Global CNS Transduction of Adult Mice by Intravenously Delivered rAAVrh.8 and rAAVrh.10 and Nonhuman Primates by rAAVrh.10

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ABSTRACT

Some recombinant adeno-associated viruses (rAAVs) can cross the neonatal blood-brain barrier (BBB) and efficiently transduce cells of the central nervous system (CNS). However, in the adult CNS, transduction levels by systemically delivered rAAVs are significantly reduced, limiting their potential for CNS gene therapy. Here, we characterized 12 different rAAV*EGFP*s in the adult mouse CNS following intravenous delivery. We show that the capability of crossing the adult BBB and achieving widespread CNS transduction is a common character of AAV serotypes tested. Of note, rAAVrh.8 is the leading vector for robust global transduction of glial and neuronal cell types in regions of clinical importance such as cortex, caudate-putamen, hippocampus, corpus callosum and substantia nigra. It also displays reduced peripheral tissue tropism compared to other leading vectors. Additionally, we evaluated rAAVrh.10 with and without microRNA (miRNA)-regulated expressional detargeting from peripheral tissues for systemic gene delivery to the CNS in marmosets. Our results indicate that rAAVrh.8, along with rh.10 and 9, hold the best promise for developing novel therapeutic strategies to treat neurological diseases in the adult patient population. Additionally, systemically delivered rAAVrh.10 can transduce the CNS efficiently and its transgene expression can be limited in the periphery by endogenous miRNAs in adult marmosets.

INTRODUCTION

The increasing prevalence of neurodegenerative diseases such as Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis (ALS) is a growing public health concern for the US population and devastating for patients and their families. By 2050, an estimation of 16 million Americans will suffer from Alzheimer's disease with total costs of $1.1 trillion (in today's dollars) [1, 2]. Conventional approaches to treat neurodegenerative diseases are limited to managing symptoms and curbing disease progression [3]. In spite of some exciting progress in the broad biomedical research field, overall efforts in developing conventional therapeutics to ameliorate or even cure neurodegenerative diseases have had limited clinical success. CNS-directed gene therapy is a promising approach for treating neurodegenerative diseases and offers some advantages over conventional remedies, which could ultimately lead to tissue specific, long-term delivery of therapeutic agents and curative measures [4-9].

A significant barrier of CNS gene-delivery is the blood brain barrier (BBB), which prevents large or hydrophilic molecules such as dopamine, chemotherapeutics and viruses from passively entering the brain [10, 11]. On the other hand, rAAVs hold promise for efficient, stable, and safe gene delivery to a wide range of tissues including the CNS [4, 8, 12-15], but methods of delivery for rAAV-mediated gene transfer remain an intricate and demanding aspect of vector-based CNS gene therapy. Direct stereotactic injections of rAAVs are ideal for diseases with local CNS pathology, but cannot transduce the CNS broadly [16]. This can be accomplished by systemic rAAV delivery that utilizes the ramified capillary network of the CNS to ameliorate widespread pathologies in the CNS. Although systemic delivery of the first generation of rAAV2 to the CNS has proven to be difficult, it can be marginally improved by transient pharmacological modulation of the BBB, producing a limited CNS transduction [17].

Recent discoveries of new clades of primate AAVs have substantially expanded potential applications of rAAVs in preclinical and clinical gene therapy [14, 18-21]. Importantly, one of these novel primate serotypes, rAAV9, can achieve robust transduction of neuronal and glial cell types in the CNS of murine neonates following intravenous injections and has shown encouraging results in treating a number of CNS diseases that require early-life intervention [4, 6, 8, 15, 22]. Studies in adult animals, however, demonstrated significantly restricted entry of viral particles into the CNS and declined transduction efficiency due to the advanced BBB development [15, 23]. In searching for means to overcome this limitation, a subsequent study by our group has identified several additional rAAVs that are as or more effective as rAAV9 in transducing the neonatal CNS [22], which motivated our selection of rAAVs for adult transduction in the current study.

A serious challenge to rAAV9-mediated systemic gene delivery to the CNS is the lack of CNS-restricted tropism and therapeutic functionality, which results in simultaneous robust transduction of peripheral tissues such as liver, heart, lung, muscle and pancreas [23-25]. Although endogenous miRNA-regulated rAAV9 is capable of detargeting transgene expression from peripheral tissues towards CNS-restricted transgene expression in mice [26], an expanded repertory of rAAV vectors with discriminating tropism for the CNS and a reduced affinity for peripheral tissues will be desirable to limit potential peripheral toxicity, allow for greater therapeutic control and flexibility as well as bypassing possible pre-existing immunity to rAAV9 in patient populations [27-29].

In order to identify highly CNS tropic rAAV(s) with naturally reduced peripheral tissue tropism for systemic gene delivery to the CNS, we compared the CNS transduction efficiencies of 12 different rAAVs (rAAV 1, 2, 5, 6, 6.2, 7, 8, 9, rh.8, rh.10, rh.39 and rh.43) in 10 weeks old adult mice following systemic delivery and performed a proof-of-concept study of systemically

delivered rAAVrh.10 with and without miRNA-regulated transgene expression in nonhuman primates. We report that rAAVrh.8 is a novel and superior vector, displaying strong tropism towards clinical relevant regions of the CNS with reduced dissemination to peripheral tissues in mice. We also demonstrate in a proof-of-concept study for the first time the successful CNS gene transfer by systemically delivered rAAVrh.10 and miRNA–regulated transgene expression in nonhuman primates (NHPs).

RESULTS

Seven rAAV vectors can achieve efficient global transduction of the adult mouse CNS. To determine the transduction efficiency of each serotype after I.V. injection, we detected and quantified the EGFP signal from the coronal sections of brains and transversal sections of cervical, thoracic and lumbar spinal cords (**Figs. 1-3**). Based on the density of the transduced cells (see Materials and Methods), we grouped 12 rAAVs into four groups (**Figs. 1 and 2**; **Suppl. Table 1**). Group 1 is consisted of three vectors, rAAVrh.8, rAAVrh.10 and rAAV9. This group ranked consistently as the top three in the transduction levels in both brain and spinal cord. The only exception is the spinal cord neurons, where the top seven vectors showed similar levels of transduction (**Fig. 3G**; **Suppl. Fig. 1**). Group 2 is consisted of four vectors, rAAV7, rAAV8, rAARrh.39 and rAAVrh.43. This group ranked lower than the group 1 but their overall transduction levels were still excellent. In some cases (e.g. total spinal cord and spinal cord neurons), the differences between this group and group 1 were small and insignificant (**Figs. 1, 2 and 3C, G, Suppl. Tab. 1**). All the group 1 and group 2 vectors transduced spinal cord motor neurons robustly (**Suppl. Fig. 1**). Group 3 is consisted of three vectors, rAAV1, rAAV6 and rAAV6.2. The transduction levels by this group were lower than groups 1 and 2. In most instances, rAAV6.2 ranked the highest and the AAV1 the lowest within this group (**Figs. 1-3, Suppl. Fig. 1**). This group had high transduction of blood vessels (**data not shown**). Group 4 is

consisted of two vectors, rAAV2 and rAAV5. This group had the lowest transduction levels (**Figs. 1-3**). rAAV5 transduced few or no cells as well as blood vessels in the CNS. rAAV2 transduced small populations of neurons in the dentate gyrus and the hypothalamus, and scattered astrocytes and neurons in the cortex and some ependymal cells (Data not shown). Based on these assessments, we focused our detailed analysis on the group 1 vectors and some representatives of group 2 and group 3 vectors.

rAAVrh.8 is the most efficient vector in transducing the adult CNS. The group 1 vectors transduced cells in wide areas of the CNS, including cortex, striatum, hippocampus, thalamus, hypothalamus, amygdale, corpus callosum (**Figs. 1 and 4**; **Suppl. Figs. 2 and 3**), the choroid plexus (**Suppl. Fig. 4**) and all three spinal cord regions (i.e. cervical, thoracic and lumbar) (**Fig. 2**; **Suppl. Fig. 1**). Overall rAAVrh.8 appeared to transduce most robustly relative to other vectors. rAAVrh.8 consistently ranked first in different regions and in different cell types (**Fig. 3A-I**) by a semi-quantitative scoring system (see Materials and Methods) [16]. The only exception was in the spinal neurons, where the top 7 serotypes had essentially the same transduction efficiency (**Fig. 3G**).

The group 1, 2 and 3 vectors transduce multiple cell types and various neuronal populations in the CNS. To identify the cell types that the vectors transduce, we first carried out morphological analysis on the CNS tissue sections stained for GFP. By high magnification microscopy we observed extensive transduction of neurons, astrocytes and oligodendrocytes in various areas of the CNS (**Figs. 3** and **4**; **Suppl. Figs. 1-3**). For example, the group 1 vectors transduced granular cells in dentate gyrus (**Fig. 4B**, **Suppl. Figs. 2** and **3B**), pyramidal neurons in CA2 (**Fig. 4D**, **Suppl. Figs. 2** and **3D**), medial spiny neurons and astrocytes in striatum (**Fig. 4E**; **Suppl. Figs. 2** and **3E**), Purkinje neurons in cerebellum (**Fig. 4 Cerebellum**; **Suppl. Figs. 2 and 3J**), neurons and astrocytes in thalamus (**Fig. 4H**; **Suppl. Fig. 2 and 3H**), hypothalamus (**Fig. 4G**; **Suppl. Fig. 3G**), amygdala (**Fig. 4I**; **Suppl. Fig. 3I**), layer 3-6 of the cortex (**Fig. 4 A, F**;

Suppl. Figs. 2 and **3 A, F**), substantia nigra (**Fig. 4 SN**; **Suppl. Fig. 3K**), ventral tegmental area (not shown) and lateral septum (**Fig. 4 LSN**; **Suppl. Fig. 3L**). Additionally, they also transduced oligodendrocytes in corpus callosum (**Fig. 4C**; **Suppl. Figs. 2 and 3C**). A similar cellular transduction pattern was also observed for group 2 and 3 vectors, albeit that the density of transduced cells was lower than group 1 vectors (**Figs. 1** and **2**; **Suppl. Fig. 2**). To verify the transduced cell types, we performed double fluorescence staining on the rAAVrh.8-treated CNS tissues. Using markers for specific cell types, including NeuN for neurons, GFAP for astrocytes, APC for oligodendrocytes, Iba1 for microglia and CD31 for blood vessels, we verified that EGFP was expressed in neurons, astrocytes, oligodendrocytes and blood vessels, but not in microglia (**Fig. 5**). We also verified EGFP expression in several specific neuronal subpopulations, including Purkinje cells (calbindin), motor neurons (ChAT), medial spiny neurons (Darpp-32) and dopaminergic neurons (tyrosine hydroxylase) (**Suppl. Fig. 5**).

rAAVrh.8 injections result in less peripheral tissue dissemination compared to rAAV9 and rAAVrh.10. In addition to strong and widespread transduction in the CNS, we consider low peripheral tropism to be an important property of an ideal viral vector for systemically delivered and CNS-targeted gene therapy. To characterize biodistribution profiles of I.V. delivered rAAV in various tissues of the injected mice, qPCR quantification of rAAV genomes was carried out for the group 1 vectors in brain, spinal cord, lung, kidney, pancreas, skeletal muscle, heart and liver tissues (**Fig. 6A**). The copy numbers of persistent rAAVrh.8 genomes were similar to those of rAAV9 and rAAVrh.10 in the brain but significantly lower than rAAV9 and rAAVrh.10 in the liver. To assess the degree of transduction in the brain relative to the peripheral tissues, we calculated the ratios between the genome copies in the brain and those in the periphery. We did not detect a significant difference between the vectors in the group 1 probably due to a high degree of variation among the injected animals (**Fig. 6B**). Nevertheless, we observed a trend

towards relatively high CNS transduction over the periphery for rAAVrh.8 compared with rAAVrh.10 and rAAV9.

Systemically delivered rAAVrh.10 transduces the motor neurons of adult marmosets. To determine CNS transduction efficiency in NHPs by I.V. injection, we conducted a proof-of-concept experiment. We intravenously dosed two adult marmosets, one with rAAVrh.10*EGFP* and the other with rAAVrh.10*EGFP*-miR-1BS-miR-122BS3 (rAAVrh.10*EGFP*-miRBS). The latter vector expresses EGFP and carries three repeated copies of miRNA binding sites in its 3'-UTR for miR-1 that is abundant in heart and muscle and miR-122 that is abundant in liver [30]. Consequently, these endogenously expressed miRNAs can bind to the transgene/*EGFP* mRNA in heart, skeletal muscle and liver and thus repress the transgene expression. A third marmoset was injected with rAAVrh.10*EGFP* intrathecally for comparison. The animals were euthanized 2 weeks after injection and the EGFP expression in both CNS and peripheral tissues were analyzed. The results of CNS transduction for the intrathecally injected monkey were published elsewhere [27]. The data for CNS transduction in two I.V. injected animals and peripheral tissue transductions in all 3 monkeys are presented below.

I.V. injection of rAAVrh.10*EGFP* to the one adult marmoset generated striking EGFP transduction in motor neurons throughout the entire length of the spinal cord (**Fig. 7A-F**) and oculomotor nucleus resided in the ventral midbrain (**Fig.7G-I**). Additionally, the axon fibers in the posterior horn and cuneate fasciculus at the cervical and thoracic levels robustly expressed EGFP (**Suppl. Fig. 6A, C**), indicating that the neurons in the dorsal root ganglia (DRG) were well transduced at these regions. The axon fibers in the posterior horn and cuneate fascicules at the lumbar level and the neurons and astrocytes in the intermediate zone of the spinal cord also expressed EGFP, although the expression in these regions was not as strong as the ventral horns at all levels and the dorsal horns at the cervical and thoracic levels (**Fig. 7A-F**; **Suppl. Fig. 6**). The brain was overall sparsely transduced but focal regions with strong EGFP expression

could be observed, e.g. spinal trigeminal nucleus and tract (**Suppl. Figs. 7 A, G** and **9**). Other areas with noticeable transduction were vestibular nucleus, hypoglossal nucleus, inferior olive nucleus, lateral reticular nucleus, red nucleus and substantial nigra (**Suppl. Figs. 7** and **8**). The rest brain areas including cerebral cortex, cerebellum and hippocampus were sparsely transduced (**Suppl. Fig. 9**).

I.V. injection of rAAVrh.10*EGFP*-miRBS to the second adult marmoset resulted in generally sparse EGFP transduction throughout the CNS except for some focal regions of hypoglossal nucleus, nucleus of vagus, gigantocellular reticular nucleus and inferior olive (**Suppl. Figs. 10-12**). The inferior olive area also showed high vascular transduction (**Suppl. Fig. 12I**).

The peripheral tissue transduction profiles showed differences among the three marmosets. While all three injected animals showed little muscle transduction (data not shown), liver transduction was robust in the animal treated with I.V. rAAVrh.10*EGFP* (**Fig. 8A**) but substantially reduced in the animal treated with I.V. rAAVrh.10*EGFP*-miRBS (**Fig. 8C**), thus suggesting that the incorporation of the endogenous miRNA-targeting sites can de-target rAAV expression in liver. Likewise, physically restricted CNS gene delivery by intrathecal injection of rAAVrh.10*EGFP* remarkably limited EGFP transduction in the liver (**Fig. 8C**) despite its robust transduction of motor neurons and dorsal root ganglion neurons throughout the spinal cord [27]. All three animals showed poor heart transduction (data not shown), thus we could not assess whether a particular treatment method reduced heart transduction. Interestingly, regardless of miRNA regulation or different routes of vector administration, the adrenal cortex expressed EGFP robustly in all 3 marmosets (**Fig. 8B, D, F**).

DISCUSSION

It is well documented that intravascular delivered rAAVs are excellent vectors for CNS gene therapy in neonatal animals [4, 15, 31]. However, earlier studies in adult mice by I.V. injected rAAVs generated limited CNS transduction due to the advanced BBB development in adult animal models [17, 22, 32]. Here we demonstrate that several rAAVs are capable of crossing the BBB efficiently following I.V injection by using a semi-quantitative scoring system for a quick assessment of cell transduction efficiency. We opted for this method to evaluate rAAV transduction efficiency since it has been successfully applied and published previously [22, 33]. However, we recognize that the semi-quantitative scoring system has disadvantages in assessing absolute differences between serotypes. Thus, to differentiate the fine points among the rAAV serotypes, more extensive quantitative analyses will be needed. Another caveat of this study is the number of animals per group (n=3). Although, 3-4 animals per group are widely used to evaluate the transduction efficiency of rAAV [16, 17, 23, 25, 33], animal-to-animal variations even with inbred animals might influence the final results. To minimize this possibility, we injected male mice only. Despite the limitations of the method, the semi-quantitative characterization provides useful reference points for the future use of these AAV serotypes for I.V. delivery.

Of all the rAAV serotypes tested, the most robust in crossing the BBB are the group 1 vectors consisted of rAAV8, rh.10 and 9 (**Figs. 1-3**; **Suppl. Table 1**). Although less robust than the group 1 vectors, the group 2 vectors, consisted of rAAV8, 7, rh.43 and rh.39, also show strong capability of transducing cells in broad CNS areas following I.V. injection. These results suggest that the group1 and group 2 vectors achieve similar results and expand the repertoire that may be used as alternative vectors to deliver therapeutic genes for treating CNS diseases via I.V. injection. It is worthwhile to point out that the grouping should not be interpreted as if there are absolute boundaries between the groups, but rather, it is used as a means to facilitate the

description of the large number of AAV serotypes tested and to provide a useful guide for selecting the most suitable vectors in future experimentation.

Among the top vectors, a particular standout is the rAAVrh.8. With its global and robust transduction of the CNS cells, rAAVrh.8 consistently ranked first in broad CNS areas and in different cell populations (**Fig. 3**). A particularly attractive feature for rAAVrh.8 is its naturally lower peripheral tissue dissemination than other vectors, particularly in the liver (**Fig. 6**), which enhances the safety profile of systemic gene delivery to the CNS. These qualities suggest that rAAVrh.8 is a top candidate vector for treatment of the CNS diseases that afflict broad areas of the CNS. For example, the broad transduction of the forebrain areas is a property that can be applied to treat Alzheimer's disease, which causes neurodegeneration in forebrain areas [34]. Similarly, the robust transduction throughout the spinal cord, brainstem and cortex are characteristics desirable for treatment of amyotrophic lateral sclerosis (ALS) and Canavan's disease. ALS causes motor neuron degeneration in cortex, brainstem and spinal cord [35]. Canavan's disease causes neurodegeneration throughout the CNS due to deficiency in aspartoacylase [4]. Of note, rAAV9 displays similar transduction qualities by I.V. injection in the spinal cord of adult mice, as reported previously [36], although it ranked slightly below the performance of rAAVrh.8 in side-by-side comparisons (**Fig. 2** and **3**). However, a direct comparison with previously published data is often complicated due to possible differences in vector production, dose and quantification methods [36].

The robust transduction of the CNS cells following I.V. injection is a surprising but useful character of some rAAV vectors. How these rAAVs cross the BBB remains elusive [32, 37, 38]. Nevertheless, our results suggest two possible routes for the rAAVs to enter and transduce the CNS. First, the rAAVs might cross the BBB by exploiting the fenestrated capillary vessels of circumventricular organs [39]. This is supported by the fact that we consistently observed strong EGFP expression in or near circumventricular organs such as the choroid plexus (**Fig. 1**; **Suppl.**

Fig. 4) in lateral (not shown), 3rd (**Suppl. Fig. 4**) and 4th (not shown) ventricles, organum vasculosum of the lamina terminalis (OVLT, not shown) and the sub-fornical organ (SFO, not shown). Upon close examination of the choroid plexus and its surrounding areas, we noticed EGFP-expressing ependymal cells (e.g. see ventricle below corpus callosum in the rAAVrh.43 and rAAVrh.10 panels in **Suppl. Fig. 2**). Additionally, we also noticed the intense EGFP expression in neurons and glia near the 3rd ventricle (e.g. the area between the corpus callosum and the third ventricle in the AAVrh. 8 panel in **Suppl. Fig. 2**). The second possibility could be the direct transcytosis of rAAVs via endothelia [38], which represent the most inner layer of blood vessels. Perivascular astrocytes have been shown to regulate the tightness and exchange of molecules across the BBB [11, 40, 41]. Therefore, the presence of intensely stained blood vessels and their surrounding astrocytes suggests that rAAVs use this second route of entry into the CNS (**Fig. 4** and **Suppl. Figs. 2** and **3)**. Further studies will be needed to understand the transduction mechanism of rAAVs.

A critical step in the clinical development of systemic gene delivery to the CNS by rAAV therapeutics is its translation from small (i.e. murine) to large animal models (i.e. NHP). The phylogenetic and physiological adjacency of nonhuman primates (i.e. marmoset) to humans supports NHP as an adequate model for evaluating gene transfer efficacy and safety. For systemic delivery, marmosets (Callithrix jacchus) are advantageous as an experimental animal model for their small body size and remarkably reduced vector needs. Marmosets have been extensively used for the investigation and treatment of CNS-related pathologies such as Parkinson's and Huntington's diseases [24, 42-44]. Previously, systemically delivered rAAV9 has been evaluated in NHP animals for CNS targeting with promising results [16-18]. In our proof-of-concept study, we systemically delivered rAAVrh.10 for the first time to the CNS of the NHP marmosets and noticed some unique characteristics in the resulting transduction pattern. I.V. injection of rAAVrh.10 robustly transduced the motor neurons in the spinal motor column

and brainstem but scattered neurons and astrocytes in the forebrain and cerebellum (**Fig. 7; Suppl. Figs. 6-11**). These findings contrast with what was reported for intravascular delivered rAAV9 in cynomologus macaques, which transduced mostly astrocytes in the brain [15, 23, 45]. The causes for these differences are not clear but could be attributed to differences in the vector biology between rAAV9 and rAAVrh.10, animal species (i.e. cynomologus *versus* marmoset) and age, etc. In any case, the data generated from a single marmoset with known high animal-to-animal variations in outbred NHP animals is a major caveat of our NHP study. More extensive studies will be required to confirm the vector biology of rAAVrh.10, and perhaps more importantly, to assess the performance of the rAAVrh.8 vector in marmosets or other NHP species.

In this study, we also attempted translating the design of miRNA-regulated transgene expression from murine to a NHP model. Endogenous miRNA-mediated posttranscriptional regulation of transgene expression is an attractive method to detarget rAAV transduction from non-target tissues and cell types and will be desirable to prevent potential peripheral transgene toxicity as well as transgene directed immune response in peripheral tissue [29]. This strategy is effective in mice as we and others have demonstrated previously [26, 46-48]. Considering the conservation of miRNA sequences between human, marmoset and mouse, particularly the high homology of mir-122 and mir-1 (about 99%) [49], we tested this design in marmoset, which is the first application of this kind in rAAV gene transfer in primates. Indeed, our data demonstrated the effectiveness in detargeting of rAAVrh.10 expression from major peripheral tissues e.g. liver. It is rather interesting that rAAVrh.10 robustly transduced adrenal gland regardless of miRNA-detargeting and route of administration, which may hold promise for some clinical applications such Addison's disease [50]. However, the CNS transduction of miRNA-regulated vectors seemed to be underwhelming as compared to the vector without miRNA binding sites. Again, it is difficult to draw a firm conclusion from an experiment that includes only

one animal in each experimental group. Nevertheless, our findings suggest that combining the capability of I.V. rAAVrh.10 with miRNA-mediated tissue detargeting could be an effective means in tailoring rAAV and their tropism to reduce unwanted peripheral transgene toxicity.

In summary, our study has revealed that many rAAVs are capable of crossing the BBB and transducing the CNS cells. Of these, the group 1 vectors, including rAAVrh.8, rAAVrh.10 and rAAV9 have demonstrated the strongest capacity for transducing CNS cells after the systemic I.V. injection. Importantly, rAAVrh.8 appears superior to its group 1 peers in its global CNS transduction and decreased peripheral tissue targeting, and thus, is the best rAAV for delivering CNS gene therapy via I.V. injection. Finally, the synergy of spinal motor neuron tropism of systemically delivered rAAVrh.10 and miRNA-silenced transgene expression in the peripheral tissues of primates may offer a promising treatment for a wide spectrum of degenerative motor neuron diseases.

MATERIALS AND METHODS

Vector cloning and production. The structure of the *EGFP* reporter vector genome was the same as previously described (Zhang et al., Mol. Ther. 2011). The miRNA-regulated EGFP construct was created by inserting 3 copies each of miR-1 and miR-122 target sites in tandem in the 3' UTR of *EGFP* reporter gene as previously described (Xie et al., Mol. Ther. 2011). Production and quality control testing of rAAV vectors were the same as previously described (Zhang et al., Mol. Ther. 2011). Multiple batches of all different serotypes were prepared and injected.

Animal injections. All animal procedures were approved by the Institutional Animal Care and Use Committees of University of Massachusetts Medical School (mouse study) and Harvard University Medical School (Marmoset study). For the mouse study, 10 week-old C57BL/6 mice

(n=3) were treated with rAAV vectors via tail vein injections at a dose of 4 x 10e12 GCs/mouse in 100 µl (2 x 10e14 GC/kg) and an average body weight of 20 gram. For the marmoset study, 4-6 year-old marmoset monkeys were first screened for serologically neutralizing antibody against rAAVrh.10. One sero-negative animal each was selected for the intravenous injection of rAAVrh.10 *EGFP* or rAAVrh.10*EGFP-3XMRBS* via an intravenous catheter placed in the saphenous vein at a dose of 5x10e13 GCs/kg.

Histological processing. Mice were anesthetized 21 days post-injection and transcardially perfused with 15 mL of cold PBS followed by 15 mL of fixation solution containing 4% paraformaldehyde (PFA) (v/v) in PBS. Brain and spinal cord were extracted and post fixed in 4% PFA overnight (n=3). Marmosets were euthanized at day 14 post-vector dosing and subjected to perfusion and fixation with 4% paraformaldehyde for histological analysis. The mouse and marmoset tissues were cryoprotected in 30% sucrose (w/v) in PBS, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in a dry ice/ethanol bath. Serial 40 µm floating sections of the entire brain as well as 3 mm segments from cervical, thoracic and lumbar regions of the spinal cord were cut with a Cryostat (Thermo Microm HM 550).

Immunohistochemistry. Free-floating sections of the Brain and spinal cord were incubated in blocking buffer containing 0.1% Triton-X100 (v/v) (Fisher, Pittsburg, PA) and 10% goat serum (v/v) (Invitrogen) for 1h at room temperature. The sections were then incubated at 4C overnight with primary antibodies diluted in blocking buffer. The following day tissue sections were washed twice in 0.05% Tween-20 (v/v) in PBS (PBST) and once with PBS, with each washing step lasting 10 min. Sections were subsequently incubated with appropriate secondary antibodies in blocking buffer for 1 h at room temperature. For fluorescent detection, sections were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and analyzed using an epifluorescent microscope (Nikon Eclipse Ti). For Avidin Biotin Complex-3,3'-Diaminobenzidine (ABC-DAB) detection, sections were incubated in 0.1% peroxide/PBS and

biotin/avidin blocking solution prior to primary antibody incubation to quench endogenous peroxidase and biotin activity. After incubation with a biotinylated secondary antibody, sections were washed and incubated in ABC reagent (PK-6200, VectorLabs) for 30 minutes at room temperature. After a quick wash in PBS, sections were developed with DAB solution (SK-4105, VectorLabs) for 60 seconds followed by 3 consecutive washes in PBS lasting 5 minutes each. Sections were subsequently dehydrated in a graded ethanol series of 50%, 70%, 95%, 100%. After 2 changes of Xylenes, sections were coverslipped with permount mounting media (Fisher) and left overnight to dry. The primary antibodies used in this study were as follows: rabbit anti-GFP (Invitrogen), goat anti-ChAT and mouse anti-NeuN (both from Millipore, Billerica, MA), mouse anti-GFAP (Cell signaling, Danvers, MA), rabbit anti-CD31 (Abcam, Cambridge, MA), mouse anti-Calbindin D-28k (Sigma, St Louis, MO) and mouse anti-tyrosine hydroxylase monoclonal antibody (Millipore, Billerica, MA).

Semi-quantitative analysis of EGFP positive cells transduced by rAAVs. We reviewed clinically relevant regions including cortex, corpus callosum, hippocampus, striatum, cerebellum, thalamus and medulla of the brain as well as cervical, thoracic and lumbar sections of the spinal cord (**Figs. 1, 2** and **4; Suppl. Figs. 1, 2** and **3**). To provide a semi-quantitative measure of the amount and type of cells that were transduced by rAAVs in the CNS, we used a previously described [22] scoring system that was originally developed by Cearley et al. [33]. Briefly, the number of (+) corresponds to the number of EGFP positive cells where (+) means very few positive cells, (++) some positive cells, (+++) many positive cells, (++++) large areas of positive cells and (+++++) saturated with positive cells. Regions with no EGFP expression is marked as (-). Mean scores were calculated by averaging plus signs for all brain and all spinal cord areas (n=3). The cell morphology of these sections was used to identify EGFP positive astrocytes, oligodendrocytes and neurons in multiple sections of any given region for each serotype. Sections of rAAVrh.8-transduced cells were stained for different cell markers to confirm the cell

identity (see **Fig. 5**). Statistical analysis was performed using one-way ANOVA test for comparison of semi-quantitative scores as well as biodistribution data.

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FIGURE LEGENDS

Figure 1. General transduction characteristics of 12 rAAVs in the adult mouse brain. rAAV*EGFP*s were injected into the tail vein of 10 week-old mice at $4x10^{12}$ genome copies (GCs) per mouse (n=3). Forty micrometer frozen sections of CNS tissues were obtained 21 days post injection and stained with antibody against EGFP. Staining was visualized with an Avidin-Biotin Complex / 3,3'-Diaminobenzidine substrate system. Shown are composite images of bregma -2.00mm brain sections of rAAV injected mice organized into four groups based on EGFP semi-quantitative score and extend of distribution**.** Bar = 1000µm.

Figure 2. General transduction characteristics of 12 rAAVs in the adult mouse spinal cord. rAAV*EGFP*s were injected into the tail vein of 10 week-old mice at $4x10^{12}$ genome copies (GCs) per mouse (further description see Fig. 1, n=3). Shown are composite images of CSC (cervical spinal cord), TSC (thoracic spinal cord) and LSC (lumbar spinal cord) sections of rAAV injected mice organized into four groups based on EGFP semi-quantitative score and extend of distribution. Bar = 250µm.

Figure 3. Quantification of transduced cell types in the CNS of adult mice.

Semi-quantitative scores of neuronal and glia cell types transduced by different rAAV*EGFP*s. EGFP positive CNS cell types are microscopically scored and the means of rAAV transduced astrocytes, neurons and oligodendrocytes in the brains and spinal cords are shown (n=3). ** $p<0.0033$, *** $p<0.001$, **** $p<0.0001$, ns = non-significant.

Figure 4. Transduction profile of various neuronal populations by rAAVrh.8.

rAAVrh.8 vectors were injected into the tail vein of 10-week-old mice at $4x10^{12}$ genome copies per mouse. Staining for EGFP was carried out as described in Fig. 1 legend. Shown are the

compilation of high magnification images of CNS cell transduction and their corresponding locations in a low magnification view at bregma -2.00mm (**A-I**) as well as some other clinical relevant brain regions (i.e. OB=olfactory bulb, SN=substantia nigra and LSN=lateral septal nucleus and cerebellum) that were not present in the lower magnification view of the selected brain section. DG=dentate gyrus, CC=corpus callosum, CA2=CA2 region of hippocampus. Bars in A, C, H = 10µm. Bars in D, F, I = 15µm and bars in B, E, G, J, K, L = 20µm

Figure 5. Identifying CNS cell types transduced in the adult mice that received I.V. injection of rAAVrh.8*EGFP*. Sections were stained with antibodies against EGFP, NeuN (neurons), GFAP (astrocytes), APC (oligodendrocytes), Iba1 (microglia), CD31 (blood vessels). Objective 60x. Bar = 20um

Figure 6. Biodistribution profiles in mice. (A) Biodistribution profiles of rAAV*EGFP*s in the adult mice after I.V. injections. Persisted rAAV*EGFP* vector genomes in 8 different tissues of rAAV treated mice were quantified by qPCR using primers/probe set targeting poly A region of the vector genome (n=3). **(B).** Ratios of vector genomes detected in the brains to those in the liver, heart, lung and pancreas tissues respectively (n=3). **** $p < 0.0001$, ns = non significant

Figure 7. Robust EGFP transduction in the spinal cord and midbrain of an adult marmoset received I.V. injected rAAVrh.10*EGFP*. A 4 year-old male marmoset was I.V. injected with rAAVrh.10CB*EGFP* at a dose of 5x10e13 GCs/kg and necropsied 2 weeks later. The CNS tissues were isolated, fixed, sectioned, and stained for EGFP as described in Methods and Fig 1 legend. All sections were counterstained with Haematoxylin. (A, C, E) Low magnification images of cervical, thoracic and lumbar spinal cord. (B, D, F) High magnification of the boxed areas showing the transduced motor neurons in A, C and E, respectively. (**G, H**) Low magnification images of ventral midbrain. (**I**) High magnification of the boxed areas in G

and H showing well transduced motor neurons in the oculomotor nucleus (3N). Bars in **A, C, E, G, H** = 200μm. Bars in **B, D, F, I** = 100μm.

Figure.8. EGFP transduction of peripheral tissues in adult marmosets treated with I.V. or I.T. injected rAAVrh.10*EGFP* or *EGFP3XMRBS* vector. Male adult marmosets were I.V. injected with rAAVrh.10CB*EGFP* or rAAVrh.10CB*EGFP-(miR-1BS)^3-(miR-122BS)^3* at a dose of 5x10e13 GCs/kg or I.T. injected with rAAVrh.10CB*EGFP* at a dose of 2.7x10e12 GCs/kg. Eight micrometer sections of peripheral tissues were counter stained with DAPI fluorescence and microscopically evaluated for native EGFP expression. Presented are the fluorescent images of native EGFP expression (overview 100X, insets 400X objective) of liver (**a, c, e**) and adrenal gland (**b, d, f**) tissues of adult male marmosets received rAAVrh.10*EGFP* by I.V. (**a, b**) or I.T. (**e, f**) injection or rAAVrh.10*EGFP3XmiRBS* by I.V. injection (**c, d**). Bars in a-i = 50 μm. Bars in insets 10 μm.

Supplementary Figure 1. Motor neuron transduction patterns of different rAAV*EGFP*s in the lumbar spinal cords of adult mice. rAAVs were injected into the tail vein of 21 day-old mice at $4x10^{12}$ genome copies per mouse (n=3). Staining and visualization was performed as described in legend of Fig. 1. The microscopic images of lumbar spinal cord sections are shown for each rAAV. Black arrows indicate motor neurons expressing EGFP. Black boxes are representative high magnification views of motor neurons expressing EGFP. Bar = 200μm.

Supplementary Figure 2. Regional transduction patterns of rAAVs in the adult mouse brains. rAAV*EGFP*s were injected into the tail vein of 10 week-old mice at $4x10^{12}$ genome copies per mouse. Forty micrometer frozen sections were obtained 21 days post injection and stained and visualized for EGFP as described in Fig. 1 legend. Cortex, corpus callosum, hippocampus, striatum, cerebellum, thalamus and medulla are shown for each rAAV-injected mouse. 10x objective. Bar = 100μm.

Supplementary Figure 3. Transduction profiles of various neuronal populations by rAAVrh.10 and 9. rAAVrh.10 and 9 vectors were injected into the tail vein of 10-week-old mice at $4x10^{12}$ genome copies per mouse (n=3). Staining for EGFP was carried out as described in Fig. 1 legend. Shown are the compilation of high magnification images of CNS cell transduction and their corresponding locations in a low magnification view at bregma -2.00mm (**A-I**) as well as some other clinical relevant brain regions (i.e. cerebellum, substantia nigra and lateral septal nucleus) that were not present in the lower magnification view of the selected brain section (**J-L**). For localization of anatomical regions see Fig. 4. Bars in A, C, H = 10μm. Bars in D, F, I = 15μm and bars in B, E, G, J, K, L = 20μm

Supplementary Figure 4. Choroid plexus transduction pattern of different I.V. delivered rAAV serotypes in mouse. Shown are representative EGFP staining signals in coronal

sections of the corpus callosum and third ventricle demonstrating the transduction of the choroid plexus by different rAAV serotypes. Bar = 200µm

Supplementary Figure 5. Identifying neuronal populations transduced in the adult mice that received I.V. injection of rAAVrh.8*EGFP*. Sections were stained with antibodies against EGFP, Calbindin (Purkinje cells), ChAT (motor neurons), Drapp-32 (medial spiny neurons) and tyrosine hydroxylase (TH; dopaminergic neurons). Objective 60x. Bar = 20um.

Supplementary Figure 6. EGFP transduction of different regions in the spinal cords of an adult marmoset received I.V. injected rAAVrh.10*EGFP*. High magnification images of the posterior horn and intermediate zone regions of spinal cord sections presented in **Fig. 7**. **(A, B)** Cervical spinal cord, **(C, D)** thoracic spinal cord, and **(E, F)** lumbar spinal cord. **(A, C, E)**: posterior horn and **(B, D, F):** intermediate zone. Bars=50µm.

Supplementary Figure 7. EGFP transduction of different regions in the medulla oblongata of an adult marmoset received I.V. injected rAAVrh.10*EGFP*. Low magnification images of **(A)** dorsolateral, **(B)** dorsal median and **(C)** ventrolateral medulla oblongata. The red boxes in **A, B** and **C** were enlarged in **D, E** and **F**, respectively. The blue boxes in **A, B** and **C** were enlarged in **G, H** and **I**, respectively. **VeN**: vestibular nucleus; **12N**: hypoglossal nucleus; **py**: pyramidal tract; **IO**: inferior olive; **icp**: inferior cerebellar peduncle; **Sp5**: spinal trigeminal nucleus; **sp5**: spinal trigeminal tract; **LRt**: lateral reticular nucleus. Bars in **A-C =** 200µm, Bars in **D-I =** 100µm.

Supplementary Figure 8. EGFP transduction of different regions in the ventral midbrain of an adult marmoset received I.V. injected rAAVrh.10*EGFP*. High magnification images of selected regions in ventral midbrain shown in **Fig.7 G and H. (A, B)**: Red nucleus (RN). **(C)**

Ventrolateral midbrain region with substantia nigra (SN) marked with a blue box. **(D)** An enlarged view of SN region marked with a blue box in **(C)**. Bars in A, B, D = 100μm. Bar in C =200μm.

Supplementary Figure 9. Sparse neuronal EGFP transduction of some brain regions in an adult marmoset treated with I.V. injected rAAVrh.10*EGFP*. (A, C, E) are low magnification images of cerebellum, hippocampus and cortex, respectively. **(B, D, F)** are enlarged views of the red boxes in **A, C** and **E**, respectively. Bars in **A, C, E** = 100μm, Bars in **B, D, F** = 50μm.

Supplementary Figure 10. Sparse EGFP transduction of the CNS tissues in an adult marmoset treated with I.V. injected rAAVrh.10*EGFP3XmiRBS*. (A, B, C) show low magnification images of EGFP transduction in the thoracic and lumbar spinal cord, and midbrain regions, respectively **(D, E, F)** are high magnification views of the red boxes in **A, B** and **C**, respectively. **(G, H, I)** are high magnification images of the blue boxes in **A, B** and **C**, respectively. **3N**: oculomotor nucleus. Bars in **A-C** = 200μm; Bars in **D-I** = 50μm.

Supplementary Figure 11. EGFP transduction of several brain regions in an adult marmoset treated with I.V. injected rAAVrh.10*EGFP3XmiRBS*. **(A, C, E)** are low magnification images of cerebellum, hippocampus and cortex regions, respectively. **(B, D, F)** are enlarged views of the red boxes in **A, C** and **E**, respectively. Bars in **A, C, E** = 200μm. Bars in **B, D, F** = 50μm.

Supplementary Figure 12. EGFP transduction of the CNS in adult marmosets treated with I.V. injected *EGFP3XMRBS* vector. Male adult marmosets were I.V. injected with rAAVrh.10CB*EGFP-(miR-1BS)^3-(miR-122BS)^3* at a dose of 5x10e13 GCs/kg. Low magnification images (**A**) cervical spinal cord, (**B**) dorsolateral and (**C**) ventrolateral medulla oblongata. (**D, E, F**) High magnification images of the red boxed areas in A, B and C, respectively. (**G, H, I**) High magnification images of the blue boxed areas in A, B and C, respectively. 12N: hypoglossal nucleus; 10N: nucleus of vagus. Gi: gigantocellular reticular nucleus; IO: inferior olive. Bars in a-c = 200μm. Bars in d-I = 50μm.

Supplementary Table 1. The 12 different rAAV serotypes are listed according to the semi-quantitative analysis.