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A combination of mutations enhances the neurotropism of AAV-2

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Abstract

There is strong interest in developing practical strategies for gene delivery to the central nervous system (CNS). Direct delivery into the brain or spinal cord is highly invasive as well as inefficient or hazardous using most current vector systems. Our objective was to generate innocuous gene vehicles that would be effectively taken up by axons and then home to the neuron cell bodies. Vectors derived from Adeno-Associated Virus (AAV), a harmless human parvovirus, offer strong starting candidates for deriving such vehicles. Enhancing the axonal uptake of AAV, and conferring more efficient retrograde transport capabilities upon the virus, should produce near ideal gene transfer vehicles for the CNS. To enhance retrograde transport of the virus, peptides mimicking binding domains for cytoplasmic dynein were inserted in the capsid by directed mutagenesis. In separate clones, peptides derived from an NMDA receptor antagonist were also introduced to provide a specific affinity for this receptor. When combined, these two functionally distinct classes of mutation enabled efficient gene transfer into neurons under conditions not permissive for standard AAV-2 vectors prepared under the same conditions. These results hold strong promise for the development of safe, convenient vehicles to target genes and other sequences to neurons, enabling new and novel approaches for the treatment of multiple neurological disorders.

Keywords: AAV; Gene therapy; Chimera; Neurons; Dynein motor complex; Retrograde transport; NMDA receptor; Histogranin

Introduction

There is keen interest in developing practical strategies for gene delivery into the central nervous system (CNS). In addition to many obvious applications for basic research, the ability to safely and efficiently engineer genes and other sequences into CNS cell populations will generate major new therapeutic possibilities for chronic as well as acute neurological disorders. Unfortunately, the distinctive features of neurons, and their complex interconnections impose stringent requirements upon gene delivery systems, rendering most current viral and other types of vectors poorly suited for this purpose. For example, gene vectors do not penetrate the tegument surrounding the spinal cord, and direct injection into the cord tends to produce spots of localized intense gene transfer, with minimal lateral diffusion. Recently, osmotic pumps have been used to infuse vectors through the cerebrospinal fluid (CSF) with some success (Cunningham et al., 2000). However, the present state of affairs remains far from ideal, particularly where the goal is to engineer new sequences into motor or sensory neurons, and less invasive strategies are highly desirable. A convenient, minimally invasive approach would enable a vector to be delivered by simple intramuscular (IM) or intravenous (IV) injection, after which the virus would home to and be absorbed by targeted axons and transported back to the neuronal cell bodies. Such, after all, are the natural routes of infection of several pathogenic neurotropic viruses, upon reaching the blood or epithelial linings (Leopold et al., 2000; Jacob et al., 2000). HSV derivatives as well as lentiviral vectors pseudotyped with rabies envelope glycoprotein can travel efficiently along neuronal axons, but bring their own particular limitations and issues (Mikkers and Berns, 2003; Fortunato and Spector, 2003; Pakzaban et al., 1994).

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For a number of reasons, recombinant gene vectors derived from Adeno-Associated Virus (rAAV) offer a strong set of starting candidates for applications in the CNS. Derived from a family of small non-pathogenic human parvoviruses, AAV vectors are capable of efficiently delivering gene cassettes of up to about 5 kb. Recombinant AAV (rAAV) carry no viral coding sequences, so no viral products are synthesized in the target cells. Unlike complex lentiviruses and simpler murine retroviruses, integration is not a prerequisite for transcription of AAV gene cassettes. Integration by standard AAV vectors, as opposed to wild type (wt) AAV, is slow, inefficient, and non-specific, and the majority of transgenes persist as highly stable, actively transcribed episomes, minimizing concerns about insertional mutagenesis. As a consequence, recombinant AAV are poorly suited for long-term gene transfer into rapidly dividing cells, but will readily persist for months to years in slowly dividing or non-dividing lineages. A further advantage is the low immunogenicity of the AAV capsid compared with other viral vectors such as Adenovirus (Bessis et al., 2004). Although some variability has been reported with strain of animal and site of delivery, exposure to rAAV, particularly a single administration, does not typically elicit destructive cellular immune responses against transduced cells in immunocompetent animals.

Although AAV is a human virus, AAV vectors function equally effectively in cells of many other species, including rodents, dogs, and other primates, streamlining transitions from animal model systems to clinical trials. All these features have served to make rAAV increasingly popular as research tools and for gene therapy applications, as rAAV gain increasing acceptance for use in human trials (Kay et al., 2000; Athanasopoulos et al., 2000; Mandel and Burger, 2004).

The natural cellular tropism of AAV is broad, and standard AAV vectors have been used to transduce a broad spectrum of cell types, especially in vitro, including neurons. Unfortunately for applications in the CNS, standard vectors derived from AAV-2, the most commonly used and best characterized serotype, do not disperse widely after injection in the brain and do not reach neuron cell bodies very efficiently following injection into peripheral sites. For example, after IM injection, rAAV are principally absorbed by myocytes. At least two studies failed to detect the vector in spinal cord neurons following IM injections of rAAV-2 (Martinov et al., 2002, Wang et al., 2002). Deficient retrograde transport in brain neurons has also been reported (Chamberlin et al., 1998). Recently, Kaspar et al. (2003) demonstrated retrograde transport of an AAV-2 IGF-1 vector to motor neurons after injection in SODG93A mice, sufficient for biological effects of the treatment to be apparent, but still at low levels that served to emphasize the overall inefficiency of the process.

Two distinct properties crucial to viruses targeting neurons appear lacking in AAV-2, a specific means of entry, and an efficient mechanism for keying into a retrograde transport pathway. Despite the susceptibility of neurons to the virus, AAV-2 may not be absorbed well by neuronal axons and dendrites. Examples of viruses which exhibit efficient uptake and retrograde transport along neural axons include the rabies viruses, herpes viruses, and even certain pathogenic parvoviruses. In some instances, particular features of their capsids which confer these traits have been identified, enabling attempts at re-engineering such properties into more innocuous vectors such as AAV. This is assisted by the availability of detailed X-ray crystallographic structures, particularly for AAV-2 (Xie et al., 2002), as well as the identification of domains in the major capsid protein (VP3) which will tolerate short peptide inserts (Shi et al., 2001; Wu et al., 2000; Grifman et al., 2001; Nicklin et al., 2001). This is the approach we adopted to enhance the neurotropism of rAAV-2.

Results

Mutagenesis of the AAV capsid

As distinct attributes, it is problematic to imagine a single small peptide insert conferring neuron-specific uptake as well as efficient retrograde transport. However, new functional epitopes need not be reiterated in every capsid component for powerful effects to be achieved. Each AAV capsid is assembled from about 60 building blocks of the major capsid protein VP3, as well as lesser amounts of other subunits produced by alternative splicing of the *cap* mRNA. A mix of capsid gene plasmids incorporating different changes will, when transfected together into packaging cells, therefore result on average in many copies of each mutation being expressed on the surface of most virus particles.

Our strategy for modifying the AAV-2 capsid was two-tiered. Initially, we incorporated peptides derived from the sequence of histogranin (HN) (Lemaire et al., 1993, 1995). A short 15-amino acid peptide, HN is a potent NMDA receptor antagonist, and efficiently displaces NMDA receptor ligand binding (Lemaire et al., 1993; Shukla et al., 1995). The specificity of HN for NMDA receptors is demonstrated by its ability to protect against NMDA-induced convulsion, but not convulsion induced by other ionotropic glutamate receptor agonists such as AMPA or kainate (Lemaire et al., 1995). NMDA receptors are expressed across wide swaths of the CNS, and can be found at high levels on axons as well as cell bodies on pyramidal neurons in the brain (Conti, 1997; Moriyoshi et al., 1991), on ventral horn motor neurons (O'Donnell et al., 2004), and on dorsal ganglion sensory neurons (Ma and Hargreaves, 2000), among others. Peptides mimicking either the natural HN sequence, [Met^1]HN, or an analog, [Ser^1]HN, with a single amino acid substitution that results in a somewhat higher binding affinity and increased stability

as a free peptide (Rogers and Lemaire, 1993) were inserted in position 587 in VP3. This domain in loop IV is recognized as a tolerant site in the capsid, involved in the interaction of AAV-2 with Heparin Sulfate Proteoglycan (HSPG) in the normal binding of the virus to the host cell (Girod et al., 1999; Grifman et al., 2001; Shi et al., 2001; Ried et al., 2002). Small disruptions in this domain can result in reduced HSPG binding, but need not interfere with virus assembly.

In a second phase, to achieve more efficient retrograde transport, we attempted to confer on the capsid a specific affinity for cytoplasmic dynein. Many cellular proteins as well as neurotropic viruses (Mueller et al., 2002; Topp et al., 1994) rely upon cytoplasmic dynein, one of two major types of Dynein Motor Complex (DMC), for retrograde transport (Schnapp and Reese, 1989). Cytoplasmic dynein is a large protein complex composed of multiple subunits, with the heavy chains containing the motor domains, while intermediate and light chains serve to bind the complex to different cargo proteins (Susalka and Pfister, 2000; Pazour et al., 1998). Recent studies have identified specific components of several light chains which are in direct association with different cargo proteins (Jacob et al., 2000; Rodriguez-Crespo et al., 2001; Mueller et al., 2002). For example, using a pepscan technique, Rodriguez-Crespo et al. (2001) identified two consensus motifs, GIQVD and KSTQT, across a panel of 10 cargo proteins all interacting with an 8-kDa light-chain component (LC8). In particular, the KSTQT motif was common to proteins found in several neurotropic viruses, including Mokola virus, rabies virus, and African swine virus (Rodriguez-Crespo et al., 2001). In a different study, an SKCSR motif within the poliovirus receptor CD155 was shown to interact with a different dynein light chain, Tctex-1 (Mueller et al., 2002). None of these motifs are normally displayed on the AAV capsid. To enhance retrograde transport, sequences encoding peptides derived from several of these motifs were inserted into separate clones of VP3, again centered at position 587, to yield clones AAV-DMC1 (KSTQT), AAV-DMC2 (SKCSR), and AAV-DMC3 (GIQVD).

In addition to the disruption of the native sequence at this site, a specific deletion was introduced in most of the mutants, encompassing residues 584–589, including two arginines at 585 and 588. Two recent mutagenesis studies implicated these residues in the efficient interaction of the capsid with HSPG, although other sites were also important (Opie et al., 2003; Kern et al., 2003). See Fig. 1 and Materials and methods for additional details of the constructs and mutagenesis.

Next, vector preparations were generated in which a lacZ cassette was packaged in either class of mutant capsid alone, or in a 1:1 mix of an HN insert together with a DMC binding peptide, or in the standard AAV-2 capsid as a control. All mutants packaged with near-normal efficiencies, with typical genomic titers insignificantly different from the wild type. Viruses were purified by passage over iodixanol gradients followed by ion exchange chromatography, a methodology not dependent on the affinity of the virus for heparin. Elution over 1-ml heparin columns revealed modest (less than 1 log) differences between mutants and standard AAV-2 in heparin binding affinity (not shown).

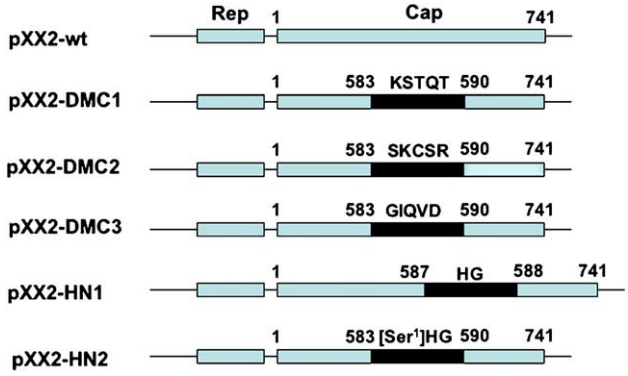

Fig. 1. Changes introduced in the AAV-2 capsid. Illustration of mutations introduced in the VP3 sequence in separate clones of plasmid pXX2, encoding the AAV-2 *rep* and *cap* functions.

Gene transfer into PC-12 cells by capsids with engineered peptide motifs

The properties of rAAV bearing these different peptides, alone or in combination, were contrasted against those of the standard AAV-2 vector in differentiated PC-12 cells, a rat pheochromocytoma line retaining many characteristic neuronal properties, including expression of NMDA receptors (Casado et al., 1996). In differentiated PC-12s, expression of the transgene following exposure to the standard rAAV-2 lacZ was poor (1–3%) to negligible, consistent with previous experiences of our group and others. Modest but reproducible improvements in efficiency were seen with vectors bearing either the KSTQT motif (DMC1) or an HN peptide insert alone. Exposure to these vectors resulted in up to several fold more lacZ expressing cells than the standard AAV-2, with up to 10–15% of the cells expressing beta-galactosidase (beta-gal) at an MOI of about 100. However, after exposure to an equivalent dose of a double mutant vector bearing both the DMC1 and HN1 motifs, the numbers of lacZ expressing cells were at least 6- to 8-fold higher compared with vectors displaying either motif alone, with greater differences evident at lower input doses. Representative results are shown in Fig. 2. Comparisons between the 2 variants of the HN motif in PC-12s did not reveal significant phenotypic differences. Capsids bearing the DMC2 or DMC3 motifs did not display phenotypes clearly distinctive from AAV-2 in initial trials, and were not characterized further.

To confirm that the enhanced gene expression by the double mutant was mediated at least in part via NMDA receptors, the transduction was competed with excess HN peptide (Bachem, Torrence, CA). Pre-incubation with the HN reduced the transduction efficiency of AAV-HN1/DMC1 in PC-12s back to low levels (see Fig. 3A). Selected cultures

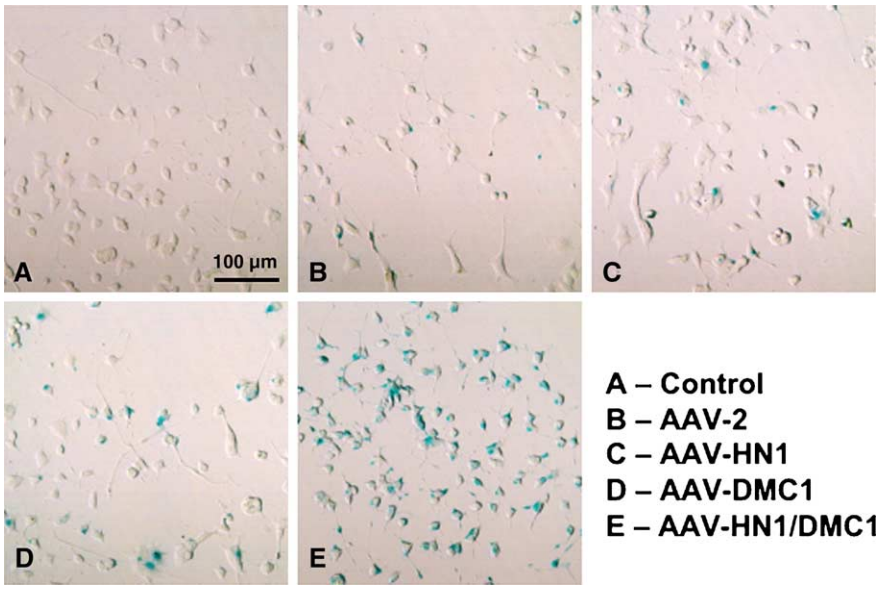

Fig. 2. Transduction of differentiated PC-12s with mutant AAV-2 vectors. After treatment with NGF for 7 days, differentiated PC-12s reached a final density of approximately 10^6 cells/well. The cells were then either mock transduced (A) or received 10 μl of either standard rAAV-lacZ (B) or an engineered vector with the indicated capsid insert (C–E). The cells were fixed and stained with X-gal 48 h later.

were also pretreated with sodium vanadate, a potent inhibitor of the dynein motor complex. This treatment also suppressed gene transfer by the double mutant, as shown in Fig. 3B. A similar effect of vanadate upon gene transfer by standard rAAV-2 in susceptible cell lines such as 293 was not observed (not shown).

Gene transfer into dorsal root ganglia neurons

The AAV-DMC1 and AAV-HN1 viruses, as well as the chimeric combination, were next evaluated in cultures of sensory neurons isolated from neonatal rat dorsal root ganglia (DRG). Gene transfer by standard rAAV-2 in dissociated DRG was very poor, resulting in only a few percent of the cells expressing beta-gal after exposure at high MOI. A vector bearing both the HN1 and DMC1 motifs transduced with much higher efficiency, with at least 50% of the cells expressing the transgene at a similar input dose. Vectors bearing single mutations yielded intermediate efficiencies, similar to the findings in PC-12s (see Fig. 4).

To demonstrate the specific contribution of the KSTQT motif in AAV-DMC1 in enhancing retrograde transport, the viruses were next applied selectively to the axons of the dorsal root ganglia, by culturing the cells in the Campenot format (Campenot, 1977, 1994). In these cultures, the cells are added to a central well partitioned with watertight barriers from separate chambers on either side. The neuron cell bodies remain isolated in the center well, but their axons and associated glia extend through the junctions into the side chambers due to a gradient of nerve growth factor (NGF). In this way, axons and neuron cell bodies become sequestered in separate fluid environments (see Fig. 5A). The Campenot format serves as a stringent in vitro test for both efficient axon

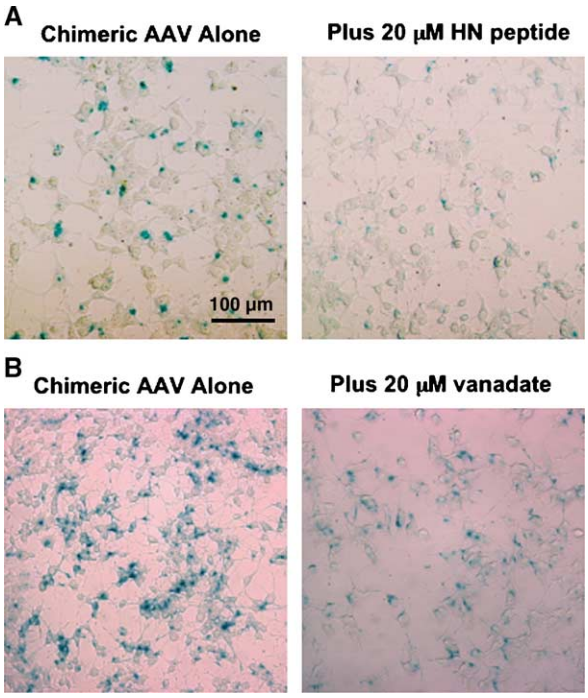

Fig. 3. Inhibition of NMDA-R-dependent vector uptake and DMC-dependent transport in PC-12 cells. (A) PC-12s were pre-incubated with 20 μM histogranin peptide for 15 min before addition of 10 μl of the AAV-HN1/DMC1 chimera carrying a lacZ transgene (right panel). Control cultures received AAV with no peptide (left panel). The medium was changed after 18 h and the cells fixed and stained with X-gal 2 days later as described in Fig. 2. (B) PC-12s were pretreated with 20 μM sodium orthovanadate (Na_3VO_4) for 2 h prior to addition of AAV-HN1/DMC1 (right panel). Control cultures received AAV with no vanadate (left panel). The medium was changed 14 h later and the cells were fixed and stained as in panel A.

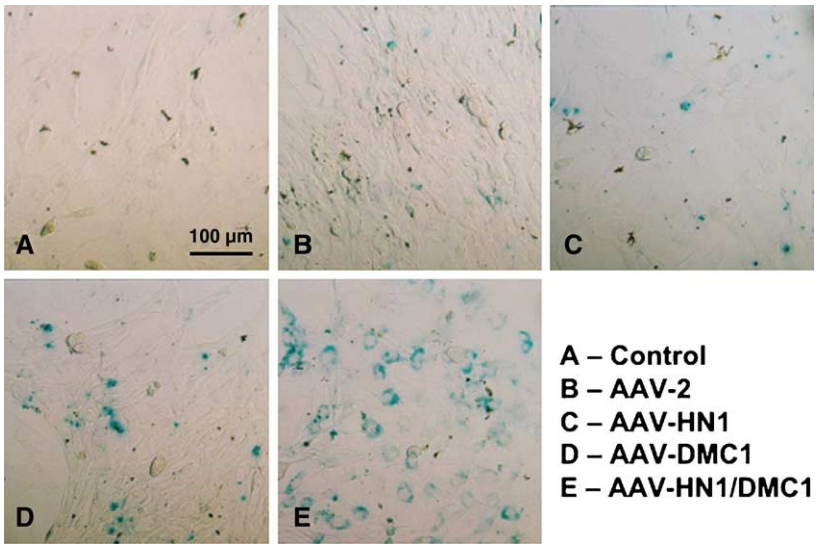

Fig. 4. Gene transfer into dissociated dorsal root ganglia cultures using mutant AAV vectors. 2×10^5 neurons/well were plated initially, and cultured for 7 days. The cells were then either mock transduced (A) or received 10 μl of the standard rAAV-lacZ (B) or one of the modified lacZ vectors (C–E), as indicated. The cells were fixed and stained 48 h after transduction.

uptake and retrograde transport, as a failure in either will prevent successful gene transfer into the neurons.

Eight days after establishment, addition of the standard AAV-lacZ to selected side chambers failed to produce any lacZ expression in the central wells, in repeated trials. Exposure of axons to AAV capsids bearing either the KSTQT or HN motifs alone also produced either no or very few (2–4) beta-gal expressing cells in the corresponding central wells, although many cells in the treated side chambers expressed beta-gal, particularly after exposure to AAV-DMC1. Only after chimeric AAV-HN1/DMC1 capsids were added to side chambers did significant numbers of central well cell bodies express beta-gal (about 350 in one trial) (see Fig. 5B). No clustering of beta-gal positive neurons near the boundary with the treated side chamber was seen. Short pretreatment of the side chambers with HN peptide prior to addition of the AAV-HN1/DMC1 effectively blocked lacZ expression in the central wells (see Fig. 5C). Limited trials conducted with a different indicator, Red Fluorescent Protein (RFP), produced similar patterns.

That the expression pattern of the chimeric AAV-HN1/DMC1 vector in the Campenot cultures correlated with many more copies of its transgene reaching the central wells was confirmed by real-time PCR. Heightened levels (1–2 logs) of transgene DNA were detected in central chambers 2 days after addition of the chimera to the axon chambers, compared with exposure to standard AAV-2 — 44.8×10^3 copies versus 1.36×10^3, respectively, in one trial. No virus signal was detected in culture medium from any of the central wells, or from side chambers not exposed to an rAAV, verifying the integrity of the watertight seals between the chambers, and confirming the appearance of the transgene and its protein product in neurons was not due to leakage of virus across the seals.

Binding of mutant AAV capsids to cytoplasmic dynein

To verify the phenotypic changes in retrograde transport and transduction efficiency produced by the DMC1 peptide insert correlated with a new binding affinity of these capsids for a specific component of cytoplasmic dynein, plasmids encoding either the standard AAV-2 *rep* and *cap* gene sequences or the mutant bearing the DMC1 motif in *cap* were transfected into 293 cells. Cell lysates were prepared 24 h later and immunoprecipitated under non-reducing conditions with an antibody against the AAV capsid protein. The immunoprecipitation products were then run on gels and visualized using a specific antiserum against LC8, the cytoplasmic dynein light chain to which the KSTQT motif in DMC1 was designed to bind. As shown in Fig. 6, immunoprecipitation of the standard capsid did not co-immunoprecipitate LC8, as expected. But immunoprecipitation of the AAV-DMC1 capsid also brought down LC8, confirming binding of the mutant to this DMC polypeptide.

Gene transfer into other cell types using mutant AAV capsids

As noted in Campenot experiments, direct exposure to rAAV bearing the DMC1 motif produced more extensive transgene expression in side chamber cells exposed to these vectors than exposure to standard rAAV-2. More glial cells were transduced using either AAV-DMC1 or AAV-HN1/DMC1, compared with the standard rAAV-2 or AAV-HN1 vectors. The phenotypes of these capsids were compared next across a panel of non-neuronal cell lines, including 293, HeLa, CEM (a human T cell line), and DITNC (an immortalized rat astrocyte cell line). No enhancing effect of the HN peptide insert was observed in these cell types, in keeping with its specific affinity for NMDA receptors. In

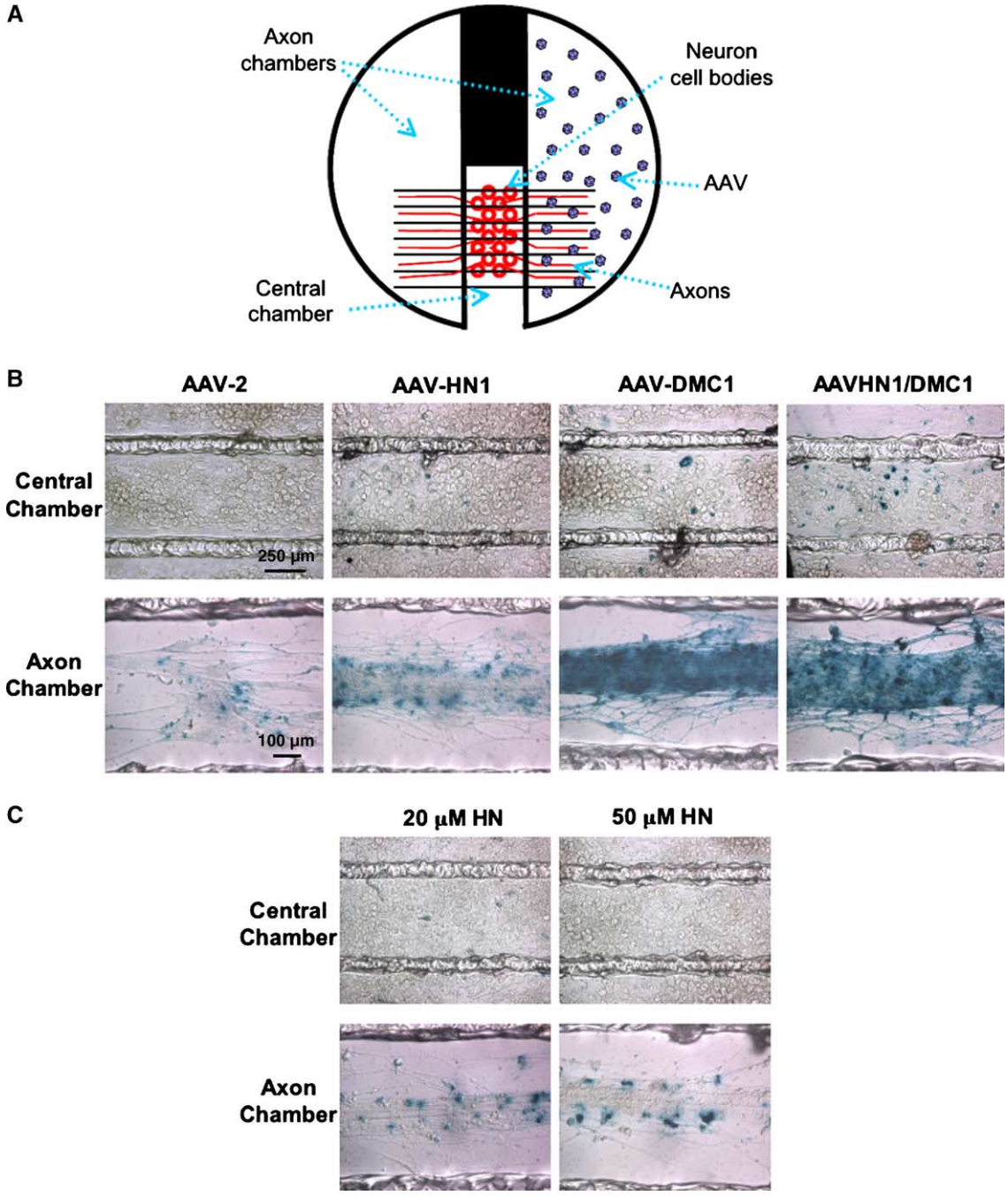

Fig. 5. Enhanced gene transfer into Campenot cultures of dorsal root ganglia neurons by mutant AAV vectors. (A) Diagram of Campenot format. Two side chambers separated from a central well were established using Teflon dividers attached with grease to a 35-mm dish. 10^5 neurons were plated initially in each central chamber. Guided by an NGF gradient between the central and side chambers, axons extend into the side chambers along parallel scratches etched in the plastic. Cell survival after 8 days was estimated at 50%. (B) AAV-mediated gene transfer into Campenot cultures. Eight days after establishment of the cultures in panel A, 10 μl of each vector was added to one side chamber of each culture. The other side chamber was left untreated. 48 h later, the central and side chambers were fixed and stained for beta-gal expression. Horizontal lines visible across the central chambers are grooves etched in the plastic as a guide for neurite outgrowth. (C) Histogranin inhibition of gene transfer mediated by a chimeric AAV vector. Histogranin peptide was added to one axon chamber of different Campenot cultures at a final concentration of either 20 or 50 μM, 10 min prior to addition of 10 μl of AAVHN1/DMC1. After 18 h, the medium was changed in this chamber and the culture was maintained for an extra 30 h before fixing and staining as described in panel B.

contrast, transgene expression after transduction with AAV-DMC-1 was reproducibly higher in some lines than after exposure to standard AAV-2, particularly at lower input MOI. Differences were marginal in CEMs, but more notable in others, ranging from less than 2- to more than 5-fold. Results from several trials are shown in Fig. 7.

Retrograde transport mediated by cytoplasmic dynein is not only active in neurons. To look more closely at this effect

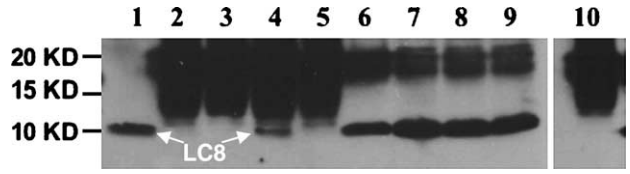

Fig. 6. Co-immunoprecipitation of LC8 light chain with an AAV capsid antibody. 293 cells were transfected with either the standard pXX2 or mutant pXX2-DMC1 plasmids. Untransfected cells served as a negative control. 24 h later, clarified cell lysates were prepared under non-denaturing conditions and immunoprecipitated with an AAV capsid antibody (A20). The precipitated immune complexes (lanes 2 to 5) as well as their respective supernatants (lanes 5 to 9) were resolved by SDS-PAGE and immunoblotted with anti-LC8 antibody. Lane 1, input control lysate; lane 2, IP of control lysate; lane 3, IP of lysate from cells transfected with pXX2; lane 4, IP of lysate from cells transfected with pXX2-DMC1; lane 5, same as lane 4 except the anti-AAV antibody was omitted; lanes 6 to 9, supernatant from IPs corresponding to lanes 2 to 5, respectively; lane 10, protein G-agarose alone.

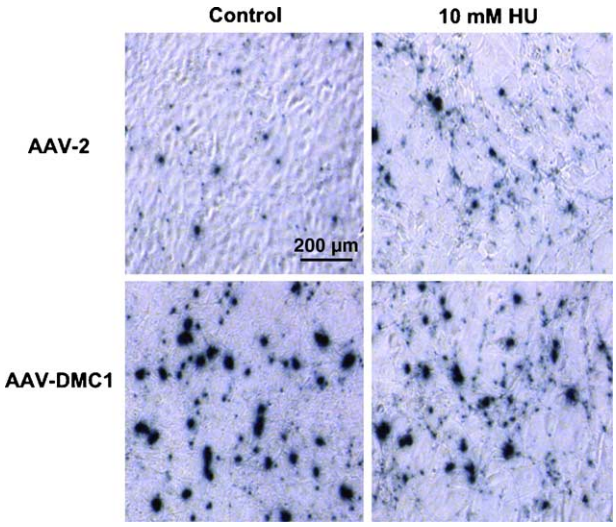

Fig. 8. Transduction of 3T3 cells by standard rAAV-2 or AAV-DMC1. 3T3 cells were incubated in the presence or absence of 10 mM HU for 2 h prior to rinsing with PBS and replenishment with new medium. The HU-treated, as well as the untreated control cells, were then transduced with 10 μl of either the standard AAV-2 or AAV-DMC1 lacZ vectors. The cells were fixed and stained with X-gal 48 h after transduction.

of the DMC1 binding motif on transduction of non-neuronal cells, AAV-DMC1 was tested on a cell type strongly resistant to standard AAV-2, murine 3T3 cells. Unlike cells that simply lack viral receptors, 3T3s are not impaired for binding or entry of AAV-2, yet still transduce very poorly. After internalization, AAV virions are localized initially in early endosomes from which they cannot escape. In 3T3s, further maturation of these endosomes is impeded, compared with more easily transduced cell types such as 293 (Hansen et al., 2000; Hansen et al., 2001). This block to transduction can be overcome by pretreatment of 3T3s with agents such as hydroxyurea (HU) which promote endosome acidification. Following HU treatment of 3T3s, AAV particles can be found in late endosomes and lysosomes from which escape is unimpaired. Because the rate-limiting step is a block in intracellular trafficking, and the insert in the DMC1 capsid appeared to affect transport of the virus, the susceptibility of 3T3s to gene transfer mediated by this virus was compared against standard AAV in the presence or absence of HU. As shown in Fig. 8, the standard AAV-2 lacZ vector transduced the cells at only a very low level, as expected. Pretreatment with HU increased the number of cells expressing beta-gal several fold. However, the percentage of cells expressing the transgene after delivery in the DMC1 capsid was much higher, even without HU, and the transduction was not enhanced further by HU treatment.

Injection of standard rAAV-2 and a chimeric capsid in a mouse tongue model

As an initial test of efficacy in vivo, either the AAV-HN1/DMC1 mutant or regular rAAV-2 were applied to a standard animal model for retrograde transport. Ten microliters of each vector was injected into the tongues of two groups of mice. Selected animals were then sacrificed at several time

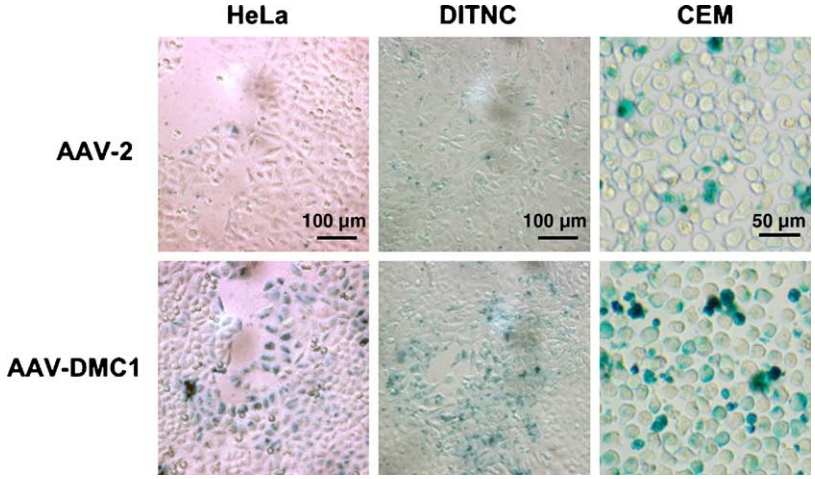

Fig. 7. Transduction of non-neuronal cells with standard rAAV-2 or AAV-DMC1. HeLa cells, rat astrocytes, or CEM cells were transduced with either of the two vectors, and fixed and stained with X-gal 48 h later. An MOI of about 50 was used for the cell lines except CEM, where the MOI was 100.

Table 1
AAV copy numbers in brain stems of tongue-injected mice

Vectors[a]	Brain stem[b]			Tongue[c]		
	24 h	48 h	72 h	24 h	48 h	72 h
AAV-2	0	0	0	98,492	4306	2435
AAV-HN1/DMC1	0	490	2771	146,944	18,078	677

Values reflect transgene copy numbers per tissue block. All values were subtracted from the mean of the negative control samples. Values less than 1 SD from the mean of the negative controls were regarded as insignificant.
[a] 10 μl of the indicated vector was injected into the front half of each tongue.
[b] 20 mg of brain stem tissue including both hypoglossal nuclei from each mouse was dissected for DNA extraction at the indicated times.
[c] 20-mg samples were also collected from each tongue near the injection site.

points. The tongues as well as brain stems were collected, and DNA was extracted for real-time PCR. Samples of brain and tongue tissue from control mice were also collected as negative controls. As expected, large quantities of vector DNA were present in tongues of animals receiving either vector, particularly immediately after injection. No signal above baseline was detected in brain stems of animals receiving only standard rAAV-2 at any time point. Following injection of AAV-HN1/DMC1, no signal was present after 24 h, but brain stems collected after 48 or 72 h were found to harbor up to several thousand copies of the vector transgene (see Table 1).

Discussion

Successful attempts to alter the tropism of AAV have typically taken the form of small discrete changes in the major *cap* gene sequence intended to confer a single new property. In this study, two distinct types of capsid mutation were introduced in separate VP3 clones, and then combined to yield chimeric virions exhibiting enhanced gene transfer capabilities in NMDA-R expressing neurons. This new tropism was achieved not by mixing two motifs intended to influence the same property, but rather by peptide inserts designed to affect distinctly different characteristics of the capsid. The full impact of the phenotypic changes was only apparent in the context of neurons, and was most evident in instances where successful transduction was heavily dependent upon axonal uptake as well as efficient retrograde transport of the particles.

Synergistic effects of the mutations were seen in differentiated PC-12 cells and in cultures of dorsal root ganglia neurons. The PC-12s and DRG were only poorly susceptible to standard rAAV-2-mediated gene transfer, and in the Campenot format the latter also offered a rigorous test of each vector's capacity for retrograde transport. The results in the Campenot cultures also provide insight into the constraints upon standard rAAV-2-mediated gene transfer in neurons. In theory, the lack of transgene expression in the neuron cell bodies following axonal exposure to standard rAAV-2 could result from limited binding or uptake of the virus by the axons, inefficient retrograde transport, or both. Comparing the performance of the different mutant capsids against that of the standard rAAV-2 indicates both are important contributors to the poor performance of the standard vector in this context. Providing a new affinity for a specific receptor on the axons, via the HN motif, did not in and of itself notably enhance transduction of the neurons. Conversely, when the vector was altered to home to an efficient retrograde transport pathway, minimal neuronal transgene expression occurred in the absence of the other peptide conferring affinity for NMDA-R.

The enhanced expression conferred by the DMC1 motif alone in some other cell types is also interesting, since this peptide insert is not designed to affect entry of the virus. AAV trafficking to the perinuclear region subsequent to endocytosis is mediated via endosomes, from which the virus then escapes to localize in the nucleus where uncoating and second-strand DNA synthesis occur. In 3T3 cells, where normal maturation of these endosomes is impaired, the effect of the DMC1 motif was dramatic, and essential for vigorous transduction. Details regarding the intracellular transport of AAV-DMC1 remain to be determined; the trafficking even of standard AAV remains an active area of investigation. But these observations are in line with its conferred ability to bind the LC8 light chain of cytoplasmic dynein, and consistent with the peptide insert in this capsid directly affecting trafficking of the virus.

This capability of the DMC1 motif suggests that this type of mutation could be valuable for other gene therapy applications unrelated to the central nervous system. There have been recurring difficulties in practice with many strategies designed to target AAV as well as other vectors to heterologous receptors. In some cases, this was due to an innate attribute of the targeted receptor, such as very slow uptake of ligand–receptor complexes. However, when a viral vector is redirected to a foreign receptor, some or most of the particles may traverse the intracellular pathway normally taken by its receptor–ligand complexes after uptake, which may be incompatible with expression of the genetic payload of the vector. This has been a problem with attempts to re-target viruses to, for example, the EGF receptor (Cosset et al., 1995; Erlwein et al., 2002). By virtue of its ability to override some non-productive pathways, as in 3T3 cells, and redirect the virus to the DMC, motifs such as DMC1 could be beneficial to other targeting strategies.

In summary, the HN and DMC1 mutations, and their combination, represent a highly promising set of engineered gene vectors. The ability to deliver therapeutic sequences in vehicles specifically tailored to CNS populations would enable new and novel approaches to treating an array of serious debilitating neurological disorders, providing strong motivation for further research in this direction. Additional characterization of such chimeras will in turn lead to further refinements to optimize performance. For example, the chimeric virions are not a pure population. Individual

virions incorporate the different capsid mutants in a range of different ratios. It is possible a specific capsid ratio contributes disproportionately to the novel phenotype observed with the population as a whole. Gigout et al. recently reported that mosaic virions containing a mix of a capsid mutant together with the wild-type capsid protein had properties distinct from pure virions of either type alone (Gigout et al., 2005).

These findings also further expand the possibilities available for generating novel chimeric AAV. With clones from a rapidly increasing number of distinct AAV becoming available, derived from human as well as non-human sources (Mori et al., 2004), investigators have already begun to explore the properties of chimeric capsids containing components of more than one type of AAV (Hauck et al., 2003; Rabinowitz et al., 2004) or even recombinant capsid proteins bearing epitopes from more than one strain (Bowles et al., 2003). The ability to mingle different mutant capsids together to generate viruses with unique functionalities further expands the pool of AAV chimeras available for research and therapy. This remarkable plasticity of the vector system should greatly increase the pace with which valuable derivatives of AAV with properties tailored for specific applications are identified.

Materials and methods

Primary cells and cell lines

293 cells, an adenovirus-transformed human embryonic kidney cell line, were maintained in Eagle's minimal essential medium (Mediatech Cellgro, Herndon, VA, USA) containing 10% fetal bovine serum (FBS) (Gibco BRL Life Technologies, Grand Island, NY, USA) and 100 U/ml penicillin–100 μg/ml streptomycin (pen-strep). HeLa, DITNC, and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) containing 10% FBS and pen-strep. PC-12 cells were maintained in RPMI 1640 (Cellgro) containing 10% heat-inactivated horse serum (Gibco BRL), 5% FBS, and pen-strep. To induce differentiation, PC-12s were plated onto 24-well plates pre-coated with collagen at a starting density of 20,000 cells/well in maintenance medium overnight, and then received 50 ng/ml NGF in RPMI 1640 containing 1% heat-inactivated horse serum and pen-strep for the next 7 days. NGF was replenished every 2 days. CEM cells were also cultured in RPMI 1640 with 10% FBS and pen-strep.

Preparation of Campenot cultures

Primary dorsal root ganglion (DRG) neurons from the superior cervical ganglia of newborn Sprague–Dawley rats were isolated as described previously (Heerssen et al., 2004). Campenot cultures were established from some preparations of DRG as described (Campenot, 1977, 1994). Briefly, after isolation, about 10^5 neurons were plated in central compartments formed by Teflon dividers placed across parallel scratches made on 35-mm dishes. The central compartments contained DMEM, with pen-strep, and 100 nM AraC supplemented with 10 ng/ml nerve growth factor (NGF), while the side compartments contained the same medium supplemented with 100 ng/ml NGF. This gradient of NGF across the 2 compartments guided the growth of neurites from the central chambers into the side compartment along the scratches. On the 6th day of culture, the concentration of NGF in the central compartment was further reduced to 1 ng/ml. The cultures were used for experiments on Day 8. Survival at this stage was estimated at 50% of the original cells.

Detection of beta-galactosidase

Expression of beta-gal, was assessed by X-gal staining as described previously (Madry et al., 2003). Briefly, cells were fixed with 2% formaldehyde and 0.2% gluteraldehyde in PBS (pH 7.6) at 4 °C for 5 min followed by 3 washes with PBS. The cells were then incubated with 1 mg/ml X-gal, 1.64 mg/ml potassium ferricyanide, 2.12 mg/ml potassium ferrocyanide, and 2 mM magnesium chloride in PBS (pH 7.6) at 37 °C for 12 h.

DNA extraction from tissue and real-time PCR

Male, 5-week-old Balb/C mice were anesthetized by i.p. injection of 100 mg/kg of ketamine/xylazine (1:1). Approximately 10 μl of standard or mutant AAV vector was injected in the front half of the tongue. The mice were allowed free access to food and water after recovering from anesthesia. At 24 h, 48 h, or 72 h after tongue injection, mice from each group received an i.p. overdose injection of pentobarbital. A 20 ± 2 mg tissue block was quickly dissected from the tongue around the injection site, and the brain stem containing both sides of the hypoglossal nuclei was collected from each mouse. Samples of cerebral cortex were collected from other mice as negative controls. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA). The copy number of the vector transgene in each sample was assayed by real-time PCR using a primer and probe set and cycling conditions previously described (Lewis et al., 2002).

Construction and packaging of rAAV

The lacZ gene sequence was cloned into an AAV-based vector plasmid, pACP, which has been described previously (Cucchiarini et al., 2003).

Mutagenesis of the AAV capsid was carried out using the ExSite PCR-based Site-directed Mutagenesis Kit (Strata-

gene, La Jolla, CA) and pXX2 (Xiao et al., 1998) as the template plasmid.

Primers used for constructing HN1 were —
Forward 5′ ggccgcactttgtacggattcggcttgtcgagacaagcagctaccgca 3′
Reverse 5′ ttggcccttaagtgtatagttcataccggtgttgcctctctggaggtt 3′
Primers used for constucting HN2 were —
Forward 5′ aagggacagggacgcactttgtacggattcgggttgtcagcagctaccgcagatgtcaac 3′
Reverse 5′ aagtgtatagttcgatccggtgaggttggtagatacagaacc 3′
Primers used for construction of DMC1 were —
Forward 5′ actcagactactagtgggttgtcagcagctaccgcagatgtcaac 3′
Reverse 5′ cgacttatcttctccggtgaggttggtagatacagaacc 3′

In each primer, bases complementary to the pXX2 template are underlined. In addition to the functional epitope, each insert included flanking Thr-Gly and Gly-Leu-Ser residues 5′ and 3′ to the inserts, respectively (Shi et al., 2001), for flexibility. A unique restriction site was included in each insert and/or deletion for screening purposes. All constructs were also verified by sequencing.

Packaging of all rAAV was carried out according to standard protocols with some modifications (Xiao et al., 1998). Briefly, vectors were packaged in a 3 plasmid system by co-complementation of the AAV vector plasmid with a second plasmid, pXX2, or one of its derivatives, encoding the AAV-2 replication and encapsidation functions, together with a 3rd plasmid, pXX6, carrying essential adenoviral helper functions. Purification of the vector preparations was achieved by a combination of passage over an iodixanol gradient followed by ion exchange chromatography using a 1- or 5-ml HiTrap Q column (Amersham Bioscience, Piscataway, NJ) as previously described (Zolotukhin et al., 2002). rAAV vector stocks were titered by real-time PCR using the ABI Prism 7700 Sequence Detection System from Perkin-Elmer Applied Biosystems (Foster City, CA, USA), as previously described (Clark et al., 1999). rAAV doses were calculated based on real-time PCR titers. Functional titers of rAAV vector preparations after purification were on the order of 10^{10} per ml. MOI was defined as number of transgenes rather than virus particles.

Western blotting and immunoprecipitation

293 cells ($6–7 \times 10^6$ per 100-mm dish) were transfected with 10 μg of each AAV vector plasmid using a standard calcium phosphate precipitation method. 24 h after transfection, the cells were scraped on ice into phosphate buffered saline (pH 7.4) containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM benzamidine, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 200 nM staurosporine, and 3.3 U/ml apyrase. The cell suspensions were homogenized using a 1-ml Wheaton homogenizer for 10 strokes, and centrifuged at 10,000×*g*, 4 °C for 15 min. Control cell lysate was prepared in the same way from untransfected cells. The clarified lysates were then used for immunoprecipitation. The lysates were pre-cleared with control rabbit antiserum and protein G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then incubated with a mouse monoclonal antibody against an epitope of the AAV-2 capsid, A20 (American Research Products, Inc., Belmont, MA), together with protein G on a rocker overnight at 4 °C. The immunocomplexes were then pelleted by centrifugation at 2,500 rpm for 30 s, washed with PBS and repelleted 3 times. The pellets were then run on SDS-PAGE, and immunoblotted with a rabbit polyclonal antibody against LC8 to check for co-precipitation of this protein.

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