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Intravenous Infusion of AAV for Widespread Gene Delivery to the Nervous System

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Abstract

The central nervous system (CNS) is a fascinating and intricate set of biological structures that we have yet to fully understand. Studying the *in vivo* function of the CNS and finding novel methods for treating neurological disorders have been particularly challenging. One difficulty is correcting genetic disorders afflicting the CNS in a targeted manner. Recombinant adeno-associated viruses (rAAVs) have emerged as promising therapeutic tools for treating genetic defects of the CNS, due to their excellent safety profile and ability to cross the blood-brain barrier (BBB). While stereotactic injection of AAV is promising for localized gene delivery, it is less desirable for some applications because of the technique's invasiveness and limited intraparenchymal spread.

¹Long-term expression of transgenes can be blocked by the host immune response. The transgene product can be recognized as nonself, triggering the activation of both humoral and cell-mediated immune responses. This is both problematic for completely foreign gene products, as well as those that are mutated forms of endogenously expressed proteins. Transgene cassettes that carry miR-BS to recruit the antigen presenting cell (APC)-enriched miR-142, thereby blocking expression in dendritic cells, have shown great promise [50, 51]. Such constructs are within the size limitations of rAAV and are compatible with ubiquitous or CNS-specific promoters that can achieve high-level expression.

³There is strong evidence that many standard animal models harbor preexisting antibodies against AAV serotypes AAV1, AAV2, AAV6, and AAV9 [53]. Neutralizing antibodies (NAbs) against AAVs are present in the sera of mice, rats, rabbits, dogs, sheep, and pigs. The CNS has traditionally been considered an immune privileged organ. However, autoimmune diseases such as multiple sclerosis suggest that the CNS is not completely protected from inflammation [55]. Because targeting the CNS via IV injection will expose animals to vector on a systemic level, immune response should be heavily considered. Therefore, prescreening of animals for NAbs is recommended to assess vector effectiveness in all animal subjects, regardless of species. Of note, we and others have found that even NAb titers between 1:2 and 1:5 can prevent *in vivo* transduction by intravascular AAV9 [53]. Several studies have focused on developing novel capsids that can efficiently evade host immunity. High-throughput human IgG screening via directed evolution of capsids, in combination with the development of humanized liver mouse models, has identified capsids that can transduce human liver as well as exhibit low immunogenicity [56]. However, a capsid that can both bypass the BBB and evade the human host immune system has yet to be developed.

Alternatively, intravascular administration can achieve widespread delivery of rAAV to the CNS. In this chapter, we will discuss the prevalent routes of administration to deliver rAAV to the CNS via intravenous (IV) injection in mice. We will highlight key considerations for using rAAV, and the advantages and disadvantages of each administration method. We will also briefly discuss intravenous delivery in larger animal models, factors that may impact experimental interpretations, and outlooks for clinical translation.

Keywords

CNS; Mouse; Adeno-associated virus; Blood-brain barrier; Neutralizing antibodies; AAV vector design

1 Introduction

1.1 General Introduction

The central nervous system (CNS), which consists of the brain and spinal cord, is arguably the most intricate organ in vertebrates. Its diversity of subanatomical structures and variety of specialized and subspecialized cell types define the complex nature of this system of tissues. The high-level organization of the CNS presents many difficulties in studying CNS function and understanding neurological diseases. While certain in vitro models may recapitulate some aspects of CNS function, full understanding of the CNS requires study in vivo. This notion is particularly true for investigating intercellular mechanisms of the CNS and the development of therapies for neurologic diseases.

Recombinant adeno-associated viruses (rAAVs), which are derived from apathogenic, non-genotoxic parvoviruses that are gutted of their natural DNA genomes, have not only become a powerful tool for studying the brain, but are also among the most promising therapeutic vehicles for treating genetic diseases that affect the nervous system. A clinical trial using systemic delivery of rAAV to treat spinal muscular atrophy (SMA) in infants has shown remarkable safety and therapeutic efficacy [1]. Although there is substantial recent excitement surrounding the potential for rAAVs and their use as biotherapies, there are still challenges associated with their use in treating neurological disease. In addition, there are many factors that investigators must consider in the design of vectors and their mode of delivery in order to efficiently transduce tissues of the CNS.

Stereotactic intracerebroventricular (ICV) and intrathecal (IT) injections to deliver rAAV via the cerebrospinal fluid (CSF) for widespread distribution to the CNS, or direct intraparenchymal stereotactic administration, are the preferred methods of delivering rAAV to specific brain regions. Although these approaches may be advantageous for diseases with local CNS pathology, intraparenchymal and ICV injections are invasive, and it may not be desirable to only transduce a restricted area of the brain. For example, in disease models where rAAV requires targeting of both CNS and peripheral tissues (e.g., liver, spleen, kidney, skeletal muscle), single intravenous (IV) infusions under a ubiquitous promoter may be preferred. Notably, many disorders, such as spinal muscular atrophies, neuronal ceroid lipofuscinoses, and spinocerebellar degenerations, require widespread treatment of the entire

CNS [2–4]. Additionally, neurological disorders may display a primary CNS phenotype, but also present a secondary etiology that affects the entire organism (CNS and peripheral organs). Intravascular delivery in these cases, as well as in their preclinical model counterparts, has substantial advantages. Utilizing the naturally existing vasculature, IV delivery of rAAV can achieve widespread CNS transduction and deliver therapeutic vectors to the entire CNS.

Although IV administration is minimally invasive and simultaneously targets both peripheral organs and tissues of the CNS, intravascular delivery unfortunately also has its own set of challenges. Namely, the ability of intravenously administered drugs to efficiently reach the CNS is hindered by the blood-brain barrier (BBB). This is less of a challenge for certain drugs (e.g., small molecules and compounds with ideal hydrophobicity and lipophilicity profiles [5]), but is a substantial barrier for large hydrophilic molecules (e.g., oligonucleotides, genes, proteins, and viruses) [6, 7]. Furthermore, attempting to target the CNS through intravascular delivery leads to a wider distribution of rAAV, which may carry the risk of off-target gene delivery to the tissues of peripheral organs, inducing potential immunotoxicities.

A chief advantage of rAAVs as therapeutic vectors is that some AAV serotypes are capable of crossing the BBB and transducing the CNS after IV delivery with relatively high efficiencies, especially when administered to neonatal animals [8–10]. We will discuss three modes of IV rAAV delivery to target the CNS: facial vein, retro-orbital, and lateral tail vein. Importantly, we will outline considerations prior to any in vivo experiment. We will primarily focus on experimental considerations in mouse models, as the mouse is the most commonly used animal model for in vivo gene transfer via rAAV administration. It should be noted that CNS gene transfer by some rAAV vector serotypes is largely consistent between rodents and large animals, but enough interspecies differences in transduction efficiency among serotypes have been reported to warrant careful consideration [11].

1.2 Natural and Engineered AAV Capsids

This section, along with Subheadings 1.3 and 1.4, will cover vector design considerations that must be tailored for the investigator's specific experimental aims. In addition, the researcher should take note that the rAAV gene therapy field is rapidly evolving. Below, we highlight concepts that stand out as critical factors for vector design for the explicit purpose of transducing the CNS via IV administration. The details herein are best used as a general starting guide for researchers that are new to this field.

One valuable property of AAV is the inherent capsid diversity observed in naturally occurring AAV. This advantage has led to the discovery and design of new AAV capsids with improved tissue tropism and potentially reduced immunogenicity [12]. New capsids can be developed by three existing strategies: (1) natural discovery of AAV capsids from pro-viral DNA isolated from host species [13, 14], (2) directed evolution [15, 16], and (3) rational capsid design, which includes the conversion of residues to paralogous motifs that are unique to other serotypes to gain different tropism profiles [17, 18], and the fusion of peptide sequences to the capsid structure to alter tissue tropism [19–22] (*see* Chapter 3).

Multiple AAV serotypes and variants have been extensively evaluated under a variety of administration parameters. The first widely used AAV serotype, AAV2, does not efficiently cross the BBB. Early studies utilized direct injection of rAAV into the parenchyma or ventricles to target the CNS, resulting in limited CNS distribution and regional transgene expression [23, 24].

The discovery of new serotypes has opened the door to vectors with improved ability to cross the BBB, providing an opportunity to treat CNS diseases via intravenous delivery of therapeutic rAAVs that achieve long-term expression in large areas of the brain. Several AAV serotypes have been shown by different research groups to cross the BBB [13, 19, 25–28]. In fact, with the exception of rAAV2 and rAAV5, it has been demonstrated that the capacity to cross the adult BBB is a common feature among natural AAV serotype isolates [26, 27]. After crossing the BBB, serotypes can display different tropisms [27]. Certain capsids stand out for efficient CNS transduction following systemic IV injection. Notably, rAAV9, rAAVrh.8, rAAVrh.10, rAAVrh.39, and rAAVrh.43 demonstrate robust global transduction of glial and neuronal cell types within the hypothalamus, medulla, striatum, hippocampus, choroid plexus, caudate-putamen, corpus callosum, and substantia nigra [26, 27, 29], with variable transduction in the cortex, habenular nucleus, cornu ammonis, dentate gyrus, thalamus, cerebellum, and olfactory bulb.

In adult animals, rAAV9 transduces mostly astrocytes and brain endothelial cells [10, 26]. rAAVrh.10 vectors can also efficiently transduce a range of cell types of the CNS, including neurons. rAAV1 transduces primarily granule cells in the cerebellum, while rAAV6 and rAAV6.2 transduce mostly Purkinje cells [27]. Of note, rAAVrh.10, rAAV9, rAAV7, and rAAVrh.39 show high transduction efficiencies in motor neurons [27]. However, these vectors have very broad tropisms that extend beyond neuronal cell types. In contrast, rAAVrh.8 exhibits naturally lower peripheral tissue dissemination than other AAV serotypes, particularly in the liver, enhancing the safety profile of systemic gene delivery to the CNS [26].

Several groups have taken a directed evolution approach to develop capsids that can cross the BBB with efficiencies greater than natural isolates. By applying selective pressure to a library of artificially created AAV capsid variants, novel vectors that cross the BBB and transduce specific cell types of the murine brain can be developed. Using such methods, Deverman et al. engineered the nonnatural AAV capsid AAV-PHP.B, which has the capacity to transduce astrocytes, neurons, oligodendrocytes, cerebellar Purkinje cells, and several interneuron populations across multiple brain regions after IV administration, and demonstrates greater CNS transduction and reduced peripheral transduction relative to AAV9 [19]. Interestingly, Hordeaux et al. recently demonstrated that AAV-PHP.B underperforms relative to AAV9 in another commonly used mouse strain, BALB/cJ, as well as in nonhuman primates [30]. The authors argue that a directed evolution approach that selects high performance capsids in the C57BL/6 J mouse strain may introduce strain-specific properties.

Extra caution should be considered when utilizing capsids selected or tested exclusively in one model system. All rAAVs delivered intravascularly to target the CNS should first be

tested with standard gene reporter systems, such as EGFP or luciferase, before proceeding with the transgene of interest.

1.3 Regulatory Elements for Controlling Gene Expression

A major drawback among current rAAVs that have strong tropism for CNS is their lack of CNS-restricted tropism. For example, rAAV9 also confers robust transduction of peripheral tissues such as liver, heart, lungs, muscle, and pancreas [31–33]. To tailor rAAV-delivered gene expression for the intended therapeutic target, the use of tissue- or cell type-specific promoters, as well as tissue-specific miRNA-mediated de-targeting elements, are attractive vector design strategies.

Adaptation or native promoter sequences for tissue- or cell type-specific expression of vector transgenes may be preferred over ubiquitous and constitutively active promoter cassettes. Both muscle- or liver-specific expression have strongly relied on the use of natively derived promoters such as muscle creatine kinase (CKM) or thyroxine-binding globulin (TBG) promoters [34, 35]. Multiple CNS cell type-specific promoters are available and have been utilized to limit transgene expression to certain cell types (Table 1). However, further testing of promoters is often required. Common challenges for promoter and rAAV compatibility are: (1) the limited availability of well-described regulatory cassettes for CNS-specific expression, (2) the packaging capacity of AAV, which requires that the transgene expression cassette, including promoter, transgene, polyA sequence, and other auxiliary elements, be below a limit of 4.7 kb, (3) changes in the metabolic state of the cell and environmental dynamics that may alter promoter activities, and (4) the fact that promoters which appear specific for one CNS cell type after brain injection may also express in a subset of peripheral tissues after IV delivery. Optimization of CNS-specific rAAV regulatory cassettes is complicated by the diversity of cell types in the CNS, with new cell types continuing to be identified [36–38]. The CNS can be divided into four main cell types: (1) neurons, (2) astrocytes, (3) oligodendrocytes, and (4) microglia. Current knowledge of gene regulation in the diverse cell populations of the CNS is incomplete, and the design and selection of promoters that can truly confer cell type-specific transgene expression is an ongoing challenge.

Many cell- or tissue-specific promoters fail to achieve experimental or therapeutic levels of transgene expression. In such cases, strong ubiquitous and constitutively active regulatory cassettes, such as the CMV-enhancer/chicken β -actin (CBA) promoter, are popular in rAAV vector design. Additionally, no cell- or tissue-specific promoter is guaranteed to be devoid of leaky off-target activation. The suppression of peripheral organ expression in these situations can be achieved by miRNA-mediated de-targeting elements [39]. This strategy involves modification of the rAAV transgene by incorporating miRNA binding sites (miR-BS) into the 3'-UTR to recruit endogenous cell type-specific, highly expressed miRNAs to inhibit expression. For example, to mitigate potential protein toxicity from unintentional transduction of peripheral organs and from leaky promoter activity, miR-BSs designed to recruit miR1 or miR122 to exclude expression from striated muscle and liver, respectively [40], can be introduced. Such designs have shown remarkable ability to specifically de-target expression from peripheral organs while maintaining expression in the CNS [8,40].

Importantly, to circumvent innate immunity, vector design incorporating miR-BSs that utilize miRNAs present in antigen presenting cells (APCs) such as miR-142 have also been used with success (*see* Note 1) [41,42].

1.4 Dose and Timing

Intravenous administration of rAAVs circulates the virus through the vascular system. Therefore, rAAV is disseminated throughout the peripheral organs, substantially reducing the number of rAAV particles that can reach and cross the BBB. Transduction efficiency of the CNS is therefore intrinsically dose dependent [29, 32]. Intravenous delivery at high doses might lead to transgene expression in undesirable cell types, potentially causing unwanted side effects. Dose also seems to influence the transduction pattern of subanatomical CNS regions. While a high dose such as 2×10^{14} vector genomes per kilogram (vg/kg) is efficiently able to achieve widespread transduction and has been successfully translated into humans, a lower dose (2×10^{12} vg/kg) may only reach CNS regions close to blood vessels, such as choroid plexus [1].

Permeability for certain molecules across the BBB seems to be age-independent, and differences between the neonatal and the adult BBB have been suggested to influence IV delivery of AAV. The transduction efficiency of rAAVs is dependent on the interplay between cellular and humoral host factors and capsid properties. During growth and development, the physiological profile of the BBB changes, and expression of molecules facilitating or preventing AAV from crossing the BBB may change as well [43]. Age-dependent differences in CNS transduction efficiency and cellular tropism [10, 26, 27, 44] observed after IV administration of rAAVs support this notion. Notably, pre-infusion with mannitol has been shown to increase permeability of the BBB for AAV2 in adult mice [45]. Additionally, certain diseases require early intervention and thus neonatal administration might be preferred. However, because AAV genomes predominantly persist in the nucleus as nonreplicating episomes [46], growth of the host tissue may dilute the transgene and reduce vector potency during normal aging. This is a problem for neonatal targeting of organs that consist of cells with high replicative rates, such as liver, but is less of an issue for the CNS.

With these considerations in mind, it is particularly important to conduct systematic dose assessments at suitable ages, to determine which conditions confer the desired strength and distribution of gene expression for each rAAV vector.

2 Materials

1. Alcohol prep pads.
2. 70% isopropyl alcohol.
3. Absorbent gauze pads.

¹Long-term expression of transgenes can be blocked by the host immune response. The transgene product can be recognized as nonself, triggering the activation of both humoral and cell-mediated immune responses. This is both problematic for completely foreign gene products, as well as those that are mutated forms of endogenously expressed proteins. Transgene cassettes that carry miR-BS to recruit the antigen presenting cell (APC)-enriched miR-142, thereby blocking expression in dendritic cells, have shown great promise [50, 51]. Such constructs are within the size limitations of rAAV and are compatible with ubiquitous or CNS-specific promoters that can achieve high-level expression.

4. Isoflurane.
5. Anesthesia machine.
6. Anesthesia chamber and nose cone.
7. 50 mL conical tube.
8. Mouse Tail Illuminator restrainer.
9. Ice.
10. rAAV vector.
11. Filter-sterilized PBS.
12. Neonatal (0–2 days) or adult mice.
13. 0.3 mL 31G insulin syringes.
14. 0.5 mL 28G insulin syringes.
15. Padded injection platform.
16. Heating pads or hand warmers.
17. Eye ointment.

3 Methods

In this section, we will focus specifically on techniques utilized for mouse models, since they are currently the standard and first-pass in vivo system for preclinical investigation of therapeutic rAAVs. There are three standard approaches for IV delivery to target rAAV to the mouse CNS: facial vein (Subheading 3.2), retro-orbital (Subheadings 3.3 and 3.4), and lateral tail vein (Subheading 3.5). Each method has advantages and disadvantages, and preference is primarily dependent on the age of mice at treatment. For neonatal treatments, the superficial temporal/facial vein is visible and accessible posterior to the eye. In adults, lateral tail vein administration is more convenient. Retro-orbital injection can be performed at all ages, and is required in adult disease animal models that must be treated at young ages when tail vein administration is not possible.

Translation to larger animals requires additional considerations (*see* Note 2).

Several aspects hinder extrapolation of rAAV function from mice to larger animal models: (1) preexisting immunity in large animals that necessitate prescreening for neutralizing antibodies (NAbs) (*see* Note 3), (2) inherent differences in humoral or cellular immunity

²Although rAAV tropism profiles are often recapitulated across strains of mice and different species, differences in tropism between mammalian species do exist. Even strain-to-strain differences can impact experimental interpretation. For example, AAVPHP.B demonstrated robust CNS transduction in the C57/BL6 strain after IV administration, but comparatively low CNS transduction in the BALB/cJ strain [30]. Additionally, PHP.B also demonstrates weak BBB permeability in nonhuman primates (NHPs), including marmosets and rhesus macaques [30, 52]. Tropism differences are often explained by differences in immunity (*see* Notes 1 and 3). Of note, the impact of NAbs against current AAV serotypes is lowest in rat and sheep [53]. Consequently, studies to validate the translational potential of rAAVs warrant evaluation in model systems that best recapitulate the human patient, such as nonhuman primates. However, it should be noted that transgene-directed immune responses can be mounted against rAAV-delivered transgenes that produce foreign or human proteins in NHPs [54].

between species (*see* Note 1), (3) differences in brain size, (4) longevity of transduced vectors, (5) faithful translation of disease models, and (6) genomic diversity of outbred strains common with large animal models. These factors should be carefully considered before translation to larger animals, and steps taken to minimize the risk of immune response and toxicity (*see* Notes 4 and 5).

3.1 Preparation of rAAV Vectors for Injection

Recombinant AAV vectors can be produced in-house by triple transfection of human embryonic kidney cells (HEK293) [47] (*see* Chapters 3, 7, 19, 21, 22, and 23). Alternatively, vectors can be obtained from a local vector core or commercial manufacturer. High-quality rAAV vector formulations for injection should be free of contaminants that might harm animals, such as bacteria, endotoxins, or cellular proteins. Low abundance of empty virions in preparations is also desirable. Analysis of rAAV purity by silverstained SDS-PAGE and assessment of empty particles by electron microscopy is recommended before sample preparation for injection. In addition, newer methods for assessing vector heterogeneity such as Adeno-Associated- Virus Genome Population sequencing (AAV-GPseq) and sequencing of single-stranded DNA virus preparations (SSV-Seq) (*see* Chapter 5), which employ highthroughput sequencing to quantify and identify the abundance of contaminating DNAs or truncated genomes, are becoming the standard for many quality control pipelines to ensure genomic homogeneity in vector preps [48, 49].

³There is strong evidence that many standard animal models harbor preexisting antibodies against AAV serotypes AAV1, AAV2, AAV6, and AAV9 [53]. Neutralizing antibodies (NAbs) against AAVs are present in the sera of mice, rats, rabbits, dogs, sheep, and pigs. The CNS has traditionally been considered an immune privileged organ. However, autoimmune diseases such as multiple sclerosis suggest that the CNS is not completely protected from inflammation [55]. Because targeting the CNS via IV injection will expose animals to vector on a systemic level, immune response should be heavily considered. Therefore, prescreening of animals for NAbs is recommended to assess vector effectiveness in all animal subjects, regardless of species. Of note, we and others have found that even NAb titers between 1:2 and 1:5 can prevent *in vivo* transduction by intravascular AAV9 [53]. Several studies have focused on developing novel capsids that can efficiently evade host immunity. High-throughput human IgG screening via directed evolution of capsids, in combination with the development of humanized liver mouse models, has identified capsids that can transduce human liver as well as exhibit low immunogenicity [56]. However, a capsid that can both bypass the BBB and evade the human host immune system has yet to be developed.

⁴Immune tolerance can be achieved by delivery of rAAV to the liver to harness its tolerogenic nature [57]. Hepatocytedirected targeting induces antigen-specific regulatory T cells (Tregs) to modulate immune tolerance [58–60]. Although the exact mechanism underpinning immune tolerance by the liver is not fully understood, this method has been shown to be highly effective [57, 61]. Liver APCs, which play roles in the induction and maintenance of tolerance, consist of nonconventional hepatic APCs that include Kupffer cells (KCs), liver sinusoidal endothelial cells (LSEC), and hepatic stellate cells that express low levels of major histocompatibility complex (MHC)-I/II and co-stimulatory molecules [57]. T lymphocytes are also found within the liver, but are much different than those found in lymph nodes, spleen, and peripheral blood. For CNS transduction, stimulation of immune tolerance has many practical applications. For example, in the mouse model of Pompe disease, investigators showed that liver-directed AAV prevented production of antibodies against the transgene, acid alpha-glucosidase (GAA) [62]. Transduction of both liver and CNS to simultaneously induce tolerance and express the enzyme in neuronal tissue may perhaps be the most effective method to ensure sustained transgene expression in the CNS after IV delivery [57], so long as the transgene is not toxic to the liver. Investigators interested in exploring immune tolerance should be cautioned that strain-to-strain differences can impact results. For example, C57BL/6 mice demonstrate transgene tolerance after systemic AAV delivery, but the same is not true for BALB/c mice, which show impaired tolerance. Studies conducted in dogs and nonhuman primates (NHP) show that AAV-mediated liver-induced tolerance is dependent on the transgene and its species of origin [57].

⁵Systemic AAV9 administration has achieved successful motor neuron transduction in a variety of animal models. However, recent studies testing AAV9 variants in NHPs and piglets have raised concerns for potential toxicity. Hinderer et al. demonstrated that administration of 2×10^{14} vector genome copies per kilogram body weight of an AAV9 variant (clade F) vector resulted in severe toxicity with marked transaminase elevations [63]. Histological lesions within the central and peripheral nervous system were also observed. Interestingly, the authors found no evidence for T-cell responses to the vector capsid or transgene product in NHPs, suggesting that the observed toxicity was independent of an immune response to the capsid or transgene product. However, it was surmised that vector-related activation of cellular stress led to hepatocyte damage and subsequent activation of systemic inflammation. Piglets demonstrated no evidence of hepatic toxicity but exhibited proprioceptive deficits and ataxia [63]. The mechanistic cause for vector-related adverse effects was not entirely explored, but these findings highlight concerns that the risks of high-dose IV administration to target the CNS is still not fully understood. Researchers are encouraged to explore the extent that cellular or systemic toxicity may impact experimental interpretations regardless of the AAV capsids or species utilized.

3.2 Facial Vein Injection (Fig. 1)

1. Thaw rAAV vector(s) (if previously frozen) and keep on ice.
2. Calculate the total amount of vector needed for experiments and prepare enough volume for 1 extra pup. 25–50 μL of vector can be injected per pup. Use filter-sterilized $1 \times \text{PBS}$ to dilute to the desired dose, if needed (*see Note 6*).
3. Load 25–50 μL of AAV into a 0.3 mL 31G insulin syringe.
4. Separate neonates from the dam.
5. Working with one pup at a time, immobilize neonates by placing them on a chilled paper towel on top of ice for 30–60 s. Wait until the pup's color takes on a bluish hue and the animal exhibits less movement. Avoid prolonged exposure to ice to avoid mortality.
6. Alternatively, add 0.5 mL of isoflurane to tissue paper in an anesthesia chamber (50 mL conical tube) and tighten the cap to allow isoflurane to vaporize for 1 min. Hold the chamber horizontally, place a pup inside, and tighten the cap. Remove the pup immediately once it has completely stopped moving. Before proceeding, check that the pup is alive by checking for rise and fall of the rib cage.
7. Hold the pup by the thumb and index finger (of nondominant hand) and gently spread the skin of the area of the injection site to visualize the facial vein (Fig. 1a). Avoid exerting excessive pressure on the pup to avoid crushing the animal.
8. Sterilize the injection site with an alcohol pad.
9. Support elbows on a stable surface. Grip the syringe with the thumb, middle finger, and ring finger. Poise the index finger over the plunger.
10. Position the needle tip at a $\sim 10^\circ$ angle over the superficial temporal vein, partially insert the needle tip into the vein, adjust the angle of the needle to $0\text{--}5^\circ$, slowly insert the needle approximately 3 mm into the vein track, and slowly inject the 25–50 μL vector volume (Fig. 1b).
11. Wait 5 s, slowly withdraw the needle, and immediately compress the injection site using an absorbent gauze to stop any bleeding. A successful injection will show a black blemish at the point of injection (Fig. 1c).
12. Ensure the pup's body temperature and color return to normal. Dampen tissue paper with 70% EtOH and gently rub the pup (Fig. 1d). Then rub the pup with used bedding to mask foreign odors that may lead to rejection of the pup (Fig. 1f).

⁶One major drawback for rAAV administration to neonatal mice via facial vein or retro-orbital injection is that the total delivery volume should never exceed 100 μL (25–50 μL is recommended), thus relatively high-titer vector preparations ($\sim 10^{13}$ vg/mL) are required for the delivery of a high dose.

13. Place the cage on warm heating pad to aid neonate recovery. Rub the dam's nose with tissue paper dampened with 70% EtOH (Fig. 1f) and return the pup to the dam.

3.3 Retro-Orbital Injection of Neonates (Fig. 2) (See Note 7)

1. Thaw rAAV vector(s) (if previously frozen) and keep on ice.
2. Calculate the total amount of vector needed for experiments and prepare enough volume for 1 extra pup. 50 μ L of vector can be injected per pup (use filter-sterilized 1 $\times$ PBS to dilute to the proper dose).
3. Load 50 μ L of vector into a 0.3 mL 31G insulin syringe.
4. Separate neonates from the dam.
5. Working with one pup at a time, immobilize neonates by placing them on a chilled paper towel on top of ice for 30–60 s. Wait until the pup's color takes on a bluish hue and the animal exhibits less movement. Avoid prolonged exposure to ice to avoid mortality.
6. Alternatively, add 1 mL of isoflurane to tissue paper in an anesthesia chamber (50 mL conical tube) and tighten the cap to allow isoflurane to vaporize for 1 min. Hold the chamber horizontally, place a pup inside, and tighten the cap. Remove the pup immediately once it has completely stopped moving. Before proceeding, check that the pup is alive by checking for rise and fall of the rib cage.
7. Construct a padded injection platform to position the pup (Fig. 2a, b).
8. Place the pup in a lateral position and grasp the head with the thumb and index finger of the nondominant hand. Avoid exerting excessive pressure on the pup to avoid crushing the animal (Fig. 2c).
9. Sterilize the injection site with an alcohol pad.
10. Support wrists on a stable surface, and grip the syringe with the thumb, middle finger, and ring finger, with index finger poised over the plunger.
11. Place the needle bevel on the eye at a 3 o'clock position, insert approximately 3 mm at a 30° angle into the center of the retro-orbital sinus, and slowly inject the 50 μ L vector volume (Fig. 2d).
12. Wait 5 s and slowly withdraw the needle, keeping the bevel outward to protect the eye from being scratched. Immediately compress the injection site using absorbent gauze to stop any bleeding.

⁷There are several considerations for retro-orbital injections. First, anesthesia is required. Second, procedural frequency should be limited to one injection per eye per day, and there should be at least a 1-day interval between injections. The injection volume should never exceed 100 μ L per eye in neonates (50 μ L is recommended). Trauma to the eye and swelling around the injection site may be caused by incorrect placement of the needle. Once inserted into the retro-orbital sinus, it is essential that minimal movement of the needle occurs, in order to prevent vessels from rupturing, causing bleeding and loss of vector. Juvenile and adult mice can also be subjected to retro-orbital injections by following procedures outlined for neonates.

13. Ensure the pup's body temperature and color return to normal. Dampen tissue paper with 70% EtOH and gently rub the pup. Then rub the pup with used bedding to mask foreign odors that may lead to rejection of the pup.
14. Place the cage on a warm heating pad to aid neonate recovery. Rub the dam's nose with tissue paper dampened with 70% EtOH and return the pup back to the dam.

3.4 Retro-Orbital Injection of Adults (Fig. 3) (See Note 7)

1. Thaw rAAV vector(s) (if previously frozen) and keep on ice.
2. Calculate the total amount of vector needed for experiments and prepare enough volume for 1 extra mouse. 150–200 μ L of vector can be injected per mouse (use filter-sterilized 1 $\times$ PBS to dilute to the proper dose).
3. Load 150–200 μ L of vector into a 0.5 mL 28G insulin syringe.
4. Sedate the mouse with isoflurane in an anesthesia chamber. Wait until breathing slows, then retrieve the mouse from the chamber.
5. Keep the mouse under general anesthesia by using a nose cone to administer 2% isoflurane in medical-grade 21% oxygen at a flow of 2 L/min. Monitor movement and ensure there is no limb withdraw reflex to toe pinch. Grasp the mouse so that its back rests on the palm of the nondominant hand with its head toward the thumb. The thumb is placed just lateral to the animal's trachea and the fur on the animal's head is drawn toward the palm. This causes the animal's eye to bulge slightly (Fig. 3a).
6. Support wrists on a stable surface. Grip the syringe with the thumb, middle finger, and ring finger. Poise index finger over the plunger.
7. Under gentle pressure to bulge the eye, direct the needle into the medial canthus (junction of eyelids closest to the animal's nose) with the bevel facing outward (lateral toward globe). Insert the needle approximately 5 mm at a 45° angle into the center of the retro-orbital sinus and slowly inject the 150–200 μ L volume (Fig. 3b).
8. Withdraw the needle carefully and slowly. Ensure the needle does not scratch cornea. Use sterile gauze to stop any bleeding and apply ointment liberally to the eye to aid recovery (Fig. 3c).
9. After the injection, return the mouse to its cage. It will take approximately 1 min to recover from isoflurane.

3.5 Lateral Tail Vein Injection (Fig. 4)

1. Thaw rAAV vector(s) (if previously frozen) and keep on ice.
2. Calculate the total amount of vector needed for experiments and prepare enough volume for 1 extra mouse. 300 μ L of vector can be injected per mouse (use filter-sterilized 1 $\times$ PBS to dilute to the proper dose).

3. Load 300 pL of vector into a 0.5 mL 28G insulin syringe.
4. Place the mouse into the mouse tail illuminator restrainer apparatus (Fig. 4a), Do not over-restrain the mouse as this will constrict the animal from breathing.
5. Spray 70% isopropyl alcohol on the tail.
6. Hold the tail straight and taut (Fig. 4b). There are two lateral veins on each side of the tail (*see* Note 8).
7. Partially insert the needle tip at a 30° angle into the vein. Once the tip has penetrated the vein halfway up the bevel, readjust the needle so that it is in-line with the length of the vein (Fig. 4c). If the needle is inserted properly, there should be no pressure when the syringe plunger is depressed.
8. Inject the 300 µL volume slowly. The vein will blanch. After approximately 4 s the vein color will turn to normal.
9. Carefully withdraw the needle and compress the injection site with absorbent gauze for 10 s to stop any bleeding.
10. Return the mouse to its cage.

References

1. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, Lowes L, Alfano L, Berry K, Church K, Kissel JT, Nagendran S, L'Italien J, Sproule DM, Wells C, Cardenas JA, Heitzer MD, Kaspar A, Corcoran S, Braun L, Likhite S, Miranda C, Meyer K, Foust KD, Burghes AHM, Kaspar BK (2017) Single-dose gene-replacement therapy for spinal muscular atrophy. *N Engl J Med* 377(18):1713–1722. 10.1056/NEJMoal706198 [PubMed: 29091557]
2. Hebert MD, Whittom AA (2007) Gene-based approaches toward Friedreich ataxia therapeutics. *Cell Mol Life Sci* 64(23):3034–3043. 10.1007/s00018-007-72936 [PubMed: 17828464]
3. Hobert JA, Dawson G (2006) Neuronal ceroid lipofuscinoses therapeutic strategies: past, present and future. *Biochim Biophys Acta* 1762 (10):945–953. 10.1016/j.bbadis.2006.08.004 [PubMed: 17049436]
4. Suzuki K, Kastuno M, Banno H, Sobue G (2009) Pathogenesis-targeting therapeutics for spinal and bulbar muscular atrophy (SBMA). *Neuropathology* 29(4):509–516. 10.1111/j.1440-1789.2009.01013.x [PubMed: 19486304]
5. Daneman R, Prat A (2015) The blood-brain barrier. *Cold Spring Harb Perspect Biol* 7(1): a020412 10.1101/cshperspect.a020412 [PubMed: 25561720]
6. Banks WA (2009) Characteristics of compounds that cross the blood-brain barrier. *BMC Neurol* 9(Suppl 1):S3 10.1186/1471-2377-9-S1-S3 [PubMed: 19534732]
7. Hawkins BT, Davis TP (2005) The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev* 57(2): 173–185. 10.1124/pr.57.2.4 [PubMed: 15914466]
8. Ahmed SS, Li H, Cao C, Sikoglu EM, Denninger AR, Su Q, Eaton S, Liso Navarro AA, Xie J, Szucs S, Zhang H, Moore C, Kirschner DA, Seyfried TN, Flotte TR, Matalon R, Gao G (2013) A single intravenous rAAV injection as late as P20 achieves efficacious and sustained CNS Gene therapy in Canavan mice. *Mol Ther* 21(12):2136–2147. 10.1038/mt.2013.138 [PubMed: 23817205]
9. Ahmed SS, Schattgen SA, Frakes AE, Sikoglu EM, Su Q, Li J, Hampton TG, Denninger AR, Kirschner DA, Kaspar B, Matalon R, Gao G (2016) rAAV gene therapy in a Canavan's disease

⁸For tail vein injections, the lateral veins are very superficial and easily accessible. The total injection volume should not exceed 0.1–0.3 mL, depending on the size of the mouse (20–40 g). Thus, high-titer vectors (~10¹³ vg/mL) are required for the delivery of high doses. Adult mice 4 weeks or older are recommended.

mouse model reveals immune impairments and an extended pathology beyond the central nervous system. *Mol Ther* 24 (6):1030–1041. 10.1038/mt.2016.68 [PubMed: 27039844]

10. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK (2009) Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 27(1):59–65. 10.1038/nbt.1515 [PubMed: 19098898]
11. Asokan A, Schaffer DV, Samulski RJ (2012) The AAV vector toolkit: poised at the clinical crossroads. *Mol Ther* 20(4):699–708. 10.1038/mt.2011.287 [PubMed: 22273577]
12. Castle MJ, Turunen HT, Vandenberghe LH, Wolfe JH (2016) Controlling AAV tropism in the nervous system with natural and engineered capsids. *Methods Mol Biol* 1382:133–149. 10.1007/978-1-4939-3271-9_10 [PubMed: 26611584]
13. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM (2003) Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci U S A* 100(10):6081–6086. 10.1073/pnas.0937739100 [PubMed: 12716974]
14. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* 99(18):11854–11859. 10.1073/pnas.182412299 [PubMed: 12192090]
15. Grimm D, Lee JS, Wang L, Desai T, Akache B, Storm TA, Kay MA (2008) In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. *J Virol* 82(12):5887–5911. 10.1128/JVI.00254-08 [PubMed: 18400866]
16. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV (2006) Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol* 24(2):498–504. 10.1038/nbt1182 [PubMed: 16680123]
17. Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam HJ, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ (2010) Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nat Biotechnol* 28(1):79–82. 10.1038/nbt.1599 [PubMed: 20037580]
18. Wang D, Li S, Gessler DJ, Xie J, Zhong L, Li J, Tran K, Van Vliet K, Ren L, Su Q, He R, Goetzmann JE, Flotte TR, Agbandje-McKenna M, Gao G (2018) A Rationally engineered capsid variant of AAV9 for systemic CNS-directed and peripheral tissue-detargeted gene delivery in neonates. *Mol Ther Methods Clin Dev* 9:234–246. 10.1016/j.omtm.2018.03.004 [PubMed: 29766031]
19. Deverman BE, Pravdo PL, Simpson BP, Kumar SR, Chan KY, Banerjee A, Wu WL, Yang B, Huber N, Pasca SP, Gradinaru V (2016) Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol* 34(2):204–209. 10.1038/nbt.3440 [PubMed: 26829320]
20. Kotterman MA, Schaffer DV (2014) Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet* 15(7):445–451. 10.1038/nrg3742 [PubMed: 24840552]
21. Bartel M, Schaffer D, Buning H (2011) Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity. *Front Microbiol* 2:204. 10.3389/fmicb.2011.00204 [PubMed: 22065962]
22. Asokan A (2010) Reengineered AAV vectors: old dog, new tricks. *Discov Med* 9 (48):399–403 [PubMed: 20515607]
23. Su X, Kells AP, Huang EJ, Lee HS, Hadaczek P, Beyer J, Bringas J, Pivrotto P, Penticuff J, Eberling J, Federoff HJ, Forsayeth J, Bankiewicz KS (2009) Safety evaluation of AAV2-GDNF gene transfer into the dopaminergic nigrostriatal pathway in aged and parkinsonian rhesus monkeys. *Hum Gene Ther* 20(12):1627–1640. 10.1089/hum.2009.103 [PubMed: 19671001]
24. Davidson BL, Stein CS, Heth JA, Martins I, Kotin RM, Derksen TA, Zabner J, Ghodsi A, Chiorini JA (2000) Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 97(7):3428–3432. 10.1073/pnas.050581197 [PubMed: 10688913]
25. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM (2004) Glades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol* 78 (12):6381–6388. 10.1128/JVI.78.12.6381-6388.2004 [PubMed: 15163731]

26. Yang B, Li S, Wang H, Guo Y, Gessler DJ, Cao C, Su Q, Kramer J, Zhong L, Ahmed SS, Zhang H, He R, Desrosiers RC, Brown R, Xu Z, Gao G (2014) Global CNS transduction of adult mice by intravenously delivered rAAVrh.8 and rAAVrh.10 and nonhuman primates by rAAVrh.10. *Mol Ther* 22 (7):4299–1309. 10.1038/mt.2014.68
27. Zhang H, Yang B, Mu X, Ahmed SS, Su Q, He R, Wang H, Mueller C, Sena-Esteves M, Brown R, Xu Z, Gao G (2011) Several rAAV vectors efficiently cross the blood-brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. *Mol Ther* 19(8):1440–1448. 10.1038/mt.2011.98
28. Choudhury SR, Fitzpatrick Z, Harris AF, Maitland SA, Ferreira JS, Zhang Y, Ma S, Sharing RB, Gray-Edwards HL, Johnson JA, Johnson AK, Alonso LC, Punzo C, Wagner KR, Maguire CA, Kotin RM, Martin DR, Sena-Esteves M (2016) In vivo selection yields AAV-B1 capsid for central nervous system and muscle gene therapy. *Mol Ther* 24 (7):1247–1257. 10.1038/mt.2016.84 [PubMed: 27117222]
29. Miyake N, Miyake K, Yamamoto M, Hirai Y, Shimada T (2011) Global gene transfer into the CNS across the BBB after neonatal systemic delivery of single-stranded AAV vectors. *Brain Res* 1389:19–26. 10.1016/j.brainres.2011.03.014 [PubMed: 21397590]
30. Hordeaux J, Wang Q, Katz N, Buza EL, Bell P, Wilson JM (2018) The neurotropic properties of AAV-PHP.B are limited to C57BL/6J mice. *Mol Ther* 26(3):664–668. 10.1016/j.ymthe.2018.01.018 [PubMed: 29428298]
31. Bevan AK, Duque S, Foust KD, Morales PR, Braun L, Schmelzer L, Chan CM, McCrate M, Chicoine LG, Coley BD, Porensky PN, Kolb SJ, Mendell JR, Burghes AH, Kaspar BK (2011) Systemic gene delivery in large species for targeting spinal cord, brain, and peripheral tissues for pediatric disorders. *Mol Ther* 19 (11):1971–1980. 10.1038/mt.2011.157 [PubMed: 21811247]
32. Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ (2011) Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther* 19 (6):1058–1069. 10.1038/mt.2011.72 [PubMed: 21487395]
33. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE (2008) Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* 16(6):1073–1080. 10.1038/mt.2008.76 [PubMed: 18414476]
34. Moscioni D, Morizono H, McCarter RJ, Stern A, Cabrera-Luque J, Hoang A, Sanmiguel J, Wu D, Bell P, Gao CP, Raper SE, Wilson JM, Batshaw ML (2006) Long-term correction of ammonia metabolism and prolonged survival in ornithine transcarbamylase-deficient mice following liver-directed treatment with adeno-associated viral vectors. *Mol Ther* 14(1):25–33. 10.1016/j.ymthe.2006.03.009 [PubMed: 16677864]
35. Salva MZ, Himeda CL, Tai PW, Nishiuchi E, Gregorevic P, Allen JM, Finn EE, Nguyen QG, Blankinship MJ, Meuse L, Chamberlain JS, Hauschka SD (2007) Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. *Mol Ther* 15(2):320–329. 10.1038/sj.mt.6300027 [PubMed: 17235310]
36. Marques S, Zeisel A, Codeluppi S, van Bruggen D, Mendanha Falcao A, Xiao L, Li H, Haring M, Hochgerner H, Romanov RA, Gyllborg D, Munoz-Manchado AB, La Manno G, Lönnerberg P, Floriddia EM, Rezayee F, Ernfors P, Arenas E, Hjerling-Lefler J, Harkany T, Richardson WD, Linnarsson S, Castelo-Branco G (2016) Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science* 352(6291):1326–1329. 10.1126/science.aaf6463 [PubMed: 27284195]
37. Hochstim C, Deneen B, Lukaszewicz A, Zhou Q, Anderson DJ (2008) Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* 133(3):510–522. 10.1016/j.cell.2008.02.046 [PubMed: 18455991]
38. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, Levi B, Gray LT, Sorensen SA, Dolbeare T, Bertagnoli D, Goldy J, Shapovalova N, Parry S, Lee C, Smith K, Bernard A, Madisen L, Sunkin SM, Hawrylycz M, Koch C, Zeng H (2016) Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat Neurosci* 19 (2):335–346. 10.1038/nn.4216 [PubMed: 26727548]
39. Xie J, Ameres SL, Friedline R, Hung JH, Zhang Y, Xie Q, Zhong L, Su Q, He R, Li M, Li H, Mu X, Zhang H, Broderick JA, Kim JK, Weng Z, Flotte TR, Zamore PD, Gao G (2012) Long-term,

- efficient inhibition of microRNA function in mice using rAAV vectors. *Nat Methods* 9(4):403–409. 10.1038/nmeth.1903 [PubMed: 22388288]
40. Xie J, Xie Q, Zhang H, Ameres SL, Hung JH, Su Q, He R, Mu X, Seher Ahmed S, Park S, Kato H, Li C, Mueller C, Mello CC, Weng Z, Flotte TR, Zamore PD, Gao G (2011) MicroRNA-regulated, systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. *Mol Ther* 19(3):526–535. 10.1038/mt.2010.279 [PubMed: 21179009]
 41. Brown BD, Venneri MA, Zingale A, Sergi L, Naldini L (2006) Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* 12(5):585–591. 10.1038/nm1398 [PubMed: 16633348]
 42. Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Della Valle P, D'Angelo A, Naldini L (2007) A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 110(13):4144–4152. 10.1182/blood-2007-03-078493 [PubMed: 17726165]
 43. Zhao Z, Nelson AR, Betsholtz C, Zlokovic BV (2015) Establishment and Dysfunction of the Blood-Brain Barrier. *Cell* 163(5):1064–1078. 10.1016/j.cell.2015.10.067 [PubMed: 26590417]
 44. Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, Fyfe J, Moullier P, Colle MA, Barkats M (2009) Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol Ther* 17(7):1187–1196. 10.1038/mt.2009.71 [PubMed: 19367261]
 45. Fu H, Muenzer J, Samulski RJ, Breese G, Sifford J, Zeng X, McCarty DM (2003) Self-complementary adeno-associated virus serotype 2 vector: global distribution and broad dispersion of AAV-mediated transgene expression in mouse brain. *Mol Ther* 8(6):911–917 [PubMed: 14664793]
 46. Penaud-Budloo M, Le Guiner C, Nowrouzi A, Toromanoff A, Cherel Y, Chenuaud P, Schmidt M, von Kalle C, Rolling F, Moullier P, Snyder RO (2008) Adeno-Associated virus vector genomes persist as episomal chromatin in primate muscle. *J Virol* 82(16):7875–7885. 10.1128/JVI.00649-08 [PubMed: 18524821]
 47. Gao G, Sena-Esteves M (2012) Introducing genes into mammalian cells: viral vectors. In: *Molecular cloning: a laboratory manual*, vol 2 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 1209–1313
 48. Lecomte E, Tournaire B, Cogne B, Dupont JB, Lindenbaum P, Martin-Fontaine M, Broucque F, Robin C, Hebben M, Merten OW, Blouin V, Francois A, Redon R, Moullier P, Leger A (2015) Advanced characterization of DNA molecules in rAAV vector preparations by single-stranded virus next-generation sequencing. *Mol Ther Nucleic Acids* 4:e260 10.1038/mtna.2015.32 [PubMed: 26506038]
 49. Tai PWL, Xie J, Fong K, Seetin M, Heiner C, Su Q, Weiland M, Gao G (2018) Adeno-Associated virus genome population sequencing achieves full vector genome resolution and reveals human-vector chimeras. *Mol Ther Methods Clin Dev* 9:130–141 [PubMed: 29766023]
 50. Boisgerault F, Gross DA, Ferrand M, Poupiot J, Darocha S, Richard I, Galy A (2013) Prolonged gene expression in muscle is achieved without active immune tolerance using microRNA 142.3p-regulated rAAV gene transfer. *Hum Gene Ther* 24 (4):393–405. <https://doi.org/10.1089/hum.2012.208> [PubMed: 23427817]
 51. Majowicz A, Maczuga P, Kwikkers KL, van der Marel S, van Logtenstein R, Petry H, van Deventer SJ, Konstantinova P, Ferreira V (2013) Mir-142–3p target sequences reduce transgene-directed immunogenicity following intramuscular adeno-associated virus 1 vectormediated gene delivery. *J Gene Med* 15 (6–7):219–232. 10.1002/jgm.2712 [PubMed: 23658149]
 52. Matsuzaki Y, Konno A, Mochizuki R, Shinohara Y, Nitta K, Okada Y, Hirai H (2018) Intravenous administration of the adeno-associated virus-PHP.B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neurosci Lett* 665:182–188. 10.1016/j.neulet.2017.11.049 [PubMed: 29175632]
 53. Rapti K, Louis-Jeune V, Kohlbrenner E, Ishikawa K, Ladage D, Zolotukhin S, Hajjar RJ, Weber T (2012) Neutralizing antibodies against AAV serotypes 1,2,6, and 9 in sera of commonly used animal models. *Mol Ther* 20 (1):73–83. 10.1038/mt.2011.177 [PubMed: 21915102]
 54. Samaranch L, Sebastian WS, Kells AP, Salegio EA, Heller G, Bringas JR, Pivrotto P, DeArmond S, Forsayeth J, Bankiewicz I-S (2014) AAV9-mediated expression of a non-self protein in nonhuman primate central nervous system triggers widespread neuroinflammation driven by

- antigen-presenting cell transduction. *Mol Ther* 22(2):329–337. 10.1038/mt.2013.266 [PubMed: 24419081]
55. Schwartz M, Deczkowska A (2016) Neurological disease as a failure of brain-immune crosstalk: the multiple faces of neuroinflammation. *Trends Immunol* 37(10):668–679. 10.1016/j.it.2016.08.001 [PubMed: 27616557]
 56. Paulk NK, Pekrun K, Zhu E, Nygaard S, Li B, Xu J, Chu K, Leborgne C, Dane AP, Haft A, Zhang Y, Zhang F, Morton C, Valentine MB, Davidoff AM, Nathwani AC, Mingozi F, Grompe M, Alexander IE, Lisowski L, Kay MA (2018) Bioengineered AAV capsids with combined high human liver transduction in vivo and unique humoral seroreactivity. *Mol Ther* 26(1):289–303. 10.1016/j.ymthe.2017.09.021 [PubMed: 29055620]
 57. Keeler GD, Markusic DM, Hoffman BE (2017) Liver induced transgene tolerance with AAV vectors. *Cell Immunol*, 10.1016/j.cellimm.2017.12.002
 58. Breous E, Somanathan S, Vandenberghe LH, Wilson JM (2009) Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology* 50(2):612–621. 10.1002/hep.23043 [PubMed: 19575456]
 59. Cao O, Loduca PA, Herzog RW (2009) Role of regulatory T cells in tolerance to coagulation factors. *J Thromb Haemost* 7(Suppl 1):88–91. 10.1111/j.1538-7836.2009.03417.x [PubMed: 19630776]
 60. Dobrzynski E, Herzog RW (2005) Tolerance induction by viral in vivo gene transfer. *Clin Med Res* 3(4):234–240 [PubMed: 16303889]
 61. Kumar SRP, Hoffman BE, Terhorst C, de Jong YP, Herzog RW (2017) The balance between CD8(+) T Cell-mediated clearance of AAV-encoded antigen in the liver and tolerance is dependent on the vector dose. *Mol Ther* 25 (4):880–891. 10.1016/j.ymthe.2017.02.014 [PubMed: 28284982]
 62. Sun B, Bird A, Young SP, Kishnani PS, Chen YT, Koeberl DD (2007) Enhanced response to enzyme replacement therapy in Pompe disease after the induction of immune tolerance. *Am J Hum Genet* 81(5):1042–1049. 10.1086/522236 [PubMed: 17924344]
 63. Hinderer C, Katz N, Buza EL, Dyer C, Goode T, Bell P, Richman LK, Wilson JM (2018) Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Hum Gene Ther* 29(3):285–298. 10.1089/hum.2018.015 [PubMed: 29378426]
 64. Kugler S, Meyn L, Holzmüller H, Gerhardt E, Isenmann S, Schulz JB, Bahr M (2001) Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol Cell Neurosci* 17(1):78–96. 10.1006/mcne.2000.0929
 65. Shevtsova Z, Malik JM, Michel U, Bahr M, Kugler S (2005) Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system in vitro and in vivo. *Exp Physiol* 90(1):53–59. 10.1113/expphysiol.2004.028159 [PubMed: 15542619]
 66. Stierl M, Penzkofer A, Kennis JT, Hegemann P, Mathes T (2014) Key residues for the light regulation of the blue lightactivated adenylyl cyclase from *Beggiatoa* sp. *Biochemistry* 53(31):5121–5130. 10.1021/bi500479v [PubMed: 25046330]
 67. Hedegaard C, Kjaer-Sorensen K, Madsen LB, Henriksen C, Momeni J, Bendixen C, Oxvig C, Larsen K (2013) Porcine synapsin 1: SYN1 gene analysis and functional characterization of the promoter. *FEBS Open Bio* 3:411–420. 10.1016/j.fob.2013.10.002
 68. McLean JR, Smith GA, Rocha EM, Hayes MA, Beagan JA, Hallett PJ, Isacson O (2014) Widespread neuron-specific transgene expression in brain and spinal cord following synapsin promoter-driven AAV9 neonatal intracerebroventricular injection. *Neurosci Lett* 576:73–78. 10.1016/j.neulet.2014.05.044 [PubMed: 24882721]
 69. Gessler DJ, Li D, Xu H, Su Q, Sanmiguel J, Tuncer S, Moore C, King J, Matalon R, Gao G (2017) Redirecting N-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues Canavan disease. *JCI Insight* 2(3):e90807 10.1172/jci.insight.90807 [PubMed: 28194442]
 70. Cho W, Hagemann TL, Johnson DA, Johnson JA, Messing A (2009) Dual transgenic reporter mice as a tool for monitoring expression of glial fibrillary acidic protein. *J Neurochem* 110 (1):343–351. 10.1111/j.1471-4159.2009.06146.x [PubMed: 19457099]

71. von Jonquieres G, Frohlich D, Klugmann CB, Wen X, Harasta AE, Ramkumar R, Spencer ZH, Housley GD, Klugmann M (2016) Recombinant human myelin-associated glycoprotein promoter drives selective AAV-mediated transgene expression in oligodendrocytes. *Front Mol Neurosci* 9:13 <https://doi.org/10.3389/fnmol.2016.00013> [PubMed: 26941604]
72. Chen CT, Gottlieb DI, Cohen BA (2008) Ultraconserved elements in the Olig2 promoter. *PLoS One* 3(12):e3946 10.1371/journal.pone.0003946 [PubMed: 19079603]

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Fig. 1.

Facial vein injection of neonatal mice, **(a)** Neonatal mouse preinjection. Arrow indicates visible facial vein, **(b)** Image of injection. Note angle of needle and blanching of facial vein during injection (arrow), indicating successful delivery of vector, **(c)** Black blemish (arrow) at the site of injection post-administration is another indication of successful delivery, **(d)** The pup is rolled in alcohol-soaked tissue, followed by **(e)** rolling in dirty bedding before returning to the dam. **(f)** The dam is also dampened on the nose with alcohol to mask foreign odors

Fig. 2.

Retro-orbital injection of neonatal mice, **(a, b)** Construction of a padded injection platform (10 cm $\times$ 5 cm $\times$ 3 cm [L $\times$ W $\times$ H]). Pads are stacked and wrapped with a paper towel, **(c)** The pup is placed in a lateral position and grasped at the head with the thumb and index finger. Arrow indicates visible superficial temporal vein. **(d)** The needle is inserted with bevel down, at the 3 o'clock position, into the eye approximately 3 mm at a 30° angle. Blanching of the vein (arrow) indicates successful injection

Fig. 3.

Retro-orbital injection of adult mice. **(a)** Manipulation of the mouse to cause slight bulge of the eye. The thumb is placed just lateral to the animal's trachea and the fur on the head is drawn toward the palm of the hand. **(b)** The needle is inserted approximately 5 mm at a 45° angle into the center of the retro-orbital sinus, **(c)** Ointment is applied liberally to the eye to aid recovery

Fig. 4.

Lateral tail vein injection, **(a)** The animal is positioned securely in the restrainer. The tail is fitted through the aperture and held straight and taut, **(b)** Two lateral veins are visible on each side of the tail. Arrow indicates a single vein, **(c)** The needle tip is partially inserted at a 30° angle into the vein. Once the tip has penetrated the vein, readjust the needle so that it is in-line with the length with the vein. Blanching of the tail vein indicates successful injection

Table 1

Lists of CNS promoters and cell targets

Promoter	Gene name	Cell type	Citations
NSE	Neuron specific enolase	Neuron	[64,65]
SYN1	Synapsin 1	Neuron	[66–68]
GFAP	Glial fibrillary acidic protein	Astrocyte	[69, 70]
MBP/MAG	Myelin basic protein/Myelin-associated glycoprotein	Oligodendrocyte	[71]
OLIG2	Oligodendrocyte transcription factor 2	Oligodendrocyte	[72]
POMC	Proopiomelanocortin	Neurons/hypothalamus	RIKEN
SOD1	Superoxide dismutase 1	Neurons/spinal cord	RIKEN