Unique Glycan Signatures Regulate Adeno-Associated Virus Tropism in the Developing Brain

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ABSTRACT

Adeno-associated viruses (AAV) are thought to spread through the central nervous system (CNS) by exploiting cerebrospinal fluid (CSF) flux and hijacking axonal transport pathways. The role of host receptors that mediate these processes is not well understood. In the current study, we utilized AAV serotype 4 (AAV4) as a model to evaluate whether ubiquitously expressed 2,3-linked sialic acid and the developmentally regulated marker 2,8-linked polysialic acid (PSA) regulate viral transport and tropism in the neonatal brain. Modulation of the levels of SA and PSA in cell culture studies using specific neuraminidases revealed possibly opposing roles of the two glycans in AAV4 transduction. Interestingly, upon intracranial injection into lateral ventricles of the neonatal mouse brain, a low-affinity AAV4 mutant (AAV4.18) displayed a striking shift in cellular tropism from 2,3-linked SA⁺ ependymal lining to 2,8-linked PSA⁺ migrating progenitors in the rostral migratory stream and olfactory bulb. In addition, this gain-of-function phenotype correlated with robust CNS spread of AAV4.18 through paravascular transport pathways. Consistent with these observations, altering glycan dynamics within the brain by coadministering SA- and PSA-specific neuraminidases resulted in striking changes to the cellular tropisms and transduction efficiencies of both parental and mutant vectors. We postulate that glycan signatures associated with host development can be exploited to redirect novel AAV vectors to specific cell types in the brain.

IMPORTANCE

Viruses invade the CNS through various mechanisms. In the current study, we utilized AAV as a model to study the dynamics of virus-carbohydrate interactions in the developing brain and their impact on viral tropism. Our findings suggest that carbohydrate content can be exploited to regulate viral transport and tropism in the brain.

Viruses enter the central nervous system (CNS) by exploiting a variety of transport pathways that hinge on preliminary infection of peripheral nerve endings or through the blood by infecting circulating leukocytes or brain endothelial cells. Subsequent spread within the brain is achieved by axonal transport and trans-synaptic spread (1). A key step in viral entry into the CNS and subsequent directional transport is the recognition of specific cell surface membrane glycoproteins as receptors. For instance, polioviruses utilize CD155 as a receptor (2), while alpha herpesviruses exploit nectin-1 for CNS entry (3); both of these proteins are members of the immunoglobulin superfamily. Several membrane-associated components have also been implicated in rabies virus CNS entry (4). Prior to engagement of such host membrane proteins, viruses often bind to cell surface glycans for attachment. One of the most versatile host glycans that have been exploited as viral attachment factors are the family of sialic acids (SA) (5–7). For instance, SA receptors have been implicated in the neurovirulence of reoviruses and polyomaviruses (8, 9). Modulating SA binding affinity has also been shown to influence the pathogenicity of the neurovirulent strain of the minute virus of mice (MVM) (10).

While no natural isolates from brain tissue have been reported thus far, adeno-associated viruses (AAV), which are helper-dependent parvoviruses, display a broad spectrum of CNS tropisms following intracranial or systemic administration in different hosts (11–17). The cellular tropisms of different AAV strains observed in these studies were mostly neuronal, with a few excep-

tions that can transduce glial cells like astrocytes as well. Similar to their helper viruses such as *Adenoviridae* or *Herpesviridae* (1), AAV strains undergo interstitial as well as axonal transport within the CNS (17). However, the molecular bases of this diversity in AAV transport mechanisms and CNS tropisms are not well understood. Within this framework, AAV isolates have been shown to utilize three different glycans—SA, galactose (Gal), and heparan sulfate (HS)—for cell surface attachment (10). In addition, several growth factor receptors and integrins have been identified as being essential for AAV cell entry (18). Our laboratory and others have recently demonstrated the roles of SA and Gal in determining the systemic fate of different AAV serotypes in mouse models (19–21).

The African green monkey isolate AAV serotype 4 (AAV4) is one of the evolutionarily and structurally most distinct serotypes known to date and displays selective tropism for the ependymal

Received 8 October 2014 Accepted 15 January 2015

Accepted manuscript posted online 28 January 2015

Citation Murlidharan G, Corriher T, Ghashghaei HT, Asokan A. 2015. Unique glycan signatures regulate adeno-associated virus tropism in the developing brain. *J Virol* 89:3976–3987. doi:10.1128/JVI.02951-14.

Editor: M. J. Imperiale

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doi:10.1128/JVI.02951-14

lining following intracerebroventricular (ICV) administration in neonatal and adult mice (22). In addition, AAV4 particles directly injected into the subventricular zone can transduce astrocytes forming glial tubes within the rostral migratory stream (RMS). The functional cell surface attachment factor for AAV4 is O-linked α 2,3-SA (mucin) (21, 23, 24). We previously identified a novel AAV4 mutant (AAV4.18) that displays decreased affinity toward 2,3-SA and a transduction-deficient phenotype following systemic administration in mice (21). In the current study, we identified a novel glycan that differentially regulates the CNS transport and cellular tropism of AAV4 and the laboratory-derived mutant strain. Unlike AAV4, which displays restricted tropism for the ependymal lining, the laboratory-derived AAV4.18 mutant spreads throughout the brain parenchyma and can selectively infect migrating progenitors in the rostral and caudal directions from a unilateral ICV injection in neonatal mice. Further biochemical characterization of AAV4 and AAV4.18 in the mouse brain confirmed a switch in receptor specificity from α 2,3-linked SA to α 2,8-linked PSA, a well-established marker of neurogenesis.

MATERIALS AND METHODS

Recombinant AAV vector production. Recombinant AAV4 and mutant AAV4.18 vectors were generated using an updated triple-plasmid transfection method (25). Briefly, this involved transfection of (i) the pXR4 helper plasmid (26) or the mutant pXR4.18 helper plasmid (21), (b) the adenoviral helper plasmid pXX6-80, and (c) pTR-CBA-tdTom or pTR-CBA-Luc plasmids encoding the tdTomato (tdTom) or luciferase (Luc) reporter genes driven by the chicken beta actin (CBA) promoter and flanked by inverted terminal repeats (ITRs) derived from the AAV2 genome. Vector purification was carried out using cesium gradient ultracentrifugation, and viral titers were obtained by quantitative PCR using a Roche Lightcycler 480 (Roche Applied Sciences, Pleasanton, CA) with primers (IDT Technologies, Ames, IA) designed for the CBA promoter (forward, 5'-CGT CAA TGG GTG GAG TAT TT-3'; reverse, 5'-GCG ATG ACT AAT ACG TAG ATG-3').

In order to generate AAV particles packaging thymidine analog 5-bromo-2'-deoxyuridine (BrdU)-labeled genomes, we adapted a modified vector production protocol described earlier (27, 28). Briefly, at 1 h after triple-plasmid transfection, HEK293 producer cells were treated with a 10:1 molar mixture of BrdU and 5-fluoro-2'-deoxyuridine at a final concentration of 10 μ g of BrdU/ml of medium (Invitrogen, Camarillo, CA). Vectors packaging BrdU-labeled genomes were purified and quantified as described above.

Cellular transduction assays. CV-1 cells (African green monkey kidney fibroblasts) were seeded at a density of 5×10^4 cells per well in 24-well plates and were maintained at 37°C and 5% CO₂. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin, 100 μ g/ml of streptomycin, and 2.5 μ g/ml of amphotericin B (Sigma-Aldrich, St. Louis, MO). For transduction assays, cells were first exposed to the different enzymatic treatments as described below.

To cleave long PSA chains, cell cultures were treated with endoneuraminidase-N (ABC Scientific, Los Angeles, CA) diluted to 1:5,000 in DMEM supplemented with 10% FBS for 12 h at 37°C and 5% CO₂. To cleave terminal SA residues, neuraminidase III (neuraminidase) (Sigma-Aldrich) was diluted to 50 mU/ml in serum-free DMEM and cells were treated for 3 h at 37°C and 5% CO₂. After these treatments, cells were washed three times with phosphate-buffered saline (1× PBS) and the medium was replaced with fresh DMEM plus 10% FBS containing AAV4 or AAV4.18 vectors packaging the firefly luciferase transgene, driven by the CBA promoter at a multiplicity of infection (MOI) of 1,000 viral genomes (vg) per cell. Cells were lysed at 24 h posttransduction, and luciferase transgene expression was quantified using a Victor2 luminometer (PerkinElmer, Waltham, MA) with D-luciferin as a substrate.

Intracerebroventricular (ICV) injections. All animal experiments were carried out with BALB/c mice bred and maintained in accordance to NIH guidelines and as approved by the UNC Institutional Animal Care and Use Committee (IACUC). Neonatal postnatal day 0 (P0) pups were rapidly anesthetized by hypothermia by placement on ice for 1 min followed by stereotaxic intraventricular cerebral injections. A Hamilton 700 series syringe with a 26-gauge needle (Sigma-Aldrich) was attached to a KOPF-900 small animal stereotaxic instrument (KOPF instruments, Tujunga, CA), and the mice were injected unilaterally in their left lateral ventricle with a dose of 1×10^9 vector genome-containing particles (volume, 3 μ l) of AAV4 or AAV4.18 vectors packaging the CBA-tdTom reporter cassette. Developing mouse brains (P14) were harvested, postfixed, and immunostained as described in detail below. For tracking BrdU-labeled viruses, 7.4×10^8 vector genome-containing particles in a volume of 5 μ l were injected into the left lateral ventricles of P0 mice. Neonatal brains were harvested 2 h postinjection, postfixed in paraformaldehyde, sectioned, and immunostained as described below. For recombinant sialidase coinjection experiments, the vectors were mixed with either 5.2 mU of neuraminidase type III (sialidase; Sigma-Aldrich) or 1.45 U of endoneuraminidase-N (ABC Scientific, Los Angeles, CA) to a total injection volume of 4.3 μ l. All neonatal injections were performed 0.5 mm relative to the sagittal sinus, 2 mm rostral to transverse sinus, and 1.5 mm deep. Following vector administration, mice were revived under a heat lamp and rubbed in the bedding before being placed back with the dam.

Tissue processing, confocal microscopy, and immunofluorescence analysis. Two-week-old mice were sacrificed with an overdose of tribromoethanol (Avertin) (0.2 ml of 1.25% solution) followed by transcardial perfusion of 4% paraformaldehyde in PBS. The brains were removed and postfixed for 24 h, and 50- μ m-thick sections were obtained using a Leica VT 1000S vibrating blade microtome (Leica Biosystems, IL). Free-floating brain sections were blocked in 10% goat serum and 1% Triton X (Sigma-Aldrich) in PBS for 1 h prior to overnight incubation with primary monoclonal antibodies at 4°C. The following primary antibodies were utilized: rabbit anti-S100 β (Sigma; 1:1,000), mouse anti-GFAP (Abcam-10062, 1:1,000), rabbit anti-Dcx (Abcam-18723, 1:1,000), goat anti-phosphohistone H3 (anti-PH3) (Millipore; 1:1,000), mouse anti-BrdU (Invitrogen; 033900, 1:2,500), rabbit anti-NeuN (Abcam; 104225, 1:750), mouse anti-PSA-NCAM (DSHB; 1:750), and mouse anti-Rc2/nc82 (DSHB; 1:750). Secondary antibodies were raised in goats and conjugated to Alexa 488 or Alexa 647 (Abcam; 1:500). For jacalin staining, we followed the blocking step with 1.5 h of incubation of free-floating mouse brain sections in fluorescein isothiocyanate (FITC)-jacalin at room temperature (Vector Laboratories, Burlingame, CA; 1:40). Jacalin was diluted to a working concentration of 20 μ g/ml in 3% goat serum in PBS-Tween (PBS-T). Immunostained brain sections were visualized using a Zeiss CLSM 700 confocal laser scanning microscope and analyzed with Zen Black software. Colocalization (expressed as percent) of tdTomato reporter expression with different cell type-specific markers was derived from the ratio of the number of transduced cells (tdTom⁺) that were S100 β /GFAP/Dcx/PH3⁺ and the total number of transduced cells (tdTom⁺). Cells were counted in nonoverlapping fields of view of a 200- μ m² area in the subependymal zone, rostral migratory stream, olfactory bulb (OB), or other pertinent regions in the P14 mouse brain.

RESULTS

Substrate-specific neuraminidases have differential effects on AAV4 transduction *in vitro*. In most mammalian tissues, SA occupies the terminal position originating from asparagine-linked (N-) or serine/threonine-linked (O-) glycoprotein glycans. However, in the CNS, sialylated glycans are expressed in two forms, α 2,3- and α 2,6-sialylated glycosphingolipids (gangliosides), as well as long polymeric chains of α 2,8-linked PSA bound to neural cell adhesion molecule (NCAM) (29) (Fig. 1A). Importantly, the expression of PSA is known to regulate neural plasticity and play

FIG 1 Effects of substrate-specific neuraminidases on AAV4 and AAV4.18 transduction *in vitro*. (A) Schematic representation of O-linked α 2,3- and α 2,6-sialic acid (top) and α 2,8-polysialic acid (bottom) on mammalian cell surfaces. The symbol key defines the different glycan symbols. Black and white arrows represent the cleavage sites of neuraminidase (Neu) and endoneuraminidase-N (Endo-N) enzymes, respectively. Effects of neuraminidase and endoneuraminidase-N treatment on AAV4 (B and C) as well as AAV4.18 (D and E) are also shown. CV-1 cells pretreated with each enzyme were incubated with AAV4 or AAV4.18 vectors packaging a CBA-Luc transgene (MOI = 1,000 vg/cell), and luciferase activity, in relative light units (RLU), was measured at 24 h postinfection. Error bars represent standard deviations ($n = 3$). n.s., not statistically significant; *, $P < 0.05$ as determined by Student *t* test.

an indispensable role in embryonic and adult neurogenesis in the mammalian brain (30).

It is well known that AAV4 utilizes α 2,3-linked SA as the mammalian cell surface receptor (23). In order to understand whether α 2,8-linked PSA on cell surfaces could also affect AAV4 transduction, we performed specific neuraminidase treatments to alter the relative levels of cell surface SA and PSA *in vitro*. We chose parental African green monkey kidney CV-1 cells (precursor to Cos cells) for these experiments due to their highly permissive nature toward AAV4 transduction (23). As depicted in Fig. 1A, two classes of neuraminidase enzymes were used: neuraminidase (Neu), which specifically cleaves α 2,3 and α 2,6 linkages on SA, and endoneuraminidase-N (endo-N), which targets α 2,8 linkages on the polymeric PSA chain. We observed that cleavage of SA by neuraminidase treatment significantly reduced AAV4 transduction (>1 log order of magnitude reduction in luciferase activity; $P < 0.005$) (Fig. 1B). In contrast, endoneuraminidase-N-mediated cleavage of α 2,8-linked PSA significantly enhanced AAV4

transduction (>7 -fold increase; $P < 0.05$) (Fig. 1C). Taken together, these preliminary results suggested that levels of SA and PSA on cell surfaces might differentially regulate AAV4 transduction. In contrast to AAV4, we observed no significant changes in the *in vitro* transduction efficiency of AAV4.18 virions arising from neuraminidase or endoneuraminidase-N treatments (Fig. 1D and E). However, we interpreted these results with caution due to the inherently low transduction efficiency of the AAV4.18 mutant in these cells. Earlier studies from our laboratory have demonstrated that the mutant AAV4.18 is likely transduction deficient *in vitro* due to its low binding affinity for SA on the cell surface (21).

Ependymal transduction efficiency by AAV4.18 is similar to that of parental AAV4 in neonatal mice. Previous studies have demonstrated that ICV administration of AAV4 results in robust gene transfer in the ependymal cells and astrocytes lining the neonatal mouse cerebral ventricles (22). We injected P0 mice with identical titers of AAV4 or AAV4.18 packaging the tdTomato (tdTom) reporter gene driven by chicken beta actin (CBA) pro-

FIG 2 Ependymal transduction in the neonatal mouse brain by AAV4 and the AAV4.18 mutant. P0 mice were injected with 1×10^9 vg of AAV4 (A) or mutant AAV4.18 (B) packaging a CBA-tdTom transgene into the left lateral ventricle. At 2 weeks postinjection, mice were sacrificed and paraformaldehyde-fixed brains were sectioned and immunostained. Brain sections were imaged using a Zeiss CLSM 700 confocal laser scanning microscope equipped with 488-nm and 555-nm excitation filters. Confocal micrographs show tdTom transgene expression in red. Global brain sections (confocal image stitches of representative 50- μ m vibratome sagittal sections) and SEZ regions show tdTom expression in the subependymal zone. Immunocolocalization of tdTom gene expression (red) with ependymal cells (S100 β ; green) (D and E) and primary astrocytes (GFAP, green) (G and H) is indicated by yellow pseudocolor within the S100 β and GFAP merged images. Quantitative assessments of the AAV4-transduced (dark gray bars) or AAV4.18-transduced (light gray bars) cells in the dorsal and ventral SEZ are indicated by the number of tdTom $^+$ cells (C) and percent colocalization with S100 β $^+$ cells (F) or GFAP $^+$ cells (I). Error bars represent standard deviations ($n = 4$). n.s., not statistically significant; *, $P < 0.05$ as determined by Student t test. Representative confocal images are shown.

motor via the ICV route. At 2 weeks postinjection, we carried out confocal microscopy analysis of sagittal mouse brain sections. Both vectors displayed efficient tdTom expression (tdTom $^+$) in the subependymal zone (SEZ) (Fig. 2A and B, top row). In order to further characterize the tdTom $^+$ cells at a cellular level, we performed immunocolocalization with markers for ependymal cells (S100 β) and primary astrocytes (GFAP). As can be seen in Fig. 2D and E, tdTom $^+$ cells (red) within the SEZ in both AAV4- and AAV4.18-injected mouse brains show significant colocalization with S100 β $^+$ cells (green). Similarly, comparable levels of colocalization of tdTom $^+$ cells (red) and GFAP $^+$ cells (green) were also observed in the SEZs of AAV4- and AAV4.18-treated mice (Fig. 2G and H). These observations were further supported by quantitative and statistical analyses that showed no significant differences in tdTom $^+$ cells within the SEZ (Fig. 2C) or the percentage of colocalization with cellular markers (S100 β and GFAP) from AAV4 or AAV4.18 injections (Fig. 2F and I). These results indicate that both AAV4 and 4.18 vectors can efficiently transduce neonatal mouse ependyma. Although not relevant to the current study focused on the developing brain, it is noteworthy to mention that similar ependymal transduction profiles for AAV4 and AAV4.18 vectors were observed in adult mouse brains (data not shown).

The AAV4.18 mutant displays expanded tropism for migrating progenitors. Neuronal progenitors in the SEZ are known to migrate via the RMS to the OB, where they differentiate into interneurons of the granular and periglomerular layers in developing and adult rodent brains (31). Confocal microscopy analysis of sagittal sections of postnatal mouse brains imaged at 2 weeks postinjection revealed strikingly distinct patterns of transduction between AAV4 and AAV4.18 vectors. Notably, AAV4.18-injected mice showed significantly more tdTom expression in the RMS and OB (~ 3 - and ~ 6 -fold increases, respectively; $n = 4$ mice) regions than did AAV4-injected mice (Fig. 3A and B). We then performed immunostaining for the migrating neuroblast marker doublecortin (Dcx) and proliferating cell marker phospho-histone H3 (PH3) to assess the cell types associated with the tdTom expression in the RMS and OB. As seen in Fig. 3D and E, tdTom $^+$ cells (red) within the RMS and the OB in AAV4.18-injected mouse brains show significantly increased colocalization with Dcx $^+$ cells (green) compared to that in AAV4-injected brains. A similar trend showing increased colocalization of tdTom expression with the PH3 $^+$ cells was observed in the RMS and, to a lesser level, in the OB of AAV4.18-injected brains (Fig. 3G and H). These observations were corroborated by quantitative and statistical analyses (Fig. 3C, F, and I; *, $P < 0.05$).

FIG 3 The AAV4.18 mutant exhibits selective tropism for migrating progenitors. P0 mice were injected with 1×10^9 vg of AAV4 (A, D, and G) or AAV4.18 (B, E, and H) packaging the tdTomato reporter transgene driven by a chicken beta actin promoter into the left lateral ventricle. tdTom expression (red) in sagittal sections of the developing mouse brain, including the subependymal zone (SEZ), rostral migratory stream (RMS), and olfactory bulb (OB) regions, is shown. Immunocolocalization of AAV4-mediated (A) or AAV4.18-mediated (B) gene expression (tdTom; red) with the migrating neuroblast marker, doublecortin (Dcx; green) (D and E), and the proliferative cell marker, phospho-histone H3 (PH3; green) (G and H), is shown. White arrows indicate the locations shown at higher magnification (insets), and immunocolocalized regions are depicted in yellow. Quantitative analyses of the number of tdTom⁺ cells (C) and percent colocalization with Dcx⁺ processes (F) and PH3⁺ cells (I) in the RMS and OB regions of AAV4-treated (dark gray bars) or AAV4.18-treated (light gray bars) mice are shown. Error bars represent standard deviations ($n = 4$). n.s., not statistically significant; *, $P < 0.05$ as determined by Student *t* test. Representative confocal images are shown.

Mutant AAV4.18 virions display enhanced CNS spread. In order to understand the mechanisms underlying the selective tropism of AAV4.18 for progenitors and neuroblasts in the postnatal CNS, we tracked the distribution of each AAV vector in the mouse brain parenchyma following ICV injections. To achieve this, we injected AAV vectors packaging genomes that were labeled with the thymidine analog bromodeoxyuridine (BrdU) through ICV injections in neonatal mice. Brains were harvested as early as 2 h after vector administration and immunostained with an anti-BrdU antibody to visualize the biodistribution of AAV genomes in the brain parenchyma. AAV4-injected mice exhibit robust BrdU staining in the immediate vicinity of the site of injection in the SEZ and the outer meninges of the neonatal brain, presumably due to cerebrospinal fluid (CSF) transport (Fig. 4A, middle, arrow). In contrast, the AAV4.18 vector shows a remarkably diffuse distribution pattern of BrdU-labeled viral particles not only in the SEZ (Fig. 4A, right, arrow) but also through the brain parenchyma and particularly in the cortical regions (Fig. 4A, right, arrowhead). Immunostaining analysis of brain sections with the endothelial cell marker CD31 revealed BrdU⁺ AAV4.18 genomes (green) arranged alongside CD31⁺ processes (red) in the cortical regions of the mouse brain (Fig. 4B, right). In contrast, AAV4 genomes did not show this phenotype in the cortex (Fig. 4B, middle). It should be noted that despite the expanded spread of the AAV4.18 genomes, complete colocalization with endothelial cells was not observed (Fig. 4B, right, inset). This suggests that the selective tropism for migrating progenitors can, in part, be attributed to the ability of AAV4.18 to spread across the neonatal brain parenchyma.

Selective enzymatic removal of PSA expands CNS tropism of AAV4 vectors. The polysialylated form (PSA; 2,8-linked sialic

acid) of neural cell adhesion molecule (NCAM) is expressed in migrating progenitor cells of the RMS during OB neurogenesis (32). PSA-NCAM plays a pivotal role in mediating rostral migration of olfactory bulb precursor cells, whereas deficiencies in either PSA or NCAM cause accumulation of progenitor cells in the SEZ and RMS, resulting in aberrant olfactory histogenesis (32, 33). We coinjected neuraminidase, which selectively cleaves terminal SA residues or endoneuraminidase-N, which cleaves the polymeric PSA chain with AAV4 packaging the CBA-tdTom reporter gene in neonatal P0 mice via the ICV route. Loss of terminal SA residues due to neuraminidase treatment was confirmed by a significant reduction in fluorescein isothiocyanate (FITC)-labeled jacalin staining in the mouse brain (data not shown). Control injections of AAV4 alone resulted in highly localized tdTom transgene expression in the ependymal lining, which was almost completely abrogated in neuraminidase-coinjected mice (Fig. 5A and C, left images). We observed minimal tdTom transgene expression in migrating progenitors within the RMS and OB by AAV4, regardless of treatment with neuraminidase (Fig. 5A and C, middle and right images). Further, no colocalization with PSA-NCAM immunostaining was observed (Fig. 5B and D). In contrast, endoneuraminidase-N treatments increased AAV4-mediated tdTom transgene expression in the RMS, but more strikingly in the OB (Fig. 5E, middle and right images) and without affecting ependymal tdTom transgene expression (Fig. 5E, left image). A concomitant and significant reduction in PSA-NCAM immunostaining in the RMS and OB upon endoneuraminidase-N treatment was also observed (Fig. 5D and F). Taken together, these results suggest that 2,8-linked PSA negatively regulates AAV4

FIG 4 AAV4.18 particles show enhanced CNS spread. P0 mice were injected with 7.4×10^8 vg of AAV4 or AAV4.18 packaging BrdU-labeled genomes into the left lateral ventricle. At 2 h postinjection, mice were sacrificed and paraformaldehyde-fixed brains were sectioned, immunostained, and imaged as outlined in Materials and Methods. (A) Global anti-BrdU immunostaining (green) in sagittal sections of the brain obtained from mock-treated or AAV4- or AAV4.18-injected mice into the left lateral ventricle (white arrows). The positions of the cortical regions shown in higher magnification in panel B are indicated by white arrowheads in panel A. (B) Immunocolocalization of BrdU⁺ viral genome-containing particles (green) and anti-CD31, an endothelial cell marker for immunostaining of blood vessels (red), in the cortex. All experiments were carried out in triplicate; representative images are shown.

spread and potentially competes for 2,3-linked SA binding sites on the AAV4 capsid.

To further characterize the expanded transduction profile of AAV4 observed following endoneuraminidase-N coinjections, we carried out immunocolocalization with several cellular markers. As discussed above, PSA-NCAM immunostaining was abrogated due to efficient cleavage of PSA residues from such treatments (Fig. 6A), and no colocalization with tdTom⁺ cells was observed, as expected. Interestingly, we observed that AAV4-mediated transgene expression (tdTom⁺) significantly colocalized with NeuN⁺ neurons in the OB (Fig. 6B, arrows, right image). However, we did not observe colocalization of tdTom⁺ cells with the astrocytic marker, GFAP, or the radial glial marker, RC2/nestin (Fig. 6C and D). These results indicate that under conditions displaying reduced PSA levels, AAV4 can efficiently transduce ma-

ture OB neurons. These data are consistent with observations from cell culture studies described earlier. Thus, it appears that modulation of PSA levels can influence AAV4 tropism in the mammalian brain.

The low-affinity AAV4.18 mutant displays expanded tropism for migrating progenitors by exploiting high PSA levels. In order to dissect the possible mechanism underlying the expanded tropism displayed by AAV4.18 from ependymal cells toward migrating neuroblasts, we subjected the mutant strain to similar enzymatic modulation of SA and PSA levels. Similar to the case with untreated controls (Fig. 7A and B), coinjection of neuraminidase III with AAV4.18 did not alter the extent of colocalization between tdTom⁺ and PSA-NCAM⁺ cells in the RMS or the OB (Fig. 7C and D). In contrast, coadministration of endoneuraminidase-N dramatically altered PSA-NCAM staining throughout the mouse

FIG 5 SA and PSA play opposing roles in AAV4 transduction within the neonatal mouse brain. P0 mice were coinjected with mixtures of 1×10^9 vg of AAV4 mixed with PBS (control) (A and B), 3.5 mU of neuraminidase (cleaves 2,3- and 2,6-linked sialic acids) (C and D), or 1.45 U of endoneuraminidase-N (cleaves 2,8-linked polysialic acid) (E and F) into the left lateral ventricle. At 2 weeks postinjection, mice were sacrificed and paraformaldehyde-fixed brains were sectioned and immunostained. tdTom transgene expression patterns (red) and PSA-NCAM immunostaining (green) resulting from enzymatic desialylations are shown in the global context or as higher magnifications of the RMS and OB regions. White arrows indicate the locations shown at higher magnification (insets). All experiments were carried out in triplicate; representative images are shown.

FIG 6 Removal of PSA expands AAV4 tropism to mature OB neurons. P0 mice were coinjected with 1×10^9 vg of AAV4 mixed with 1.45 U of endoneuraminidase-N into the left lateral ventricle. At 2 weeks postinjection, mice were sacrificed and paraformaldehyde-fixed brains were sectioned and immunostained for different cellular markers, PSA-NCAM (A), NeuN (B), GFAP (C), and RC2/nestin (D), with specific antibodies as outlined in Materials and Methods. Sagittal sections of the mouse brain featuring progenitor migration in the rostral migratory stream (RMS) and olfactory bulb (OB) and immunocolocalization of tdTom transgene expression (red) with each cellular marker (green) are shown. White arrows indicate the locations shown at higher magnification (insets) or immunocolocalized regions (shown in yellow). All experiments were carried out in triplicate; representative images are shown.

brain, particularly within the migrating progenitor continuum, and completely abrogated tdTom reporter gene expression in AAV4.18-injected mouse brains (Fig. 7E and F). Taken together, these results support the notion that AAV4.18 has undergone a complete switch in glycan receptor specificity from terminal 2,3-linked SA to the polymeric 2,8-linked PSA chain owing to a loss in SA binding affinity. In turn, AAV4.18 appears to exploit the PSA glycosylation pattern in the developing brain to efficiently transduce migrating progenitors.

Further characterization of AAV4.18-treated mice coinjected with neuraminidase revealed a striking correlation between the pattern of tdTom⁺ cells and PSA-NCAM staining along the migrating progenitor RMS-OB continuum (Fig. 8A). No apparent colocalization was observed with the mature neuronal marker, NeuN, in the PSA-NCAM-labeled region (Fig. 8B). However, it is noteworthy that some colocalization was observed between tdTom⁺ and NeuN⁺ cells in the periphery of OB (data not shown). Several tdTom⁺ cells along the migratory pathway colocalized with the astrocyte marker, GFAP, but not the radial glial marker, RC2/nestin, in the RMS as well as OB (Fig. 8C and D). Taken together, these results corroborate the notion that modulating SA and PSA levels in the brain can be differentially exploited

by AAV4 and AAV4.18 to attain strikingly distinct transduction patterns targeting the ependymal lining, neurons within the OB, or migrating progenitors in the RMS and OB regions within the neonatal brain.

DISCUSSION

Successful infection by parvoviruses such as AAV involves a series of carefully orchestrated events, including cell surface receptor binding, endocytic uptake, capsid uncoating, nuclear entry, and genome release followed by second-strand synthesis and subsequent transcription. The first step, i.e., parvoviral attachment to the host cell surface, is mediated by different glycans (10). In the brain, AAV capsid interactions with heparan sulfate (HS) have been particularly well studied. Direct parenchymal injection of AAV serotype 2, which utilizes HS as a primary receptor (34), results in a prominently neuronal transduction profile (11, 35). Coinjection of soluble heparin has been shown to improve the CNS spread and, consequently, transduction efficiency of AAV2 following intracranial injections in rodent models (36, 37). The ability to bind HS also appears to restrict the CNS transduction profile of AAV serotype 6 (38). However, this effect can be reversed in part by mutating a lysine residue (K531) on the capsid

FIG 7 The AAV4.18 mutant selectively exploits PSA to transduce migrating progenitors. P0 mice were coinjected with mixtures of 1×10^9 vg of AAV4.18 mixed with PBS (control) (A and B), 5.2 mU of neuraminidase (cleaves 2,3- and 2,6-linked sialic acids) (C and D), or 1.45 U of endoneuraminidase-N (cleaves 2,8-linked sialic acid/polysialic acid) (E and F) into the left lateral ventricle. Postfixed sagittal sections of P14 mouse brains displaying tdTom transgene expression patterns resulting from enzymatic desialylation are shown in the global context or as higher magnifications of the RMS and OB regions. White arrows indicate the locations shown at higher magnification (insets), and immunocolocalization of tdTom expression (red) with PSA-NCAM staining (green) is depicted in yellow (B, D, and F). All experiments were carried out in triplicate; representative images are shown.

FIG 8 AAV4.18 selectively transduces migrating progenitors expressing polysialylated NCAM and GFAP. P0 mice were coinjected with 1×10^9 vg of AAV4.18 mixed with 5.2 mU of neuraminidase into the left lateral ventricle. Postfixed P14 mouse brains were sectioned and immunostained for different cellular markers, PSA-NCAM (A), NeuN (B), GFAP (C), and RC2/nestin (D), with specific antibodies as outlined in Materials and Methods. Sagittal sections of the mouse brain featuring progenitor migration in the rostral migratory stream (RMS) and olfactory bulb (OB) and immunocolocalization of tdTom transgene expression (red) with each cellular marker (green) are shown. White arrows indicate the locations shown at higher magnification (insets) or immunocolocalized regions (shown in yellow). All experiments were carried out in triplicate; representative images are shown.

surface, which abolishes HS binding (38, 39). These earlier studies highlight the potential for glycan expression patterns to regulate viral spread and tropism in the brain.

In the current study, we have characterized a novel AAV mutant that selectively transduces migrating progenitors in the neonatal mouse brain. This mutant was originally discovered from a randomly mutated AAV4 capsid library and characterized as an SA binding-deficient mutant. When administered systemically, the AAV4.18 mutant displays attenuated cardiopulmonary tropism in mice due to the low binding affinity toward O-linked 2,3-SA, the cognate receptor for the parental AAV4 serotype (21). In the neonatal mouse brain, the natural isolate AAV4 exclusively transduces ependymal cells following ventricular injection (22). Interestingly, when injected directly into the subependymal zone (SEZ), AAV4 can transduce type B astrocytes and glia overlying the RMS (22). Our results now show that this dichotomy potentially arises from the high binding affinity of AAV4 capsids for SA and PSA, which likely restricts transduction to the ependymal lining following ICV administration. In contrast, the low-affinity AAV4.18 mutant can penetrate the ependymal barrier into the

brain parenchyma following a single ICV injection and selectively transduce migrating neuroblasts and proliferating cells, apparently due to interactions with PSA alone (Fig. 1 to 3). It is noteworthy that the enhanced spread of AAV4.18 particles to distal regions of the mouse brain does not result in successful transduction of mature neurons within these regions. Rather, immunohistochemical analysis suggests that AAV4.18 particles that reach the cortex are closely associated with the brain microvasculature without actually transducing endothelial cells (Fig. 4). This observation suggests that interstitial solutes such as viral particles might exploit paravenous efflux or the “glymphatic” clearance pathway (40). Correspondingly, we postulate that AAV4.18 particles with low binding affinity are more likely to be affected by interstitial fluid transport through white matter tracts and perivascular spaces, leading to enhanced penetration of brain parenchyma.

We further expanded these findings by evaluating the effect of selective enzymatic removal of 2,3- or 2,8-linked SA from the murine brain by ICV injection of substrate-specific neuraminidases. While AAV4 transduction is completely abrogated by selective SA removal, AAV4.18 transduction of a subset of cells in the ependy-

mal wall, the choroid plexus, and PSA-NCAM⁺ migrating progenitors remains unaffected (Fig. 5 to 8). This selective requirement of polymeric 2,8-linked PSA chain rather than terminal SA residues for AAV4.18 transduction was clearly demonstrated by enzymatic removal. Taken together, these results support the notion that AAV4.18 can not only spread throughout the brain parenchyma but also selectively exploit PSA (2,8-linked SA) to transduce postnatal migrating progenitors in the mouse forebrain.

The expanded receptor usage and selective cellular tropism displayed by AAV4.18 particles are not a mere coincidence. It is well known that the linear homopolymer of α 2,8-linked sialic acid (PSA) plays an indispensable role in embryonic and adult neurogenesis. Two regions of the brain, namely, the OB and hippocampal dentate gyrus, are persistently neurogenic and undergo constant progenitor chain migration into adulthood in rodents (41). Despite multiple differences between adult and embryonic neurogenesis, consistent PSA-NCAM expression is a feature observed in both of these regions through adulthood (42). The biochemical properties of PSA make it a potent negative regulator of cell-cell adhesion. This is important for successful migration of precursor cells during neurogenesis (43). This is potentially the reason PSA-NCAM is highly expressed in the neuronal precursor cells during olfactory neurogenesis (43). Furthermore, the enzymatic removal of PSA using endoneuraminidase-N treatment disrupts the RMS, leading to neuroblast dispersion to unspecific regions like the cortex and striatum (32). Thus, the selective transduction of migrating progenitors by AAV4.18 can be directly attributed to the expression patterns of this unique glycan attachment factor that can vary with the developmental stage of the host organism.

Certain gaps still remain in our understanding of the proposed AAV-PSA interactions. First, PSA does not appear to functionally influence the tropism or transduction efficiency of AAV4 or related mutants in physiological settings other than the CNS, such as the heart and lung following intravenous administration (21). It is likely that the developing brain provides a unique setting for this novel virus-glycan interaction. Second, the structural coordinates that mediate PSA recognition by AAV4 and the mutant virion remain to be determined. Preliminary structural modeling revealed altered surface electrostatics for the AAV4.18 mutant in comparison with the parental AAV4 strain (data not shown). It is tempting to speculate that manipulation of capsid surface charge density might decrease affinity for branched or linear 2,3-linked SA glycans while simultaneously imparting the expanded potential to recognize the negatively charged PSA glycopolymer. These hypotheses warrant further structural and biophysical analysis outside the scope of the current study. Nevertheless, our overall approach helps understand the functional implications of altering virus-glycan interactions in the CNS and the impact of developmentally regulated or disease-specific glycan expression profiles on virus neurotropism. Simultaneously, we provide a roadmap for engineering viruses to favor certain glycan architectures for gene transfer applications in the brain.

ACKNOWLEDGMENTS

This study was supported by NIH grants awarded to A.A. (R01HL089221 and P01HL112761) and H.T.G. (R01NS062182).

G.M., H.T.G., and A.A. designed the overall study. G.M. and A.A. wrote the manuscript. G.M. and T.C. carried out viral vector production, animal studies, and immunohistology.

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