 2**). Enzyme activity in tissue extracts from IDUA deficient control animals was undetectable, while in Aldurazyme tolerized animals, enzyme levels ranged from 4- to 1000-fold that of control heterozygous animals (**Figure 2A**). In animals administered CP, enzyme levels ranged from about 100–1,000-fold above normal (**Figure 2B**). Surprisingly, animals that did not receive CP also showed very high levels of enzyme activity that were similar to those of

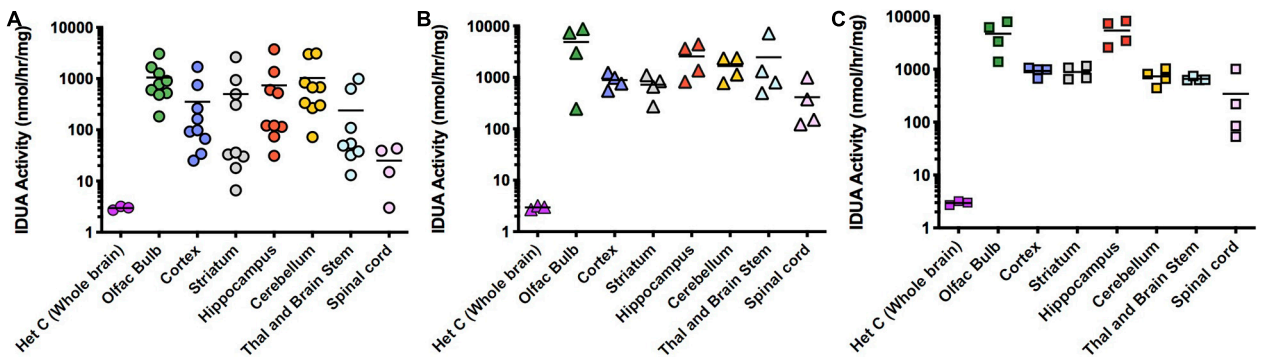

FIGURE 2 | IDUA activity in the brain after ICV administration of AAV9-IDUA vector. Brains were microdissected and assayed for IDUA enzyme activity. Each data point indicates a value from a single animal with the mean indicated by the short horizontal line. **(A)** Animals were immunotolerized with Aldurazyme (laronidase) (n = 9). **(B)**. Animals were immunosuppressed with cyclophosphamide (n = 4). **(C)** Animals were not immunomodulated (n = 4). Widespread enzyme activity was seen in all ICV treated groups compared to heterozygote normal controls (n = 3) regardless of whether they were immunomodulated or not. Enzyme was not detected in untreated MPS I animals (<0.02 nmoles/h/mg protein) (n = 3).

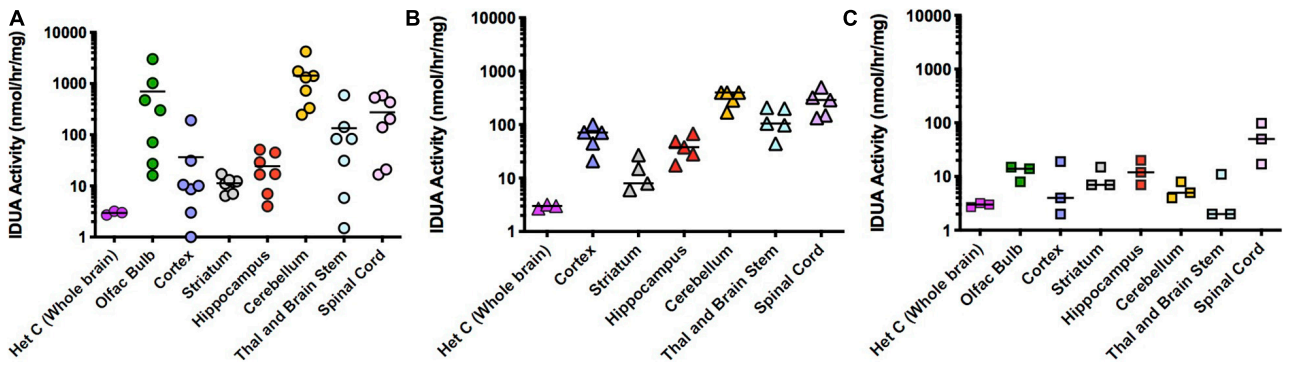

FIGURE 3 | IDUA Activity in the brain after IT administration of AAV9-IDUA vector. Brains were microdissected and assayed for IDUA enzyme activity. Each data point indicates a value from a single animal with the mean indicated by the short horizontal line. **(A)** Animals were immunotolerized with Aldurazyme (laronidase) (n = 9). Widespread enzyme activity was seen in the immunotolerized IT treated group compared to heterozygote normal controls (n = 3). **(B)** Animals were immunosuppressed with cyclophosphamide (n = 5). Animals that were immunosuppressed had lower levels of activity in the cerebellum, compared to immunotolerized animals, although the difference was not significant. P-values for treated animals were < 0.01 compared to untreated controls. **(C)** Animals were not immunomodulated (n = 3). Activities in these animals were lower for several areas of the brain, notably the olfactory bulb, cortex, cerebellum, thalamus and brain stem. Levels of enzyme activity were close to that of normal heterozygote controls. Enzyme was not detected in untreated MPS I animals (<0.02 nmoles/h/mg protein) (n = 3). P-values for treated animals were < 0.05 compared to untreated controls.

CP administered animals (**Figure 2C**). Enzyme activities were observed to be evenly distributed among the various parts of the brain, with slightly lower levels detected in the spinal cord.

IT administration of AAV9-IDUA into IDUA deficient mice also resulted in high levels of enzyme expression, although activity levels were lower than those observed after direct ICV administration to the brain (**Figure 3**). IDUA expression in Aldurazyme-tolerized animals ranged from normal to 1,000-fold higher than normal (**Figure 3A**). In CP administered animals, levels ranged from normal to 100-fold above normal (**Figure 3B**), while in animals that were neither immunotolerized nor immunosuppressed, IDUA activities were much lower, around wild type levels (**Figure 3C**).

AAV9-IDUA was also intranasally instilled into CP immunosuppressed MPS I mice, subsequently assaying for IDUA enzyme activity in the brain. Observed IDUA levels in most parts of the brain were around that of normal heterozygotes, much lower than those observed in ICV or IT-injected animals, while IDUA activity in the olfactory bulb was 100 times higher than the heterozygote level (**Figure 4**). This is consistent with our previous histological demonstration that AAV9 transduction is limited to the olfactory bulb after intranasal administration (Belur et al., 2017).

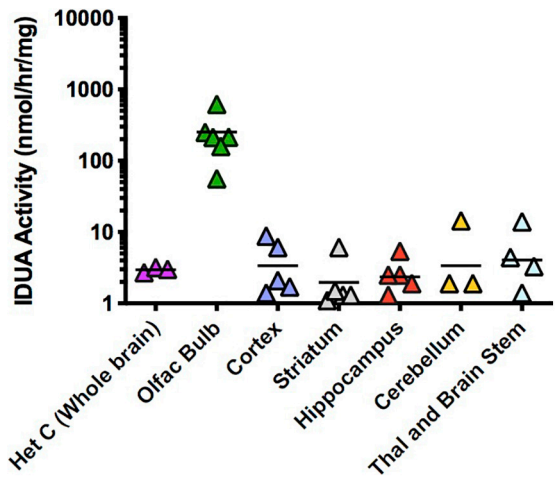

FIGURE 4 | IDUA activity in the brain after IN administration of AAV9-IDUA vector. Brains were microdissected and assayed for IDUA enzyme activity (n = 7). Each data point indicates a value from a single animal with the mean indicated by the short horizontal line. All animals were immunosuppressed with cyclophosphamide, and showed levels of activity that were similar to or a fraction of normal heterozygote controls, except for the olfactory bulb, which exhibited enzyme levels that were 100 times that of normal controls. Enzyme was not detected in untreated MPS I animals (< 0.02 nmoles/hr/mg protein) (n = 3). P-values ranged from < 0.001 (olfactory bulb) to not significant (other parts of the brain).

Correction of GAG Storage Material

High level expression of IDUA enzyme resulted in reduced accumulation of GAG storage material in the brain (**Figure 5**). Untreated MPS I mice had high levels of GAGs throughout the brain which was normalized in animals administered AAV9-IDUA intracerebroventricularly (**Figure 5A**). GAG levels were also normalized throughout the brain in IT treated animals except in the cortex and in the thalamus/brain stem, where storage was a slightly higher than normal in some animals but reduced compared to that of untreated MPS I animals (**Figure 5B**). For animals instilled with AAV9-IDUA intranasally, GAGs were substantially reduced and, in some cases, normalized in all parts of the brain (**Figure 5C**).

AAV9-IDUA Vector Biodistribution

Homogenates of tissues from ICV, IT and IN administered animals were extracted for DNA and assayed for the presence of vector sequences by qPCR for the human IDUA sequence

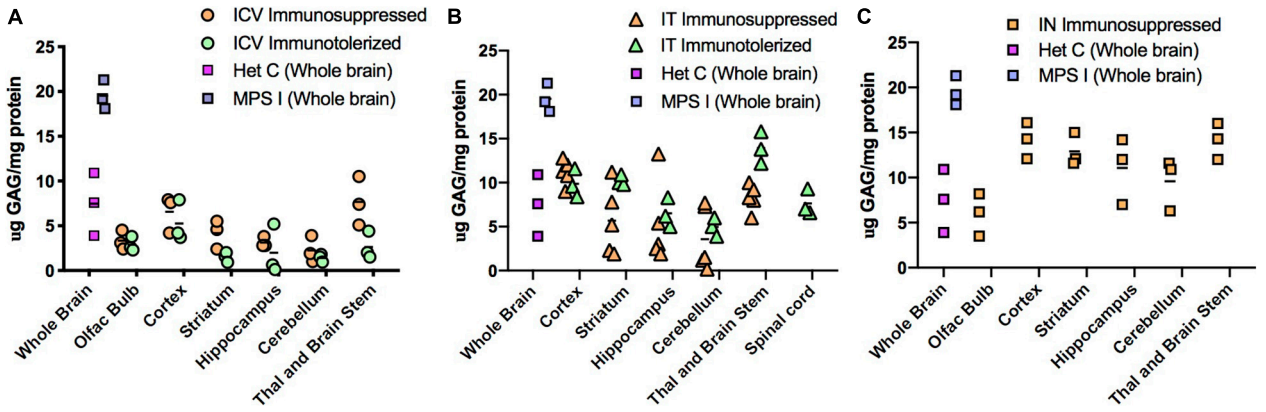

FIGURE 5 | Glycosaminoglycan (GAG) storage in brain post-AAV administration. Tissue lysates from different parts of the brain were assayed for GAG storage. The levels of GAG found in whole brain of untreated MPS I mice averaged around 20 μg GAG/mg protein, and in normal heterozygotes ranged from 4 to 12 μg GAG/mg protein. Each data point indicates a value from a single animal with the mean values represented by horizontal lines. **(A)** GAG accumulation in brain following ICV administration. GAG levels from both immunosuppressed and immunotolerized animals were normalized across the brain. There was no significant difference between the 2 immunomodulated groups. *P*-values were <0.0001 for treated animals compared to untreated controls. **(B)** GAG accumulation in brain following IT administration. GAG levels from both immunosuppressed and immunotolerized animals were normalized across the brain. Levels of GAG were slightly higher than ICV GAGs, but still in the normal range. Thalamus and brain stem GAG levels from immunotolerized animals were slightly higher than normal levels, although lower than untreated MPS I animals. There was no significant difference between the 2 immunomodulated groups. *P*-values were < 0.001 for treated animals compared to untreated controls. **(C)**. GAG accumulation in brain following IN administration. GAG levels from immunosuppressed animals were normalized in the olfactory bulb, and while some animals were not normalized in other parts of the brain, levels were lower than untreated MPS I animals. *P*-values ranged from < 0.001 to < 0.05 for treated animals compared to untreated controls.

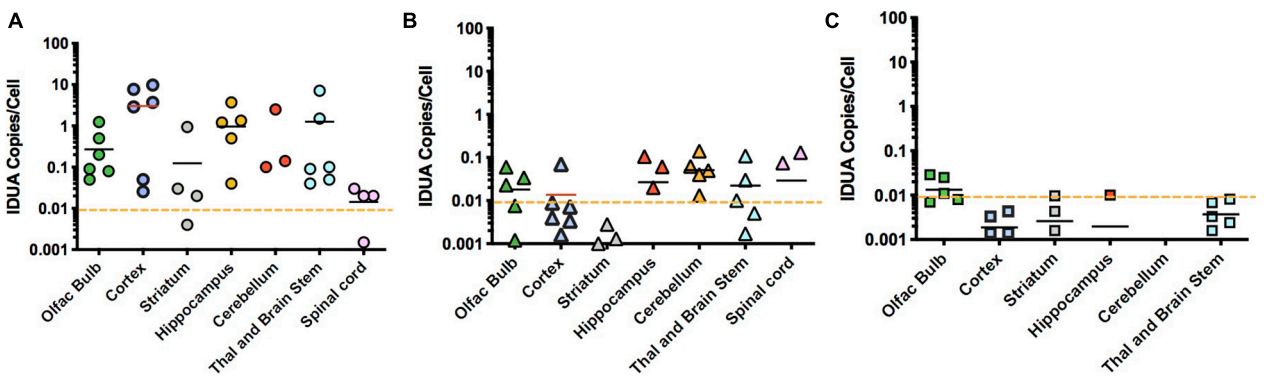

FIGURE 6 | Vector biodistribution. Tissue DNA extracts were assayed for the presence of IDUA sequences by quantitative PCR. Each symbol represents 1 animal. Dashed line indicates the lower limit of detection analyzed from genomic DNA samples collected from heterozygote controls (<0.01). **(A)** IDUA vector sequences after ICV administration. Vector copy numbers (VCNs) from immunotolerized animals ranged from 0.01 to 10 copies per cell in ICV administered animals. Average VCNs were highest in cortex and hippocampus, while they were lowest in the spinal cord, close to the limit of detection of 0.01. **(B)** IDUA vector sequences after IT injection. VCNs from immunotolerized animals were lower in IT injected animals, ranging from 0.01 to 0.1 copies per cell. **(C)** IDUA vector sequences after IN administration. The pattern of VCN was similar to that of enzyme data. As expected, the only VCNs that were above the level of detection were in the olfactory bulb.

(**Figure 6**). The pattern of vector copy number was similar to that of enzyme data obtained from animals administered vector via the different routes of administration. ICV-administered animals showed a broad range of vector copies, sometimes reaching 10 vc/cell (**Figure 6A**). Very high vector copy number is consistent with close proximity of brain tissues to the ICV route of administration. IT-administered animals exhibited a much lower level of vector, with a maximum of around 0.1 vc/cell (**Figure 6B**). This lower copy number is consistent with diffusion required through the cerebrospinal fluid from the lumbar site of injection to the brain. Intranasally administered animals had low level vector of 0.01 vc/cell in the olfactory bulb, with other parts of the brain below 0.01 copies/cell (**Figure 6C**). We previously reported the presence of transduced cells limited to the olfactory bulb in mice treated intranasally with AAV vector transducing IDUA or GFP sequences (Belur et al., 2017), consistent with the qPCR results shown here.

Immunohistochemistry (IHC)

Expression of IDUA enzyme in the brain was detected by IHC using an antibody specific for human IDUA. Similar to the

distribution of enzyme activity, scattered IDUA labeling was observed throughout the brain following ICV administration, while labeling following IT delivery was more limited (**Figure 7**). We observed colocalization of IDUA and NeuN labeling in cortex of ICV-treated mice, which demonstrated expression of IDUA within neurons. In addition to brain parenchyma, IDUA labeling was also observed in cells of the choroid plexus. In spinal cord, IDUA labeling was observed following IT but not ICV delivery and colocalized with labeling for LAMP-1 (**Figure 8**), suggesting lysosomal subcellular localization of IDUA. Consistent with our previous observations, AAV delivery within cerebrospinal fluid via ICV or IT injection resulted in redistribution of viral particles to the systemic circulation as indicated by IDUA expression in the liver (**Figure 9**). Limited IDUA labeling was also seen in lung.

Prevention of Neurocognitive Deficit

At 10 months of age, all experimental and control animals were evaluated for neurocognitive function using the Barnes maze, a test of spatial navigation and memory (**Figure 10**). Animals were evaluated in 6 trials a day for a total of 4 days. During this time, normal animals showed an improvement in spatial navigation, requiring an average of ∼30 s to locate the escape hole by day 4, while untreated animals showed

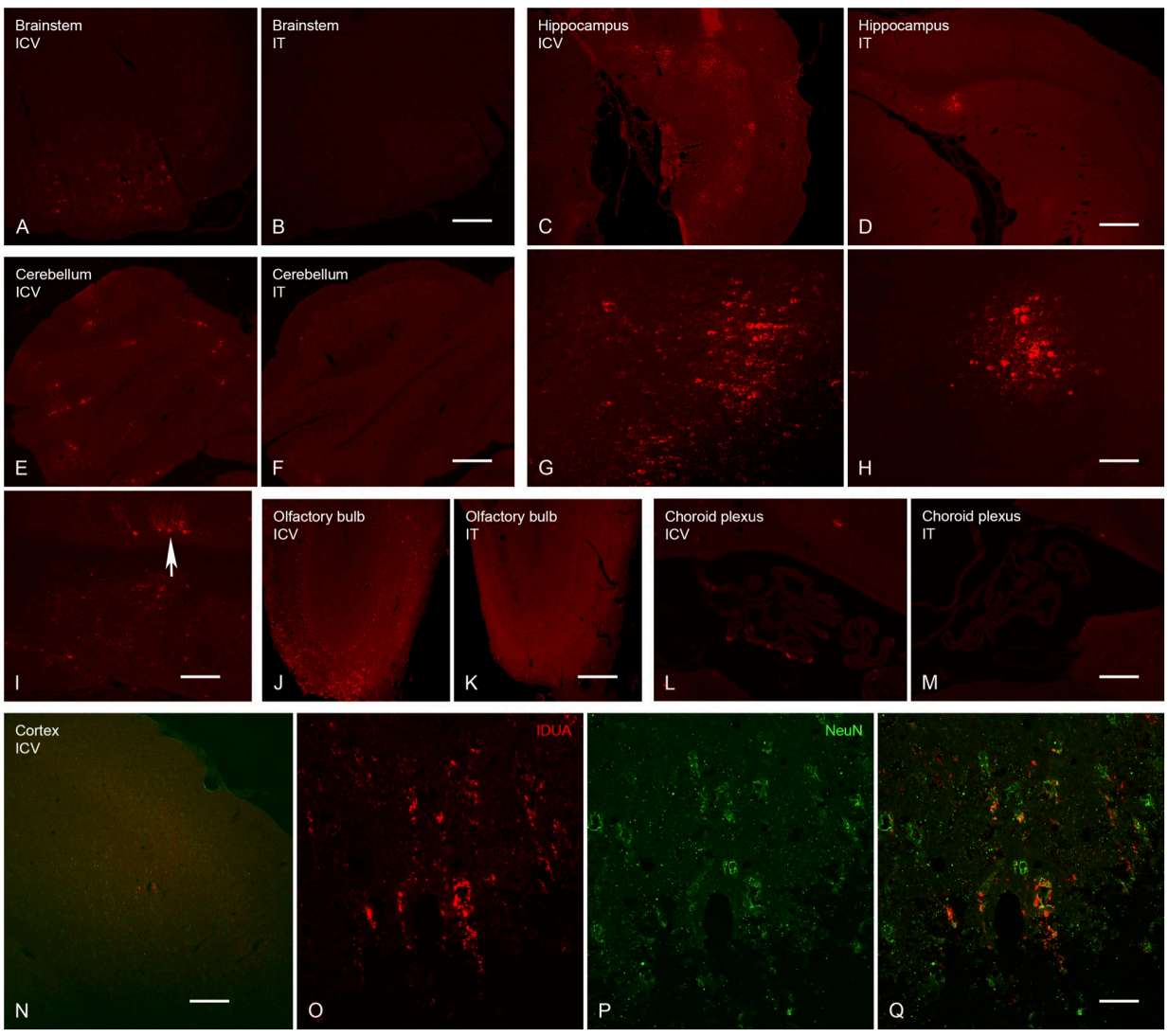

FIGURE 7 | Localization of IDUA immunolabeling in brain following ICV and IT delivery. **(A,B)** Select neurons in the brainstem showed IDUA-ir following ICV **(A)** but not IT **(B)** delivery of the vector. **(C,D,G,H)** Cells of the hippocampus showed IDUA-ir following both ICV **(C,G)** and IT **(D,H)** delivery, although many more hippocampal neurons were labeled after ICV delivery. **(E,F,I)** Cells of the cerebellum showed IDUA-ir following ICV **(E,I)** but not IT **(F)** delivery of the virus. The majority of the labeled cells in the cerebellum were Purkinje cells (arrow in I). **(J,K)** Many IDUA-ir cells of the olfactory bulb were seen following ICV **(J)** delivery as opposed to IT **(K)** delivery. Most transduction was seen in the glomerular layer. **(L,M)** A subset of cells of the choroid plexus showed IDUA-ir after ICV **(L)** delivery but not IT delivery **(M)**. **(N–Q)** IDUA-ir was seen in the cortex of animals following ICV **(N,O,Q)** but not IT (not shown) delivery. Colocalization of IDUA (red) and NeuN (green, P and Q) labeling in cortex suggests that IDUA-ir could be seen in neurons. Scale bars: **(A–F,J–N)**, 150 μm; **(G,H,I)** 75 μm; O, P, Q 25 μm.

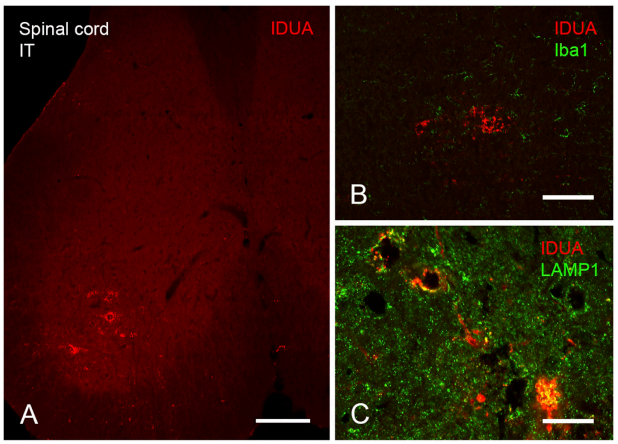

FIGURE 8 | Localization of IDUA immunolabeling in spinal cord following IT delivery. **(A)** Motor neurons of the ventral horn of sacral spinal cord show IDUA labeling. **(B)** IDUA staining is restricted to neurons and is not colocalized with the microglial marker Iba1. **(C)** IDUA is colocalized within neurons with the lysosomal marker LAMP1. Scale bars: **(A)** 150 μm; **(B)** 25 μm; **(C)** 75 μm.

a significant deficit in this test, requiring an average of ∼90 s. In contrast, animals treated with AAV9-IDUA vector by either ICV or IT routes of administration exhibited significantly improved neurocognitive skills that were similar to normal heterozygous controls, with an escape time of about 30 s by day 4. Additionally, we have previously demonstrated improved neurocognitive function in animals treated intranasally with AAV9-IDUA (Belur et al., 2017). We conclude that emergence of neurocognitive dysfunction in MPS I mice is prevented by ICV, IT, or IN administration of AAV9-IDUA.

DISCUSSION

In the present study, we compared and evaluated the effectiveness of different routes of vector administration for IDUA gene delivery to the CNS. We observed supraphysiological and highest levels of enzyme expression in the brain following ICV injection, lower levels with IT administration, and lowest (wild type) levels after IN delivery of vector. GAG levels were normalized following ICV and IT delivery, while after IN delivery GAG levels were normalized in the olfactory bulb and close to normal in other parts of the brain. All 3 routes of administration prevented neurocognitive defect as assessed by the Barnes maze.

The effectiveness of ERT and HSCT in the treatment of MPS I and other lysosomal diseases is based on the concept of metabolic cross-correction, whereby enzyme that is either directly infused or expressed by donor cells is taken up by host cells and trafficked to lysosomes, subsequently contributing to lysosomal metabolism (Fratantoni et al., 1968). The concept of metabolic cross-correction also underlies the anticipated effectiveness of genetic therapies for MPS I and other MPS diseases, wherein the missing enzyme is expressed from genetically transduced cells either infused into the host or generated *in vivo* after vector infusion into the host. Previous studies have demonstrated the effectiveness of *in vivo* and *ex vivo* IDUA gene transfer using retroviral (Ponder et al., 2006; Herati et al., 2008a,b; Metcalf et al., 2010), lentiviral (Visigalli et al., 2010, 2016), AAV (see below), and *Sleeping Beauty* transposon vectors (Aronovich et al., 2007, 2009) in mouse, dog and cat models of MPS I. This has resulted in the initiation of human clinical trials testing *in vivo* AAV mediated IDUA transduction targeting the liver (Sangamo Therapeutics, SB-318, ClinicalTrials.gov Identifier: NCT02702115), targeting the CNS (REGENXBIO, RGX-111, ClinicalTrials.gov Identifier: NCT03580083), and *ex vivo* lentiviral transduction of autologous hematopoietic stem cells (Orchard Therapeutics, OTL-203).

The blood brain barrier sequesters the brain from systemically administered enzyme replacement therapy (ERT), resulting in challenges for the treatment of neurologic disease in MPS I patients. These challenges can potentially be overcome by gene delivery with the use of AAV vector serotypes that are capable of crossing the blood brain barrier. While we have demonstrated that systemic intravenous delivery of AAV9 and AAVrh10 vector delivers substantial IDUA enzyme activity to the CNS (Belur et al., 2020), direct administration to the CNS ensures vector delivery and subsequent IDUA expression in the brain. There are several strategies by which vector can be delivered directly to the brain. For MPS I, correction of neuropathology after intraparenchymal injection of AAV2 and AAV5 has been reported in murine, feline, and NHP animal models (Desmaris et al., 2004; Ciron et al., 2009; Ellinwood et al., 2011). Delivery directly into the CSF via intracerebroventricular (ICV) injection (Wolf et al., 2011; Janson et al., 2014; Hordeaux et al., 2018) or intrathecal (IT) injection either into lumbar or cisternal spaces in murine, feline, canine, and NHP models has been reported with varying levels of success for MPS I (Watson et al., 2006; Hinderer et al., 2014a, 2015; Hordeaux et al., 2019).

In order to suppress the immune response in animals elicited by the human IDUA protein (Aronovich et al., 2007, 2009), we explored two immunomodulation approaches. The first was immune suppression with cyclophosphamide, while in the second we immunotolerized animals beginning at birth by administering IDUA enzyme protein, Aldurazyme (laronidase). In animals that received ICV injection of vector, we found that levels of enzyme expression in the brain were roughly equivalent, regardless of whether the animals were immunosuppressed or immunotolerized. Surprisingly, control ICV injected animals with no immunomodulation did not show a decrease in enzyme activity but exhibited IDUA levels in the brain that were equivalent to the other two groups. Animals that received IT injections showed similar results in comparision to animals either immunosuppressed or immunotolerized. However, control IT injected animals that received no immunomodulation showed a significant decrease in enzyme activity, with enzyme levels that were similar to or slightly higher than normal heterozygotes. This difference in response between the non-immunosuppressed ICV and IT groups could be explained by a greater amount of vector released into the circulation after IT injection

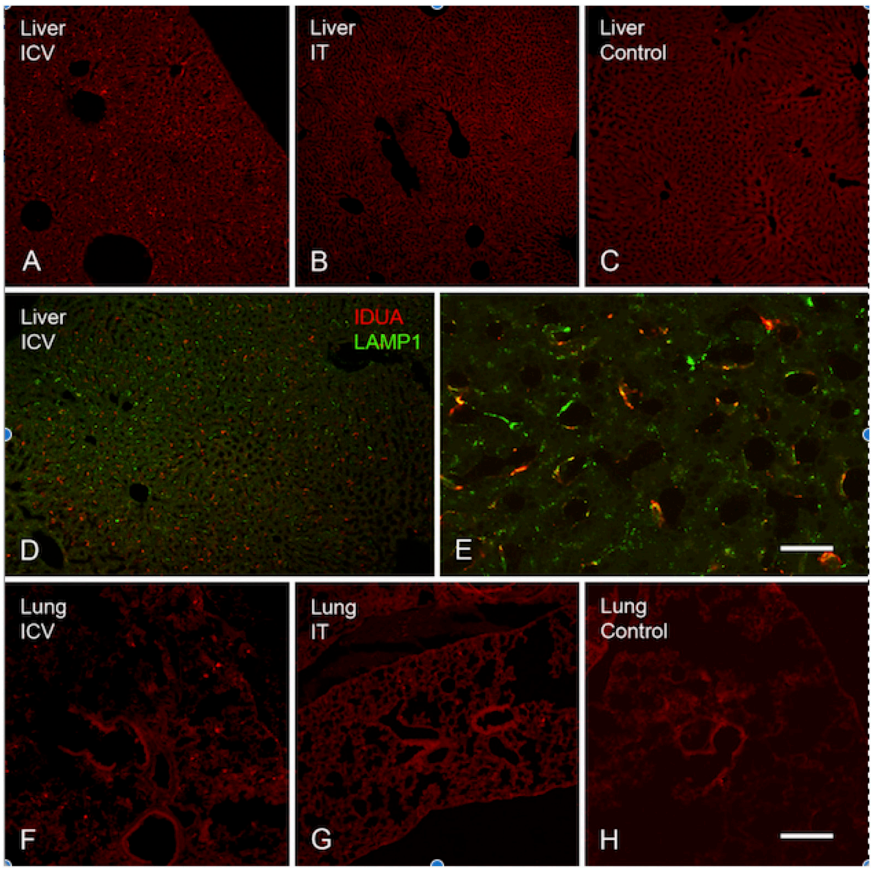

FIGURE 9 | Localization of IDUA immunolabeling in liver and lung. **(A–C)** IDUA labeling was seen in the liver of ICV-treated **(A)** and to a lesser extent IT-treated **(B)** mice, compared to control **(C)**. **(D,E)** IDUA labeling in liver colocalized with LAMP-1 labeling. **(F–H)** Sparse IDUA labeling was seen in lung of ICV-treated **(F)** and to a lesser extent IT-treated **(G)** mice, compared to control **(H)**. Scale bars: **(A–D,F–H)** 150 μm; **(E)** 25 μm.

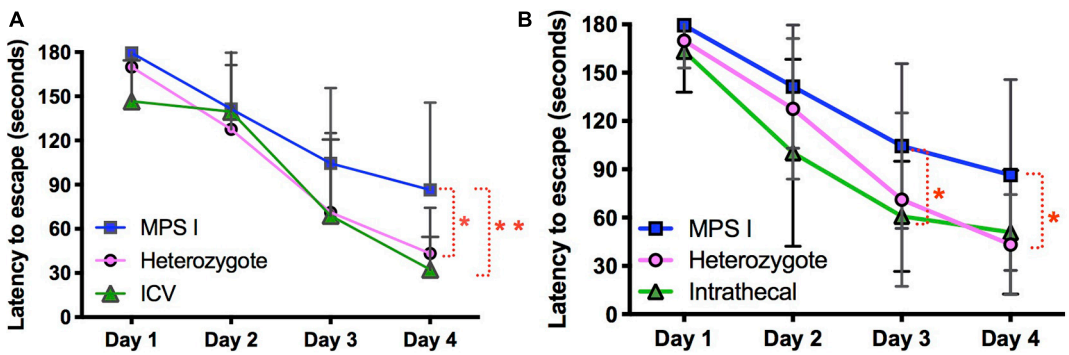

FIGURE 10 | Assessment of neurocognitive improvement. The Barnes Maze was used to assess spatial learning and memory in immunomodulated animals treated with vector using ICV and IT routes (n = 9 in both groups). We have previously demonstrated improvement of neurocognitive deficit in IN administered animals (Belur et al., 2017) (*$p < 0.05$, **$p < 0.01$). **(A)** ICV treated animals were significantly improved based on the Barnes maze. Latency to escape in ICV treated animals was not significantly different from heterozygote controls, and was significantly improved compared to untreated MPS I animals. **(B)** IT treated animals showed the same pattern of cognitive improvement in the Barnes maze exhibiting significantly better performance compared to untreated MPS I controls.

compared to ICV administration. We did not test for anti-IDUA antibody levels.

Improved neurologic outcomes have been reported in MPS I mice administered aldurazyme starting early and/or at high dose when tested soon after (Baldo et al., 2013) or during ERT administration (Ou et al., 2014). However, Schneider et al. (2016), reported that in MPS I mice undergoing ERT from birth, interruption of treatment for a period from 2 to 4

months of age compromised neurobehavioral outcome at 6 months of age, indicating deterioration in brain function. Had ERT not been restored from 4 to 6 months in that study, the brain and associated neurocognition would likely have deteriorated further. In our study, we observed normalized time to escape for immunotolerized, AAV9-IDUA treated animals evaluated in the Barnes maze 5 months after the withdrawal of enzyme at 5 weeks of age. We did not include a control group of MPS I mice that were administered Aldurazyme but not administered AAV9-IDUA, so it is formally possible that some of the improvement seen in the Barnes maze is attributable to enzyme alone. However, the fact that performance in the Barnes maze was not reduced in comparison with wild-type animals argues that this normalized neurocognitive function is at least partially attributable to AAV9-IDUA treatment of MPS I mice at 3 months of age. Moreover, we have recently carried out experiments in MPS I animals immunosuppressed with CP and treated with AAV9-IDUA intrathecally and intravenously. We observed complete retention of cognitive function in these animals, thus demonstrating that treatment with AAV9-IDUA in the absence of enzyme therapy prevents neurodegeneration in this animal model (Belur et al., 2021).

Results from IDUA immunofluorescence analysis were consistent with the biochemical data obtained from animals administered AAV9-IDUA via the different routes of administration. ICV administered animals demonstrated the highest levels of IDUA expression, with a high percentage of IDUA positive cells widespread throughout the brain. IT injected animals showed IDUA positive cells scattered throughout the brain, with most of the labeling confined to the hindbrain. Intranasal administration led to IDUA positive cells localized exclusively in the olfactory bulb (Belur et al., 2017). The pattern of vector copy biodistribution by qPCR also reflected that of IDUA enzyme activities observed in different parts of the brain after vector infusion via the different routes of administration, with ICV administered animals showing the highest vector copy number, followed by IT injected animals, followed by IN administered animals. Results from the Barnes maze indicated that the levels of enzyme in the brain after vector treatment were sufficient to rescue animals from the neurocognitive deficit observed in untreated affected animals.

This is the first study comparing different routes of vector delivery at the same dose directly to the brain in adult MPS I mice. Our data demonstrate that ICV injection of vector, although invasive, results in very high and widespread distribution of enzyme in the brain. Lumbar IT injections result in high levels of enzyme in the hindbrain that are comparable to ICV levels, but enzyme levels were lower in the forebrain and midbrain compared to ICV administration. Intranasal administration showed the lowest enzyme levels of the three routes of delivery, but nevertheless, resulted in enzyme levels that were sufficient to reverse neurocognitive deficit (Belur et al., 2017). These results have considerable clinical implications for the treatment of MPS diseases using AAV 9 vectors: REGENXBIO is currently enrolling patients in a Phase I/II gene therapy clinical trial of RGX-111 delivered through the CSF to the CNS for treatment of MPS I[1].

Our results thus support the prospect of developing a non-invasive approach for IDUA gene delivery to the CNS for high level enzyme expression and prevention of neurologic disease in human MPS I.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Animal work reported in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

AUTHOR CONTRIBUTIONS

LB planned and carried out experiments, collected, analyzed, and interpreted data, and wrote the manuscript. MR and JL carried out biochemical assays. KP-P performed animal immunomodulation and assisted with neurobehavioral testing. ZN performed ICV administrations. MR and LV performed IHC staining, image collection, analysis, and interpretation. KKi carried out IT injections. CF, KFK, PO, WF, WL, and RM provided expertise and critical feedback. RM conceived and designed the experiments, provided input on data analysis and interpretation, and supervised the project. All authors contributed to the article and approved the submitted version.

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[1]https://clinicaltrials.gov/ct2/show/NCT03580083?term=RGX-111&rank=1

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Complete Correction of Brain and Spinal Cord Pathology in Metachromatic Leukodystrophy Mice

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder characterized by accumulation of sulfatides in both glial cells and neurons. MLD results from an inherited deficiency of arylsulfatase A (ARSA) and myelin degeneration in the central and peripheral nervous systems. Currently, no effective treatment is available for the most frequent late infantile (LI) form of MLD after symptom onset. The LI form results in rapid neurological degradation and early death. ARSA enzyme must be rapidly and efficiently delivered to brain and spinal cord oligodendrocytes of patients with LI MLD in order to potentially stop the progression of the disease. We previously showed that brain gene therapy with adeno-associated virus serotype rh10 (AAVrh10) driving the expression of human ARSA cDNA alleviated most long-term disease manifestations in MLD mice but was not sufficient in MLD patient to improve disease progression. Herein, we evaluated the short-term effects of intravenous AAVPHP.eB delivery driving the expression of human ARSA cDNA under the control of the cytomegalovirus/b-actin hybrid (CAG) promoter in 6-month-old MLD mice that already show marked sulfatide accumulation and brain pathology. Within 3 months, a single intravenous injection of AAVPHP.eB-hARSA-HA resulted in correction of brain and spinal cord sulfatide storage, and improvement of astrogliosis and microgliosis in brain and spinal cord of treated animals. These results strongly support to consider the use of AAVPHP.eB-hARSA vector for intravenous gene therapy in symptomatic rapidly progressing forms of MLD.

Keywords: metachromatic leukodystrophy, aav, gene therapy, intravenous injection (i.v.), sulfatide accumulation, lysosomal storage disease

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INTRODUCTION

Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder (LSD) caused by an inherited deficiency of arylsulfatase A (ARSA; EC 3.1.6.8) (Gieselmann and Krägeloh-Mann, 2006). ARSA enzyme catalyzes the first step in the degradation pathway of 3-O-sulfogalactosylceramides (sulfatides). Patients with MLD develop neurological symptoms that result from sulfatide accumulation in oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nerves (PNS). Sulfatides also accumulate in brain neurons, contributing significantly to additional pathology (Wittke et al., 2004; Gieselmann and Krägeloh-Mann, 2006). Three clinical forms of MLD have been described, based on the age of symptom onset: late infantile, juvenile

and adult forms (Gieselmann and Krägeloh-Mann, 2010; Van Rappard et al., 2015). In the case of late-onset forms (first symptoms after 4 years), which are more variable and progress more slowly, allogenic hematopoietic stem cell transplantation (Allo-HSCT) may modify their natural history if performed in pre-symptomatic or early symptomatic patients (Boucher et al., 2015; Groeschel et al., 2016). In the early onset forms (first symptoms before 4 years, the most frequent phenotype of the disease), the disease progresses very rapidly toward severe motor and cognitive regression and premature death, no available therapy being effective once patients are symptomatic. A clinical trial using autologous hematopoietic stem cell transplantation of CD34 cells corrected with lentiviral vector overexpressing human ARSA (HSCT-GT) has shown very encouraging results and provides strong evidence of clinical benefit in pre-symptomatic patients with late infantile MLD (LI-MLD) and pre or early symptomatic patients with early juvenile MLD (EJ-MLD; NCT01560182[1]) (Biffi et al., 2013; Sessa et al., 2016). This treatment will likely become the gold standard treatment for pre-symptomatic MLD patients. However, HSCT-GT failed to stop or slow-down the disease in LI-MLD once patients are symptomatic. Enzyme replacement therapy is another option (Platt and Lachmann, 2009), but the lysosomal enzyme does not cross the blood-brain-barrier (BBB) efficiently, even if results obtained *in vitro* and in MLD mice suggest that ARSA is able to cross the BBB to some extent (Matzner et al., 2009; Matthes et al., 2011). Enzyme replacement therapy using intrathecal delivery of recombinant ARSA enzyme could be an alternative. Results of a phase I-II trial, performed in early symptomatic LI-MLD patients has been published (í Dali et al., 2020), vouching for a good safety profile and some trend to a less pronounced motor function decline over time in patients receiving the highest dose. A phase II-III clinical trial is ongoing (NCT03771898; see text footnote 1) to evaluate the efficacy profile.

However, no effective treatment is currently available for symptomatic patients with early onset forms of MLD. Arresting their rapid neurological degradation by delivering rapidly and efficiently ARSA enzyme into oligodendrocytes, and likely into neurons in brain but also in spinal cord and peripheral nerves, before irreversible damage occurs is crucial.

Previously, we demonstrated that intracerebral gene therapy using an adeno-associated vector of serotype 5 (AAV5) or AAVrh10 to express the human ARSA had a long-term efficacy in MLD mice (Sevin et al., 2006, 2007; Piguet et al., 2012), and more recently the efficacy of these vectors in normal non-human primates (Colle et al., 2009; Zerah et al., 2015). A long-term decrease in sulfatide storage was observed in the brain of treated mice at the neuropathological and biochemical levels, and effect of neuroinflammation was demonstrated in the brain. A Phase I/II clinical trial was initiated in four MLD children (aged between 9 months and 5 years, either pre-symptomatic or early symptomatic), using intracerebral delivery of AAVrh10-hARSA (NCT01801709; see text footnote 1). Despite long-lasting restoration of ARSA activity in the cerebrospinal fluid (CSF, to 20–70% of values in controls), treatment was not able to prevent or even stabilize the disease (Sevin et al., 2018). Lessons of this trial was that intracerebral delivery is probably not enough to achieve sufficient ARSA activity rescue in the whole brain but also in the spinal cord.

Among new serotypes of AAV, AAVPHP.eB was recently described as strongly efficient to cross the BBB after intravenous delivery and lead to an efficient brain and spinal cord transduction in mouse models (Deverman et al., 2016; Chan et al., 2017).

Therefore, as an intravenous delivery of this vector could potentially be more efficient than an AAVrh10 vector, we evaluated, at the neuropathological and biochemical levels, the short-term efficiency of a single intravenous injection of AAVPHP.eB-hARSA-HA vector in 6-month-old MLD mice that have already accumulated marked amounts of sulfatides. Herein, we demonstrate that a single intravenous injection of AAVPHP.eB-hARSA-HA results in robust and diffuse expression of ARSA enzyme in the brain and the spinal cord of MLD treated animals. Importantly, the injection allowed us to correct, within 3 months, the sulfatide accumulation and the neuropathology in brain and spinal cord.

MATERIALS AND METHODS

Adeno-Associated Viral Vector Construction and Production

AAV vectors were produced and purified by Atlantic Gene therapies (Translational Vector Core Research grade services, Nantes, France). AAVPHP.eB-CAG ARSA-HA was produced by cloning the HA tag to the ARSA sequence under the CAG promoter (Piguet et al., 2012). The viral constructs for pAAVPHP.eB-CAG-hARSA-HA contained the expression cassette consisting of the human ARSA genes, driven by a CMV early enhancer/chicken β-actin (CAG) synthetic promoter surrounded by inverted terminal repeats (ITR) sequences of AAV2. Plasmid for AAVPHP.eB was obtained from Addgene (United States). The final titer of the batch was 4.10^{12} vector genomes (vg)/ml.

Animal Model

All animal studies were performed in accordance with local and national regulations and were reviewed and approved by the relevant institutional animal care and use committee. The experiments were carried out in accordance with the European Community Council directive (2010/63/EU) for the care and use of laboratory animals. Our protocol was approved by the European community council directive (2010/63/EU) (No. 17303). Moreover, the use of GMOs has been validated by the Haut Commissariat aux Biotechnologies (Number 5463).

ARSA-deficient mice (KO ARSA mice) were bred from homozygous founders on a 129/Ola strain (Hess et al., 1996) and heterozygous mice were generated to be control mice. Mice

[1]http://clinicaltrials.gov

were housed in a pathogen free animal facility in a temperature-controlled room and maintained on a 12-h light/dark cycle. Food and water were available *ad libitum*.

Injection of AAV Vector

Female and male KO ARSA mice were anesthetized by isoflurane (2% induction). Animals were injected at 6 months of age by intravenous retro-orbital delivery (Yardeni et al., 2011) with saline (NaCl 0.9%) solution or AAVPHP.eB-hARSA-HA (5.10^{11}vg total). Retro-orbital administration of the vector was preferred to tail vein injection based on preliminary results that demonstrated a better efficiency to target CNS and decrease liver transduction. Three groups of animals were performed: wild-type (WT, $n = 3$), untreated (NT, $n = 8$) and treated (AAV, $n = 8$) KO ARSA mice. For each group males and females were equally divided so that treatment efficacy is evaluated in both genders. The injected dose was determined according to previous results of dose ranging study on WT animals and evaluation of transduction efficacy.

Tissue Preparation

Animals were sacrificed by an intraperitoneal administration of a lethal dose of Euthasol (180 mg/kg, Vetcare) 3 months after treatment. Mice were perfused intracardiacally with phosphate buffered saline (PBS). Brain, spinal cord, sciatic nerve, heart, liver, gall gladder, lung, spleen and kidney were collected for analysis. Different structures of a cerebral hemisphere (cortex, striatum, cerebellum, pons and rest of brain) were dissected and frozen in liquid nitrogen. Sciatic nerve, heart, liver, lung, spleen and kidney were directly frozen in liquid nitrogen and stored in −80°C. For DNA and protein extraction from the same samples, tissue samples were crushed in liquid nitrogen and divided into two equals parts. A cerebral hemisphere, a portion of spinal cord, sciatic nerve and gall bladder were post-fixed overnight in 4% paraformaldehyde (PFA)/PBS1X. Samples were rinsed three times in PBS 1X and cryoprotected in 30% sucrose/PBS1X. Tissue are embedded Tissue-Tek OCT compound (VWR International) and cut into 14-μm sagittal section of brain or transversal section of spinal cord or 4 μm longitudinal section of sciatic nerve or transversal section of gall bladder in cryostat (Leica, Langham, TX). Cryosections were dried at room temperature and stored at −20°C.

Quantitative PCR

DNA was extracted from brain, spinal cord and peripheral organs using chloroform/phenol protocol. AAVPHP.eB-hARSA-HA vector genome copy numbers were measured by quantitative PCR in cortex, striatum, cerebellum, pons, rest of brain, spinal cord and peripheral organs using the Light Cycler 480 SYBR Green I Master (Roche, France) as described (Sevin et al., 2006). The results (vector genome copy number per cell) were expressed as n-fold differences in the transgene sequence copy number relative to the Adck3 gene copy as internal standard (number of viral genome copy for 2N genome). Primers sequence for qPCR were: *human Arsa* (forward 5′-TCA CTG CAG ACA ATG GAC CTG A-3′, reverse 5′-ACC GCC CTC GTA GGT CGT T-3′) and *Adck3* (forward 5′-CCA CCT CTC CTA TGG GCA GA-3′, reverse 5′-CCG GGC CTT TTC AAT GTC T-3′).

Protein Extraction and ARSA Expression and Activity Quantification

Samples were homogenized in 0.3 ml of lysis buffer (100 mM Trizma base, 150 mM NaCl, 0.3% Triton; pH 7) and incubated for 30 min on ice and centrifuged. The supernatant was collected for the determination of (1) protein content (bicinchoninic acid [BCA] protein assay kit; Pierce Biotechnology/Thermo Fisher Scientific, Rockville, IL); (2) ARSA activity, using the artificial p-nitrocatechol sulfate (pNCS) substrate assay (Sigma-Aldrich, France) (Bass et al., 1970; Piguet et al., 2012). Assays were performed in triplicate and results are expressed as nanomoles of 4-nitrocatechol (4NC) per hour per milligram of protein. And (3) the concentration of recombinant hARSA using an indirect sandwich ELISA specific for human ARSA as described (Matzner et al., 2000), using 2 specific non-commercial antibodies (Kind Gift from Pr. Gielselmann, Bonn). Assays were performed in duplicate and results are expressed as nanograms of hARSA per milligram of protein. All samples were quantified in duplicates.

Histopathology

To evaluate sulfatide storage, frozen sections were postfixed in 4% PFA, stained with Alcian blue (A5268; Sigma-Aldrich) (0.05% in 0.025 M sodium acetate buffer, pH 5.7, containing 0.3 M MgCl2 and 1% PFA), rinsed in the same buffer without dye, counterstained with fast red (229113; Sigma-Aldrich) and mounted as previously described (Piguet et al., 2012).

Immunohistochemical labeling was performed with the ABC method. Briefly, tissue sections are treated with peroxide (0.9% H_2O_2/0.3% Triton/PBS) for 30 min to inhibit endogenous peroxidase. Following washes with PBS, sections are incubated with the blocking solution (10% goat serum in PBS/0.3% TritonX-100) for 1 hr. The primary antibodies [rabbit anti-Calbindin (CB38; Swant, 1:10 000); mouse anti-GFAP (G3893, Sigma-Aldrich; 1:400); rabbit anti-Iba1 (019-19741, Wako; 1:500)] are diluted in blocking solution and incubated on tissue sections overnight at 4°C. After washes in PBS, sections are sequentially incubated with goat anti-rabbit or anti-mouse antibody conjugated to biotin (Vector Laboratories) for 30 min at room temperature, followed by the ABC complex (Vector Laboratories). After washes in PBS, the peroxidase activity is detected with diaminobenzidine as chromogen (Dako, Carpinteria, CA). In some cases, slides are counterstained with hematoxylin. The slides are mounted with Eukitt (VWR International). Slices are acquired at 20X by using a slide scanner (NanoZoomer2.ORS, Hamamatsu).

Tissue cryosections were permeabilized with PBS/0.3%TritonX-100 for 15 min and saturated with PBS/0.3% Triton/10% horse serum (HS) for 45 min. The primary antibodies were diluted in the saturation solution and incubated 1 h at 37°C. After washes in PBS/0.1% Triton,

the secondary antibodies and DAPI were diluted in PBS/0.1% Triton/10% HS and added for 1 h at room temperature. After washes in PBS/0.1% Triton, the slides were mounted with fluorescent mounting medium (F4680; Sigma-Aldrich). Primary antibodies for immunofluorescence were rabbit polyclonal anti-hARSA (kind gift from V. Gieselmann and U. Matzner, Bonn, Germany; 1:1,000) and mouse anti-Lamp1 (1D4B, DSHB, 1:200). Secondary antibodies were diluted 1:1,000 and were donkey anti-rabbit/AlexaFluor 594 and anti-mouse/AlexaFluor 488. Pictures were taken with a Confocal SP8 Leica DLS Inverted (Leica). For all images, brightness and contrast were adjusted with Image J software after acquisition to match with the observation. All histological studies were assessed blinded by two investigators.

Stereological Cell Counts

Stereological counts were performed by two independent investigators, blind for both genotypes and treatments, using Image J software. All quantifications were done on three sections of brain and of spinal cord for each animal (n = 3 for WT, n = 8 for NT, and n = 3 for AAV). For Alcian staining, the number of sulfatide storage inclusions were quantified in three random areas of the cortex, the corpus callosum and the fimbria. For GFAP and Iba1 labeling, the number of positive cells were evaluated in three random areas of the cortex and corpus callosum or the center of cerebellum white matter.

In the spinal cord, for sulfatide storage, GFAP and Iba1, a hemi-section was counted. All results were assessed per mm^2 and expressed as the mean ± SEM.

Statistical Analysis

Data were analyzed using GraphPad Prism 8 software. The statistical significance of values among groups was evaluated by ANOVA, followed by the least significant difference t-test. All values used in figures and text are expressed as mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

For all graphs, a special symbol has been assigned for each individuals so that it is possible to correlate between ARSA expression and astrogliosis, sulfatide accumulation and so.

RESULTS

Validation of pAAV-CAG-hARSA-HA Plasmid *in vitro*

After hARSA-HA cloning in the pAAV plasmid and validation by sequencing, an *in vitro* assay based on 293T cells transfection with pAAV-CAG-hARSA-HA plasmid was performed. We demonstrated hARSA-HA expression using HA staining, in transfected cells as well as a significant increase in ARSA activity in supernatant of transfected cells, up to 90-folds compared to non-transfected cells, 72 h after transfection (**Supplementary Data** and **Supplementary Figure 1**). These data confirmed the functionality of the pAAV-CAG-hARSA-HA plasmid. The AAVPHP.eB-hARSA-HA vector was produced as described.

Widespread Distribution and Expression of AAVPHP.eB-hARSA-HA in the CNS of KO ARSA Mice

Treated KO ARSA mice (n = 8) mice received a single intravenous injection of AAVPHP.eB-hARSA-HA. Treatment was well tolerated, no adverse event was observed in mice injected with the AAVPHP.eB vector, attesting for the safety of the procedure. Vector injection resulted in a widespread transduction of CNS, with a mean of 2.71 ± 0.76 vector genome copies per cell (VGC) in the cortex, 1.6 VGC ± 0.90 in the striatum, 2.3 VGC ± 0.96 in the pons, 1.2 VGC ± 0.37 in the remaining forebrain, 0.5 VGC ± 0.17 in the cerebellum and 1.6 VGC ± 0.50 in the spinal cord (**Figure 1A**) in treated mice. The mean number of VGC in peripheral organs was less than 0.05 VGC, indicating a low peripheral transduction of the vector (**Figure 1B**).

In accordance with the biodistribution profile, hARSA expression was detected by immunofluorescence studies in several areas of brain (**Figure 1C**) of treated KO ARSA mice, such as striatum (**Figure 1D**), hippocampus, thalamus (**Figure 1E**), corpus callosum (**Figures 1F,J**), pons and cerebellum (**Figures 1G,K**). Moreover, hARSA-positive cells were also detected in the spinal cord (**Figure 1L**) of treated mice. As a negative control, hARSA protein expression was not detected in untreated KO ARSA mice (**Figure 1H**). To be active, ARSA enzyme needs to be targeted to the lysosome. Proper lysosomal localization was confirmed by co-staining with anti-hARSA and anti-Lamp1 (lysosomal marker) antibodies, performed on brain and spinal cord sections of treated KO ARSA mice. A colocalization of hARSA and Lamp1 was observed in different areas of CNS (Inset, **Figures 1I–K**), indicating hARSA is correctly localized in lysosomes and thus could catabolize sulfatides.

hARSA Activity and Expression in CNS of Treated KO-ARSA Mice

To validate the functionality of recombinant hARSA in treated KO ARSA mice, ARSA activity was measured in different structures of the CNS. We demonstrated a clear trend to ARSA over activity in the cortex, pons, cerebellum and spinal cord in treated KO ARSA mice (**Figure 2A**). Moreover, expression of recombinant hARSA assessed by ELISA in several structures of brain and the spinal cord with a mean to 326 ng ARSA/mg protein in treated KO mice whereas it was not detected in WT and untreated mice (**Figure 2B**). We demonstrated a high hARSA expression in the brain and the spinal cord in treated KO ARSA. To conclude, treated mice with AAVPHP.eB-hARSA-HA express high levels of functional ARSA enzyme.

Significant Improvement of MLD Pathophysiology in Treated KO ARSA Mice With AAVPHP.eB-ARSA-HA

Complete Correction of Sulfatide Storage in CNS of Treated KO ARSA

Sulfatide accumulation starts during fetal development, is obviously detectable at 3 months of age in the CNS of KO

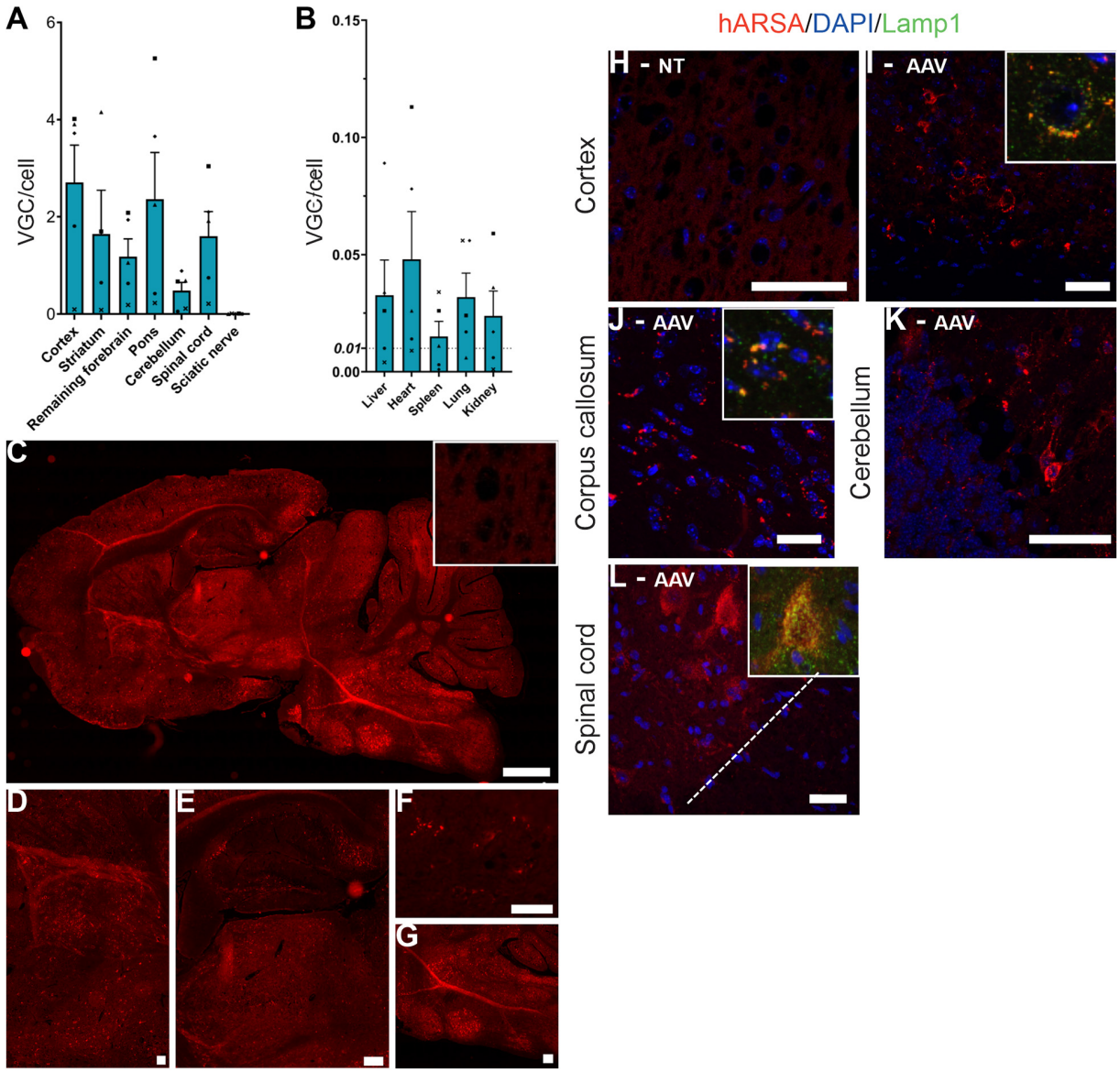

FIGURE 1 | AAVPHP.eB-hARSA-HA efficiently transduce central nervous system. **(A,B)** Biodistribution of the AAVPHP.eB-hARSA-HA in central nervous system **(A)** and peripheral organs **(B)** in 9-month-old KO ARSA mice, 3 months after intravenous injection of AAVPHP.eB-hARSA-HA (n = 5). VGC for vector genome copy number per 2n genome. **(C–G)** Immunofluorescence detection of hARSA (red) on sagittal sections in the brain **(C)** and high magnification of different brain areas **(D–G)** i.e., Striatum **(D)**, hippocampus and thalamus **(E)**, corpus callosum **(F)** and cerebellum **(G)** in 9-month-treated KO ARSA mice with AAVPHP.eB-hARSA-HA. Inset is a sagittal section of cortex in control mice. **(H–L)** Immunofluorescence detection of hARSA (red) and of Lamp1 (green) on sagittal sections in the cortex **(H,I)**, corpus callosum **(J)** and cerebellum **(K)** and on coronal sections of spinal cord **(L)** in 9-month-old untreated **(H)** or treated **(I–L)** KO ARSA mice with AAVPHP.eB-hARSA-HA. Nuclei are stained in blue. Insert in **(I**,**J**,**L)** shows a co-localization of hARSA in lysosome (Lamp1, green). Data are represented as mean ± SEM. Scale Bars: 1,000 μm **(C)**; 200 μm **(E,G)**; 100 μm **(E)** and 50 μm **(F,H–L)**.

ARSA mice (corpus callosum and pons) and then increases progressively with age (personal non-published data). To assess the efficiency of intravenous administration of AAVPHP.eB-ARSA-HA vector to decrease sulfatide storage, alcian staining was performed in the brain, spinal cord, sciatic nerve and gall bladder sections of untreated and treated KO ARSA animals and compared to wild-type control. Nine-month-old untreated KO ARSA mice display massive sulfatide storage in brain, spinal cord, sciatic nerve and gall bladder compared to WT mice (**Figure 3** and **Supplementary Figure 2**). In 9-month-old treated animals, 3 months after intravenous injection, AAVPHP.eB-ARSA-HA vector had significantly decreased the sulfatide storage in brain and spinal cord of treated KO ARSA mice (**Figures 3C,G,K,O**). This was confirmed by the quantification of the number of sulfatide storage inclusions in the cortex, corpus callosum, fimbria and spinal cord (**Figures 3D,H,L,P**). Indeed, a

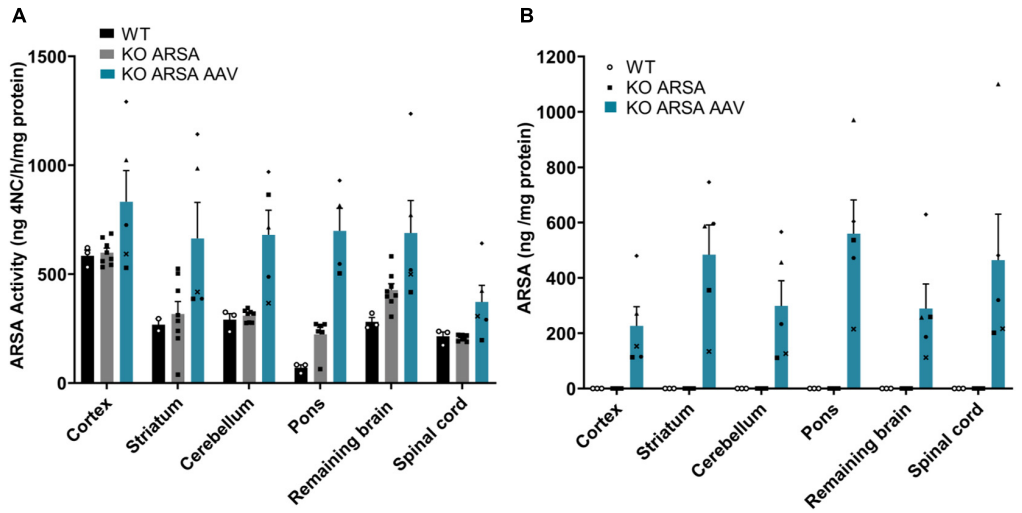

FIGURE 2 | (A) ARSA activity in several brain regions and spinal cord in 9-month-old wild-type (n = 3), untreated (n = 7) and treated KO ARSA (n = 5) mice with AAVPHP.eB-hARSA-HA. **(B)** Arylsulfatase A (ARSA) expression (ng/mg protein) assessed by ELISA in several brain regions and spinal cord in 9-month-old wild-type (n = 3), untreated (n = 3) and treated KO ARSA (n = 5) mice with AAVPHP.eB-hARSA-HA. Data are represented as mean ± SEM.

tremendous decrease of sulfatide accumulation was observed in treated mice that were almost similar to WT mice for brain and spinal cord. However, sulfatide storage was not improve in gall bladder and sciatic nerve of treated animals that remained similar to untreated animals (**Supplementary Figure 2**).

No Abnormalities of Purkinje Cells in KO ARSA Mice

In this mouse model, the abnormalities of Purkinje cells are detected around 12 months (Hess et al., 1996). To confirm the absence of abnormalities in Purkinje cells of 9-month-old mice, an immunochemical staining was performed with anti-calbindin antibody on the brain sections of different groups. No abnormalities in the number of Purkinje cells were observed in 9-month-old KO ARSA mice (data not shown). This result confirms the absence of phenotype in cerebellum of 9-month-old KO ARSA mice.

AAVPHP.eB-hARSA-HA Treatment Rescue Neuroinflammation in MLD Mouse Model

Astrogliosis and microgliosis are two hallmarks of MLD pathology that are present in MLD mouse model (Sevin et al., 2006, 2007; Piguet et al., 2012). To assess the effect of AAVPHP.eB-hARSA-HA treatment on astrogliosis and microgliosis in KO ARSA mice, an immunohistochemical staining was performed with anti-GFAP or anti-Iba1 antibodies on the brain and spinal cord sections of different groups of animals. A significant increase of GFAP-positive cells was observed in cortex (**Figures 4A–D**) and spinal cord (**Figures 4M–P**) of 9-month-old untreated mice compared to WT mice. In the cerebellum, an increase of GFAP-positive cells was also detected in KO ARSA mice, compared to WT animals, even if not significant (**Figures 4I–L**). No astrogliosis was detected in the corpus callosum of untreated mice (**Figures 4E–H**). Three months after injection with AAVPHP.eB-hARSA-HA, a significant decrease of astrogliosis was observed in the spinal cord of treated KO ARSA mice, as well as a trend to improvement in the cortex and cerebellum, vouching for a clear therapeutic effect (**Figure 4**).

A significant increase of Iba1-positive cells was observed in cortex and corpus callosum of untreated KO ARSA mice, compared to WT mice (**Figures 5A–H**). This was not observed in the cerebellum and in spinal cord of untreated mice compared to WT mice (**Figures 5M–P**), even if microglia had tendency to increase in both these structures (**Figures 5I–L**). Three months after AAVPHP.eB-hARSA-HA injection, a significant decrease in microgliosis was observed in both cortex and corpus callosum of treated mice (**Figures 5A–H**), indicating a positive therapeutic effect. No difference of microgliosis was shown in cerebellum and spinal cord of treated mice compared to two other groups.

In summary, makers of neuroinflammation observed in the CNS of 9-month-old KO ARSA mice was significantly reduced after intravenous administration of the AAVPHP.eB-hARSA-HA therapeutic vector.

DISCUSSION

Treating early onset forms of MLD is a huge challenge, given the rapidity of the neurologic deterioration in these forms of the disease. Given the rapid and devastating progression of cerebral disease in patients, the therapeutic challenge is to deliver rapidly and efficiently ARSA enzyme or gene in both neurons and oligodendrocytes and not only in the brain but also in the spinal cord. This is notably the case for patients already symptomatic in which HSCT or HSCT-GT are insufficient (Sessa et al., 2016).

Very promising results let us consider that HSCT-GT may become in the future the standard of care for patients with early onset MLD at a pre-symptomatic stage, justifying an expansion of

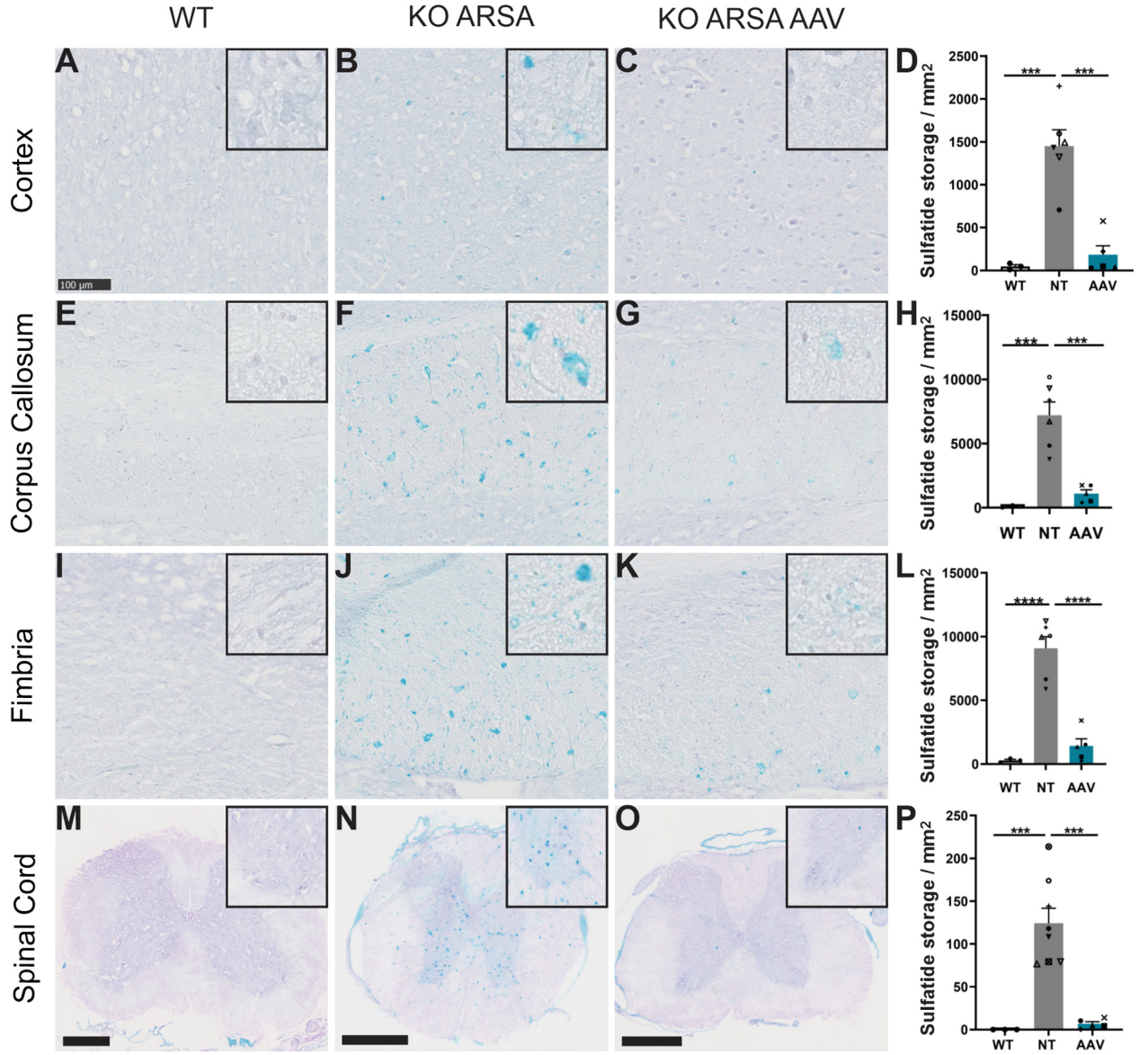

FIGURE 3 | Correction of sulfatide storage in brain and spinal cord of treated KO-ARSA mice, 3 months after treatment **(A–C,E–G,I–K,M–O)**. Alcian blue staining in cortex **(A–C)**, corpus callosum **(E–G)**, fimbria **(I–K)** and spinal cord **(M–O)** of wild-type (WT; **A,E,I,M**), untreated (KO ARSA; **B,F,J,N**) and AAVPHP.eB-hARSA-HA treated (KO ARSA AAV; **C,G,K,O**) KO ARSA mice. Inserts are high magnification of tissue section to show the absence or the presence of sulfatide storage. **(D,H,L,P)** Quantification of sulfatide storage per mm^2 in cortex **(D)**, corpus callosum **(H)**, fimbria **(L)** and spinal cord **(P)** of WT ($n = 3$), untreated (NT, $n = 6–8$) and treated (AAV, $n = 5$) KO ARSA mice. Data are represented as mean ± SEM. Scale bars: 100 µm expected for **(M–O)**, scale bars: 500 µm. $^{***}p < 0.001$; $^{****}p < 0.0001$.

the newborn screening for MLD. However, its effect is dependent on a delayed onset of action (12–18 months), which is a problem for these very rapidly progressing diseases. Thus, up to now, no therapy is available once symptoms are already present, which is the main clinical situation in the daily life for pediatricians. Intrathecal ERT, currently under clinical evaluation, could be helpful and act very quickly but would require lifelong repeated injections.

In vivo gene therapy is attracting growing interest for the treatment of neurodegenerative diseases, including MLD, with the goal to act quickly (few weeks) and achieve, after a "one-shot" procedure, long-lasting expression of the therapeutic gene. In our intracerebral AAVrh.10 gene therapy trial (NCT01801709), despite long-lasting restoration of ARSA activity in the CSF, we failed to demonstrate any clinical effect, even in pre-symptomatic patients with LI-MLD (Sevin et al., 2018). Inconstant results were observed in clinical trials using intracerebral GT for other lysosomal diseases (MPSIIIA, MPSIIIB, LINCL) (Tardieu et al., 2014, 2017; Sondhi et al., 2020). New routes of administration (intra-CSF, intravenous)

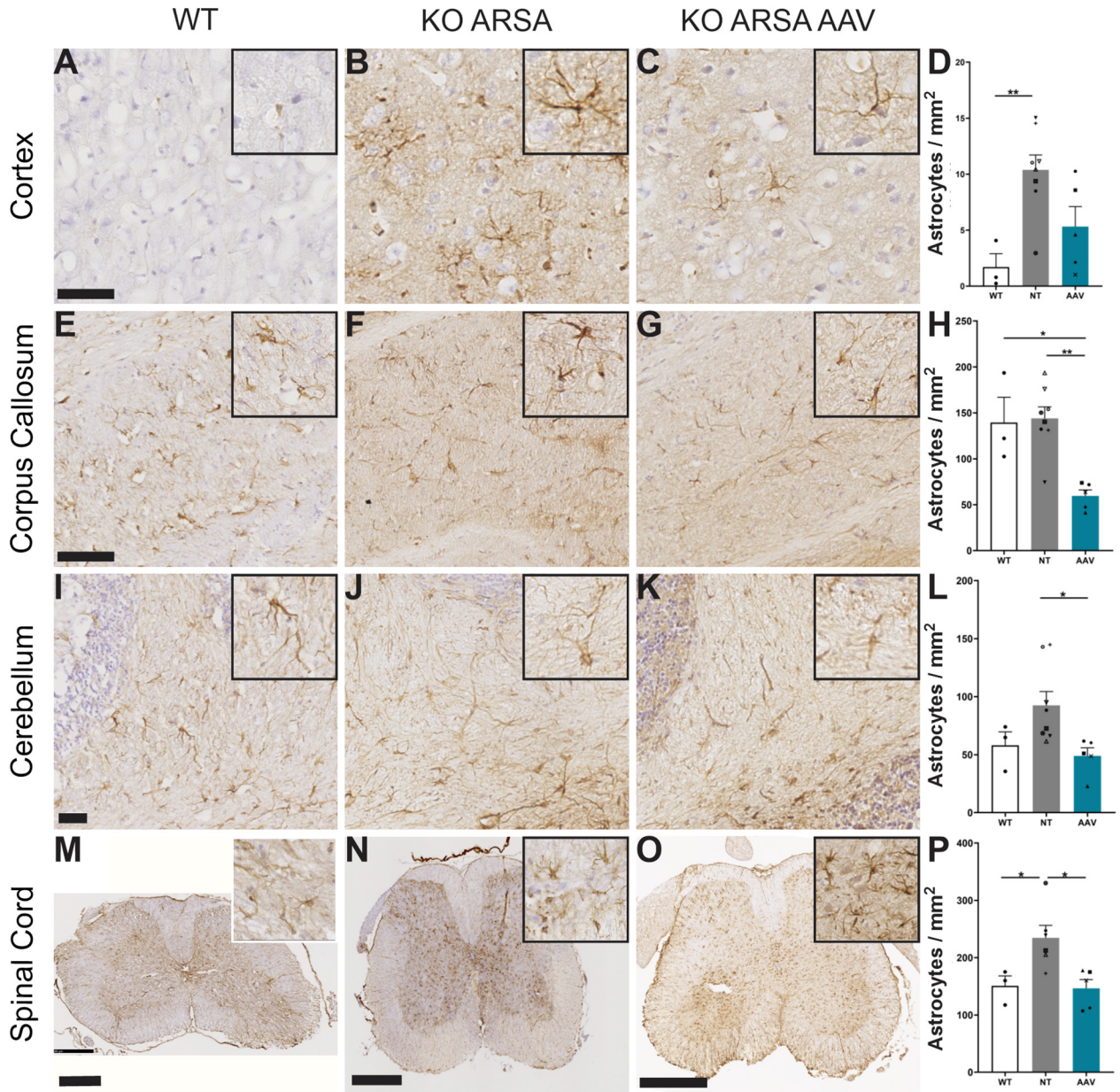

FIGURE 4 | Correction of astrogliosis in brain and spinal cord of treated KO-ARSA mice, 3 months after treatment. **(A–C,E–G,I–K,M–O)** Immunohistochemistry of GFAP in cortex **(A–C)**, corpus callosum **(E–G)**, cerebellum **(I–K)** and spinal cord **(M–O)** of wild-type (WT; **A,E,I,M**), untreated (KO ARSA; **B,F,J,N**) and AAVPHP.eB-hARSA-HA treated (KO ARSA AAV; **C,G,K,O**) KO ARSA mice. Insets are high magnification of tissue section. **(D,H,L,P)** Quantification of GFAP-positive cells per mm^2 in cortex **(D)**, corpus callosum **(H)**, cerebellum **(L)** and spinal cord **(P)** of WT (n = 3), untreated (NT, n = 6–8) and treated (AAV, n = 5) KO ARSA mice. Data are represented as mean ± SEM. Scale bars: 50 μm **(A–C)**, 100 μm **(E–H)**, 500 μm **(M–O)**. *$p < 0.05$; **$p < 0.0$.

are currently under clinical evaluation, most of them using AAV9 vector. Interestingly, a combined approach, using both intravenous gene therapy (with an AAVrh.10 vector) and allogenic HSCT is currently in clinical trial in pre-symptomatic children with infantile Krabbe disease (NCT04693598).

Here, we proposed an intravenous gene therapy approach to rapidly and efficiently deliver ARSA expression both in brain and in spinal cord, using intravenous administration of AAVPHP.eB-hARSA-HA in MLD mice. AAVPHP.eB was recently described as strongly efficient to cross the BBB after intravenous delivery and lead to an efficient brain and spinal cord transduction in mouse models (Deverman et al., 2016; Chan et al., 2017). Moreover, the ARSA KO mouse strain is 129/Ola, which is known to express the LY6A receptor (Hordeaux et al., 2018; Mathiesen et al., 2020), and thus ability of AAVPHP.eB to transduce the BBB.

Our results demonstrate clearly that intravenous AAVPHP.eB-hARSA-HA delivery in MLD mice lead to a

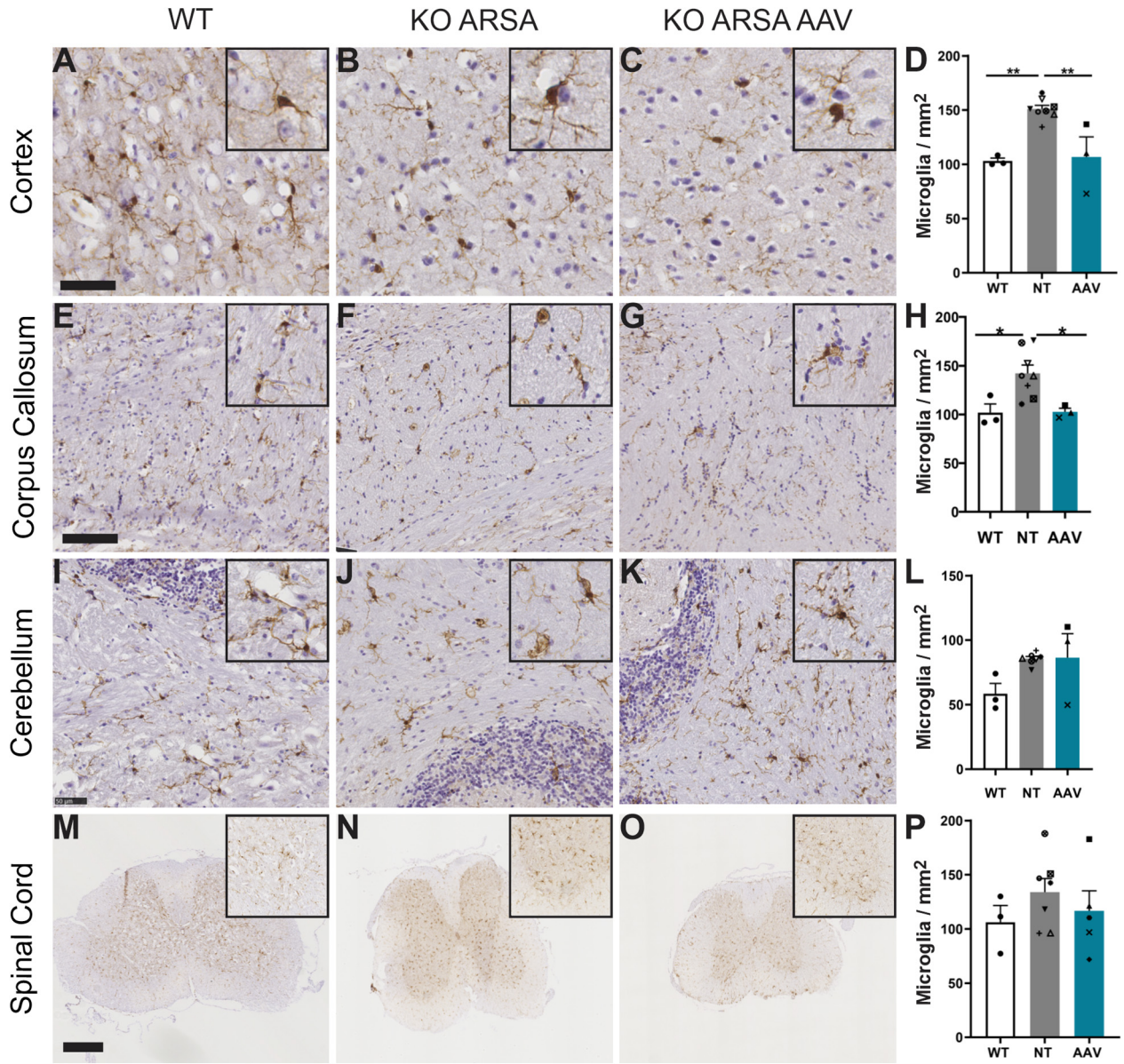

FIGURE 5 | Correction of microgliosis in cortex and corpus callosum of treated KO-ARSA mice, 3 months after treatment. **(A–C,E–G,I–K,M–O)** Immunohistochemistry of Iba1 in cortex **(A–C)**, corpus callosum **(E–G)**, cerebellum **(I–K)** and spinal cord **(M–O)** of wild-type (WT; **A,E,I,M**), untreated (KO ARSA; **B,F,J,N**) and AAVPHP.eB-hARSA-HA treated (KO ARSA AAV; **C,G,K,O**) KO ARSA mice. Insets are high magnification of tissue section. **(D,H,L,P)** Quantification of Iba1-positive cells per mm^2 in cortex **(D)**, corpus callosum **(H)**, cerebellum **(L)** and spinal cord **(P)** of WT (n = 3), untreated (NT, n = 6–8) and treated (AAV, n = 5) KO ARSA mice. Data are represented as mean ± SEM. Scale bars: 50 μm **(A–C,I–K)**, 100 μm **(E–H)**, 500 μm **(M–O)**.*$p < 0.05$; **$p < 0.01$.

broad transduction of brain and spinal cord (**Figure 1**) without major transduction in peripheral organs (**Figure 1**), confirming the CNS tropism described by Deverman et al. (2016). No behavioral tests were done in this study due to the age of the mice at the necropsy as rotarod alterations are known to be clear around 12–18 months of age (Sevin et al., 2006, 2007).

Moreover, compare to our previous studies with AAVrh10, mean levels in the brain (striatum, cortex and rest of the brain) is similar between intravenous delivery of AAVPHP.eB-hARSA (330 ng ARSA/mg of protein) than with AAVrh10 intracerebral delivery (325 ng/mg of proteins). However, in the cerebellum, brainstem and spinal cord where no ARSA expression was detected with AAVrh10 (Piguet et al., 2012), intravenous delivery of AAVPHP.eB-hARSA lead to high expression of the hARSA (around 300 ng/mg of protein in the cerebellum; 560 ng/mg of protein in the brainstem and 460 ng/mg of protein in the spinal cord). This strongly demonstrates the efficacy of the intravenous delivery of the AAVPHP.eB to achieve a broad and strong expression of ARSA, which is suitable for therapeutic development.

Intravenous delivery of AAVPHP.eB-hARSA-HA in MLD mice leads to a rapid and complete correction of brain sulfatide storage, one of the hallmarks of the disease (Gieselmann and Krägeloh-Mann, 2006), as our previous approach with intracerebral delivery of AAVrh10-ARSA (Piguet et al., 2012). However, we can demonstrate here a complete correction of sulfatide storage in spinal cord of treated animals. This has never been observed previously with both our AAV5 and AAVrh10 intracerebral approach (Sevin et al., 2006, 2007; Piguet et al., 2012) and even never reported in MLD mice after enzyme replacement therapy (Matzner et al., 2009; Matthes et al., 2012). In addition, the treatment was administrated in 6-month-old mice, an age where the sulfatide storage is already clearly detectable (Hess et al., 1996) and its effectiveness was evaluated only 3 months after injection for which the complete rescue was observed in the CNS. This indicates us that a strong expression of ARSA leads to a rapid reversal of sulfatide storage, which is a crucial advantage for rapidly progressive form of MLD.

The second hallmark of the disease on which we focused was the astrogliosis and microgliosis. Neuroinflammation is a hallmark of many LSDs with CNS involvement, including MLD and has emerged as a key factor in promoting neurodegeneration in these diseases. Astrogliosis and microglia activation are observed in the brain of MLD patients and mice, associated with increased inflammatory cytokines (Jeon et al., 2008; Stein et al., 2015; Thibert et al., 2016). The most commonly accepted hypothesis is that neuroinflammation is due to secondary activation of microglia following phagocytosis of myelin debris containing undegraded material. However, microglia activation and elevation of cytokines have been shown to precede demyelination in MLD mice, suggesting that neuroinflammation may also be a primitive phenomenon (Stein et al., 2015). In our study, we demonstrated a significant improvement of astrogliosis and microgliosis in AAVPHP.eB-hARSA-HA treated mice compared to untreated MLD mice, as efficiently as our previous approach with intracerebral delivery of AAVrh10-ARSA ARSA (Piguet et al., 2012). However, we can demonstrate, in addition, a clear reduction/normalization of neuroinflammation in spinal cord which was never observed previously with our AAV5 and AAVrh10 intracerebral approach (Sevin et al., 2006, 2007; Piguet et al., 2012) neither with the enzyme replacement therapy (Matzner et al., 2009; Matthes et al., 2012).

Altogether, the intravenous injection of AAVPHP.eB-hARSA-HA vector resulted in an unprecedented level of sulfatide and neuropathology corrections, not only in the brain but also in the spinal cord. Those results provide strong support for implementing intravenous AAVPHP.eB gene therapy in MLD patients with rapidly progressive forms of the disease after disease onset, but also in other rapidly progressing leukodystrophies like Krabbe disease. Tolerance and efficacy studies are currently in progress in non-human primates before translation to human patients, in particular to evaluate the dose of AAVPHP.eB that would be required for therapeutical benefit. Preliminary results demonstrated a capacity of the AAVPHP.eB to cross brain barrier and transduce CNS in NHP without sign of toxicity. In NHP, there is no direct homolog of LY6A receptor, however, other key factor could share properties and allow AAVPHP.eB passage across BBB (Huang et al., 2019). In the optic of a clinical application, the existence of preexisting antibodies is a remaining question as AAV is highly prevalent in humans (Fu et al., 2017), that would anyway need a serology prior to injection. This has not been evaluated in mouse has there is no preexisting immunity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by CE17 Darwin. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

EA and FP designed the experiments. EA, FP, VO, and BM performed the experiments. EA, FP, and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.677895/full#supplementary-material

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REVIEW
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Gene Therapy for Neurodegenerative Disease: Clinical Potential and Directions

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The pathogenesis of neurodegenerative diseases (NDDs) is complex and diverse. Over the decades, our understanding of NDD has been limited to pathological features. However, recent advances in gene sequencing have facilitated elucidation of NDD at a deeper level. Gene editing techniques have uncovered new genetic links to phenotypes, promoted the development of novel treatment strategies and equipped researchers with further means to construct effective cell and animal models. The current review describes the history of evolution of gene editing tools, with the aim of improving overall understanding of this technology, and focuses on the four most common NDD disorders to demonstrate the potential future applications and research directions of gene editing.

Keywords: gene editing, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis

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INTRODUCTION

Neurodegenerative disease (NDD) refers to a group of chronic disorders characterized by progressive loss of neurons in the brain and spinal cord. Due to technical limitations, our initial understanding of NDD was initially restricted to the pathological manifestations of abnormal protein aggregation, such as Aβ protein in Alzheimer's disease (AD), huntingtin (HTT) protein in Huntington's disease, α-synuclein in Parkinson's disease, and neurofilament in amyotrophic lateral sclerosis. However, treatments targeting abnormal protein levels have constantly faced setbacks in clinical trials. By the end of the 20th century, revolutionary advances in sequencing techniques offered a novel perspective to interpret the mechanisms underlying progression of NDD and gene mutations were identified as drivers of phenotype changes. Thereafter, several studies on NDD at the gene level were conducted. With progression of sequencing methods to third-generation technology, numerous NDD-related mutations and single nucleotide polymorphism (SNP) sites were progressively identified. However, gene mutations cannot explain 100% of NDD cases and sporadic cases exist, even for Huntington's disease (HD) that is generally considered an autosomal dominant disorder. Thus, in recent years, research focus has expanded from direct gene expression to regulation of expression, which encompasses the fields of transcriptomics, proteomics, and epigenomics.

The concept of gene therapy, first proposed in 1972 (Friedmann and Roblin, 1972), refers to targeted changes in gene sequences through molecular means.In a narrow sense, gene editing is primarily achieved through inducing specific DNA double-strand breaks (DSB) to replace or modify target genes based on the donor sequence.From zinc finger endonuclease to the CRISPR/Cas9 system, the rapidly progressing geneediting field has significant therapeutic

potential. The CRISPR/Cas system is particularly outstanding with the advantages of minimal molecular weight and natural existence. Free of artificial design, its convenience and effectiveness has greatly aided in providing insights into the mechanisms underlying NDD, reduced the cost of gene editing, and enabled construction of disease models in both cell and animal systems, in addition to facilitating multiple gene editing valuable for complex diseases such as NDD.

This report provides an overview of the history of gene editing and recent research focus on the four most common NDD disorders, specifically, AD, Parkinson's disease (PD), HD, and amyotrophic lateral sclerosis (ALS).

EVOLUTION OF GENE EDITING

Homologous Recombination

Homologous recombination is the earliest genome editing technique based on natural DNA damage and the cellular repair system. Chemicals, radiation, and by-products of cellular biological processes, such as reactive oxygen species produced during aerobic respiration, nitrogen compounds produced by inflammatory cells and free radicals generated in hydrolysis reactions, can lead to DNA damage (Jackson and Bartek, 2009). Cells in the human body experience tens of thousands of DNA lesions per day (Lindahl and Barnes, 2000). Among the multiple forms of damage, double-stranded breaks (DSB) are one of the most toxic and difficult to repair (Khanna and Jackson, 2001).

Cells have evolved various mechanisms to repair DSB, two of the most important being homologous recombination (HR) and non-homologous end-joining (NHEJ) (Lieber, 2010). Compared with template-free NHEJ, HR is confined to S and G2 phases (Hustedt and Durocher, 2016; Zhao et al., 2017; Murray and Carr, 2018) while NHEJ is prevalent over the entire cell cycle (Symington and Gautier, 2011). As an important method of allelic exchange, HR also plays a key role in meiosis and mitosis (San Filippo et al., 2008). NHEJ is the dominant repair pathway in mammals and HR competes with other mechanisms (Symington and Gautier, 2011; Kowalczykowski, 2015; Haber, 2016). The repair process of HR is based on multiple pathways, starting from Mre11-Rad50-Nbs1 (MRN) binding to the DSB site followed by 5′-to-3′ resection to generate single-stranded DNA (ssDNA), eventually initiating sub-pathway repair (Mehta and Haber, 2014; Skoneczna et al., 2015; Haber, 2016). HR uses homologous sequences of the sister chromatid as templates in the natural route (San Filippo et al., 2008; Heyer et al., 2010). In genetic engineering, plasmid or viral vector-mediated sequences homologous to DSB ends serve as the new template. However, due to competition with NHEJ, the natural frequency of HR repair alleles in eukaryotes is extremely low (Allen et al., 2002), greatly limiting the efficiency of gene editing, which could be augmented with human intervention in the future.

Evidence indicates that DSBs trigger HR (Rouet et al., 1994; Choulika et al., 1995) and an enzyme designated "meganuclease" or "homing endonuclease" that is naturally present in mitochondria and chloroplasts of microorganisms specifically recognizes 12–30 bp DNA sequences for cleavage without affecting the whole genome (Colleaux et al., 1986; Thierry and Dujon, 1992; Choulika et al., 1994). Owing to the advantages of long recognition sites and 3′ overhang production after DNA cleavage, meganucleases exhibit lower toxicity and better precision than other restriction enzymes (Kc and Steer, 2019). While hundreds of meganucleases have been identified to date, the likelihood of locating the enzyme required for a specific site remains low. On the other hand, since DNA binding and cleavage sites of meganuclease are interspersed in the same domain and are difficult to separate, tailoring the required meganuclease through engineered modifications remains a big challenge (Khan et al., 2018). Overall, the clinical application of meganuclease continues to face technical difficulties (Gaj et al., 2016).

Zinc Finger Nucleases

In the 1990s, the discovery of Flavobacterium okeanokoites (*Fok*I) enzyme (Sugisaki and Kanazawa, 1981; Li et al., 1992; Kim and Chandrasegaran, 1994) and zinc finger structure (Fegan et al., 1985; Lee et al., 1989) promoted further development of the gene editing technique. Hydrolyzed *Fok*I enzyme (a type IIS restriction endonuclease) in Flavobacterium contains N-terminal DNA-binding and C-terminal domains with non-specific DNA cleavage activity (Li et al., 1992, 1993) that can be easily separated (Waugh and Sauer, 1993). Owing to the modularity of *Fok*I enzyme, engineering is relatively simple.

Zinc finger is an independently folded binding domain that coordinates zinc ions to stabilize the structure. Repeated zinc-binding motifs were first reported in Xenopus transcription factor IIIA (TFIIIA) (Miller et al., 1985). After the first single zinc finger was described in 1989 (Lee et al., 1989), vast complexes were successively identified. Considering Cys2-His2, the most common zinc finger domain as an example, a zinc finger is composed of about 30 amino acids in a conserved ββα configuration (Beerli and Barbas, 2002) and builds contacts with three base pairs in DNA sequences. Zinc finger proteins with conserved sequences are arranged in a certain order followed by attachment of *Fok*I to the 3′ end of the protein, ultimately generating a zinc-finger nuclease (ZFN) consisting of both DNA binding and DNA cleavage domains that recognizes a 9–18 bp sequence (Liu et al., 1997). After binding of ZFN to DNA, the *Fok*I nuclease induces cleavage as a dimer, resulting in DSB. HR and NHEJ are activated to complete gene editing *via* the intracellular DNA repair mechanism (Bitinaite et al., 1998; Smith et al., 2000). Theoretically, ZFNs recognize almost all 64 possible nucleotide triplets but several of these fail in terms of pairing, design and selection (Ramirez et al., 2008; Kim et al., 2010). The specificity and affinity of ZFN is also an issue although optimal fingers have a certain affinity for similar sequences. Increasing the number of zinc fingers can improve specificity and affinity but also raises the issue of inability to access sequences at certain sites, such as those with close chromatin structure and DNA modification (Carroll, 2011). Additionally, ZFNs are reported to exert a significant cytotoxic effect (Khalil, 2020).

Transcription Activator-Like Effector Nuclease

The discovery of transcription activator-like effector in the plant pathogen Xanthomonas (Boch et al., 2009; Moscou and Bogdanove, 2009) promoted the development of transcription activator-like effector nuclease (TALEN) (Miller et al., 2011), a second-generation nuclease editing technique. The central structure of TALEN protein is a highly conserved sequence of 33–35 amino acids with two variable residues at positions 12 and 13, referred to as repeat variable di-residues (RVD). Each motif relies on RVDs to recognize a single nucleotide (Deng et al., 2012). Similar to ZFN, construction of TALEN is based on the modularity and DNA cleavage function of FokI (Sun and Zhao, 2013). TALEN is easier to design and produce than ZFN but requires about three times as many coding genes. Moreover, its higher molecular weight makes transfection into mammalian cells difficult, especially using virus vectors with limited packaging capacity (Chandrasegaran and Carroll, 2016; Maeder and Gersbach, 2016).

CRISPR

Clustered regularly interspaced palindromic repeat (CRISPR) was first described in 1987 (Ishino et al., 1987) and its gene editing ability confirmed in human cells in 2013 (Cho et al., 2013). CRISPR exists in 40% bacteria and 90% archaeal genomes and functions as an adaptive immune defense system (Horvath and Barrangou, 2010). The CRISPR system can specifically capture gene sequences adjacent to protospacer adjacent motif (PAM) for cleavage using Cas nuclease into spacer segments derived from the exogenous genome. Spacers are subsequently incorporated into the CRISPR locus of host cells, separated by palindromic sequences, and eventually transcribed to CRISPR RNA (crRNA) with spacer characteristics (Barrangou et al., 2007; Garneau et al., 2010; Horvath and Barrangou, 2010). CrRNA pairs with invading foreign gene sequences in a complementary manner. Simultaneously, Cas nuclease destroys target DNA and completes the entire immune response. By capturing exogenous gene segments from invading phages, viruses and plasmids and incorporating them into host genomic loci, the CRISPR/Cas system sustains acquired immune function (Barrangou et al., 2007; Garneau et al., 2010; Horvath and Barrangou, 2010).

Type II CRISPR/Cas9 composed of Cas9 endonuclease, crRNA and trans-activating crRNA (tracrRNA) is the most commonly used system in genetic engineering (Jinek et al., 2012). Cas nuclease is the core functional element of the CRISPR system. TracrRNA and precursor crRNA (pre-crRNA) bind *via* base pairing, are trimmed by RNaseIII, self-folded into a partial double-stranded RNA structure, and interact with Cas9 to form a complex with DNA cleavage ability. The crRNA-tracrRNA duplex functions as a single guide RNA (sgRNA) that effectively pairs with the target sequence. After binding to the target site, Cas9 undergoes conformational changes and induces DSBs 3–4 nucleotides upstream of PAM (Jinek et al., 2012; Nishimasu et al., 2014). Domains in Cas9 not only interact with the PAM motif but also assist with sgRNA binding to the target sequence (Nishimasu et al., 2014; Barman et al., 2020).

Compared with ZFN and TALEN, the editing horizon of CRISPR is elevated from protein to RNA and technical difficulties from design to assembly are greatly simplified. In terms of target recognition, specificity is higher and binding is more stable. In addition, Cas9 acts as monomer in contrast to *Fok*I, which only cleaves DNA in a dimeric form (Bitinaite et al., 1998; Smith et al., 2000). However, CRISPR does not alter the nature of HR induction through DSBs. In fact, NHEJ is a more prevalent pathway for DSB repair in the entire cell cycle (Chapman et al., 2012). Although NHEJ inhibitors (e.g., Scr7) (Maruyama et al., 2015) and HR promoters (e.g., Cas9-RecA fusion protein) (Cai et al., 2019) are expected to improve the efficiency of HR, CRISPR technology requires further improvement to improve the accuracy of gene editing.

LATEST DEVELOPMENTS IN GENOME EDITING

In 2017, a study published in Nature reported a technique denoted "Base Editor" (BE) that achieved single base conversion independently of DSB and HD (Komor et al., 2016; Gaudelli et al., 2018). Based on a complex composed of dCas9 (inactive or dead Cas9) or Cas9n (Cas9 nickase with single-strand DNA incisional enzyme activity), cytosine deaminase (yCD), uracil DNA glycosylase inhibitor (UGI) and sgRNA, BE can achieve four types of accurate base substitution between C/T and G/A (Komor et al., 2016). A new technique known as "Prime Editor" (PE) was reported in 2019 (Anzalone et al., 2019) involving coupling of Cas9 protein with reverse transcriptase. Under guidance of prime editing guide RNA (pegRNA), the PE complex cuts a single strand of DNA at the target site and synthesizes new sequences with the aid of reverse transcriptase. Unpaired sequences in pegRNA are used as templates. The newly synthesized sequence is finally incorporated into the host genome for completion of the gene editing process. PE is free of DNA template and can achieve precise single nucleotide substitutions in sequences inaccessible for BE. While the advent of BE and PE has created new possibilities for gene editing, several concerns remain. For instance, dependence on the Cas9 enzyme limits their recognition window, since Cas9 can only act on sequences adjacent to PAM. BE converts all editable bases in the editing window in a non-specific manner. Moreover, BE can achieve transition of purine–purine and pyrimidine–pyrimidine but not transversion of purine-pyrimidine. In addition, BE and PE only perform edits on single nucleotides and are unable to achieve targeted integration of DNA. The off-target effects of BE and PE in practical applications remain to be established.

In 2019, a new technique using CRISPR-associated transposon (CAST) for DNA transposition was reported in Science (Strecker et al., 2019) and another similar report published in Nature (Klompe et al., 2019). CAST utilizes the ability of Tn7-like transposons to recruit the CRISPR/Cas system in bacteria (Peters et al., 2017). After instrumentalization, Tn7-like transposons can be used for targeted DNA insertion. Independent from DSBs, CAST can effectively carry cargo genes up to 10 kB,

which is far superior to the current gene knock-in tool (Hou and Zhang, 2019).

ALZHEIMER'S DISEASE

Alzheimer's disease is a clinical syndrome characterized by brain amyloid-beta (Aβ) protein deposition in senile plaques (SPs), downstream neuronal degeneration, and tau protein hyperphosphorylation (p-tau) forming neurofibrillary tangles (NFTs) (McKhann et al., 2011). Over the past 20 years, the amyloid cascade hypothesis has dominated research on the pathogenesis of AD. However, identification of mutations within three autosomal dominant genes, specifically, *APP* on chromosome 21 (Goate et al., 1991), *PSEN1* on chromosome 14 (Sherrington et al., 1995), and *PSEN2* on chromosome 1 (Levy-Lahad et al., 1995), has significantly changed research perspective. Subsequent genome-wide association studies (GWAS) have resulted in the identification of another risk gene, ApoE4, and further AD-related SNP sites. These genes encode proteins implicated in various biological processes of AD, which may serve as future editing targets.

Recent GWAS have led to the discovery of dozens of risk loci (Lambert et al., 2013; Kunkle et al., 2019; Vacher et al., 2019; Kunkle et al., 2021). Among these, clear evidence of function has been obtained for ApoE, ABCA7, BIN1, TREM2, SORL1, ADAM10, SPI1, and CR1. In particular, ApoE4 has been extensively characterized in different disease models (Burnham et al., 2020). A recent study showed that Klotho hormone in its biological form reduces risk of AD onset in individuals carrying ApoE4. Moreover, a heterozygous state of KL-VS (KL-VSHET+) genotype was suggested in association with reduced burden of AD and Aβ protein (Belloy et al., 2020). However, no association of KL-VS, the variant of Klotho, with cognitive decline of patients was observed in another clinical study (Porter et al., 2019). The potential contribution of ApoE4 to AD was further examined from multiple perspectives. REST, a central regulator of neural differentiation, is suggested to be related to the ApoE4-induced phenotype (Meyer et al., 2019). Moreover, considering the energy metabolism failure in patients with AD, the ApoE4 genotype may have a regulatory effect on metabolism. These metabolic changes have additionally been linked to gender differences (Arnold et al., 2020). One advantage is that ApoE4 has only one nucleotide difference from its allele ApoE3. Therefore, it is feasible to induce single nucleotide changes, especially with the PE technique. Triggering receptor expressed on myeloid cells 2 (TREM2) is a genetic locus shared by AD and PD. Aggravated neurodegeneration has been detected in TREM2-deleted mice, which may be related to microglial activation (Guo et al., 2019). Another *in vivo* study exhibited transformational value for clinical treatment. Researchers successfully improved Aβ pathology in APP transgenic mice (Nagata et al., 2018; see **Table 1**).

A number of studies have also focused on the genetic background of AD pathological manifestations. For instance, CHRFAM7A exerts an antagonistic effect on cholinergic receptors in induced pluripotent stem cell (iPSCs) transfected with TALEN (Szigeti et al., 2020). PSENLIN2 is associated with greater amyloid β protein accumulation than PSENLIN1 (Lessard et al., 2019). However, editing the C-terminus of APP *via* CRISPR led to successful reduction of Aβ protein generation in iPSCs (Sun et al., 2019). The Aβ protein-related phenotype was also inhibited by phosphorylation of Threonine 205 (T205) in APP transgenic mice. The post-synaptic mitogen-activated protein kinase (MAPK), p38γ, is further proposed to be involved in regulation (Ittner et al., 2020). Other epigenetically dysregulated loci have been described in a genetic model of *Caenorhabditis elegans*. In another study, mimicking of phosphorylation of Threonine 231 (T231) and acetylation of Lysine 274 (K274) and Lysine 281 (K281) in *C. elegans* was associated with age-related reduction in touch sensation and neuronal morphological abnormalities (Guha et al., 2020).

In addition to the loci that have been extensively investigated, other risk loci identified by GWAS remain to be validated in cell/animal models. A number of studies have included peripheral tissues (such as skin tissue) for analysis. However, the pathogenic significance of these newly identified genes in AD remains to be confirmed (Gerring et al., 2020).

PARKINSON'S DISEASE

Parkinson's disease, another important age-related chronic progressive neurodegenerative disorder, is characterized by aggregation of α-synuclein protein. Numerous studies have focused on PD patients with family history-specific mutations in LRRK2, PARK2, DJ-1, PINK1, and SNCA (Sundal et al., 2012). SNCA is directly related to expression of α-synuclein and one of the most significant prediction sites for sporadic PD (Ferreira and Massano, 2017). Mutation and triplication of SNCA A53T affects nucleocytoplasmic transport mediated by α-synuclein (Chen V. et al., 2020). This regulation has been further confirmed in CRISPR-edited iPSCs (Barbuti et al., 2020). Another newly discovered α-syn SNP site, rs12411216, is reported to regulate the function of glucocerebrosidase, which promotes distribution of α-syn protein (Jiang et al., 2020). An improved SCNA-specific CRISPR technique has been applied to generate a PD cell model (Arias-Fuenzalida et al., 2017). A novel CRISPR-based lentiviral vector has additionally been designed to downregulate transcription and expression though targeted methylation of intron 1 of SNCA (Kantor et al., 2018). Another study showed that cell lines depleted of SNCA present resistance to Lewy pathology (Chen X. et al., 2020).

P13, PINK, and PARKIN are additionally highlighted as therapeutic targets on account of their involvement in regulation of mitochondrial function. Several groups have investigated the effect of PARKIN mutation on expression of PD-related proteins in iPSCs lines (Suda et al., 2018). Decreased expression of P13 is reported to exert neuroprotective effects on genetic PD and toxin-induced PD models. In contrast, overexpression of P13 has been shown to promote the emergence of phenotypes in toxin-induced PD mice (Inoue et al., 2018). Some researchers have proposed references for the construction of a LRRK2-related PD stem cell model through cytogenetic analysis (Vetchinova

et al., 2018). Another LRRK2 iPSC model constructed with the TALEN technique could additionally serve as a reference (Ohta et al., 2020). In an interesting study, PARKIN, DJ-1, and ATP13A2 genes were deleted using the CRISPR/Cas system in nigral dopaminergic neurons (DN). Through integration of transcriptome and proteome data, oxidative stress was identified as the common dysregulation pathway of all the isogenic cell lines (Ahfeldt et al., 2020). With elucidation of the molecular mechanisms underlying PD, traditional clinical typing may no longer be applicable. More precise delineation of PD subtypes is required, whereby knowledge of molecular etiology could provide further therapeutic perspectives that may be applicable to all NDD disease types.

A novel mutation in DNAJC6 potentially contributes to early impairment of PD in human embryonic stem cells (hESC) (Wulansari et al., 2021). Moreover, PD-related behavioral deficits have been reported in LIN28A knockout mice (Chang et al., 2019). Similar to AD, several recent GWAS for PD have been conducted (Chang et al., 2017; Nabais et al., 2021). However, the issue of whether these disclosed mutations are valuable for clinical prediction requires further study in cell/animal models.

HUNTINGTON'S DISEASE

Huntington's disease, a hereditary neurodegenerative disorder characterized by involuntary dance movements and continuous deterioration of behavior and cognition, is commonly associated with disability and early death. HD is distinguished by neuronal loss and astrocytosis in terms of pathology and progressive brain atrophy on imaging. Confirmation of diagnosis is mainly based on family history, clinical symptoms and genetic mutations. Duplication of CAG trinucleotides on exon 1 of Huntington's gene (HTT) is associated with occurrence of HD (Horvath et al., 2016). Normal CAG repeats on HTT are less than 27 and complete penetration is accomplished when CAG repetition exceeds 39 (McColgan and Tabrizi, 2018). The proteins encoded by the mutated HTT gene (mHTT) cannot participate in physiological cellular mechanisms like their normal protein counterparts and additionally display cytotoxicity. As a disorder caused by single mutation and single abnormal protein, HD is an ideal environment for application of gene therapy.

The construction of HD cell models with gene editing techniques that can be used to validate the efficacy of therapeutic agents has been described in several articles (An et al., 2012, 2014; Xu et al., 2017; Dunbar et al., 2019; Ooi et al., 2019; Malankhanova et al., 2020a). Earlier studies have reported high calcium influx (Vigont et al., 2021) and ultrastructural synapse defects (Malankhanova et al., 2020b) in a HD cell model. Furthermore, the frequency of ultrastructural synapse defects is related to the number of CAG repeats (Morozova et al., 2018).

In another study, deletion of neuronal mHTT was induced *via* CRISPR/Cas9 in HD140Q-KI mice, which led to a significant reduction in reactive astrocytes and improvement of motor dysfunction in the experimental group (Yang et al., 2017). Targeting on the exon 1 of CAG repeat, another *in vivo* study successfully interfered HTT expression as well (Ekman et al., 2019; see **Table 1**). Based on current studies, although inactivation of CAG expression can effectively alleviate the HD phenotype, the apoptotic cells cannot be restored. Following the success of the non-allele-specific CRISPR system in the PD mouse model, allele-specific CRISPR was shown to be effective in two

TABLE 1 | Pre-clinical studies of gene therapy in neurodegenerative diseases.

Reference	Gene editing tool	Vector	Disease	Target	Animal model	Injections	Results
Nagata et al., 2018	CRISPR/ Cas9	px330 plasmid	AD	App 3′-UTR	NL-G-F mice	Microinjected in mice zygotes	Deletion of App 3′-UTR mitigated Aβ pathology in the App KI mice.
Inoue et al., 2018	CRISPR/ Cas9	px330 plasmid	PD	p13 exon1	C57BL/ 6J mice	Injected into the pronuclear stage eggs	Heterozygous p13 knockout prevents motor deficits and loss of dopaminergic neurons in the substantia nigra.
Yang et al., 2017	CRISPR/ Cas9	AAV	HD	CAG flanking region	HD 140Q-KI mice	Injected in striatum	Targeted inactivation of CAG repeat could reverse the neuropathological and behavioral phenotypes even in adult mice.
Ekman et al., 2019	CRISPR/ SaCas 9	AAV	HD	HTT exon 1	HDR6/2 mice	Injected in striatum	Disruption on HTT reduced mHTT protein, increased lifespan, and protected neurons from death, though lost cells were not restored.
Gaj et al., 2017	CRISPR/ Cas9	AAV	ALS	SOD1	G93A-SOD1 mice	Intravenously injected *via* the facial vein	Disruption of mutant SOD1 enhances the survival of spinal cord motor neurons and improves motor function and life span.
Lim et al., 2020	Cytidine base editors	Dual AAV	ALS	SOD1	G93A-SOD1 mice	Injected in the lumbar subarachnoid space	Base editor systems prolonged survival, protected the motor neurons and neuromuscular junctions, slowed the disease progression, decreased muscle denervation.
Duan et al., 2020	CRISPR/ Cas9	AAV	ALS	SOD1	G93A-SOD1 mice	Injected in the lateral ventricle (ICV injection)	Deletion of SOD1 delayed motor neuron degeneration and disease onset, and improved the lifespan.

AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis.

studies (Shin et al., 2016; Monteys et al., 2017). In addition to directly targeting HTT mutations, CITP2, which interacts with mutant huntingtin (Fjodorova et al., 2019), was edited. ZFN and TALEN were also applied to correct repeated expansion of CAG (Fink et al., 2016; Zeitler et al., 2019).

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis is an adult-onset, fatal neurodegenerative disorder. In this disease, apoptosis of the upper motor neurons (e.g., spinal cord, brain stem, and motor cortex) triggers progressive weakness and atrophy of muscles throughout the body, resulting in paralysis and death within 3–5 years after the onset of symptoms. Similar to many other NDDs, ALS is currently incurable. The major pathogenic genes in ALS have been identified as C9orf72, SOD1 (Rosen et al., 1993), FUS, TARDBP, and TBK1 (Müller et al., 2018). However, ALS does not have strong genetic background due to its most cases are sporadic.

Several cell and animal models targeted on SOD1 have been reported (Gaj et al., 2017; Kim et al., 2020). The adeno-associated virus (AAV)-mediated CRISPR system was applied to disrupt SOD1, leading to decreased expression of SOD1 protein in spinal cord and reduction of muscle atrophy in mice. With improvement of motor function, the average survival time of mice increased by 28–30 days (Gaj et al., 2017). Similar favorable results of SOD1 deletion were reported in other animal studies as well (Duan et al., 2020; Lim et al., 2020). Such *in vivo* studies were summarized in **Table 1**.

The G4C2 hexanucleotide repeat in C9orf72 is a newly described pathogenic factor (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Its pathogenic mechanism can be complex. Researchers found that the deletion of C9orf72 aggravated the axonal defects and thus increased cell apoptosis (Abo-Rady et al., 2020). While recent studies suggest that this pathogenic expansion can be fully corrected using the CRISPR/Cas system (Ababneh et al., 2020), expression of C9orf72 is also reported to affect efficacy of the gene editing process (Moore et al., 2019) and DSB repair (Andrade et al., 2020). The pathogenic effect may achieved through affecting the GluA Q/R site RNA editing (Konen et al., 2020; Moore et al., 2019) and mitochondrial Ca2+ uptake impairment (Dafinca et al., 2020). Many other risk sites have also been reported, such as KIF5A associating to the cytoskeletal defects in ALS (Nicolas et al., 2018). However, further in-depth studies are currently insufficient on these GWAS-identified sites.

DISCUSSION

In recent decades, integration of the fields of computer science and biology has fueled the development of bioinformatics, with significant improvements in efficacy of analysis and utilization of sequencing data. For instance, genome-wide analysis facilitates prediction of potential risk loci at low cost, which is valuable for complex diseases. To an extent, computer science has revolutionized the paradigm and efficacy of research in traditional experimental biology. These changes have significant implications for gene editing techniques. Despite the fact that gene editing tools are rapidly evolving in terms of improved ease of use and accuracy, actual editing efficacy remains unpredictable, especially *in vivo*. As an auxiliary discipline, computer science is highly valuable in helping to improve the efficacy of editing tools. To this end, researchers have integrated cell-specific information based on gene expression profiles and biological networks to further develop CRISPR sgRNA design tools and predict the efficacy of the CRISPR/Cas system (Liu et al., 2019). Computer science has become an indispensable part of biological research. With increasingly comprehensive research at the molecular level, the accuracy of interpretation of DNA and RNA sequences depends on the advancement of natural language processing techniques. Based on review of the studies on gene editing tools in NDD, we propose the following transformation of future research patterns: clinical studies provide patient information, computers process the profile and make predictions, experimentalists verify the hypotheses, and the data are collectively used to obtain meaningful conclusions.

From the earliest anti-protein treatment strategy to the gene editing technique, one common feature is the translational gap between human and animal models. Often the performance of therapy in humans does not conform to predictions, which could be attributed to the complexity of the human body. In complex organisms, expression of genes is regulated on a multiple and not linear scale. Similarly, expressed products participate in multiple regulatory mechanisms that form a regulatory DNA-RNA-protein network in the human body. Therefore, the actual results of gene editing are inconsistent due to unknown compensation effects (Sun et al., 2019). However, different types of NDD share common pathways, including mitochondrial dysfunction, cytoskeletal integrity, and DNA repair defects (Chia et al., 2018), suggesting that patient stratification *via* molecular typing or genotyping is valuable for treatment. The next step in NDD analysis is to explain the association between clinical syndromes and molecular pathogenesis. Considering that changes in the neuronal phenotype can be directly detected through knockin/knockout, gene editing tools should significantly enrich our knowledge of regulation networks within neurons. In the foreseeable future, gene sequencing will become a routine procedure that directly impacts clinical practice (Chia et al., 2018).

In general, research on gene editing techniques in neurogenerative disease has primarily centered on cell/animal models to explore the underlying biological mechanisms and involves multiple disciplines including molecular biology, cell reprogramming, computer science, statistics, and multi-omics. The major current challenge for NDD is unknown pathogenesis. Researchers have attempted to explore the pathological changes of NDD at the molecular level, whereby gene editing tools play a significant role in clarifying the gene-phenotype relationships. While gene editing tools have been updated at a rapid pace, their clinical transformation may not be easily achievable in the near future. In addition, digitalization has been explored

as a critical research direction, from designing of editing tools to construction of disease models. Further studies on NDD incorporating participants from diverse academic backgrounds with large-scale studies are warranted.

AUTHOR CONTRIBUTIONS

XZ: manuscript drafting and review and editing. YZ and XY: conceptualization. CH and HD: supervision and review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Current and Future Prospects for Gene Therapy for Rare Genetic Diseases Affecting the Brain and Spinal Cord

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In recent years, gene therapy has been raising hopes toward viable treatment strategies for rare genetic diseases for which there has been almost exclusively supportive treatment. We here review this progress at the pre-clinical and clinical trial levels as well as market approvals within diseases that specifically affect the brain and spinal cord, including degenerative, developmental, lysosomal storage, and metabolic disorders. The field reached an unprecedented milestone when Zolgensma® (onasemnogene abeparvovec) was approved by the FDA and EMA for *in vivo* adeno-associated virus-mediated gene replacement therapy for spinal muscular atrophy. Shortly after EMA approved Libmeldy®, an *ex vivo* gene therapy with lentivirus vector-transduced autologous CD34-positive stem cells, for treatment of metachromatic leukodystrophy. These successes could be the first of many more new gene therapies in development that mostly target loss-of-function mutation diseases with gene replacement (e.g., Batten disease, mucopolysaccharidoses, gangliosidoses) or, less frequently, gain-of-toxic-function mutation diseases by gene therapeutic silencing of pathologic genes (e.g., amyotrophic lateral sclerosis, Huntington's disease). In addition, the use of genome editing as a gene therapy is being explored for some diseases, but this has so far only reached clinical testing in the treatment of mucopolysaccharidoses. Based on the large number of planned, ongoing, and completed clinical trials for rare genetic central nervous system diseases, it can be expected that several novel gene therapies will be approved and become available within the near future. Essential for this to happen is the in depth characterization of short- and long-term effects, safety aspects, and pharmacodynamics of the applied gene therapy platforms.

Keywords: rare diseases, gene therapy, viral vectors, spinal muscular atrophy, personalized medicine, spinal cord, central nervous system, clinical trials

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INTRODUCTION

Classically, the majority of medical treatments have been developed for diseases affecting large number of patients and patients with chronic and recurrent treatment needs. Consequently, patients suffering from rare diseases have been left with few or no treatment options. With the advent of gene therapy and other advanced therapies a paradigm shift with more ambitious treatment goals, including disease modification and

potential cures, is on the horizon for treatment of rare diseases. Even though a rare disease encompasses few patients, the number of rare diseases amount to more than 6,000 rare diseases, and affect a total of 3.5–5.9% of all people, equating to 263–446 million people globally (Wakap et al., 2020). In addition, it is worth noting that the majority of rare diseases have a genetic and often monogenic origin (Lee et al., 2020). While there is no globally accepted definition of rare disease, there is an overall acceptance of point prevalence setting the threshold in the scientific and regulatory frameworks (Wakap et al., 2020). According to the harmonized standards in the EU regulation on orphan medicinal products, a rare disease affects <50 in 100,000 people, and as defined by the US Food and Drug Administration (FDA) in the Orphan Drug Act, a rare disease affects <200,000 people in the US alone (corresponding at present to approximately 61 in 100,000 people) (Wakap et al., 2020). The average prevalence threshold for the term "rare disease" was calculated as 40 in 100,000 by ISPOR (Rare Disease Special Interest Group) (Richter et al., 2015). Thus, it appears that the overall international consensus is that a *rare disease affects <40–60 in 100,000 people*, and this is the definition applied in the present review. The definition, including the patient numbers, is important to drug developers aspiring to enter the fast-track and orphan drug programs for development of treatments for patients with rare diseases which can include additional regulatory support and advising, as well as economic incentives, and market exclusivity. Here, we focus on the current development and prospects of gene therapies for treatment of a subgroup of rare diseases, namely, rare diseases affecting the brain and spinal cord with known genetic etiology.

A Short Overview of Gene Therapy Development

Already back in the 1970s, it was recognized that gene therapy, replacing or supplementing defective disease-causing DNA with exogenous healthy or beneficial DNA, could hold the promise of offering viable treatment options for human genetic diseases (Friedmann and Roblin, 1972). In the 1980s, the concept formed of using a virus vector for gene transfer into mammalian cells (Williams et al., 1984), and, in 1990, the first approved gene therapy trial took place with viral vector-mediated transfer of the gene encoding the enzyme adenosine deaminase (ADA) in a 4-year-old patient suffering from chromosome X-linked severe combined immunodeficiency (SCID-X1) due to ADA deficiency (Blaese et al., 1995). Hereafter followed a decade of new trials and great optimism, which culminated in two trials with unfortunate outcomes, and a transient halt of further gene therapy trials. In the first case, involving adenovirus (Ad) vector-mediated gene therapy in ornithine transcarbamylase deficiency, unexpected events led to severe vector-associated toxicity, multi-organ failure, and the death of an 18-year-old man (Raper et al., 2003). In the second case, a gamma-retrovirus (γRV) vector-mediated gene therapy encoding for interleukin-2 receptor gamma chain in patients with SCID-X1 was associated with development of genotoxic adverse events and uncontrolled clonal T-cell proliferation in six patients after RV host genome integration and the activation of LIM domain only-2 (LMO2) proto-oncogenes (Hacein-Bey-Abina et al., 2003). Hereafter followed a lock-down period of clinical trials. In the following years, new and safer viral vectors, including a large number of adeno-associated viral (AAV) vectors were discovered (Gao et al., 2005) and introduced to new gene therapy development programs. Recombinant AAVs that are deprived of viral DNA, essentially rendering them a non-replicable protein-based gene transfer carrier, have been favored in the central nervous system (CNS) gene therapy due to their desirable safety profile including low immunogenicity potential and strong neuronal tropism (Hudry and Vandenberghe, 2019). A little more than a decade later, the first gene therapy in Europe, Glybera® (alipogene tiparvovec) for treatment of lipoprotein lipase deficiency, was approved in 2012 (Watanabe et al., 2015). In 2016, the *ex vivo* hematopoietic stem and progenitor cell (HSPC) gene therapy Strimvelis® was approved for treatment of ADA-SCID (Aiuti et al., 2017) and, in 2019, Zynteglo was approved for treatment of beta-thalasemia (Schuessler-Lenz et al., 2020), both in Europe. Subsequently, Luxturna® (voretigene neparvovec), the first gene therapy against inherited eye diseases was approved in the US and Europe in 2017 and 2018, respectively, followed by approval of Zolgensma® (onasemnogene abeparvovec), a gene therapy targeting motor neurons residing in the CNS with axonal projections into the PNS, for treatment of spinal muscular atrophy in US and Europe in 2019 and 2020, respectively (Keeler and Flotte, 2019). The latest addition is the approval of Libmeldy®, an *ex vivo* gene therapy with lentivirus vector (LV)-transduced autologous CD34-positive hematopoetic stem and pluripotent cells (HSPCs) for treatment of metachromatic leukodystrophy, in Europe in 2020 (Bulaklak and Gersbach, 2020).

The current and applicable definitions of human gene therapy from the FDA (*Cellular & Gene Therapy Guidances*, July 20, 2018) and the EU commission (Directive 2001/83/EC, Part IV of Annex I) can be summed up as a biological medicinal product containing recombinant nucleic acid used in or administered to a human to regulate, repair, replace, add, or delete a genetic sequence with the aim to treat or cure diseases. The discipline of gene therapy includes: (1) *in vivo* vector-mediated gene therapy, (2) *ex vivo* cell transduction gene therapy, and (3) genome editing (Brenner et al., 2020). Treatments with antisense oligonucleotides (ASOs) are outside the scope of this review and will only be mentioned briefly when relevant.

In vivo Vector-Mediated Gene Therapy

Generally, there are two types of vectors coming from either viral or non-viral origin, and the viral vector platforms are predominantly based on Ad, AAV, or retro-/lentiviruses due to observed efficacy, safety profile, and regulatory acceptance. The objective of gene transfer is often to compensate for a pathogenic loss-of-function (LoF) mutation by delivery of a functional gene copy or to downregulate the expression of a pathogenic gain-of-toxic-function (GoTF) mutation by delivery of DNA encoding short hairpin RNA (shRNA), small interfering RNA (siRNA), microRNA (miRNA), or antisense RNA (Mitchell et al., 2010; Wang and Gao, 2014). The pharmacokinetics and tissue/cell

specificity depend on the selected vector, surface proteins, and cis-acting elements such as promotor elements.

From early on, Ad vectors were applied due to the efficient transduction of dividing and non-dividing cells, high transgene capacity, and low insertional mutagenesis rate (Gray et al., 2010). However, despite development of newer and improved generations of Ad vectors, challenges persist with pre-existing viral immunity, induction of strong innate immune responses toward capsid proteins, and adaptive immune response to viral and transgene products, which has led to Ad-based vectors no longer being preferred in trials targeting CNS disorders (Sing et al., 2018; Goswami et al., 2019). In other therapeutic areas where the associated challenges are less of a problem Ad vectors are still applied, e.g., in vaccines and oncolytic therapies.

Recombinant AAV vectors have found particular use in treatment strategies for CNS diseases (Mendell et al., 2021). AAV vectors are versatile and induce expression in both dividing and non-dividing cells and remain predominantly as single- or double-stranded DNA within the cell nucleus in episomal form (Salganik et al., 2015), although, *in vivo* and *in vitro* characterizations have suggested an integrative potential for wild-type AAV into a specific site in chromosome 19 in the human (Kotin et al., 1990, 1991). AAVs and their simple DNA genomes are well-studied, and AAV-based vectors have been shown to deliver long-term transgene expression, which has been documented up to 10 years in humans and up to 15 years in non-human primates after administration (Sehara et al., 2017; Chu et al., 2020). Several different AAV serotypes have been discovered, which differ by their specific tropism and tissue specificity linked to the diverse surface capsid proteins they express. These capsids have been discovered by (1) vectorization from natural isolates, (2) from rational designs using pre-existing capsids (Chen et al., 2009), (3) directed evolution using interative selection of mutated capsids, e.g., AAV2.7m8 (Dalkara et al., 2013), AAVPHP.B (Deverman et al., 2016), and AAV-F (Hanlon et al., 2019), (4) and by *in silico* approaches using computation tools to design novel synthetic capsids (Wang et al., 2019). So far, the approved AAV gene therapies, such as Glybera® and Luxturna®, are derived from naturally occurring variants (AAV capsid serotype 1 and 2, respectively). Currently, AAV vectors are regarded the least immunogenic and with less vector-associated toxicity, which make them preferred for many CNS diseases. Nonetheless, important safety concerns still need to be tackled, especially regarding genome integration issues, long-term sustained safety (Nguyen et al., 2021), and risk of high-dosing induced toxicity (Hinderer et al., 2018).

The retroviridae family has provided the simple γRV and the more complex lentiviruses, which have both been applied for gene therapy. Whereas the γRV was used earlier, the field has moved to prefer the lentivirus, and especially the HIV-1 virus as vector platform. LVs possess desirable characteristics, including genome integration for persistent long-term transgene expression in both postmitotic and quiescent cells (Naldini et al., 1996), low immunogenic potential (Abordo-Adesida et al., 2005), and relatively large transgene cassette capacity enabling expression of multiple genes from a single vector construct (Zhu et al., 2001; Tian and Andreadis, 2009). In contrast to the gamma-retroviral vectors, the LVs do not integrate into the genome within the proximity of oncogene transcriptional start sites, making them much less prone to oncogenic risk, and therefore they are regarded as much safer (Schröder et al., 2002; Cattoglio et al., 2007). In addition, lentivirus vectors have been modified to minimize the risk of host genome integration or to direct the insertional mutagenesis into heterochromatin regions (not affecting gene activation or silencing), for safe and stable transduction of non-dividing cells or transient transduction in actively dividing cells (Lentz et al., 2012). The development of more efficient and safer vectors over the years has resulted in LVs, which are self-inactivating and replication-incompetent (Zufferey et al., 1998). Using pseudotyping with glycoproteins have enabled specific tropisms and tissue-specificity, and have facilitated specific transduction to the retina and HSPCs (Duisit et al., 2002; MacKenzie et al., 2002). LVs have been tested in many successful clinical trials, and have become a preferred tool in particular in *ex vivo* gene therapy strategies for treating genetic diseases (see below).

Non-viral vectors with different transgene encapsulations exist, but despite that the first lipid nanoparticle-based RNA interference (RNAi) therapeutic drug was approved for treatment in 2018 (Kimura and Harashima, 2020), the viral vector-mediated gene transfers are still the preferred choice for gene therapies in the CNS. Finally, new genetic tools using *in vivo* gene therapy such as chemogenetics and optogenetics (Ingusci et al., 2019), have been developed as useful tools for basic scientific research, but could also refine gene therapy approaches to control neuronal activation for rare genetic disorders in the CNS in the future.

Ex vivo Cell Transduction Gene Therapy

Ex vivo HSPC transduction gene therapy (HSPC-GT) has played a central role in the development of gene therapies, as mentioned above, with the successful treatment of ADA-SCID with γRV-based vectors (Blaese et al., 1995; Aiuti et al., 2009) and later with the unfortunate occurrence of genotoxic events in SCID-X1 patients disrupted the immediate success (Hacein-Bey-Abina et al., 2003). This led to the increased usage of LVs derived from the human immunodeficiency virus (HIV), which are believed to possess a safer integration profile and much lower risk of insertional mutagenesis (Tucci et al., 2021). In general, gene transfer into autologous HSPCs has the potential to provide permanent therapeutic gene expression as a selective treatment in monogenic inherited disorders, and can be exploited as cell vehicles to deliver proteins into the circulation and tissues, including the CNS (Tucci et al., 2021). Briefly, patients' own cells are collected and stem cells are isolated to be mixed and transduced with a viral vector encoding a desired therapeutic gene. The transduced stem cells are later re-infused in the patient engrafted in the tissue, aiming at restoring a healthy phenotype (Penati et al., 2017). Thus, applying autologous HSPCs has become a viable treatment option for some patients with inborn errors of metabolism, providing enduring effect while reducing the risk of allogenic treatment-related toxicities and development of graft-vs.-host-disease as seen with donor HSPCs from healthy individuals (Morgan et al., 2017). HSPCs have lifelong ability to self-renew and to differentiate into

specific cell types which make them an attractive target for gene therapy. Although HSPCs are not fully characterized, the expression of a surface glycoprotein, CD34 (CD34+), and lack of another one, CD38 (CD38-), allow for selection and purification when grown *ex vivo* after collecting from the patients (Hossle et al., 2002). Furthermore, the CD34+/CD38- HSPCs can be subdivided depending on the presence or absence of CD90 and CD45RA glycoproteins which can aid in selecting the optimal population for HSPC-GT (Majeti et al., 2007). LVs derived from HIV are the preferred vectors due to superior safety and efficacy parameters, including self-inactivating configuration to minimize the risk of producing replication-competent lentiviral particles and with a safer integration profile as compared to other retroviral vectors (Tucci et al., 2021). The focus has, so far, mainly been on application in monogenic disorders affecting the lysosomal and peroxisomal metabolic activity impairing CNS functions, which leads to oxidative stress, local inflammation, microglial activation, progressive demyelination, and axonal degeneration (Tucci et al., 2021). The first clinical application of lentiviral-based gene therapy was for treatment of inherited metabolic disorders including X-linked adrenoleukodystrophy (Eichler et al., 2017), metachromatic leukodystrophy (Rosenberg et al., 2016), and mucopolysaccharidoses (Kinsella et al., 2020), with the aim of increasing enzyme bioavailability and brain entry (Begley et al., 2008) for correction of the neuropathological phenotype (see below in the disease sections for more details). Several projects appear promising based on pre-clinical and clinical data, however, challenges remain including the validation of long-term sustained efficacy and safety profile in patients who received γRV- or LV-based HSPC-GT *ex vivo*.

Genome Editing

Genome editing enables insertion, deletion, or replacement of nucleotides, but also modulation of gene expression and epigenetic editing (Duarte and Déglon, 2020). In monogenetic CNS diseases, a disease-causing mutation leading to either LoF or GoTF can be corrected by targeted editing of the specific mutation to restore a healthy phenotype. The available editing tools include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the CRISPR/Cas systems (Goswami et al., 2019; Poletto et al., 2020).

ZFNs are eukaryotic specific DNA-binding domains consisting of two anti-parallel beta-sheets and one alfa-helix, binding to triplet DNA sequences, and with intrinsic nuclease activity to open up DNA strands (Miller et al., 1985; Pavletich and Pabo, 1991). Realizing that the early modular assembly ZFNs were too error proned (Ramirez et al., 2008), the development has moved toward selection-guided assembly ZFNs (Greisman and Pabo, 1997; Cornu et al., 2008), and creation of synthetic ZFN oligomers displaying higher affinity and specificity toward larger multiple triplet basepair sequences (Urnov et al., 2010). This increase in combinatorial opportunities means that it is now possible to select ZFNs targeting almost any thinkable DNA sequence. TALENs are molecularly programmable nucleases inside of a central array of 33-35 amino acid motifs, recognizing single bases (Boch et al., 2009; Miller et al., 2011; Zhang et al., 2011). However, the cloning and protein engineering work for ZFNs and TALENs is complex and requires extensive expertise in molecular biology to take advantage of those techniques, which has limited their general distribution and application. CRISPR/Cas are based on RNA-guided nucleases and DNA-binding properties which are easily modulated by a short RNA sequence (Fineran and Charpentier, 2012; Wiedenheft et al., 2012). They can be grouped into two main classes according to their nuclease effectors (Makarova et al., 2015, 2020): The class 1 systems (types I, III, and IV) involve a large complex of several effector proteins, and the class 2 systems (types II, V, and VI) use a single Cas protein to mediate the recognition and cleavage of foreign nucleic acids. The class 2 systems are most widely used because of their simple structure, and the type II and type V CRISPR/Cas ribonucleoprotein complexes recognize specific DNA sequences through RNA-DNA base pairing and induce a double strand break. The host cell responds to this break by a mechanism known as homology-directed repair where donor DNA is offered as a template for the repair, thereby allowing gene editing and repair based on a healthy DNA template (Karimian et al., 2018; Yeh et al., 2019). Recently, the CRISPR/Cas-based genome editing has prevailed over the ZFNs and TALENs since they are easier to engineer to recognize unique sequences. The DNA-binding specificity of ZFNs and TALENs is dependent on protein-DNA interactions whereas the CRISPR/Cas is provided by the sgRNAs, which are simpler and less expensive to design (Duarte and Déglon, 2020).

Despite making progress in pre-clinical studies (Lubroth et al., 2021), only a few *in vivo* genome editing approaches utilizing ZFNs in CNS have reached clinical trials. Nonetheless, the potential of expanding into CNS diseases is clear, and there should be a great interest from the pharmaceutical industry to advance more *in vivo* genome editing into clinical stages within brain and spinal cord diseases. For this to become a reality the identified challenges and safety concerns have to be solved, especially related to immunogenicity induced by the genome editing tools (Shim et al., 2017) as well as better characterization and control of on- and off-target modifications (Mills et al., 2003).

Delivery Strategies for Gene Therapy to the Brain and Spinal Cord

Delivery of genetic material is an important issue since accessibility of systemically administered treatments targeting the CNS has long been complicated by the tightly regulated blood-brain barrier (BBB) that controls passage into the CNS (Kimura and Harashima, 2020). Direct intracerebral injection into the parenchyma, although highly invasive, is one way to circumvent this problem, which also ensures a direct match between treatment delivery and the targeted region. This method could be preferred when the target is a defined and limited area of the brain or when targeting deeper brain structures in humans such as the thalamus or putamen (Hocquemiller et al., 2016; Taghain et al., 2020). Intracerebral administration into specific brain regions will typically be associated with the administration of lower numbers of viral genomes compared to those required for systemic administration which limits the risk of toxicity. A rat study preparing for human trials for Parkinson's disease found

that the no observed adverse effect level (NOAEL) dose was 6.8 $\times$ 10^8 viral genomes of an AAV2 vector encoding glial-derived neurotrophic factor administered as a single intracerebral dose into the striatum (Terse et al., 2021). Nonetheless, toxicity and spread of transgene expression in different regions and cell types may vary extensively depending on the titer/volume injected and serotype of vectors used (Peters et al., 2021).

If it is necessary to deliver treatment to larger parts of the brain or spinal cord, it is possible to apply multiple injection sites and/or viral vectors with a larger degree of spread and retrograde transport along neuronal processes (Kimura and Harashima, 2020). Another strategy for achieving a wide spread in the brain and spinal cord could be delivered through the CSF, by intracerebroventricular (ICV), intracisterna magna, or intrathecal injections (Hocquemiller et al., 2016; Taghain et al., 2020). Intrathecal injections are conveniently achieved by lumbar puncture and achieve extensive spinal cord transduction whereas administration into the cisterna magna delivers the drug closer to the targeted brain areas and has shown transduction in the spinal cord as well as brain (Taghain et al., 2020). Studies have demonstrated that AAV vectors, when injected into the cerebrospinal fluid, deliver genes throughout the brain and spinal cord in non-human primates (Bey et al., 2020).

As the cell and tissue tropism of different AAV serotypes became better understood, it also became apparent that some serotypes are better than others at reaching the CNS after systemic administration, for example, AAV9, AAVrh8, AAVrh10, and AAVHSC15 can cross the BBB after intravenous (IV) administration, resulting in widespread transduction of the CNS and peripheral organs through a less invasive procedure (Yang et al., 2014; Ellsworth et al., 2019; Belur et al., 2020). Intravascular administration is the current delivery method for Zolgensma®, approved for treatment of SMA1, as discussed later, which utilizes the AAV9 vector capabilities of crossing the BBB (Chen, 2020). In addition, new AAV subtypes, such as AAV-PHP.B, show up to 40 times higher efficiency at transducing neurons and astrocytes when compared to IV-injections of AAV9 (Liu et al., 2021). However, it appears that AAV-PHP.B expression is species dependent, expressing at much lower levels in BALB/cJ mice and non-human primates than in C57BL/6J mice, and toxicity observed in non-human primates indicate that its usefulness in humans may consequently be limited (Hordeaux et al., 2018, 2019).

RARE DISEASES AND PROSPECTS OF UTILIZING GENE THERAPIES

We here provide an overview of the developmental progress for novel gene therapeutic treatments for rare genetic diseases in the brain and spinal cord, with a special focus on clinical development. For diseases which have not reached clinical testing, we seek to describe the current status and near-future prospects. Overviews are given of current gene therapy clinical trials from https://clinicaltrials.com (last search on the 1st of May 2021) for the therapeutic areas in **Table 1** and for the individual diseases and trials in **Table 2**.

NEURODEGENERATIVE DISORDERS

Spinal Muscular Atrophy (SMA)

SMA is characterized by degeneration of spinal cord alpha motor neurons resulting in muscular wasting. The disease impairs the patient's ability to walk, speak and breathe (Pattali et al., 2019). It affects approximately 10 in 100,000 newborns and is the most

TABLE 1 | Overview of clinical gene therapy trials for groups of rare genetic diseases affecting the brain and spinal cord found on https://clinicaltrials.gov 1st of May 2021.

Group	Disease	Prevalence per 100,000	Number of Trials	Clinical Trial Phase Reached
Neurodegenerative Disorders	Spinal Muscular Atrophy (SMA)	10	9	Gene therapy market approval by FDA and EMA (Zolgensma®)
	Multiple System Atrophy (MSA)	2	1	Gene therapy trial planned, not yet recruiting
	Amyotrophic Lateral Sclerosis (ALS)	5	1	Compassionate-use study in two patients
	Huntington's Disease (HD)	3	1	Phase 1/2 trial
Neurodevelopemental Disorders	Rett Syndrome (RTT)	10–12	0	No clinical trials with gene therapy
	Genetic Syndromes (e.g., Dravet, Lennox-Gastaut, West, and Angelman synromes)	3-15	0	No clinical trials with gene therapy
Lysosomal Storage Diseases	Batten Disease (CLN, Neuronal Ceroid Lipofuscinoses)	2–4	6	Phase 1/2 trials and LTFU
	Krabbe Disease (KD)	1	2	Phase 1/2 trials
	Metachromatic Leukodystrophy (MLD)	1–2	6	*Ex vivo* autologous hematopoietic stem cell gene therapy approved by EMA (Libmeldy®)
	Mucopolysaccharidoses (MPS)	4	19	Phase 2/3 trials
Neurometabolic Disorders	Canavan Disease (CD)	<16	3	Phase 1/2 trials
	Niemann-Pick Disease	5	0	No clinical trials with gene therapy
	X-linked Adrenoleukodystrophy (ALD)	7	5	Phase 2/3 trial
	Phenylketonuria (PKU)	10	3	Phase 1/2 trials
	Gangliosidoses (GM1/2)	1	5	Phase 1/2 trials

TABLE 2 | Overview of clinical gene therapy trials for rare genetic diseases affecting the brain and spinal cord found on https://clinicaltrials.gov 1st of May 2021.

Disease (OMIM)	Intervention and dose	Route of administration	NCT number	Phase	Participants	Ages eligible	Sponsors	Study period	Status (May 2021)
SMA (600354)	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi ($6.7 \times 10^{13} - 2.0 \times 10^{14}$ vg/kg)	IV	NCT02122952	1/2	15	≤6 months	AveXis	05/2014–12/2017	Completed
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (1.1×10^{14} vg/kg)	IV	NCT03306277	3	22	≤180 days	AveXis	10/2017–11/2019	Completed
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi ($6.0 \times 10^{13} - 1.2 \times 10^{14} - 2.4 \times 10^{14}$ vg)	IT	NCT03381729	1/2	51	6–60 months	Novartis	12/2017–06/2021	Suspended
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (LTFU, no additional dosing)	IV	NCT03421977	LTFU	13	Child- to adulthood	Novartis	09/2017–13/2033	Active not recruiting
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (1.1×10^{14} vg/kg)	IV	NCT03461289	3	33	≤180 days	AveXis	08/2018–09/2020	Completed
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (1.1×10^{14} vg/kg)	IV	NCT03505099	3	30	≤42 days	Novartis	05/2018–07/2021	Active not recruiting
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (dose unknown)	IV	NCT03837184	3	2	≤6 months	Novartis	05/2019–06/2021	Active not recruiting
	scAAV9-SMN (AVXS-101) onasemnogene abeparvovec-xioi (LTFU, no additional dosing)	IV/IT	NCT04042025	LTFU	308	Child- to adulthood	Novartis	02/2020–12/2035	Enrolling by invitation
MSA (146500)	AAV2-GDNF (dose unknown)	IC (putamen)	NCT04680065	1	9	35–75 years	Brain Neurotherapy Bio/Asklepios Biopharmaceutical	01/2021–01/2024	Not yet recruiting
ALS	AAVrh10-miR-SOD1 (4.2×10^{14} vg)	IT	n/a	n/a	2	22 and 56 years	Univ. of Massachusetts Medical School / Massachusetts General Hospital / Harvard Medical School	07/2017–05/2020	Completed
HD (143100)	AAV5-miHTT (AMT-130) ($6 \times 10^{12} - 6 \times 10^{13}$ gc)	IC (striatum)	NCT04120493	1/2	26	25–65 years	UniQure Biopharma	09/2019–05/2026	Recruiting
BD (CLN2) (204500)	AAV2-CUhCLN2 (3×10^{12} particle units)	IC	NCT00151216	1	10	3–18 years	Weill Medical College of Cornell University	06/2004–06/2019	Completed
	AAVrh10-CUhCLN2 ($2.85 \times 10^{11} - 9 \times 10^{11}$ gc)	IC	NCT01161576	1	12	2–18 years	Weill Medical College of Cornell University	08/2010–12/2020	Completed
	AAVrh10-CUhCLN2 ($2.85 \times 10^{11} - 9 \times 10^{11}$ gc)	IC	NCT01414985	1/2	8	3–18 years	Weill Medical College of Cornell University	04/2010–02/2017	Completed
BD (CLN3) (204200)	AAV9-CLN3 (AT-GTX-502) ($6 \times 10^{13} - 1.2 \times 10^{14}$ vg)	IT	NCT03770572	1/2	7	3–10 years	Amicus Therapeutics	11/2018–09/2023	Active not recruiting
BD (CLN6) (606725)	AAV9-CLN6 (AT-GTX-501) (dose unknown)	IT	NCT02725580	1/2	13	≥1 year	Amicus Therapeutics	03/2016–11/2021	Active not recruiting
	AAV9-CLN6 (AT-GTX-501) (LTFU, no additional dosing)	IT	NCT04273243	LTFU	13	≥1 year	Amicus Therapeutics	01/2020–01/2035	Recruiting
KD (245200)	AAVrh10-hGALC (FBX-101) (dose unknown)	IV	NCT04693598	1/2	6	≤12 months	Forge Biologics	01/2021–04/2023	Not yet recruiting
	AAVhu68-GALC (PBKR03) ($1.5 \times 10^{11} - 5.0 \times 10^{11}$ gc/g brain mass)	ICM	NCT04771416	1/2	24	1–9 months	Passage Bio	06/2021 - 01/2030	Not yet recruiting
MLD (250100)	LV-ARSA (OTL-200) (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT01560182	1/2	20	≤7 years	Orchard Therapeutics. Telethon	04/2010–04/2023	Active not recruiting
	AAVrh10-cuARSA ($1 \times 10^{12} - 4 \times 10^{12}$ vg)	IC	NCT01801709	1/2	5	6 months−5 years	Institut National de la Santé et de la Recherche Médicale	03/2013–04/2019	Active not recruiting

(Continued)

TABLE 2 | Continued

Disease (OMIM)	Intervention and dose	Route of administration	NCT number	Phase	Participants	Ages eligible	Sponsors	Study period	Status (May 2021)
	LV-ARSA (*ex vivo*) CD34+ HSPC ($2 \times 10^6 - 20 \times 10^6$ per kg)	IV	NCT02559830	1/2	50	2–45 years	Shenzhen Second People's Hospital	01/2015–10/2025	Recruiting
	LV-ARSA (OTL-200) (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT03392987	2	10	≤6 years	Orchard Therapeutics, Telethon	01/2018–08/2028	Active not recruiting
	LV-ARSA ($1 \times 10^9 - 2 \times 10^9$ moi/ml per site)	IC	NCT03725670	1/2	10	≥1 month	Shenzhen Geno-Immune Medical Institute	10/2018–11/2020	Recruiting
	LV-ARSA (OTL-200) (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT04283227	3	6	Child- to adulthood	Orchard Therapeutics, Telethon	12/2020–01/2032	Recruiting
MPS I (607015)	Various, venipuncture (LTFU, no additional dosing)	Various	NCT00695279	LTFU	100	Child- to adulthood	St. Jude Children's Research Hospital	01/2007–12/2036	Recruiting
	AAV6-ZFN-IDUA (SB-318) (dose unknown)	IV	NCT02702115	1/2	3	≥5 years	Sangamo Therapeutics	05/2017–01/2022	Active not recruiting
	LV-IDUA (*ex vivo*) CD34+ HSPC (target dose 8×10^6 CD34+ cells/kg)	IV	NCT03488394	1/2	8	≤11 years	IRCCS San Raffaele	05/2018–01/2023	Recruiting
	AAV9-IDUA (RGX-111) ($1 \times 10^{10} - 5 \times 10^{10}$ gc/g brain mass)	IC	NCT03580083	1/2	5	≥4 months	Regenxbio	04/2019–07/2023	Recruiting
	AAV6-ZFN-IDUA (SB-318) (LTFU, no additional dosing)	IV	NCT04628871	LTFU	13	≥18 years	Sangamo Therapeutics	11/2020–01/2030	Enrolling by invitation
MPS II (309900)	AAV6-ZFN-IDS (SB-913) (dose unknown)	IV	NCT03041324	1/2	9	≥5 years	Sangamo Therapeutics	05/2017–02/2022	Active not recruiting
	AAV9-IDS (RGX-121) ($1.3 \times 10^{10} - 2.0 \times 10^{11}$ gc/g brain mass)	IC	NCT03566043	1/2	12	4 months−5 years	Regenxbio	09/2018–12/2023	Recruiting
	AAV9-IDS (RGX-121) (6.5×10^{10} gc/g brain mass)	ICM/ICV	NCT04571970	1/2	6	5–17 years	Regenxbio	02/2021–06/2023	Recruiting
	AAV9-IDS (RGX-121) (LTFU, no additional dosing)	ICM/ICV	NCT04597385	LTFU	12	≥28 months	Regenxbio	03/2021–09/2025	Enrolling by invitation
	AAV6-ZFN-IDS (SB-913) (LTFU, no additional dosing)	IV	NCT04628871	LTFU	13	≥18 years	Sangamo Therapeutics	11/2020–01/2030	Enrolling by invitation
MPS IIIA (252900)	AAVrh10-hSGSH-IRES-SUMF1 (SAF-301) (dose unknown)	IC	NCT01474343	1/2	4	18 months−6 years	Lysogene	08/2011–05/2013	Completed
	AAVrh10-hSGSH-IRES-SUMF1 (SAF-301) (dose unknown)	IC	NCT02053064	1/2	4	Child- to adulthood	Lysogene	05/2013–06/2017	Completed
	scAAV9.U1a.hSGSH (ABO-102) ($0.5 \times 10^{13} - 3 \times 10^{13}$ vg/kg)	IV	NCT02716246	1/2	22	≥6 months	Abeona Therapeutics	03/2016–12/2022	Recruiting
	AAVrh10-hSGSH (LYS-SAF302) (dose unknown)	IC	NCT03612869	2/3	20	≥6 months	Lysogene	12/2018–03/2022	Active not recruiting
	scAAV9.U1a.hSGSH (ABO-102) (3×10^{13} vg/kg)	IV	NCT04088734	1/2	12	Child- to adulthood	Abeona Therapeutics	10/2019–12/2023	Recruiting
	LV-SGSH (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT04201405	1/2	5	3–24 months	University of Manchester, Orchard Therapeutics	01/2020–10/2024	Recruiting
	scAAV9.U1a.hSGSH (ABO-102) (LTFU, no additional dosing)	IV	NCT04360265	LTFU	50	Child- to adulthood	Abeona Therapeutics	10/2020–12/2025	Recruiting

(Continued)

TABLE 2 | Continued

Disease (OMIM)	Intervention and dose	Route of administration	NCT number	Phase	Participants	Ages eligible	Sponsors	Study period	Status (May 2021)
MPS IIIB (252920)	AAV5-hNAGLU (4×10^{12} vg)	IC	NCT03300453	1/2	4	18–60 months	UniQure Biopharma	10/2013–11/2019	Completed
	AAV9-hNAGLU ($2 \times 10^{13} - 1 \times 10^{14}$ vg/kg)	IV	NCT03315182	1/2	15	Child- to adulthood	Abeona Therapeutics	10/2017–10/2022	Recruiting
CD (271900)	AAV2-hASPA (9×10^{11} vg)	IC	n/a	1	13	4–83 months	n/a	2001–2005	Completed
	AAV2-hASPA (LTFU, no additional dosing)	IC	n/a	LTFU	13	4–83 months	n/a	2006–2011	Completed
	AAV-oligo001-ASPA (3.7×10^{13} vg)	ICV	NCT04833907	1/2	24	3–60 months	CureRare Disease LLC	04/2021–03/2024	Recruiting
ALD (300100)	LV-ALD (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT01896102	2/3	32	≤17 years	Bluebird bio	08/2013–05/2021	Active not recruiting
	LV-ABCD1 (*ex vivo*) CD34+ HSPC ($2 \times 10^6 - 2 \times 10^7$ CD34+ cells/kg)	Infusion	NCT02559830	1/2	50	2–45 years	Shenzhen Second People's Hospital	01/2015–10/2025	Recruiting
	LV-ALD (*ex vivo*) CD34+ HSPC (LTFU, no additional dosing)	IV	NCT02698579	LTFU	60	Child- to adulthood	Bluebird bio	01/2016–05/2037	Enrolling by invitation
	LV-ABCD1 ($1 \times 10^9 - 2 \times 10^9$ moi/ml per site)	IC	NCT03727555	1/2	10	Child- to adulthood	Shenzhen Geno-Immune Medical Institute	10/2018–10/2020	Recruiting
	LV-ALD (*ex vivo*) CD34+ HSPC (dose unknown)	IV	NCT03852498	3	35	≤17 years	Bluebird Bio	01/2019–02/2024	Recruiting
PKU (261600)	AAV.HSC15-PAH (HMI-102) (dose unknown)	IV	NCT03952156	1/2	21	18–55 years	Homology Medicines	06/2019–09/2021	Recruiting
	AAV.HSC15-hPAH (HMI-102) (LTFU, no additional dosing)	IV	NCT04348708	LTFU	21	18–55 years	Homology Medicines	08/2020–12/2026	Enrolling by invitation
	AAV5-PAH (BMN 307) (dose unknown)	IV	NCT04480567	1/2	100	≥15 years	BioMarin Pharmaceutical	09/2020–12/2027	Recruiting
GM1 (230500)	AAV9-GLB1 ($1.5 \times 10^{13} - 4.5 \times 10^{13}$ vg/kg)	IV	NCT03952637	1/2	45	6 months−12 years	National Human Genome Research Institute, Axovant Sciences	08/2019–05/2024	Recruiting
	AAVrh10-GLB1 (LYS-GM101) (8×10^{12} vg/kg)	ICM	NCT04273269	1/2	16	≤3 years	Lysogene	03/2021–06/2025	Active Not recruiting
	AAVhu68-GLB1 (PBGM01) ($3.3 \times 10^{10} - 1.1 \times 10^{11}$ gc/g brain mass)	ICM	NCT04713475	1/2	20	4–36 months	Passage Bio	02/2021–02/2029	Recruiting
GM2 (230600)	AAVrh8-HEXA/AAVrh8-HEXB (AXO-AAV-GM2) (dose unknown)	IC/ICM/IT	NCT04669535	1	18	6 months−12 years	Sio Gene Therapies	01/2021–06/2028	Recruiting
	AAV9-HEXA/HEXB (TSHA-101) (dose unknown)	IT	NCT04798235	1/2	6	≤12 months	Taysha Gene Therapies	03/2021–03/2027	Recruiting

AAV, adeno-associated virus; ALD, X-linked adrenoleukodystrophy; ALS, amyotrophic lateral sclerosis; ARSA, arylsulfatase; BD, Batten disease; CD, Canavan disease; CLN, ceroid lipofuscinosis neuronal; GALC, galactosylceramidase; gc, genome copies; GDNF, glial cell line-derived neurotrophic factor; GLB1, galactosidase beta 1; GM, gangliosidosis; HD, Huntington's disease; HSPC, hematopoietic stem and progenitor cells; HTT, huntingtin; IC, intracerebral; ICM, intracisterna magna; ICV, intracerebroventricular; IDS, iduronate-2-sulfatase; IDUA, α-L-iduronidase; IT, intrathecal; IV, intravenous; KD, Krabbe disease; LTFU, long-term follow-up; LV, lentivirus; MLD, metachromatic leukodystrophy; moi, multiplicity of infection; MPS, mucopolysaccharidoses; MSA, multiple system atrophy; n/a, not applicable; NAGLU, alpha-N-acetylglucosaminidase; PKU, phenylketonuria; RTT, Rett syndrome; SGSH, N-sulfoglucosamine sulfohydrolase; SMA, spinal muscular atrophy; SMN, survival motor neuron; SUMF1, sulfatase-modifying factor 1; vg, vector genomes; ZFN, zinc finger nuclease.

common monogenic disease leading to death in infants (Darras, 2015; Chen, 2020). SMA is the result of a LoF mutation in the *survival of motor-neuron 1 (SMN1)* gene. *SMN1* encodes a protein essential for survival of the alpha motor neurons. The function of the protein is not yet completely understood (Kariyawasam et al., 2018). The human genome has a similar gene, *survival of motor-neuron 2 (SMN2)*, which exists in multiple copies in the genome and is different from *SMN1* only by a few nucleotides, notably a nucleotide variant in exon 7. This variant in *SMN2* leads to exclusion of exon 7, resulting in an unstable protein. It is estimated that the protein production resulting from *SMN2* creates 90% truncated proteins (without exon 7) and generates 10% normal but still unstable SMN proteins. These proteins can partially compensate for the loss of *SMN1*, and it is argued that *SMN2* copy number in the genome determines phenotypic severity (Pattali et al., 2019; Chen, 2020). SMA is classified in different clinical phenotypes based on age at onset of symptoms, the level of motor functions achieved, and number of *SMN2* genes. Type 0 (1 *SMN2* copy): with onset *in utero*, dependent of mechanical ventilation at birth, and survival is usually below 6 months of age; type 1 (Werdnig Hoffman disease) (1-3 *SMN2* copies): onset before 6 months of age, with posture, respiratory, and feeding support required, and expected life expectancy below 2 years of age; type 2 (Dubowith disease) (2-4 *SMN2* copies): onset at 7–18 months, with inability to walk, need for respiratory and feeding support, and with life expectancy to reach adulthood; type 3 (Kugelberg-Welander disease) (3-4 *SMN2* copies): onset at 18 months, initially with ability to stand and walk, however, it is not retained, and with normal life expectancy; type 4 (4 *SMN2* copies): late onset in adulthood and with mild symptoms and normal life expectancy (Munsat and Davies, 1992; Wang et al., 2007). Patients suffering from SMA type 1 (SMA1), the most common form accounting for approximately 60% of all cases, show symptoms including hypotonia, motor delays, and breathing difficulties. The primary cause of death is respiratory failure as a result of muscle weakness. Median survival for SMA1 is estimated at 13.5 months (Rao et al., 2018).

Until recently, the only available medical care for patients suffering from SMA was supportive. In 2016 and 2017, FDA and EMA, respectively, approved nusinersen (Spinraza®), an ASO up-regulating full-length SMN2 transcription which leads to both symptom reduction and halted disease progression (Chen, 2020). Two phase 3, randomized, double-blind trials (CHERISH, NCT02292537; ENDEAR, NCT02193074) have confirmed the efficacy of nusinersen with a 47% reduction in risk of death or permanent ventilation and a favorable safety profile (Finkel et al., 2017; Kariyawasam et al., 2018; Mercuri et al., 2018). Soon after, the first gene therapy for SMA, onasemnogene abeparvovec (AVXS-101) developed by AveXis (acquired by Novartis Pharmaceuticals), was approved under the brand name Zolgensma® by the FDA and EMA in 2019 and 2020, respectively. AVXS-101 is an SMN1 gene replacement therapy delivered by a self-complementary AAV9 (scAAV9) virus that is able to cross the BBB. It has a constitutively active promotor providing persistent expression of SMN1 protein. The efficacy and safety of Zolgensma® (onasemnogene abeparvovec-xioi) have been established in three clinical studies (START, NCT02122952; STR1VE, NCT03306277; and SPR1NT; NCT03505099) and one long-term follow-up study (NCT03421977). Results have been published from the START study, an open-label, dose-escalation, phase 1/2A trial evaluating safety and efficacy of IV delivery of AVXS-101 as a treatment for SMA1 in 15 participants aged up to 6 months and compared with historical controls (Mendell et al., 2017; Al-Zaidy et al., 2019a,b; Lowes et al., 2019). All patients had SMA1, homozygous *SMN1* exon 7 deletions, and two copies of *SMN2*. Significant improvements were reported after AVXS-101 treatment, with all patients surviving past the age of 20 months without requiring permanent ventilation compared to just 8% in the historic cohort (Mendell et al., 2017). In addition, a rapid increase from baseline in the motor function score followed in the high-dose (therapeutic dose) cohort as compared with a decline in this score in the historical control cohort, indicating that of the 12 patients who had received the high dose, 11 sat unassisted, 9 rolled over, 11 fed orally and could speak, and 2 walked independently. During the 24 months follow-up period, the AVXS-101 treated patients spent less time hospitalized with lower number of admissions and length of stay as compared to historical controls (Al-Zaidy et al., 2019a). End-of-study analysis demonstrated that AVXS-101 treatment substantially improved permanent ventilation-free survival, and significantly improved motor function and motor milestone achievement in infants with SMA1 as compared with outcomes observed in the history cohort (Al-Zaidy et al., 2019b). *Post-hoc* analysis revealed that the biggest motor improvements were obtained in infants with SMA1 treated at an early age highlighting the importance of newborn screening and early treatment (Lowes et al., 2019). Therefore, the SPR1NT, a phase 3, open-label, single-arm study was conducted with one-time IV dosing of AVXS-101 in 30 infants up to 42 days of age with genetically diagnosed and pre-symptomatic SMA1 with 1-3 *SMN2* copies (most commonly 2). Results remain to be posted. Moreover, three open-label, single-arm, single-dose, phase 3 trials were conducted in the US (NCT03306277; STR1VE), Europe (NCT03461289; STRIVE-EU), and Asia (NCT03837184) after one-time IV infusion of AVXS-101. In 2021, results were published from the STR1VE study (NCT03306277) in 22 patients younger than 6 months with SMA with biallelic *SMN1* mutations (deletion or point mutations) and one or two copies of *SMN2,* and compared to untreated patients from pediatric neuromuscular clinical research dataset (Day et al., 2021). At the 18 month of age study visit, in patients treated with AVXS-101, 59% achieved functional independent sitting (0% in the control group) and 91% survived free from permanent ventilation at age 14 months (vs. 26% in the control group). The most frequently reported serious adverse events were bronchiolitis, pneumonia, respiratory distress, and respiratory syncytial virus bronchiolitis, and three serious adverse events were related or possibly related to the treatment (two patients had elevated hepatic aminotransferases, and one had hydrocephalus). Long-term benefits and risks await to be determined. The favorable benefit–risk profile suggests that AVXS-101 could provide new hope for treatment of future patients with infantile-onset SMA1. An open-label, dose-escalation, phase 1/2A trial (STRONG; NCT03381729) evaluating safety and efficacy after intrathecal

delivery of AVXS-101 (also called OAV-101 in intrathecal administration) as a treatment for SMA with 2 copies of *SMN2* and deletion of *SMN1* in 51 participants aged 6–60 months is ongoing and has recently (August 2021) been allowed by the FDA to resume after nearly a 2 year suspension due to safety concerns. Interim data published from the STRONG study imply that intrathecal administration is feasible, well-tolerated and improve motor funtions in patients with SMA1 (non-ambulatory) and SMA2 patients (Finkel et al., 2019, 2020). Two ongoing phase 4 long-term follow-up trials (NCT03421977, NCT04042025) have been initiated, enrolling participants who were treated with onasemnogene abeparvovec-xioi for SMA in the START study and previous (parent) studies for continuous monitoring of safety as well as monitoring of continued efficacy and durability of response to treatment. Despite a general favorable safety profile, some concerns have been raised, since at least three children with SMA developed thrombotic microangiopathy (TMA), after being treated with onasemnogene abeparvovec (Chand et al., 2021). All three children developed TMA approximately 1 week after treatment, and they had all contributory factors, including concurrent infections and recent vaccine exposure, which could be putatively contributing to development of TMA. Moreover, coagulation abnormalities have been reported more frequently in children with SMA (Wijngaarde et al., 2020), which could mean that they are more susceptible and precautions should be taken. They recovered after receiving plasmapheresis, high dose corticosteroids, and/or transfusions. TMA has also been reported with other gene therapies using AAV vectors including treatment of Duchenne muscular dystrophy (Chand et al., 2021). Since thrombocytopenia is a key feature of TMA, it is recommend that platelet counts are monitored after starting treatment with onasemnogene abeparvovec. Fairly recently, an indirect comparison has been attempted, which suggests that onasemnogene abeparvovec may have an efficacy advantage relative to nusinersen for overall survival, independence from permanent assisted ventilation, motor function, and motor milestones, when comparing clinical trials (NCT02122952 vs. NCT02193074) using frequentist and Bayesian approaches (Dabbous et al., 2019).

Multiple System Atrophy (MSA)

MSA is a rare neurodegenerative disorder characterized by Parkinsonism, cerebellar ataxia, and autonomic failure, impacting on striatonigral, olivopontocerebellar, and autonomic systems, with an approximate prevalence around 2 in 100,000 (Chrysostome et al., 2004). The key pathological hallmark is the presence of glial cytoplasmic inclusions with insoluble proteinaceous filaments in the oligodendrocytes, and therefore MSA is regarded as an α-synucleinopathy along with Parkinson's disease and dementia with Lewy bodies. The etiology of MSA is largely unknown, although some emerging evidence suggests the involvement of mutations in the genes *SNCA, COQ2, MAPT, GBA1, LRRK2,* and *C9orf72* (Katzeff et al., 2019). Currently, there is no treatment targeting MSA, therapeutic management is based on symptomatic treatment. MSA patients may benefit from l-dopa for the symptomatic treatment of Parkinsonism symptoms, whereas physiotherapy remains the best therapeutic option for the ataxia (Perez-Lloret et al., 2015).

So far, no gene therapies targeting the potential pathological gene variants have been accelerated into clinical testing. However, consistent with promising pre-clinical data in Parkinson's disease models after vector-mediated overexpression of glial-derived neurotrophic factor (GDNF, Axelsen and Woldbye, 2018), Brain Neurotherapy Bio is preparing for a randomized, double-blinded, placebo-controlled phase 1 trial with symptomatic AAV2-GDNF gene therapy delivered bilaterally to the putamen of adult MSA patients (NCT04680065).

Amyotrophic Lateral Sclerosis (ALS)

ALS consists of a group of rare neurological diseases that mainly involve neurons responsible for controlling voluntary muscle movement. The disease is progressive and worldwide affects about 5.4 in 100,000 (Chiò et al., 2013). ALS is characterized by progressive degeneration of motor neurons in the brain and spinal cord, causing individuals to gradually lose their strength and ability to speak, eat, move, and even breathe. Most people with ALS die from respiratory failure, usually within 3–5 years after symptoms first appear. About 5–10% of all ALS cases are familial due to mutations in more than a dozen genes. About 34% of all familial cases in Europeans are caused by defects in the *C9orf72* gene (most likely GoTF mutations; Mejzini et al., 2019). Another 15–30% of familial cases result from GoTF mutations in the gene encoding copper-zinc superoxide dismutase 1 (SOD1; Mejzini et al., 2019). Currently, there is no cure for ALS, and the two drugs approved by the FDA for treatment of ALS, riluzole, an anti-excitotoxic glutamate antagonist, and edaravone, a free radical/reactive oxygen species scavenger presumed to mitigate oxidative injury, are not effective at reversing disease progression, although the former has modest benefits on survival, and the latter may halt ALS progression during early stages (Bensimon et al., 1994; Jaiswal, 2019).

Different AAV vector-mediated and ASO strategies aiming at silencing *SOD1* or *C9orf72* as a therapeutic approach in familial ALS are currently being developed and tested in pre-clinical studies (Cappella et al., 2019; Amado and Davidson, 2021). Novartis Gene Therapies (formerly AveXis) has developed a self-complementary AAV9 vector expressing a short hairpin (sh) RNA to silence SOD1, and this vector has shown promising results after IV or intra-cisterna magna delivery in SOD1 mutant mice at different ages (Foust et al., 2013; Iannitti et al., 2018). Subsequently, the AAV9-sh-SOD1 vector was further tested successfully via subpial delivery in mice, pigs, and non-human primates (Bravo-Hernandez et al., 2020). Similarly, an AAVrh10 vector encoding artificial miRNA has been developed and delivered in a single intra-CSF injection in monkeys to silence the activity of the mutated SOD1, which significantly lowered SOD1 expression in spinal cord motor neurons, and the treatment was overall well-tolerated (Borel et al., 2016, 2018). Likewise, an AAVrh10-antisense-SOD1 vector was tested *in vivo* in adult SOD1 mutant mice through combined IV and ICV delivery (Biferi et al., 2017). The principle of targeting SOD1 expression with AAV-mediated down-regulation of malign SOD1 variants gained further support from a recently completed

compassionate-use study in two SOD1-ALS male patients aged 22 and 56 years old (Mueller et al., 2020). After a single intrathecal injection of an AAVrh10 vector (4.2×10^{14} vector genomes) encoding miRNA targeting SOD1, the 22-year-old patient showed reduced post-mortem levels of SOD1 in spinal cord tissue and a transient improvement in right leg strength without change in vital capacity, whereas the 56-year-old patient who received immunosuppressive treatment had stable composite measure ALS functional scores and a stable vital capacity during 12 months. Safety assessment included vital signs, clinical laboratory assessments, CSF SOD1 activity and protein levels, electrocardiogram, physical and neurological examination, and any adverse events. This study suggests that intrathecal AAV-mediated miRNA gene therapy could be developed for treatment of SOD1-linked ALS, but potentially requires the concomitant use of immunosuppresants, and additional studies with larger numbers of patients are needed. Finally, focusing on *C9orf72* silencing, pre-clinical testing of an AAV5-miRNA-C9ORF72 vector sponsored by UniCure showed marked reduction of *C9orf72* transcripts in ALS mouse models (Martier et al., 2019).

Huntington's Disease (HD)

This is an autosomal dominant disorder resulting from GoTF mutations in the form of CAG trinucleotide repeats in the *HTT* gene on chromosome 4p16.3 that encodes the widely expressed cytoplasmic protein, huntingtin (HTT, Jimenez-Sanchez et al., 2017). This leads to abnormal expansion of the polyglutamine sequence in HTT resulting in HTT aggregation that causes neurodegeneration, choreatic movements, as well as cognitive and behavioral disturbances. The prevalence of HD varies globally but overall affects around 2.7 in 100,000, and it commonly affects patients between the ages of 30–50 years, being most often fatal 10–15 years after diagnosis (Pringsheim et al., 2012). There is no cure for the disease, and current treatment is merely aimed at improving the quality of life and decreasing complications. With the treatment strategy to reduce the toxic effects of HTT, several clinical trials are ongoing with the use of ASOs for down-regulating HTT (Rodrigues and Wild, 2020), but recently disappointing results were reported from trials by Roche and Wave Therapeutics (Kingwell, 2021). To explore the potential of gene therapy, uniQure Biopharma, in 2019, has initiated a phase 1/2 randomized, double-blind, sham-controlled study to assess safety, tolerability and efficacy after multiple ascending doses administered into the striatum of AAV5-miHTT encoding miRNA aimed at reducing levels of HTT (AMT-130) in adult patients with early manifest HD (NCT04120493; Rodrigues and Wild, 2020).

NEURODEVELOPMENTAL DISORDERS

Rett Syndrome (RTT)

RTT is a progressive neurodevelopmental disorder with multisystem comorbidities that occurs almost exclusively in girls, affecting an estimated 10–11.8 in 100,000 females (Ip et al., 2018; Kyle et al., 2018; Fu et al., 2020). Boys typically die shortly after birth. In its classic form of RTT, girls have 6 to 18 months of apparently normal development before developing often highly debilitating problems with learning, language, coordination, autism symptomatology, and epilepsy. Several variant forms of RTT have been described which can be milder or more severe than the classic form. Up to 95% of cases of RTT are caused by LoF mutations in the X-linked gene *methyl-CpG-binding protein 2* (*MECP2*), which is a ubiquitously expressed transcriptional regulator critical for normal brain function, including the maintenance of synaptic connections (Ip et al., 2018). It remains unclear how these changes lead to the specific features of RTT. Several conditions with signs and symptoms overlapping those of RTT have been found to result from mutations in other genes. These conditions, including forkhead box protein G1 (FOXG1) syndrome and cyclin-dependent kinase-like 5 (CDKL5) deficiency disorder, were previously thought to be variant forms of RTT but are now usually considered to be separate disorders.

There is no known cure for RTT, and treatment is directed at improving symptoms. Anticonvulsants may be used to help with seizures. Many of those with the condition live into middle age. Using MECP2-knockout mice, encouraging pre-clinical results with increased survival and body weight have been seen after intracisternal delivery of AAV vectors encoding MECP2 (Gadalla et al., 2017; Sinnett et al., 2017; Sandweiss et al., 2020). An alternative approach is the use of CRISPR/Cas9 genome editing that has been shown to be efficient at correcting FOXG1 variants in human RTT patient-derived fibroblasts and induced pluripotent stem-derived neurons using AAV9 vectors (Croci et al., 2020). Human clinical trials remain to be initiated.

Genetic Epilepsy Syndromes

Epilepsy is one of the most common neurological diseases characterized by an enduring pre-disposition to generate seizures. Despite having an overall lifetime prevalence of approximately 800 in 100,000 (Beghi, 2020), it comprises a large heterogeneous group of syndromes of which some of them fulfill the definition of a rare disease in this review (i.e., <40–60 in 100,000), e.g., Dravet syndrome (severe myoclonic epilepsy in infancy; 2.5 in 100,000), Lennox-Gastaut syndrome (15 in 100,000), West syndrome (infantile spasms; 8 in 100,000), and Angelman syndrome (5–8 in 100,000) (Buiting et al., 2016; Döring et al., 2016). The majority of these genetic epilepsies are diagnosed within the first months to years of life with developmental deterioration or seizures as the first symptomatic signs. Since these syndromes are mostly drug-resistant, gene therapy could become an alternative treatment avenue (Turner et al., 2021).

The vast majority of Dravet syndrome cases are caused by a LoF mutation in one allele of the *SCN1A* gene, resulting in voltage-gated sodium channels with a non-functional NaV1.1 subunit primarily in inhibitory GABAergic neurons, leading to hyperexcitability and seizures associated with high risk of sudden infant death (Samanta, 2020). Pre-clinical data after single intrahippocampal injection of an AAV vector (ETX101) mediating increased production of functional copies of *SCN1A* in GABAergic interneurons in Dravet mouse model show decreased seizure frequency and severity as well as lower mortality (Steriade et al., 2020). Encoded Therapeutics is

currently preparing for clinical trials using ETX101 for *SCN1A*-positive Dravet syndrome patients[1]. Genome editing is also being explored with CRISPR/Cas9-based gene therapy triggering SCN1A transcription in inhibitory neurons shown to ameliorate seizures in Dravet syndrome mice (Colasante et al., 2020; Yamagata et al., 2020). Encouraging reductions in seizures and mortality were also seen in a mouse model of Dravet syndrome after ICV administration of ASO that increases expression of SCN1A transcripts by reducing non-productive splicing (Han et al., 2020). A recently initiated clinical trial sponsored by Stoke Therapeutics will test this ASO (STK-001) in Dravet syndrome patients (NCT04442295). These studies also suggest that RNAi gene therapeutic vectors targeting non-productive splicing could become relevant.

Another largely monogenic rare genetic epilepsy syndrome is that of Angelman syndrome which is most often caused by LoF mutations in the maternal *UBE3A* gene encoding ubiquitin-protein ligase E3A (Turner et al., 2021). This *UBE3A* deficiency can be potentially treated by gene replacement therapy and, indeed, intrahippocampal AAV9-UBE3A injection caused some improvements in a mouse model of Angelman syndrome (Daily et al., 2011). However, there is some concern that a gene replacement strategy could be associated with side effects if UBE3A expression levels become too high since this might result in autism (Vatsa and Jana, 2018). An alternative strategy that has been explored is to activate the dormant UBE3A gene on the paternally inherited chromosome which is silenced by expression of paternal expression of UBE3A-ATS transcripts by the use of ASO treatment targeting UBE3A-ATS (Elgersma and Sonzogni, 2021). Currently, a clinical trial with intrathecal administration of an ASO (GTX-102) is ongoing using this approach[2]. If successful, it is possible that gene therapy with vectors similarly targeting UBE3A-ATS via RNAi constructs could also come into play.

Other rare genetic epilepsy syndromes, like Lennox-Gastaut and West syndromes, can be due to mutations in multiple different genes, and the molecular mechanisms of these mutations are poorly understood (Mastrangelo, 2017; Pavone et al., 2020). Consequently, gene therapeutic pre-clinical studies are so far limited. Since more than 100 causative genes have been identified in epilepsy syndromes (Helbig and Ellis, 2020), it can, however, be expected that gene therapy will attract increasing attention for treating genetic epilepsies in the near future. One example of a gene therapeutic approach was conducted with a RNAi vector (scAAV9-miDnm1a), targeting pathogenic *DNM1* gene variants in a mouse model of developmental and epileptic encephalopathy that prevented development of lethal tonic-clonic seizures (Aimiuwu et al., 2020). *DNM1* encodes a brain-specific GTPase, dynamin-1, which mediates presynaptic endocytosis, and the few individuals identified with pathogenic *DNM1* variants suffer from developmental and epileptic encephalopathy syndromes including Lennox-Gastaut syndrome and infantile spasms. Moreover, gene therapy trials with symptomatic vector construct approaches are also on the way targeting hyperexcitability in more frequent focal epilepsies with intracerebral injections of viral vectors mediating focal overexpression of engineered Kv1.1 potassium channel (NCT04601974; lentivirus; Snowball et al., 2019) or neuropeptide Y and its antiepileptic receptor Y2 (AAV1-NPY-IRES-Y2; CG01; Szczygiel et al., 2020; Cattaneo et al., 2021). It is likely that these gene therapy vectors could also be efficacious in genetic epilepsy syndromes, by targeting the general disease mechanisms underlying seizure development instead of single mutated genes *per se*.

LYSOSOMAL STORAGE DISEASES

Batten Disease

Batten disease is the common name for a broad class of rare, fatal, inherited neurodegenerative lysosomal storage diseases affecting the nervous system and often retina, also known as neuronal ceroid lipofuscinoses (CLNs; Johnson et al., 2019). Batten disease affects 2–4 in 100,000 live births (Santavuori, 1988) and has several forms (CLN1-CLN14; Specchio et al., 2021) that share some common features and symptoms but vary in severity and age when symptoms appear. Each form is caused by LoF mutations in different genes affecting lysosomal function. Most forms of Batten disease/CLNs begin during childhood where symptoms may include vision loss, seizures, loss of previously acquired skills, dementia, abnormal movements, and greatly shortened life expectancy.

Traditional medications are available as symptomatic treatment for symptoms such as seizures, anxiety, depression, parkinsonism, and spasticity. Alternative treatments being explored include enzyme replacement therapy (for CLN1 and CLN2 diseases), stem-cell therapy (for CLN1, CLN2, CLN8 diseases), and gene therapy (for CLN1-CLN3, CLN5-CLN8, CLN10, CLN11 diseases) in pre-clinical and clinical studies (Johnson et al., 2019; Liu et al., 2020; Specchio et al., 2021). Studies of potential treatments are lacking for CLN4, CLN9, and CLN12-CLN14 diseases. So far, enzyme replacement therapy with ICV-administered cerliponase alpha for CLN2 disease is the only one that has been approved for Batten disease (Markham, 2017).

CLN1 Disease

CLN1 is caused by a LoF mutation of the gene *palmitoyl-protein thioesterase-1* (*PPT1*) that encodes the enzyme of the same name, leading to accumulation of lipopigments within cells, resulting in neuroinflammation and -degeneration. ABO-202, a scAAV9 vector that carries the *PPT1* gene, is a promising candidate for intracerebral gene therapy for CLN1. Pre-clinical studies with ABO-202 as well as various other AAV vectors encoding PPT1 have shown increased survival and improvement of neurological function in CLN1 mouse models (Shyng et al., 2017; Liu et al., 2020). ABO-202 has been granted Orphan Drug and Rare Pediatric Disease designations by FDA and Orphan

[1] *https://encoded.com/encoded-therapeutics-announces-135-million-series-d-financing-to-support-first-clinical-trials-in-scn1a-dravet-syndrome-and-advance-preclinical-pipeline-of-gene-therapies-for-debilitating-neurologic/*

[2] https://ir.ultragenyx.com/news-releases/news-release-details/genetx-and-ultragenyx-announce-positive-interim-phase-12-data#

Medicinal Product Designation from EMA. A phase 1/2 clinical trial is anticipated to start in 2021[3].

CLN2 Disease

CNL2 also known as "Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL)," derives from a defect in the lysosomal gene *CLN2* encoding the enzyme tripeptidyl peptidase 1 (TPP1) (Kohlschütter and Schulz, 2016), resulting in the lysosomal accumulation of ceroid lipofuscin. Clinical trials completed and ongoing suggest promising effects of intraventricular enzyme replacement therapy (BMN190; Markham, 2017; Schulz et al., 2018), but gene therapy is also being explored (Liu et al., 2020). AAV vectors encoding human TPP1 induced cerebral enzyme expression and increased survival in a mouse model of CLN2 disease after intracerebral administration (Passini et al., 2006; Kohlschütter and Schulz, 2016), and a human clinical trial (NCT00151216) with infusion into 12 distinct cerebral locations in 10 children suggested slowing of disease progression (Worgall et al., 2008; Souweidane et al., 2010). Two subsequent phase 1/2 trials (NCT01161576, NCT01414985) have been completed using AAVrh10-CNL2 that appeared more promising in the mouse model (Sondhi et al., 2007) and showed long-term expression and acceptable safety profile in rats and non-human primates (Sondhi et al., 2012), but, so far, no results have been published.

CLN3 Disease

This type of Batten disease derives from mutations in the *CLN3* gene that encodes a lysosomal membrane protein called battenin, the function of which is poorly understood. Based on encouraging pre-clinical data from mice (Bosch et al., 2016), a phase 1/2 gene therapy open-label, single dose, dose-escalation clinical trial (NCT03770572) has been initiated in subjects with CLN3 disease to explore safety and efficacy of intrathecal delivery in the lumbar spinal cord region of a vector encoding the normal human *CLN3* gene (scAAV9.P546.CLN3; AT-GTX-502). Using a mouse model of CLN3 disease, intraocular gene therapy with AAV-mediated expression of CLN3 may also be efficacious for treating loss of vision by preventing decline in inner retinal function resulting from the death of rod bipolar cells (Holthaus et al., 2020).

CLN5 Disease

This is caused by mutations in a lysosomal protein encoded by the gene *CLN5* (Liu et al., 2020). Gene therapy using lentivector, AAV9, or scAAV9 encoding CLN5 has shown promising results in sheep with naturally occurring CLN5 disease (Mitchell et al., 2018). The vectors were injected intraventricularly and/or directly into the brain parenchyma, and the treated sheep retained neurological and cognitive functions. So far, no human clinical trials have been initiated.

[3]*https://www.globenewswire.com/news-release/2020/08/17/2079104/0/en/Abeona-Therapeutics-and-Taysha-Gene-Therapies-Enter-into-Licensing-and-Inventory-Purchase-Agreements-for-ABO-202-a-Clinical-Stage-Novel-One-time-Gene-Therapy-for-CLN1-Disease.html*

CLN6 Disease

This condition results from mutations in an endoplasmic reticulum membrane protein encoded by the gene *CLN6* (Liu et al., 2020). ICV-administered scAAV9 encoding CLN6 drastically reduced pathology, improved memory, motor performance, and survival in CLN6 mutant mice (Cain et al., 2019). ICV delivery of scAAV9-CLN6 also slowed visual deterioration in CLN6 disease mice by preventing disease pathology in visual centers of the brain and retina (White et al., 2021). Intrathecal gene therapy with a similar vector was shown to be safe and efficiently induce expression in the brain and spinal cord in non-human primates (Cain et al., 2019). A phase 1/2 single dose study is ongoing to test intrathecal administration of scAA9-CLN6 (AT-GTX-501) in CLN6 disease children (NCT02725580) and a 15-year follow-up study will evaluate long-term safety and efficacy (NCT04273243).

CLN7 Disease

This condition can result from more than 35 different mutations in the *MFSD8* gene encoding CLN7, a lysosomal putative membrane transporter protein (Danyukova et al., 2018). Recently, a novel concept known as *N-of-1* treatment has emerged, which encompasses true personalized medicine and development of specific treatment for single patients (Mullard, 2020). This was showcased by the development within only 1 year of a new unique ASO treatment, milasen, for a young child, suffering from CLN7 due to a unique mutation that caused mis-splicing of MFSD8 (Kim et al., 2019). After dose escalation followed by maintenance dosing every 3 months, it was found that seizures were substantially decreased and several neurologic and neuropsychological subscores stabilized during 7 months after treatment (Kim et al., 2019). Mila's case has created new hope in rare and ultra-rare diseases where gene therapy can potentially be developed in cases where only one person in the world has a specific genetic mutation (Kim et al., 2019). As for CLN7 disease patients, most of them will probably not benefit from treatment strategies targeting RNA mis-splicing. Another line of clinical testing is currently recruiting for a phase 1 open-label, single-dose gene replacement therapy (AAV9-MFSD8) administered intrathecally into the lumbar spinal cord of pediatric patients with CLN7 Batten disease (NCT04737460).

CLN8 Disease

This is a condition caused by biallelic mutations in the gene *CLN8* which encodes an endoplasmic reticulum cargo receptor that regulates lysosome biogenesis (di Ronza et al., 2018). A single neonatal ICV injection of a scAAV-9 vector encoding human CLN8 has recently shown a successful degree of rescue in a mouse CLN8 model, as revealed by reduced histopathology, substantial behavioral improvement, and increased lifespan (Johnson et al., 2021). These data clearly encourage the testing of gene therapy for this disorder.

CLN10 Disease

CLN10 disease is caused by homozygous or compound heterozygous mutations of the *CTSD* gene encoding cathepsin D,

an aspartic endoprotease ubiquitously distributed in lysosomes (Liu et al., 2020). Intracerebral administration of a mosaic AAV1/2 encoding CTSD into neonatal CTSD knockout mice increased lifespan and rescued brain pathology, and CLN10-associated visceral abnormalities as well as lifespan were further improved by peripheral vector treatment (Shevtsova et al., 2010; Liu et al., 2020).

CLN11 Disease

This adult onset disease is caused by homozygous or compound heterozygous mutations in the *GRN* gene that encodes the protein granulin implicated in lysosomal function (Liu et al., 2020). Heterozygous mutations are associated with frontotemporal dementia (Baker et al., 2006; Cruts et al., 2006). Pre-clinical studies with CLN11 gene therapy using GRN knockout mice have generated conflicting results with AAV1-GRN vector injected into prefrontal cortex showing improved pathology, also outside the injected region (Arrant et al., 2018), while ICV injection of AAV9-GRN using a different promoter was associated with severe hippocampal neurodegeneration (Amado et al., 2019). Further pre-clinical studies are needed to clarify the safety and efficacy of gene therapy with GRN overexpression for CLN11 disease.

Krabbe Disease (KD)

KD is an autosomal recessive, often fatal lysosomal storage disease leading to pronounced neurodegeneration (Kwon et al., 2018). KD is also known as globoid cell leukodystrophy because of the characteristic multinucleated globoid cells found in a brain biopsy and the presence of white matter degeneration. The disease has an estimated prevalence around 1 in 100,000 (Foss et al., 2013). KD is caused by a LoF mutation at human chromosome 14, which codes for a lysosomal hydrolase known as galactosylceramide beta hydrolase (GALC), which is responsible for metabolizing galactolipids in both the central and peripheral nervous systems. KD disease is subdivided into sub-categories based on the age at presentation of symptoms. It is possible to screen newborns for KD, but current tests to identify which children are likely to develop the disease are inadequate (Kwon et al., 2018; Ehmann and Lantos, 2019). HSPC transplantation is the only available treatment for early infantile KD and should be performed before the onset of symptoms to be effective (Ehmann and Lantos, 2019).

Currently, promising pre-clinical data (Bradbury et al., 2018, 2021; Rafi et al., 2020) have enabled Forge Biologics to plan recruitment in 2021 of children below 12 months of age in a phase 1/2 clinical study, performing IV administration of an AAVrh10 vector expressing GALC (FBX-101) in newborns with early-infantile KD receiving HSPC transplantation (NCT04693598). A similar approach is applied by Passage Bio who intend to start recruitment in 2021 of 1–9 months children with early-infantile KD for treatment with intracisternal injections with the recombinant AAVhu68 vector encoding human GALC (PBKR03; NCT04771416). In both studies, the aim is to assess safety, tolerability and efficacy of escalating doses of AAVhu68-GALC gene therapy.

Metachromatic Leukodystrophy (MLD)

MLD has an incidence of 1.4–1.8 in 100,000 live births (Rosenberg et al., 2016). Leukodystrophies are genetic disorders of the CNS leading to progressive neurologic deterioration; in the case of MLD, the disease arises from a deficiency of the lysosomal enzyme arylsulfatase A (ARSA) due to LoF mutations in the *ARSA* gene. This leads to a build-up of sulfatides, resulting in cerebral demyelination and loss of neurons. It affects both oligodendrocytes and Schwann cells, thus affecting neurons in both the central and peripheral nervous system. MLD is classified depending on age at onset of symptoms, the most common form, known as late infantile form, debuts at around 2 years of age (accounting for 50% of cases). Patients die within a few years after onset and display seizures, impaired swallowing, muscle wasting, paralysis, and dementia. Sulfatides accumulate in several other organs although the effect of this accumulation has not yet been observed. This could be attributed to the short lifespan of these patients, and accumulation of sulfatides could have effects that will be revealed if survival is increased (Rosenberg et al., 2016).

Gene therapy has been utilized *ex vivo* in combination with bone marrow transplants to re-implant genetically corrected HSPCs to patients (utilizing lentivectors and insertion of healthy genes into the cell genome) (Rosenberg et al., 2016). Three children with ARSA deficiency and mutations associated with early-onset MLD were included in a phase 1/2 trial, carried out in a partnership between Orchard Therapeutics and San Raffaele-Telethon Institute for Gene Therapy, and treated at the pre-symptomatic stage with autologous CD34-positive HSPCs transduced *ex vivo* with a lentivector carrying the *ARSA* gene (OTL-200; Biffi et al., 2013). This resulted in stable engraftment of transduced HSPCs at high levels and with reconstituted ARSA activity in the cerebrospinal fluid and arrested progression of neurodegenerative disease in all patients (Sessa et al., 2016; NCT01560182). Subsequently, preliminary data from 33 early-onset MLD patients with up to 7.5 years follow-up after treatment with OTL-200 suggested a favorable safety profile (no treatment-related mortality, no malignancies, no abnormal clonal expansion, and no evidence of replication-competent lentiviruses) and efficacy at modifying the disease course of early-onset MLD patients (hematological recovery, stable OTL-200 engraftment, ARSA activity restoration, and long-term stabilization of motor functions) as compared to a national history cohort (Calbi et al., 2020). Recently, OTL-200 was approved for treatment of MLD by EMA in 2020 under the tradename Libmeldy®, but OTL-200 is not yet approved by the FDA. In addition, two studies applying the same principle, but in later onset symptomatic MLD patients, have been initiated to evaluate short- and long-term safety and efficacy (NCT02559830, NCT04283227).

In addition, treatments with *in vivo* gene transfer are being explored. Based on promising pre-clinical results in rodents and non-human primates after intracerebral injection of an AAVrh10-ARSA vector that induced high expression of ARSA in neurons and oligodendrocytes (Piguet et al., 2012; Zerah et al., 2015), a phase 1/2 clinical study of ARSA gene transfer with 12 intracerebral injections to children with early onset forms of MLD was initiated in 2013 (Penati et al., 2017; NCT01801709).

So far, results remain to be posted. Another phase 1/2 study sponsored by the Shenzhen Geno-Immune Medical Institute will test effects of ARSA gene therapy on MLD patients using a safety- and efficiency-improved self-inactivating lentivector (TYF-ARSA) after intracerebral injections (NCT03725670). Safety will be evaluated with regard to vital signs, physical examination, treatment-emergent adverse events, biochemical analysis, and magnetic resonance imaging (MRI) up to 3 years post-treatment.

Mucopolysaccharidoses (MPS)

MPS are a defined group of different lysosomal storage disorders (MPS I-IX) caused by a deficiency in lysosomal enzymes catalyzing degradation of glycosaminoglycans (GAGs) that affects 4 in 100,000 (Poswar et al., 2019). GAGs consist of long chains of sugar carbohydrates aiding the buildup of bone, cartilage, tendons, corneas, skin, and connective tissue. Lysosomal enzyme deficiencies of MPS lead to aberrant development with neurocognitive and musculoskeletal pathological abnormalities. Each MPS is clinically heterogeneous, with milder to more severe cases within each type (Terlato and Cox, 2003). Diagnosis is determined by measuring urinary GAGs, enzyme activity in blood samples, and by identification of specific gene variants related to each MPS enzyme (Kubaski et al., 2020). Current treatment consists of enzyme replacement therapy (e.g., for MPS I and II; Concolino et al., 2018) and allogeneic HSPC transplantation (Poswar et al., 2019). However, despite reduced morbidity, these do not prevent persisting neurocognitive and musculoskeletal deficits (Fraldi et al., 2018). It is, therefore, not surprising that gene therapeutic approaches are under exploration. Here we will mainly describe the gene therapy efforts in the MPS types I, II, and III which show consistent CNS involvement. In other MPS types, e.g., VI, although clinical testing has been performed, CNS is not affected and, consequently, outside the scope of this review (for review see Fraldi et al., 2018).

MPS I

This type of MPS is divided into three subtypes (Hurler, Hurler-Scheie, and Scheie syndromes) based on severity of symptoms. All three are caused by a defective gene causing alpha-L-iduronidase (IDUA) enzyme deficiency and tissue accumulation of the GAGs heparan and dermatan sulfate (Hampe et al., 2021). Several encouraging studies have been performed using animal models of MPS type 1 with intrathecally or IV administered IDUA gene replacement approach using serotype 9 or rh10 AAV vectors, including in rodents, dogs, cats, and non-human primates (Watson et al., 2006; Hinderer et al., 2014, 2015; Belur et al., 2020). In line with these promising pre-clinical data, a first-in-human gene therapy study sponsored by Regenxbio has been initiated using an AAV9-IDUA vector (RGX-111) designed to restore IDUA enzyme activity in the brain (NCT03580083). This is an open-label, dose-escalation phase 1/2 study evaluating safety, tolerability, efficacy, and pharmacodynamics after intracisternal delivery of RGX-111 to MPS I patients during the 24 weeks study period. An ongoing *ex vivo* phase 1/2 clinical trial (NCT03488394) explores safety and efficacy of IV-injected autologous HSPCs genetically modified to express IDUA using a LV in patients with the Hurler variant. A long-term follow-up study will look for adverse neurologic and other events for up to 30 years after (NCT00695279). Genome editing has also been explored as a treatment approach. Both ZFN and CRISPR-Cas9 platform studies where AAV8 vectors were injected IV to genome edit liver cells have shown encouraging results in rodents (Ou et al., 2020; Poletto et al., 2020). The latter platform may be more efficacious (Ou et al., 2020), but, so far, only ZFN genome editing has entered clinical testing using an IV-injected AAV6 vector that inserts a corrective copy of the IDUA transgene into the genome of patients' hepatocytes (NCT02702115; Harmatz et al., 2019). This is expected to provide permanent, liver-specific expression of IDUA. A 10-year long-term safety follow-up study is also ongoing (NCT04628871). Although CNS symptoms were clearly improved in a mouse model of MPS I (Ou et al., 2020), it is disputed to what extent enzymes will pass into the CNS (Poletto et al., 2020). Nonetheless, it is suggested that constant high blood levels of IDUA in the blood may cause sufficient amounts to reach the brain (Ou et al., 2020).

MPS II

This type of MPS, also known as Hunter syndrome, results from a recessive X-linked LoF mutation in the gene encoding the lysosomal enzyme iduronate-2-sulfatase (IDS; Sestito et al., 2018). Pre-clinical models of MPS II have shown success with regard to improvement of neurological symptoms using gene replacement *ex vivo* after transplantation of HSPCs modified to synthesize IDS via a lentivector or *in vivo* using intracerebral administration of an AAV-IDS vector (Gleitz et al., 2018; Sestito et al., 2018). Two phase 1/2 open-label multicenter *in vivo* gene replacement trials (AAV9-IDS; RGX-121) are currently recruiting patients. One is a dose-escalation study (NCT03566043), and the other is a single dose study (NCT04571970). A follow-up study will evaluate long-term safety and efficacy of RGX-121 over 5 years (NCT04597385). Consistent with encouraging results with ZFN-mediated *in vivo* genome editing in mouse model of MPS II (Laoharawee et al., 2018), the first human genome editing trial (CHAMPIONS) in the form of a phase 1/2, multicenter, open-label, ascending dose trial is currently investigating efficacy of genome editing with the use of an AAV6 vector delivering ZFN (SB-913) that corrects the IDS gene in hepatocytes of MPS II patients, aiming to provide permanent, liver-specific expression of IDS (NCT03041324; Muenzer et al., 2019). An additional long-term safety study will follow the patients for 10 years (NCT04628871). As argued above for MPS I genome editing targeting liver cells, it remains to be seen to what extent CNS symptomatology will be improved by the treatment (Poletto et al., 2020).

MPS III

This type of MPS is also known as Sanfilippo syndrome and exists in five different forms (A-E) that are all recessive lysosomal storage diseases primarily affecting the brain (Pearse and Iacovino, 2020). MPS type IIIA causes Sanfilippo syndrome A and is the most common and severe type of MPS III with lowest

survival rate (Pearse and Iacovino, 2020). The disease is caused by enzyme deficiency of N-sulfoglucosamine sulfohydrolase (SGSH; also known as heparan-N-sulfatase) due to LoF mutation in the *SGSH* gene, leading to the lysosomal accumulation of the GAG heparan sulfate (Winner et al., 2016). Promising results from pre-clinical studies with AAV-mediated gene transfer in animal models of MPS IIIA (Winner et al., 2016) led to initiation of phase 1/2 clinical trials in four children with MPS IIIA sponsored by Lysogene (Tardieu et al., 2014; NCT01474343, NCT02053064). The catalytic site of SGSH is activated by a sulfatase-modifying factor (SUMF1). Consequently, the vector that was injected intracerebrally in these trials encoded both SGSH and SUMF1 (AAVrh10-SGSH-IRES-SUMF1; LYS-SAF301). At 1-year follow-up moderate improvements were observed in three patients (Tardieu et al., 2014). The treatment was generally well-tolerated. An enhanced vector (AAVrh10-SGSH; LYS-SAF302) only encoding SGSH, the enzyme deficient in MPS IIIA, that induces 3-fold higher enzyme expression than LYS-SAF301 (Laufer et al., 2019) is currently being tested in an open-label single arm, phase 2/3 study after intracerebral delivery (NCT03612869; AAVance trial). A 5-year-old girl from this trial recently died several months after receiving the vector injection, and consequently the FDA has so far put the trial on hold while it is being analyzed whether the death is related to the gene therapy[4,5]. Two other trials using IV administration of a vector carrying the human *SGSH* gene (scAAV9-hSGSH; ABO-102) sponsored by Abeona Therapeutics are also currently recruiting (NCT02716246, NCT04088734). Long-term safety and efficacy will be monitored in a 5-year follow-up study (NCT04360265). Preliminary data suggest that ABO-102 is well-tolerated (Marcó et al., 2019).

Pre-clinical safety and efficacy of *ex vivo* transduction of CD34-positive HSPCs with a LV containing *SGSH* (LV-SGSH) has been demonstrated (Ellison et al., 2019). This has led to initiation of a phase 1/2 study using *ex vivo* gene therapy with LV-SGSH transduced CD34-positive HSPCs administered to MPS III A patients (NCT04201405). The study is sponsored in collaboration between the University of Manchester and Orchard Therapeutics (Kinsella et al., 2020).

MPS type IIIB causes Sanfilippo syndrome B due to deficient enzyme alpha-N-acetylglucosaminidase (NAGLU) activity (Pearse and Iacovino, 2020). A phase 1/2 clinical trial sponsored by UniQure Biopharma has tested gene replacement therapy using intracerebral administration of AAV5-hNAGLU in four MPS IIIB patients (NCT03300453). The treatment appears to be safe and well-tolerated with sustained NAGLU production in the CSF 30 months after injection (Tardieu et al., 2017). Abeona Therapeutics has sponsored a 2-year open-label, dose-escalation phase 1/2 trial using IV administration of an AAV9 vector encoding the human NAGLU gene (AAV9-hNAGLU; ABO-101; NCT03315182). No results are available yet.

MPS type IIID causes Sanfilippo syndrome D due to deficiency in N-acetylglucosamine 6-sulfatase (GNS; Pearse and Iacovino, 2020). GNS-deficient mice show lysosomal storage CNS pathology, locomotor deficits, and shortened lifespan similar to humans with MPS IIID, and intracisternal administration of a vector encoding GNS (AAV9-GNS) reversed these deficits (Roca et al., 2017). These encouraging results await clinical testing.

NEUROMETABOLIC DISORDERS

Canavan Disease (CD)

CD is a rare leukodystrophy resulting in neurodegeneration that occurs after a LoF mutation in the gene encoding aspartoacylase (ASPA). ASPA deacetylates N-acetylaspartate (NAA), and dysfunction results in accumulation of NAA in the nervous system (and in urine). The result of accumulation of NAA is dysmyelination, vacuolation of white matter and intramyelinic edema leading to hydrocephalus (Ahmed and Gao, 2013). The overall incidence is unknown. It occurs most frequently in individuals of Ashkenazi Jewish descent where it affects, at up to 16 in 100,000 (Zayed, 2015). ASPA mRNA is mainly found in oligodendrocytes while it is not present in neurons (Kirmani et al., 2002). NAA is produced in neurons and transported to the extracellular space where it is taken up by glial cells. NAA constitutes more than 0.1% of the healthy brain by weight, yet the function of NAA remains largely unknown, and the mechanism of CD pathology is unclear (Leone et al., 2012; Gessler and Gao, 2016; Gessler et al., 2017). Three subclasses exist based on onset of symptoms and severity of progression: Congenital, infantile and juvenile. CD is fatal in its congenital form where children die within days or weeks after birth (Ahmed and Gao, 2013, Gessler and Gao, 2016). Most patients suffer from the infantile form with symptoms including hypotonia, macrocephaly, blindness, and halting motor function development starting within the first postnatal months (Gessler and Gao, 2016).

The first pre-clinical gene therapy studies on CD utilized a lipid-entrapped, polycation-condensed delivery system in combination with an AAV-based plasmid encoding ASPA that was administered by intracerebral and intraventricular injections to healthy rodents and primates (Gessler and Gao, 2016). Subsequently, a proof-of-concept study with the same injection constructs was performed on two children with CD (Leone et al., 2000). Although the effect of this type of gene therapy was well-tolerated, and some biochemical and radiological parameters improved, no clinically relevant disease rescue was observed (Gessler and Gao, 2016). A few years later a follow up study conducted a phase 1 trial in a larger group of CD patients with an improved system for delivering the enzyme (AAV2-ASPA; Janson et al., 2002; Leone et al., 2012). A follow-up study found that AAV2-ASPA gene therapy slowed progression of brain atrophy, reduced seizures, and stabilized overall clinical status (Leone et al., 2012). No severe adverse events related to the administration of AAV2-ASPA into six intracerebral infusion sites were reported after a minimum of 5-years follow-up (Leone et al., 2012).

Subsequent pre-clinical studies with ASPA gene replacement therapy have shown phenotype rescue after systemic injection

[4]https://www.genengnews.com/news/lysogene-confirms-childs-death-in-phase-ii-iii-gene-therapy-trial/

[5]https://www.firstwordpharma.com/node/1730262?tsid=17

of AAV9 or other AAV serotypes (i.e., rh8, rh10) that are able to cross the BBB or after intracerebral injection of AAV-Cy5 with a promoter that specifically targets oligodendrocytes in CD mice (Gessler and Gao, 2016; von Jonquieres et al., 2018). Additional studies introducing human *ASPA* gene replacement into the astrocytes in mice has provided support for utilizing the astrocytes as a metabolic sink for clearing NAA (Gessler et al., 2017). Again, successful treatment was age-dependent, with mice receiving treatment shortly after birth showing greater improvement in motor functions and survival (Gessler and Gao, 2016). NAA ASO knockdown of expression of neuronal NAA synthesizing enzyme N-acetyltransferase 8-like in a mouse CD model also showed some effect for 2 months after administration on NAA levels (Hull et al., 2020). Using a novel capsid variant, AAV/Olig001, with oligotropism allowing the vector to mediate ASPA expression more specifically in oligodendrocytes (Francis et al., 2021), a phase 1/2 open label clinical trial sponsored by CureRareDisease LLC has recently been initiated with administration of a single ICV dose of AAV/Olig001-ASPA (NCT04833907). The trial will enroll 24 CD children aged 3–60 months.

Niemann-Pick Disease

Niemann-Pick disease occurs in 5 in 100,000 live births in Europe (Gessler and Gao, 2016). All subtypes result from acid sphingomyelinase (ASM) deficiency causing increases in in metabolic intermediates including sphingomyelin and cholesterol (Salegio et al., 2012). Different subtypes have been described: Niemann-Pick disease type A presents with cognitive decline, loss of motor function, and hepatosplenomegaly. Rapid neurodegeneration leads to death within 3 years after birth (Samaranch et al., 2019). Niemann-Pick disease type B displays milder progression and symptoms show before adulthood and neurological symptoms are less common. Niemann-Pick disease type C affects trafficking of endocytosed cholesterol, and symptoms start before adulthood and include ataxia, cognitive dysfunction, and loss of language. Patients only reach 10–25 years of age (Gessler and Gao, 2016). Type A and type B Niemann-Pick disease have a residual ASM enzyme activity of 1–2% and 5–10%, respectively, suggesting that even marginal increases in ASM activity could lead to a therapeutic beneficial effect (Salegio et al., 2012).

Enzyme replacement therapy has been suggested as a viable treatment for the peripheral symptoms of patients suffering from type A and B. Clinical trials are underway (Samaranch et al., 2019). As these do not reach the CNS, gene replacement by injection into the lateral ventricles has been suggested. Studies performed in knockout mice lacking the *ASM* gene showed some phenotypic improvements, which was not reproduced in non-human primates where poor diffusion from the ventricles into the brain parenchyma limited the spread of the transgene. Injections into the brain parenchyma of AAV vectors encoding the *ASM* gene were more effective, though only at injection sites limited by poor spread of the transgene (Samaranch et al., 2019). A pre-clinical study with intracerebral delivery of AAV2-ASM in non-human primates resulted in only low spread of expression and toxicity in the form of immunogenicity as well as abnormal gait and posture and paresis in animals treated with high doses (Salegio et al., 2012). The immunogenicity and inflammatory response was suggested to arise from ASM-induced upregulation of cytokine CCL5 that is associated with gliosis and inflammation (Salegio et al., 2012). It is not clear to what extent CCL5 upregulation can limit the usefulness of *ASM* gene replacement therapy. Delivery has been also attempted via CSF through the cerebellomedullary cistern, resulting in transgene expression and better spread to deeper brain structures (Samaranch et al., 2019). Despite the initially promising opportunity of gene replacement, pre-clinical studies have revealed challenges related to ASM-induced calcium imbalance, aberrant intracellular signaling, inflammation and cell death (Lloyd-Evans et al., 2008), which should be carefully assessed and may be eliminated by dose de-escalation. No clinical trials utilizing gene therapy in humans have yet been initiated.

X-linked Adrenoleukodystrophy (ALD)

ALD, also known as Lorenzo's oil disease, is an X-linked disease with an estimated incidence of 6.8 in 100,000 (Kemp et al., 2001). ALD is caused by a defective *ABCD1* gene that encodes a peroxisomal ATP-binding cassette transporter for transporting very long-chain saturated fatty acids (VLCFA) into the peroxisome for beta-oxidation. *ABCD1* dysfunction leads to a pathologic build-up of fatty acids damaging the myelin sheaths of the neurons in the brain, leading to cognitive and motor impairments (Turk et al., 2020). The disease primarily affects boys, though half of heterozygous females show some symptoms later in life. If left untreated, it will ultimately lead to a vegetative state and life expectancies of no longer than 10 years from time of diagnosis (Turk et al., 2020).

The *ABCD1* gene defect can be screened for in childhood genetic testing providing a short opening to start up treatment and to prevent the progressing and irreversible degenerative effects. More than 2,700 different *ABCD1* mutations have been identified, indicating a large degree of non-recurrent variations and *de novo* mutations and a low degree of phenotypic to genotypic correlation (Kemp et al., 2012; Turk et al., 2020). Current standard treatment for childhood cerebral ALD is allogeneic HSPC transplantation. However, this intervention is associated with high morbidity and long-term complications related to the concomitant chronic immunosuppression and graft-vs.-host response. Moreover, adrenal dysfunction is not corrected following the HSPC transplant for the cerebral disease.

One attempt to improve the clinical therapeutic options and decrease mortality is the execution of *ex vivo ABCD1* gene replacement in CD34-positive HSPCs in 17 ALD boys with early stage brain disease using a lentivector (Lenti-D) (NCT01896102; NCT03852498; Cartier et al., 2009; Eichler et al., 2017). The patient's own HSPCs were transduced *ex vivo*, inserting a correct version of the *ABCD1* gene into the patient's stem cells. Subsequently, the patients received chemotherapy to eradicate the host HSPCs and make room for the genetically altered cells, which were re-infused IV. As first indication, interim findings reported that 15 of the boys survived and remained free of major functional disabilities at the 2-years follow-up, with no treatment-associated death or

graft-vs.-host disease reported. One boy died of rapid neurologic deterioration, and one withdrew from the study due to rapid disease progression and was instead submitted to allogeneic stem cell transplantation. A third clinical trial is recruiting advanced stage ALD patients to test the effects of *ABCD1*-corrected CD34-positive HSPCs (NCT02559830). A multi-center, long-term safety and efficacy follow-up study will test ALD patients treated with Lenti-D vector modified HSPCs for an additional 13 years (NCT02698579).

Pre-clinical *in vivo* gene therapy has shown promising results in a mouse model of ALD where intrathecal delivery of AAV9-ABCD1 in mice improved VLCFA metabolism and behavioral parameters (Gong et al., 2015). However, so far, only lentivector-mediated gene replacement approach administered intracerebrally to ALD patients is being tested in a Shenzhen Geno-Immune Medical Institute-sponsored phase 1/2 clinical trial using a self-inactivating LV (TYF-ABCD1; NCT03727555).

Phenylketonuria (PKU)

PKU is a monogenic autosomal recessive disease caused by different LoF mutations in the *phenylalanine hydroxylase* (*PAH*) gene located on chromosome 12 with a prevalence between 6.7 and 10 in 100,000 newborns (Woo et al., 1983; Gessler and Gao, 2016). PAH catalyzes the hydroxylation of phenylalanine to tyrosine and is predominantly expressed in the liver but is also found in kidney, pancreas, and brain (Lichter-Konecki et al., 1999). The underlying disease mechanism for PKU is not fully understood, however, if left untreated, it leads to severe intellectual disability, developmental impairment, seizures, and psychosocial problems (White et al., 2010). Today's screening programs of newborn children and early nutritional intervention can reduce cognitive impairment. However, gene therapy has been explored at the pre-clinical level since gene replacement normalizing liver PAH activity and even boosting CNS PAH expression are predicted to potentially improve cognition and quality-of-life for PKU patients. Thus, both *ex vivo* and *in vivo* PAH gene replacement therapy in murine models of PKU (Gessler and Gao, 2016). For instance, *in vivo PAH* gene replacement by portal or tail vein injection in mice using an AAV8-PAH vector was associated with long-term reduction of phenylalanine levels with no elevation of markers of liver damage, inflammation, or humoral immune response against vector-mediated PAH expression (Ding et al., 2006; Harding et al., 2006).

Recently, translation into the clinic has begun with three trials currently recruiting. The first is a phase 1/2, open-label, randomized, dose-escalation study sponsored by Homology Medicines to evaluate the safety and efficacy for 1 year after a single IV-injection of an AAVHSC15 vector containing a functional copy of the human *PAH* gene (HMI-102; NCT03952156). This AAV serotype administered IV has been observed to cross the BBB and transduce neurons in the brain and spinal cord in non-human primates (Ellsworth et al., 2019) and could, consequently, further boost PAH CNS expression in addition to peripheral expression. A second clinical trial is a 5-year follow-up safety and efficacy study to the first trial (NCT04348708). The third trial, sponsored by BioMarin Pharmaceutical, is a phase 1/2 open-label, dose-escalation study which tests an AAV5 carrying a functional *PAH* gene (BMN 307; NCT04480567).

Gangliosidoses

Gangliosidoses (GM1 and GM2) are neurodegenerative lysosomal storage disorders resulting from autosomal recessive mutations causing the accumulation of lipid gangliosides (Breiden and Sandhoff, 2019).

GM1 Gangliosidosis

GM1 gangliosidosis results from LoF mutations in the *GLB1* gene, leading to deficiency in beta-galactosidase 1 (GLB1) hydrolase that results in GM1 ganglioside accumulation primarily in nervous tissue in the CNS. Incidence is estimated to be 0.5–1 in 100,000 (Tonin et al., 2019). Age of onset and progression of GM1 gangliosidosis differ, and the disease is divided into infantile (Type I), late-infantile/juvenile (Types IIa and IIb), and adult (Type III). The early forms constitute the more serious forms with multiple severe hallmark symptoms, including the typical CNS manifestations and severe cognitive and physical disabilities. The disease is uniformly fatal with no effective therapy and standard of care is limited to symptomatic medical management. Two clinical trials aiming at gene transfer with AAV vectors carrying a functional copy of the *GLB1* gene are currently ongoing. In 2019, a non-randomized, phase 1/2 clinical trial started aiming at evaluating the safety and efficacy of a single dose AAV9-GLB1 (AXO-AAV-GM-1) after IV infusion to subjects, aged 2–12 years old, with Type II GM1 gangliosidosis (NCT03952637). The study is conducted in a collaboration between the National Human Genome Research Institute and Axovant Sciences and is expected to be completed in 2023. Not long after in April 2020, Lysogene started another phase 1/2 clinical trial aiming at evaluating safety and efficacy of different doses of AAVrh10-GLB1 (LYS-GM101) infused IV to subjects with infantile Type I GM1 gangliosidosis (NCT04273269). This study is expected to be completed in 2025. Passage Bio is sponsoring a third phase 1/2, single-arm, dose escalation, multicenter study currently recruiting that will test an AAVhu68-GLB1 vector (PBGM01) delivered into the cisterna magna in infantile GM1 patients with Types I and IIa gangliosidoses (NCT04713475).

GM2 Gangliosidoses

GM2 gangliosidoses include the Tay-Sachs disease, the Sandhoff disease, and the GM2 AB, which result from LoF mutations in the genes *HEXA*, *HEXB*, and *GM2A*, respectively (Dastsooz et al., 2018). These variants of GM2 gangliosidoses are clinically indistinguishable, but are all associated with beta-hexosaminidase deficiency (Leal et al., 2020). All three variants are usually fatal by early childhood. Tay-Sachs disease, which is the more common of the GM2 gangliosidoses (0.5 in 100,000; Meikle et al., 1999), debuts around 6 months of age and results in death by the age of 4 years. Sandhoff disease (0.25 in 100,000; Meikle et al., 1999) results from LoF mutations in the *HEXB* gene on chromosome 5, critical for the lysosomal enzymes beta-N-acetylhexosaminidase A and B. The GM2 AB variant

is caused by gene mutations causing cofactor GM2 activator deficiency leading to lack of the normal beta-hexosaminidase A. With no authorized treatments available the current standard of care for GM2 gangliosidosis is limited to supportive care aimed at providing adequate nutrition and hydration. So far, no clinical gene therapy trials have been conducted in GM2 gangliosidoses. However, several pre-clinical approaches with intracerebral co-administration of AAV1, scAAV9.47, and AAVrh8 vectors have been used in Sandhoff disease mice and cats and in non-human primates to transfer the genes for the beta-hexosaminidase α and β subunits, which resulted in increased lifespan, reduced GM2 ganglioside levels, and improved motor functions (Cachon-Gonzalez et al., 2018; Leal et al., 2020). Recently, IV administered gene transfer of both the α and β subunits was performed using a bicistronic ssAAV9-HexBP2A-HexA vector, again leading to increased lifespan, reduced GM2 ganglioside brain levels, and improvement in motor performance in Sandhoff disease mice (Woodley et al., 2019). Furthermore, intracerebral administration of AAVrh8 vectors encoding the α and/or β subunits showed therapeutic effect in a Tay-Sachs disease sheep model, with slowing of disease progression and reversal of ganglioside accumulation (Gray-Edwards et al., 2018). In contrast, neurotoxic effects were observed in normal macaques after bilateral intra-thalamic infusion of a combination of two AAVr8 vectors encoding α and β subunits at three tested doses, suggesting that species differences exist with regards to effects of gene therapeutic regulations of *HEXA/HEXB* (Golebiowski et al., 2017). Nonetheless, two phase 1/2 clinical trials are currently recruiting GM2 patients for intrathecal treatment with an AAV9 carrying *HEXA* and *-B* genes (TSHA-101; NCT04798235) or bilateral intrathalamic and dual intracisterna magna/intrathecal administration of a mixture of AAVrh8-HEXA and AAVrh8-HEXB vectors (NCT04669535).

DISCUSSION

Great progress in gene therapy has been made over the last decade. From the approvals of Luxturna® (voretigene neparvovec), the first gene therapy against inherited eye diseases, Zolgensma® (onasemnogene abeparvovec) as treatment for SMA, and Libmeldy® for MLD, we stand on the brink of gene therapy to deliver on its promise to potentially cure or modulate severe diseases. The increasing number of development projects and progress highlighted in this review offers reasons for optimism toward novel gene therapy treatments being approved for rare genetic diseases of the brain and spinal cord in a foreseeable future. Fully aligned with these expectations the FDA has proclaimed that they expect to be approving 10–20 cell and gene therapies a year from 2025[6], and it is likely that some of these will fall within rare genetic diseases of the brain and spinal cord. However, for this to happen it is essential to obtain an even better understanding of the biology, pharmacodynamics, and safety of the applied systems. This became increasingly evident during the 1990s with the occurrence of firstly a fatal case of acute immunogenicity induced by a human adenovirus (type 5) vector in an ornithine transcarbamylase trial (Raper et al., 2003), and secondly genotoxicity induced by treatment with a gamma-retro virus vector in SCID-X1 (Hacein-Bey-Abina et al., 2003). These events were not expected but have had far reaching effects not only on the affected patients but also on the trajectory of gene therapy research and clinical trials. This has underlined the requirements and importance of both short-term and long-term monitoring of efficacy and safety aspects (Wilson, 2009; Somanathan et al., 2020). Recently we were once again reminded of the intrinsic uncertainties in drug development, e.g., with the Audentes Therapeutics gene therapy for treatment of X-linked myotubular myopathy being cleared to resume by the FDA after it was temporarily stopped for carefully reviewing the cases of unexpected deaths[7] (Nature Biotechnology, 2020), suggesting that studies involving high systemic doses of AAV vectors should be carefully monitored for similar toxicities (Hinderer et al., 2018). However, even intracerebral AAV administration may also cause potential risks as seen in Lysogene's phase 2/3 trial for MPS IIIA (AAVance) where an unexpected death has presently put this trial on hold, as described above. Since not all adverse events are discovered shortly after the treatment, and some could occur only many years later, long-term follow up studies are required to gain the full understanding of the gene therapy safety profiles. Thus, a long-term study over up to 10 years in dogs treated with AAV vectors systemically for hemophilia A showed that the AAV vectors inserted its own genome into genes of the dogs associated with cell growth that could potentially lead to malignancy (Nguyen et al., 2021). Still the progress made has shown that the current wave of gene therapies being developed appears safer and more promising than its predecessors (Bulaklak and Gersbach, 2020).

Most types of gene therapy up till now use the *in vivo* approach of administering vectors that transfer DNA sequences into cells of the nervous system. Nonetheless, *ex vivo* modified re-implanted genetically modified stem cells have shown promising results as evidenced by the EMA approval of Libmeldy® for MLD and completed or ongoing clinical trials in ALD and MLD. So far, genome editing holds an unredeemed potential in treating rare genetic diseases of the brain and spinal cord providing improved symptomatic alleviation or in some cases even a curative potential. Most progress has been seen with MPS I and MPS II, where *ex vivo* and *in vivo* genome editing strategies are promising and have even reached clinical testing (**Table 2**). Here, we have until now seen the ZFNs being utilized rather than CRISPR/Cas9 even though the latter can be more easily designed and implemented, which could be due to the concerns associated with the off-target effects seen with CRISPR/Cas9 (Merkle et al., 2015). In addition to potential viral vector-specific risks described above, Cas9-based *in vivo* genome editing also raises concerns as to potential immunogenic responses to a bacterial protein (Wang et al., 2015, 2020). Nonetheless, considering that rapid progress is being made with development and testing of new genome editing platforms (Poletto et al., 2020), it is likely that genome editing

[6] *https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics*

[7] *https://www.audentestx.com/press_release/audentes-therapeutics-announces-fda-lifts-hold-on-aspiro-clinical-trial-of-at132-for-treatment-of-x-linked-myotubular-myopathy-xlmtm/*

in the future will become relevant as a treatment strategy for several genetic CNS disorders (Lubroth et al., 2021). This would, however, require better understanding and solutions toward safety concerns related to treatment-induced immunogenicity (Shim et al., 2017) and expanded control of on- and off-target modifications (Mills et al., 2003).

Expanding the scope to include more broadly gene-affecting treatments, we could expect to see more development programs and approvals within gene therapy and ASOs. First line of successes appears to fall within gene replacement in diseases caused by LoF mutations as seen with Zolgensma® (onasemnogene abeparvovec) in SMA1, where we have also appreciated the viable ASO strategies affecting RNA-splicing to boost expression of healthy protein as seen with Spinraza® (nusinersen). ASOs in general have the advantage that they exert a transient effect which provides security in case of unforeseen safety issues, and the development paradigm of N-of-1 treatment has even shown that it can be advanced to clinical testing rapidly for compassionate usage, as evidenced with the success of milasen in CLN7 Batten disease developed within 1 year (Mullard, 2020). On the other hand, it does not hold a disease-modifying potential and renders the patient with life-long need for taking medication. Therefore, it is likely that gene therapy using viral DNA vectors encoding shRNA/siRNA/miRNA/antisense RNA will prove to be better suited for obtaining a more stable long-lasting effect without the need for repeated central injections as seen with ASOs. Already AAV vectors are being tested in clinical trials to reduce production of pathologic proteins resulting from GoTF mutations for ALS (AAVrh10-antisense-SOD1) and HD (AAV5-miHTT).

For the genetic diseases which are often inherited and with an early-onset of symptoms, it is crucial to initiate treatment as early as possible (often within the first year of life) in order not to lose valuable time and treatment opportunities (Gessler and Gao, 2016; Al-Zaidy et al., 2019a,b). For instance, in SMA, animal models strongly suggest a critical window for gene therapy to achieve effective rescue, and clinical trials on children with SMA1 show improved outcome when gene therapy is performed earlier in the disease course (Robbins et al., 2014; Govoni et al., 2018). The same was found in *post-hoc* analysis where the biggest motor improvements were obtained in infants with SMA1 treated at an early age, highlighting the value of newborn screening and early treatment (Lowes et al., 2019). Similarly, in CD, phenotype rescue is better in patients treated at a younger age (Gessler et al., 2017), and in MLD *ex vivo* gene therapy appears to predominantly work on pre-symptomatic or early-symptomatic patients (Penati et al., 2017).

To ensure development of treatments for rare disease, including gene therapies, biopharmaceutical companies are incentivized with the prospect of fast-track designations and longer market exclusivity. Nonetheless, current and future gene therapies are coming with very high prices, many-fold higher than for other treatment modalities, which is justified by the higher development costs and small market of rare diseases. This will most likely limit the number of patients being offered new treatments even though there is evidence of efficacy and safety. This also means that the biopharmaceutical companies will be forced to build strong cases on how they can provide value for money to receive market authorization and recommendations for public or insurance reimbursement differing from country to country. In the case of Zolgensma® for SMA, the total costs range from 4.2 to 6.6 million dollars (Malone et al., 2019), which could carry to high a cost to be offered to larger numbers of patients, especially in countries with tax-funded public health care systems and strong prioritization and decision-making authorities.

To evaluate the cost-benefit of Zolgensma® compared to Spinraza®, a model was created based on data from the clinical trials START (AVXS-101-CL-101) and ENDEAR (nusinersen). As the AVXS-101-CL-0101 trial had no deaths, life expectancy of patients was estimated based on the motor milestones achieved - Patients who achieved sitting were aligned with the survival of SMA type 2 patients who sit but never walk, and patients treated who achieved walking aligned with SMA type 3 patients, who have normal life expectancy (Malone et al., 2019). From the modeling and simulations based on parametric curves fitted to estimate the probability of patients dying during each model cycle, it was assumed that 50% of SMA patients treated with Zolgensma® will survive until the age of 35, while the corresponding simulation applied to the ASO Spinraza® showed only 50% survival to the age of 3 years (Malone et al., 2019). An increasingly common way to evaluate novel treatments is by applying the quality adjusted years (QALY) analysis, and in a comparative case between Zolgensma® and Spinraza® this yielded QALYs of 15.65 and 5.29, respectively (Malone et al., 2019).

These high costs should be evaluated in the prospects of increased QALY and lowered medicine and hospitalization expenses. Furthermore, the benefits and ethical considerations of allowing patients who would have died in early childhood to live well into adulthood are to be considered. The calculations above are based on simulations on current data since treatment has only been available for a few years and long-term data and results remain to be seen (Malone et al., 2019). Interestingly, we have seen new and atypical ways of drug development, as in the case of N-of-1 and the ASO treatment for Batten CLN7 disease, where it was possible to raise money on an individual basis. It will be important that developers and payers work together on new ways of orchestrating models of pricing and reimbursement to ensure that gene therapy treatments reach the patients in need.

In conclusion, we expect that gene therapy will become increasingly relevant for rare brain and spinal cord diseases in the coming future. Considering that the vast majority of medical treatments available for diseases of this review offer merely symptomatic alleviation without targeting the underlying pathological etiology, approval of more gene therapies by regulatory authorities could become game changers for patients affected by rare diseases. This also highlights the potential of a paradigm shift where we move from symptomatic alleviation to disease modification and even cure.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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