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Selection of Optimal AAV Serotype for Brain-Wide Gene Delivery Following Intracerebroventricular Injection in mdx52 Mice

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Abstract

Adeno-associated virus (AAV)-based gene therapy is a promising approach for the treatment of Duchenne muscular dystrophy. In addition to muscle degeneration, patients exhibit cognitive and behavioral impairments, highlighting the need for efficient brain targeting. While several AAV serotypes efficiently transduce muscle and brain tissues, their diffusion remains limited.

To address this challenge, this study aimed to compare the brain diffusion of different AAV serotypes in the *mdx52* mouse model: AAV9, a peptide-modified AAV9 (PM-AAV9), AAVDJ, and AAVrh10. We evaluated AAV distribution using a GFP reporter gene following intracerebroventricular injection in neonatal or adult mice. Brain-wide transduction was assessed by fluorescence imaging and quantitative analysis. In addition, cell-type tropism was characterized through colocalization with neuronal and glial markers in the hippocampus, cortex, and cerebellum.

Our results demonstrate that neonatal intracerebroventricular injection enhances brain-wide AAV distribution compared to adult delivery. Moreover, following neonatal intracerebroventricular injection, AAVrh10 shows superior brain diffusion and expression, with a predominant neuronal tropism across serotypes. These findings highlight the importance of serotype selection for optimizing central nervous system gene delivery. Overall, this study provides valuable insights for the development of AAV-based strategies targeting brain comorbidities in Duchenne muscular dystrophy, with potential applications to other neurological disorders.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations in the *DMD* gene, leading to the absence of a functional dystrophin. In addition to progressive muscle degeneration, 30–50% of patients present neurobehavioral comorbidities.

Although several genetic therapies have been developed to restore dystrophin expression in muscle, their ability to address CNS manifestations remains limited. In fact, over the past few years, several genetic approaches have been investigated to restore dystrophin expression in muscle tissues, including antisense oligonucleotides (ASOs) and AAV-based gene therapies, notably AAV-microdystrophin. The AAV-microdystrophin strategy consists of delivering, using an AAV vector, a shortened dystrophin-encoding sequence that retains key functional domains of the dystrophin protein while fitting within the limited packaging capacity of the vector. This strategy has received FDA approval for the treatment of DMD muscle manifestations with Elevidys (1, 2). A major advantage of AAV-based strategies is their ability to provide long-term transgene expression in target cells (3). However, this approach cannot readily be used to restore dystrophin expression in the CNS or to investigate the specific roles of individual dystrophin isoforms. Indeed, AAV vectors encoding microdystrophin are primarily designed for muscle targeting, with the transgene typically placed under the control of a muscle-specific promoter. Additionally, systemically administered AAV vectors have limited ability to cross the blood–brain barrier (BBB). In contrast to the AAV-microdystrophin approach, ASO-mediated exon-skipping strategies can

restore the expression of specific dystrophin isoforms depending on the mutation and the exon targeted for skipping. ASOs are not dependent on a specific promoter and can therefore act wherever the target dystrophin transcript is naturally expressed. ASO strategies also reached an advanced stage of clinical development for DMD, leading to the approval by the FDA of several ASO-based therapies targeting skeletal muscle, including casimersen (exon 45 skipping), eteplirsen (exon 51 skipping), golodirsen and viltolarsen (exon 53 skipping) (4, 5). ASO-based therapies have also shown therapeutic potential for several neurological disorders, particularly following direct CNS administration, including spinal muscular atrophy, SOD1-related familial ALS, Parkinson's disease and Huntington's disease (6–9). However, the therapeutic effects of ASOs are limited by their relatively transient persistence in target cells, requiring repeated administration to maintain therapeutic effect. Our group and others have previously investigated ASO-based strategy for DMD following local CNS administration, achieving encouraging results but levels dystrophin restoration remained relatively low and transient, highlighting the need for repeated administration(10–13).

To overcome these limitations while retaining the advantages of exon-skipping and AAV-based approaches, an AAV-mediated exon-skipping strategy can be used for DMD-associated brain manifestations (13, 14). This strategy consists of using an AAV vector to deliver small nuclear RNAs (snRNAs), such as modified U7 snRNAs, designed to induce exon skipping (15–17). In contrast to ASOs, AAV-mediated delivery has the potential to provide sustained expression of the exon-skipping machinery following a single administration. However, efficient and widespread delivery of AAV vectors throughout the brain remains a major challenge. Although some AAV serotypes have demonstrated the ability to cross the BBB following systemic administration, direct administration into the CNS can bypass this anatomical barrier and provide higher vector exposure to the brain. Nevertheless, the extent of vector distribution, transgene expression, and cellular tropism following administration route can vary substantially depending on the AAV capsid and the biological characteristics of the recipient (18, 19).

Several approaches have been developed to improve AAV tropism and transduction efficiency in the CNS. These include the identification of naturally occurring serotypes, the generation of chimeric capsids through recombination of different serotypes, and rational capsid engineering through peptide insertion or site-directed mutagenesis as well as directed evolution approaches and ligand- or antibody-mediated capsid conjugation to promote selective interactions with brain-specific receptors (20, 21). More recently, artificial intelligence-assisted approaches have also emerged to design novel AAV capsids with optimized CNS targeting properties.

For our study, we selected four AAV capsids that presented high potential for CNS delivery: AAV9 and AAVrh10, representing naturally occurring serotypes; PM-AAV9, a peptide-modified AAV9 variant; and AAVDJ, a chimeric capsid. Because AAV biodistribution and transduction profiles can be influenced by the genetic and pathological background of the recipient, evaluating capsid performance in a disease-relevant model is essential. We therefore selected the *mdx52* mouse model of DMD, which is widely used to investigate DMD-associated brain manifestations (10, 22–26). This model carries a deletion of exon 52 of the *dmd* gene, resulting in the loss of both Dp427 and Dp140 dystrophin isoforms in the brain. This

combined deficiency is relevant to CNS manifestations observed in DMD patients, as the loss of Dp140 in addition to full-length dystrophin has been associated with increased severity of cognitive and neurobehavioral impairments(27–30). In this study, the four selected AAV capsids were administered by intracerebroventricular injection (ICV) in adult or neonatal *mdx52* mice using a CAG promoter-driven GFP reporter cassette to characterize their brain distribution, transduction efficiency, and cellular tropism. This comparative analysis aims to identify AAV capsid properties associated with efficient CNS delivery and to provide insights for the development of future brain-targeted gene therapies for DMD-associated neurological manifestations.

Material and Methods

Animals and AAV vectors

The *mdx52* mouse model of Duchenne muscular dystrophy, characterized by a deletion of exon 52 in the DMD gene, was developed by Dr. Katsuki Motoya's team. This model was created by replacing exon 52 with a neomycin resistance gene, resulting in the absence of Dp427, Dp260, and Dp140 dystrophins, while maintaining the expression of Dp116 in peripheral nerves and Dp71 in the brain and retina. The strain was backcrossed with C57BL/6J mice for over eight generations to ensure genetic stability. This mouse line was generously shared by Prof. Toshikuni Sasaoka (Department of Comparative & Experimental Medicine/Brain Research Institute, Niigata University, Japan). Breeding pairs were provided to our laboratory by Dr. Jun Tanihata and Dr. Shin'ichi Takeda (National Center of Neurology and Psychiatry, Tokyo, Japan). In our facility at UVSQ in Montigny-le-Bretonneux (France), heterozygous females were bred with *mdx52* males to produce *mdx52* males and their C57BL/6J wild-type (WT) littermates. Genotyping was performed using PCR analysis of tail DNA from neonatal mice or ear tissue from adult mice. All animal care and experimental protocols adhered to European regulations (CEE 86/609/EEC and EU Directive 2010/63/EU) as well as French national guidelines (87/848) and were approved by the Paris Center-South Ethics Committee (no. 47).

AAV9, PM-AAV9, AAVrh10, and AAVDJ vectors were produced by triple transfection of HEK293T cells using the expression plasmid pscAAV-CAG-GFP (Addgene plasmid #83279; gift from Mark Kay), the helper plasmid pXX6 (31), and the corresponding capsid plasmid : pAAV9, pPM-AAV9 (gift from Beverley Davidson), pAAVrh10, or pAAVDJ. Seventy-two hours after transfection, cells were harvested and lysed, and AAV particles were purified by iodixanol gradient ultracentrifugation. Viral genome (vg) titers were determined by quantitative real-time PCR (qPCR) as previously described (32).

ICV injection and experimental groups

Intracerebroventricular (ICV) injections in adult male *mdx52* mice were performed by stereotaxic surgery. Seven-week-old mice were deeply anesthetized with a single intraperitoneal injection of ketamine (95 mg/kg) and medetomidine (1 mg/kg). AAV vectors were bilaterally injected into the lateral ventricles

using the following stereotaxic coordinates relative to bregma: 0.5 mm posterior, 1 mm lateral, and 2 mm ventral from the dura mater. Each lateral ventricle received 4 µL of the appropriate AAV preparation and each mice received a total dose of 1×10^{12} viral genomes (vg).

ICV injections in neonatal mice were performed on postnatal day 1 (P1). Neonatal pups were anesthetized by hypothermia on ice until complete immobility was achieved. AAV vectors were bilaterally injected into the lateral ventricles using the coordinates previously described by Kim *et al.* : 1 mm lateral to the superior sagittal sinus, midway between bregma and lambda, and 2 mm below the skull surface (33). Each lateral ventricle received 2 µL of the appropriate AAV preparation and each mouse received a total dose of 1×10^{11} viral genomes (vg).

Each mouse received AAV9, PM-AAV9, AAVrh10, or AAVDJ vector encoding GFP under the control of the CAG promoter (scAAV9-CAG-GFP, scPM-AAV9-CAG-GFP, scAAVrh10-CAG-GFP, or scAAVDJ-CAG-GFP). Following surgery, mice were housed in groups of two to five per cage under a 12 h light/dark cycle (lights on at 7:00 a.m.) with ad libitum access to food and water.

Tissue collection

Four weeks after ICV injection, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were carefully dissected, post-fixed in 4% PFA for 30 minutes at 4°C, cryoprotected overnight in 30% PBS-sucrose, and stored at − 80°C until sectioning. Coronal forebrain sections (14 µm) and sagittal cerebellum sections (14 µm) were obtained using a cryostat and processed for subsequent histological and immunofluorescence analyses.

Immunochemistry

Coronal and sagittal brain sections for forebrain and cerebellum respectively were air-dried for 10 minutes at room temperature (RT), then permeabilized in PBS containing 0.5% Triton X-100 for 15 min at RT. Slices were subsequently incubated in a blocking solution for 45 minutes at RT (5% normal goat serum, 0.1% Triton X-100, 1% BSA), followed by overnight incubation at 4°C with a rabbit polyclonal anti-GFAP antibody (Dako, ref. Z0334; 1:1000 dilution in blocking buffer) and chicken polyclonal anti-GFP antibody (Invitrogen, ref. AB13970; 1:1000). After washing in PBS, sections were incubated with secondary antibodies for 2 hours at RT: a goat anti-rabbit IgG 647 (Invitrogen, ref. A21245; 1:300), a goat anti-chicken (Molecular Probes Invitrogen, ref. A11039; 1:400), counterstained with NeuroTrace Nissl 530/615 (Thermo Fisher Scientific, ref. N21482; 1:500) and DAPI (Thermo Fisher Scientific, ref. D1306). Control sections processed without the primary antibodies showed no specific staining. Imaging was performed at equivalent anatomical locations and under identical acquisition settings using an Olympus widefield microscope with a ×10 objective.

GFP diffusion analysis

Image analysis was performed using Imaris software. The total area of each section was first segmented using the ‘Segmentation Setup’ tool with the machine learning segmentation option enabled and ‘Keep training data’ box checked. To quantify the GFP-labeled area, a second surface was created based on signal in the FITC channel. The segmentation method ‘Absolute Intensity Thresholding’ was applied, and the thresholding was manually optimized to define specific GFP labeling across sections. For brain sections, a threshold value of 240 was used and 150 for the cerebellum. The percentage of total GFP marked area was determined by area sum parameters collected from the first and second surface, following this ratio: (total area sum / GFP area sum) x 100. The GFP intensity was directly collected from the second surface.

AAV tropism analysis

Image analysis was performed using Imaris software. For each widefield image, the regions of interest (ROIs) were manually delineated (CX, HIP, and CBL) and analyzed separately. A “GFP-positive” surface was then generated using the machine-learning tool to identify GFP-positive cells within each ROI.

To identify neuronal and astrocytic populations among GFP-positive cells, Nissl (channel 3) and GFAP (channel 4) surfaces were generated using manually defined intensity thresholds for each marker. The mean fluorescence intensity was then measured within each Nissl- or GFAP-positive surface and used as the reference intensity for cell classification.

The GFP-positive cells identified by the machine-learning analysis were exported to Excel together with their mean fluorescence intensity values for channels 3 and 4. Each GFP-positive cell was classified according to its mean intensity relative to the corresponding reference intensity. Cells with a mean intensity above the reference intensity were considered positive for the corresponding marker (Nissl or GFAP) and classified as neurons or astrocytes, respectively.

The percentage of colocalization with neurons or astrocytes was calculated as follows:

Neuronal tropism (%) = (number of Nissl-positive GFP+ cells / total number of GFP+ cells) × 100

Astrocytic tropism (%) = (number of GFAP-positive GFP+ cells / total number of GFP+ cells) × 100.

Neuronal transduction efficiency analysis

Image analysis was performed using Imaris software. For each widefield image, the regions of interest (ROIs) were manually delineated (CX, HIP, and CBL) and analyzed separately. A “Spots” detection based on the Nissl signal (channel 3) was used to automatically identify neurons. Each detected spot was then manually classified as GFP-positive or GFP-negative based on the GFP signal.

The total number of neurons and the number of GFP-positive neurons were exported to Excel. The percentage of neuronal transduction was calculated as follows:

Neuronal transduction efficiency (%) = (number of GFP-positive neurons / total number of neurons) × 100.

Statistical analysis

All data are presented as mean ± SEM. The number of animals per group is indicated as “N” in the figure legends. Statistical analyses were performed using GraphPad Prism. Group comparisons were performed using one-way or two-way analysis of variance (ANOVA), with repeated-measures ANOVA applied where appropriate. To evaluate the overall effect of treatments across multiple tissues, two-way ANOVA was used, and the corresponding treatment *P*-value is reported in the text or figure legend. When data did not meet the assumptions of normality, as determined by the Shapiro–Wilk test, the non-parametric Kruskal–Wallis test was employed, followed by Dunn’s multiple comparisons test. Statistical significance was defined as follows: *p < 0.05, **p < 0.01, ***p < 0.0005 and ****p < 0.0001.

Results

AAVrh10 shows broader brain-wide GFP distribution and higher GFP expression than other serotypes following neonatal ICV administration

To identify the AAV capsid providing the most efficient CNS transduction in a DMD context, *mdx52* mice received an intracerebroventricular (ICV) injection of AAV9, PM-AAV9, AAVrh10, or AAVDJ, each carrying an identical CAG-driven GFP reporter cassette (Fig. 1A). We first evaluated these capsids following neonatal administration, with *mdx52* mice receiving an equivalent dose of 1E + 11 vector genomes (vg) at postnatal day 1 (P1). Brains were fixed and collected seven weeks after injection for histological analyses (Fig. 1B).

Whole-brain GFP immunostaining revealed marked differences in GFP distribution among the four AAV capsids (Fig. 1C and Supplementary Figure S1). AAVrh10 produced the broadest GFP-positive distribution throughout both the forebrain and cerebellum (CBL) compared with AAV9, PM-AAV9, and AAVDJ. Qualitative examination of coronal sections further showed that GFP expression was most prominent in regions located close to the ventricular system, particularly the hippocampus (HIP) and cerebral cortex (CX), whereas lower GFP expression was observed in deeper structures such as the hypothalamus. This spatial pattern was observed across all four capsids and was consistent with a progressive reduction in transgene distribution with increasing distance from the ventricles, the site of vector administration.

Quantification of the GFP-positive area confirmed these observations (Fig. 1D). In the forebrain, AAVrh10 resulted in a significantly larger GFP-positive area than AAV9 ($P = 0.0212$) and AAVDJ ($P = 0.0098$), reaching near-complete forebrain coverage. In contrast, AAV9 and AAVDJ transduced approximately half of the forebrain, while PM-AAV9 transduced approximately 60%. In the CBL, AAVrh10 also showed a significant larger GFP-positive area compared with AAV9 ($P = 0.0196$), while PM-AAV9 and AAVDJ displayed intermediate levels of coverage.

Having established differences in the extent of GFP distribution, we next quantified GFP fluorescence intensity to determine whether the capsids also differed in their overall levels of transgene expression (Fig. 1E). Consistent with the fluorescence images, AAVrh10 produced the strongest GFP signal. In the forebrain, total GFP fluorescence intensity was significantly higher following AAVrh10 administration than following AAV9 ($P = 0.0139$) or AAVDJ ($P = 0.0385$), whereas PM-AAV9 showed intermediate levels. Similarly, in the CBL, GFP fluorescence intensity was significantly higher with AAVrh10 than AAV9 ($P = 0.0055$) and AAVDJ ($P = 0.0327$), while PM-AAV9 again showed intermediate levels. Together, these results identify AAVrh10 as the most effective of the four capsids tested for achieving broad and robust transgene expression throughout the brain following neonatal ICV administration.

Neonatal ICV delivery provides broader and more robust brain transduction than adult administration

To evaluate the transduction capacity of the different AAV capsids in the adult brain and compare their performance with neonatal administration, adult *mdx52* mice received a single bilateral ICV injection of 1E + 12 vg of AAV9, PM-AAV9, AAVrh10, or AAVDJ, each carrying the same CAG-driven GFP reporter cassette (Fig. 2A–B).

Whole-brain GFP immunostaining revealed a spatial pattern broadly similar to that observed following neonatal administration (Fig. 2C and Supplementary Figure S2). Across all four capsids, GFP expression was most prominent in regions located close to the ventricular system, particularly the hippocampus (HIP) and cerebral cortex (CX), which are proximal to the site of vector administration, whereas lower expression was observed in deeper structures such as the hypothalamus. AAVrh10 again appeared to produce the broadest GFP-positive distribution throughout the forebrain and cerebellum compared with AAV9, PM-AAV9, and AAVDJ. However, quantification of the GFP-positive area showed only a trend toward greater forebrain coverage with AAVrh10, which did not reach statistical significance (Fig. 2D). Inter-individual variability was also more pronounced in adult-treated animals than in neonatally treated mice. Similarly, GFP fluorescence intensity tended to be higher with AAVrh10 in the forebrain, but differences among capsids were not statistically significant (Fig. 2E).

We next directly compared the extent and intensity of GFP expression following neonatal and adult administration for each capsid. In the forebrain, quantification of the GFP-positive area revealed significantly greater transduction following neonatal delivery than adult delivery overall ($P = 0.0077$, two-way ANOVA; Fig. 3A), and a significant capsid effect was also observed ($P = 0.0035$), with no significant age × capsid interaction ($P = 0.9415$). Among the neonatal groups, AAVrh10 achieved nearly complete forebrain coverage and showed significantly greater GFP-positive area than AAV9 and AAVDJ ($P = 0.0354$ and $P = 0.0163$ respectively). In the cerebellum, the advantage of neonatal delivery was most apparent for AAVrh10 and PM-AAV9 although not statistically different (Fig. 3B).

Comparison of total GFP fluorescence intensity further supported the benefit of neonatal administration (Fig. 3C–D). Higher GFP signal was observed after neonatal delivery, particularly for AAVrh10 and PM-

AAV9. This difference was especially pronounced in the cerebellum, where AAVrh10-mediated GFP fluorescence was significantly higher in neonatally treated than in adult-treated *mdx52* mice ($P < 0.0001$). Overall, these findings demonstrate that neonatal ICV administration results in broader and more robust brain transduction than adult delivery, while AAVrh10 provides the most extensive brain coverage among the capsids tested.

AAV capsids predominantly transduce neurons following ICV administration

As AAVrh10 exhibited the broadest brain distribution and highest GFP expression following neonatal ICV injection, we next investigated whether this enhanced transduction profile was associated with differences in cellular tropism. Since AAV cellular tropism can be influenced by both capsid and route of administration, we characterized the cellular distribution of all four AAV capsids under our experimental conditions. Previous studies have reported that AAV9 and AAVrh10 predominantly transduce neurons, with more limited astrocytic transduction following CNS delivery (18, 34, 35). We therefore assessed whether similar tropism profiles were observed for AAV9, PM-AAV9, AAVrh10, and AAVDJ. Neurons were identified using Nissl staining and astrocytes using GFAP immunostaining in *mdx52* brains following neonatal (Fig. 4A) or adult (Fig. 5A) ICV administration.

Qualitative examination revealed extensive colocalization of GFP with Nissl-positive cells, whereas colocalization with GFAP-positive cells was more limited across all four capsids, suggesting a predominantly neuronal transduction profile. We therefore quantified GFP colocalization with Nissl- and GFAP-positive cells across the hippocampus (HIP), cerebral cortex (CX), and cerebellum (CBL). Following neonatal administration, GFP colocalization was significantly higher with Nissl-positive than with GFAP-positive cells for all four capsids across the brain regions analyzed (Fig. 4B; AAV9, $P = 0.0070$; PM-AAV9, $P = 0.0014$; AAVDJ, $P < 0.0001$; AAVrh10, $P = 0.0083$; two-way ANOVA, Nissl vs GFAP), demonstrating a predominant neuronal tropism across all four capsids used. Although the extend of GFP colocalization with neuronal and astrocytic markers varied across brain regions and AAV capsids, neuronal colocalization was consistently higher.

A predominantly neuronal transduction profile was also observed following adult ICV administration (Fig. 5B). GFP colocalization with Nissl-positive cells was significantly higher than with GFAP-positive cells for AAV9 ($P = 0.0048$), PM-AAV9 ($P < 0.0001$), and AAVDJ ($P = 0.0327$). AAVrh10 showed a similar trend, although the overall difference was not statistically significant ($P = 0.1053$). Overall, these findings indicate that ICV administration of the capsids tested preferentially targets neurons over astrocytes in the *mdx52* brain, both at neonatal and adult stages.

AAVrh10 and PM-AAV9 show the highest neuronal transduction efficiency following neonatal ICV delivery

Having characterized the cellular tropism of the different AAV capsids, we next assessed their neuronal transduction efficiency. In contrast to the tropism analysis, which determines the cellular identity of GFP-positive cells, neuronal transduction efficiency was defined as the proportion of Nissl-positive neurons that were also GFP-positive, thereby reflecting the percentage of neurons successfully transduced by each capsid.

In neonatal mice, neuronal transduction efficiency varied across AAV capsids and brain regions (Fig. 6). In the cortex, AAV9, PM-AAV9, AAVDJ, and AAVrh10 transduced approximately 25%, 50%, 15%, and 40% of Nissl-positive neurons, respectively. A significant overall effect of capsid was observed ($P = 0.0014$, ordinary one-way ANOVA test), with PM-AAV9 and AAVrh10 showing the highest neuronal transduction efficiencies. Post-hoc analysis (Tukey's multiple-comparison test) confirmed significantly greater neuronal transduction with PM-AAV9 compared with AAV9 ($P = 0.0147$) and AAVDJ ($P = 0.0016$), as well as AAVrh10 compared with AAVDJ ($P = 0.0073$).

A significant overall effect of capsid was also observed in the hippocampus ($P = 0.0071$, ordinary one-way ANOVA test), where approximately 35%, 40%, 15%, and 40% of Nissl-positive neurons were transduced by AAV9, PM-AAV9, AAVDJ, and AAVrh10, respectively. Post-hoc analysis (Tukey's multiple-comparison test) shows significantly greater neuronal transduction with AAV9, PM-AAV9 and AAVrh10 compared with AAVDJ (AAV9, $P = 0.0493$; PM-AAV9, $P = 0.0099$; AAVrh10, $P = 0.0105$). However, in the cerebellum, no significant differences were observed in the percentage of Nissl-positive Purkinje neurons expressing GFP, with approximately 70%, 80%, 70%, and 90% of Purkinje neurons transduced by AAV9, PM-AAV9, AAV-DJ, and AAVrh10, respectively.

Overall, these results identify PM-AAV9 and AAVrh10 as the most efficient capsids for neuronal transduction following neonatal ICV administration, despite regional variations in transduction efficiency.

Discussion

In this study, we first assessed GFP distribution and expression in the brain of neonatal *mdx52* mice following ICV administration of four AAV capsids: AAV9, PM-AAV9, AAVDJ and AAVrh10. Although these capsids have been individually compared to AAV9 in previous studies, they have not been evaluated together in a single study. This allowed us to directly assess their relative performance under identical experimental conditions in the same DMD mouse model. Among the capsids tested, AAVrh10 showed the broadest GFP distribution and the highest GFP expression throughout the brain, while PM-AAV9 displayed an intermediate profile.

This result is consistent with previous studies reporting a more efficient CNS transduction with AAVrh10 compared with AAV9 in different species and in experimental context (36, 37), as well as with the previously reported efficient CNS transduction of PM-AAV9 (38). Our results, therefore, extend these observations to ICV administration in a dystrophic mouse model. In contrast, AAVDJ showed the lowest GFP distribution and expression under our experimental conditions. This finding was unexpected, as AAVDJ is a chimeric capsid generated from several AAV serotypes, including AAV9, and was specifically

developed to combine favorable transduction properties (39, 40). However, this difference further illustrates the context-dependent nature of AAV capsid performance. Factors such as the administration route, age at injection, vector design, mouse model and experimental readout can substantially influence the relative efficiency of a given capsid.

Given this context-dependent performance, we next investigated whether the age at injection also influenced AAV brain distribution following ICV administration under our experimental conditions. In adult *mdx52* mice, we found no significant differences in GFP distribution or expression between the capsids, together with an overall decrease compared with neonatal injection. This observation is consistent with previous studies showing that developmental stage strongly influences AAV CNS delivery. This age-dependent effect may occur at different levels of AAV delivery. First, vector distribution within the brain may be influenced by the maturation of CNS architecture. Changes in cellular density, tissue organization and the extracellular environment, including the maturation of perineuronal nets surrounding neurons, may restrict AAV dispersion in the mature brain following ICV administration. Second, age-dependent changes in cellular receptors and other factors involved in AAV uptake may affect capsid-specific transduction. Thus, developmental changes may not only restrict vector distribution but also modify the relative transduction efficiency of individual capsids. Taken together, these results indicate a more favorable brain-wide distribution and GFP expression following ICV administration in neonatal *mdx52* mice. Early administration may be particularly relevant for DMD, given the high expression of Dp140 during early brain development, suggesting that restoring dystrophin expression at an early stage could be beneficial (41, 42).

Beyond the extent of brain-wide distribution, we next sought to determine the cellular tropism of each capsid. Previous studies have shown that AAVs can preferentially transduce neurons in the CNS, with lower transduction of astrocytes, although cellular tropism can vary depending on the capsid and experimental context. Characterizing this tropism is particularly relevant in the context of *mdx52* mice, as Dp427 is predominantly expressed in neurons, whereas Dp140 is expressed in both neuronal and glial populations. We therefore characterized the cellular populations transduced by each capsid across the cortex (CX), hippocampus (HIP), and cerebellum (CBL) by assessing GFP colocalization with neuronal and astrocytic markers. For each capsid, we found a predominantly neuronal tropism, with lower astrocytic transduction in the CX, HIP, and CBL, despite some inter-individual variability. Moreover, the distribution of transduced neurons is particularly relevant when considering the therapeutic potential of these vectors. In the hippocampus, GFP expression was predominantly observed in the densely organized neuronal layers corresponding to the CA1–CA3 pyramidal cell layers and the dentate gyrus granule cell layer. These regions are key components of hippocampal circuits involved in learning and memory. This observation is of particular interest in the context of DMD, as cognitive impairments, including intellectual and memory deficits, are frequently reported in affected patients. The preferential transduction of these hippocampal neuronal populations therefore suggests that these vectors may provide relevant cellular targeting for a gene therapy approach aiming to restore Dp427 and potentially alleviate cognitive manifestations associated with its deficiency. A similar observation can be made in the cerebellum, where GFP expression was predominantly observed in Purkinje cells, which can be

readily identified by their characteristic morphology and localization at the interface between the molecular and granular layers. This finding is relevant for DMD, as one of the three Dp427 isoforms is driven by a Purkinje cell-specific promoter and is specifically expressed in this neuronal population. The observed transduction pattern may therefore be particularly appropriate for restoring Dp427 expression in Purkinje cells. This preferential targeting may also have therapeutic potential for addressing neurological manifestations associated with Dp427 deficiency. However, in the forebrain, we could not distinguish between neuronal subtypes, such as GABAergic neurons, based on their morphology.

To further assess the efficiency of neuronal targeting, we next quantified the proportion of neurons transduced by each capsid. AAVrh10 and PM-AAV9 showed the highest percentages of neuronal transduction, in line with the overall results obtained in this study. Although PM-AAV9 and AAVrh10 showed a similar percentage of neuronal transduction in the brain regions analyzed, AAVrh10 displayed a broader brain-wide distribution, suggesting that it may ultimately transduce a greater number of neurons when considering the brain as a whole. This broader distribution may be particularly relevant for targeting additional brain regions involved in DMD-associated neurological manifestations, such as the basolateral amygdala (BLA), which is implicated in anxiety and fear-related behaviors. Thus, the broader distribution of AAVrh10 may increase its potential therapeutic relevance by allowing transduction of neuronal populations beyond those assessed in the present analysis. Furthermore, AAVrh10 showed higher GFP expression than PM-AAV9 which may provide a greater potential for therapeutic benefit.

From a therapeutic perspective, AAV offers the advantage of sustained transgene expression in post-mitotic cells, potentially allowing long-term effects after a single administration. This feature is particularly attractive for CNS targeting in a chronic disease such as DMD, where repeated administration may be challenging. The feasibility of this approach is further supported by the recent approval of an intrathecal AAV9-based gene therapy for SMA (Itvisma, expressing SMN1) (43). However, although direct CNS administration represents a possible route for clinical translation, a systemic approach capable of simultaneously reaching both skeletal muscle and the CNS would be preferable for a neuromuscular disease such as DMD. Importantly, the objective of the present study was not to identify the best capsid for direct clinical translation, but rather to determine which capsid provides the most efficient brain distribution under our experimental conditions in dystrophic mice. This approach was indeed designed to support the investigation of the consequences of cerebral dystrophin restoration, including its potential impact on behavioral phenotypes, in the *mdx52* mouse model.

In conclusion, our study demonstrates that bilateral ICV administration of AAVrh10 in neonatal *mdx52* mice provides the most favorable profile among the capsids tested for brain dystrophin restoration. AAVrh10 enabled broad transgene distribution throughout the brain and high transgene expression. It showed predominantly neuronal transduction and efficiently targeted specific neuronal populations, such as Purkinje cells in the cerebellum, which are relevant for Dp427 expression. Taken together, these results support AAVrh10 as the most promising capsid tested in this study for the development of a gene therapy approach targeting brain dystrophin restoration in DMD.

Declarations

Data Availability Statement: The primary data for this study are available from the authors upon request.

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