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Tropism and toxicity of adeno-associated viral vector serotypes 1, 2, 5, 6, 7, 8, and 9 in rat neurons and glia *in vitro*

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Abstract

Recombinant adeno-associated viral (rAAV) vectors are frequently used for gene delivery to the central nervous system and are capable of transducing neurons and glia *in vitro*. In this study, seven serotypes of a rAAV vector expressing green fluorescent protein (GFP) were characterized for tropism and toxicity in primary cortical cells derived from embryonic rat brain. At 2 days after transduction, serotypes 1 and 5 through 8 expressed GFP predominately in glia, but by 6 days post-transduction expression was neuronal except for AAV5. AAV2 and 9 produced minimal GFP expression. Using cell viability assays, toxicity was observed at higher multiplicities of infection (MOI) for all serotypes except AAV2 and 9. The toxicity of AAV1 and 5–8 affected mostly glia as indicated by a loss of glial-marker immunoreactivity. A frameshift mutation in the GFP gene reduced overall toxicity for serotypes 1, 5 and 6, but not 7 and 8 suggesting that the toxicity was not solely due to the overexpression of GFP. Collectively, a differential tropism and toxicity was observed among the AAV serotypes on primary cortical cultures with an overall preferential glial transduction and toxicity.
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Keywords: AAV; Adeno-associated virus; Gene therapy; Serotype; Tropism; Toxicity; Neuron; Glia

Introduction

Adeno-associated viral (AAV) vectors are a versatile platform for gene delivery to the central nervous system (CNS) (Gao et al., 2005; McCown, 2005; Tenenbaum et al., 2004; Xiao et al., 1997). An AAV viral genome is approximately 4.7 kilobases (kb) and contains two sets of viral genes, the *rep* and *cap* genes important for replicating/packaging the viral DNA and producing the capsid proteins, respectively. The viral genes are flanked by two 145 bp inverted terminal repeats (ITRs) which are required for replication and packaging. Recombinant AAV vectors are most commonly generated by replacing all viral DNA between the ITRs with a transcriptional cassette of less than approximately 5 kb. The resulting construct is cotransfected into HEK293 cells with a plasmid containing helper virus genes and rep/cap containing plasmid (Xiao et al., 1998). The resulting rAAV lacks any viral genes and the serotype of the rAAV can be altered by using different rep/cap constructs during packaging.

Currently, over 100 non-redundant genotypes of AAV have been identified (Gao et al., 2005) and offer varied tropism. In the rodent brain, Taymans et al. (2007) compared serotypes 1, 2, 5, 7 and 8 for tropism in various brain regions and found that these serotypes were primarily neuronal (Taymans et al., 2007). Earlier studies also found neuronal expression by serotypes 1, 2, 5, 7 and 8 (Burger et al., 2004; Cearley and Wolfe, 2006; Davidson et al., 2000; Harding et al., 2006; Paterna et al., 2004). Additional expression was observed in ependymal cells by serotypes 4 and 5 (Davidson et al., 2000), astrocytes by serotypes 5–8 (Davidson et al., 2000; Harding et al., 2006), microglia by serotypes 2 and 5 (Cucchiarini et al., 2003) and oligodendrocytes by serotype 2 (Chen et al., 1999). *In vitro* studies using rAAV vectors have identified transduced neurons (Cao et al., 2004; Garrity-Moses et al., 2005; Keir et al., 1999; Kugler et al., 2003; Michel et al.,

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2005; Nomoto et al., 2003; Shevtsova et al., 2005) and glia (Kugler et al., 2003; Nomoto et al., 2003; Shevtsova et al., 2005), but these studies were conducted under different conditions with limited serotypes (2 and 5). No reports to date have compared tropism of multiple serotypes on neurons and glia *in vitro* under the same controlled conditions.

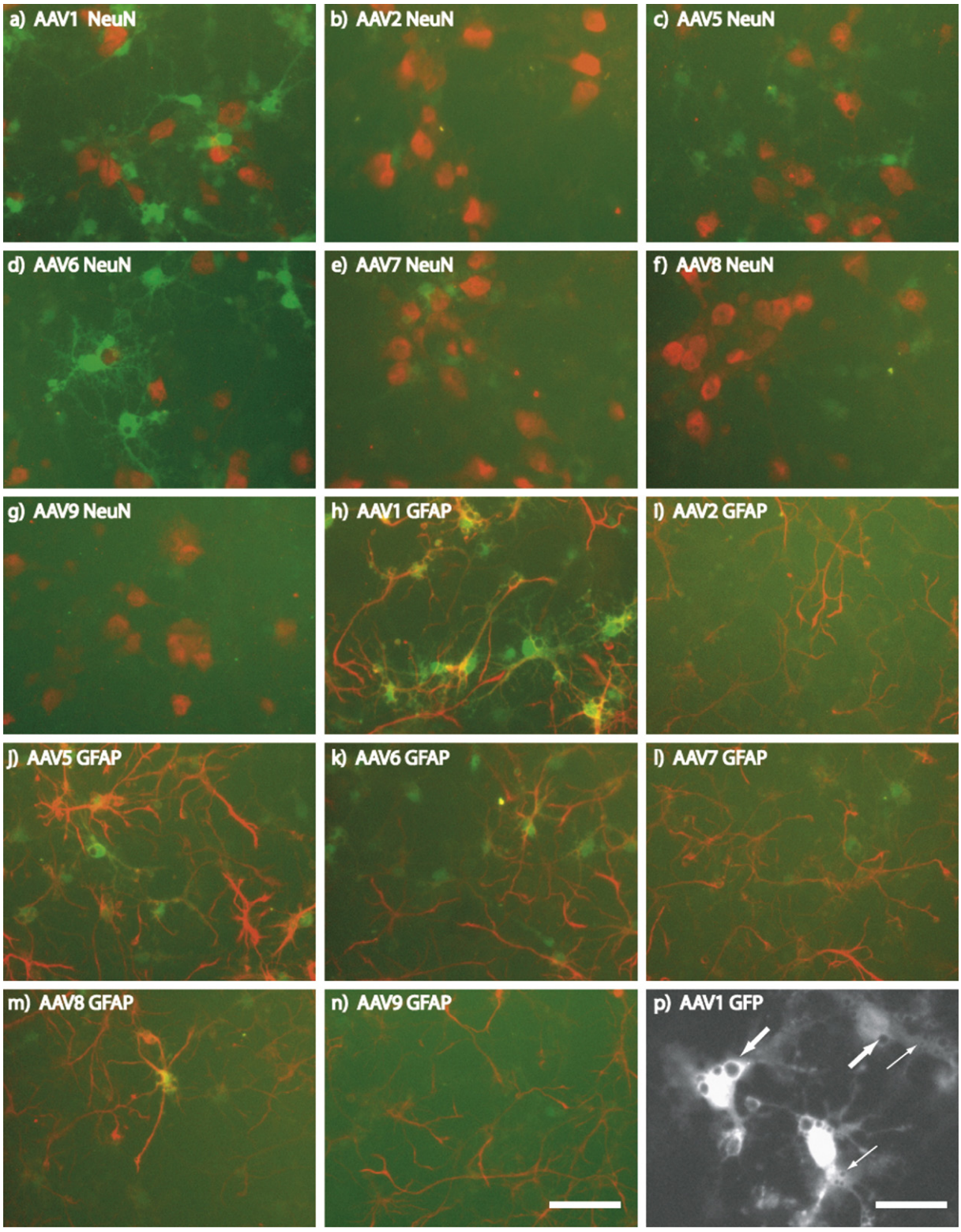


Fig. 1. Serotype-specific expression of GFP in primary cortical cultures. Primary cortical cultures (DIV6) were transduced with different serotypes of dsAAVGFP. Two days after transduction, cells were fixed and immunostained for NeuN (red; a–g) or GFAP (red; h–n). Results from MOI = 2×10^4. P) At 2×10^5 MOI, AAV1-transduced cells contained vacuoles of varying size (small and large arrows) devoid of GFP fluorescence (white) were observed. Scale bars: a–n) = 50 μm, p) = 25 μm.

In vitro studies using primary cells or tissue provide an opportunity to conduct complex analysis of molecular mechanisms and cellular processes that would be impractical to test *in vivo* initially. Neurons and glia grown *in vitro* are extensively used to study the functions of genes and their proteins for understanding basic mechanisms, modeling neurodegenerative diseases and evaluating therapeutic potentials of genes and compounds. One limitation to gene transfer studies in primary neurons is the inefficiency and toxicity associated with transfection methods (Jaworski et al., 2000; Martinez and Hollenbeck, 2003; Watanabe et al., 1999). AAV vectors can efficiently transduce primary neurons *in vitro* (see above) but the toxicity of AAV vectors on primary neurons and glia *in vitro* has not been well characterized.

One of the features that makes rAAV vectors a desired vector for gene therapy in the brain is the low toxicity and immunogenicity. Delivery of rAAV2 vector to rodent brain produces minimal cellular immune response (Feng et al., 2004; Lo et al., 1999; Mastakov et al., 2002). Lo et al. (1999) reported that circulating antibodies to capsid, but not the transgene, were detectable at 2–4 months after single injection of AAV, but there was no correlation with antibody titers and transgene expression. In a recent phase I clinical trial, a rAAV2 vector delivered intraparenchymally showed no evidence of increased neutralizing antibodies to AAV2 capsid in 7 of 10 patients and all subjects showed minimal systemic signs of inflammation or immune stimulation (McPhee et al., 2006). Although AAV vectors have been used to transduce neurons and glia *in vitro* (Cao et al., 2004; Garrity-Moses et al., 2005; Keir et al., 1999; Kells et al., 2007; Kugler et al., 2003; Michel et al., 2005; Nomoto et al., 2003; Shevtsova et al., 2005), there are no reports examining the toxicity of different serotypes nor comparing tropism under the same culture conditions. In addition to the toxicity associated with the vector, the transgene can be responsible for toxic effects associated with viral vector transduction. Previous studies have shown that overexpression of GFP can be toxic to cells (Detrait et al., 2002; Klein et al., 2006; Liu et al., 1999), therefore, it is important to differentiate the toxicity of the transgene product from the vector/cell interactions.

The purpose of this study is to evaluate the tropism and toxicity of seven serotypes (1, 2, and 5 through 9) of a double-stranded rAAV vector expressing GFP in primary cultures containing both glia and neurons. The vectors are packaged, purified, titered and applied to primary cultures under the same conditions. The toxicity related to the GFP transgene is also examined by comparison to an AAV vector containing a frameshift mutation in the GFP gene (AAVΔGFP). Our results indicate a differential expression of the GFP transgene (tropism) and toxicity from AAV serotypes 1, 2 and 5 through 9 on primary neurons and glia *in vitro*. The pattern of toxicity and tropism is consistent with the relatedness of the capsid proteins.

Results

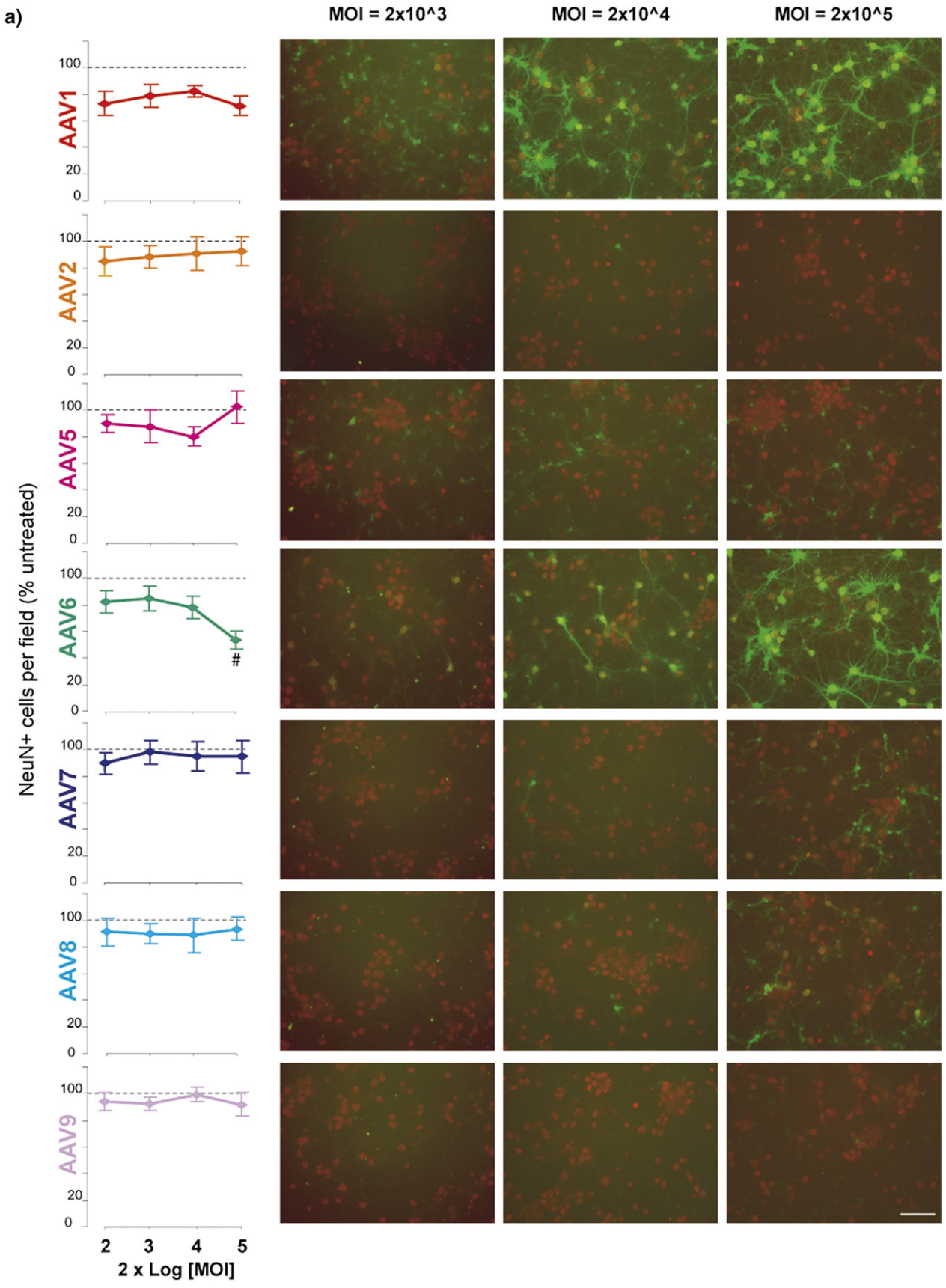
Tropism of AAV serotypes on primary cortical cultures

To broadly characterize the tropism of the various serotypes of dsAAVGFP, primary cortical cultures were transduced on the 6th day *in vitro* (DIV) at multiplicities of infection (MOIs) of 2×10^2, 2×10^3, 2×10^4 and 2×10^5 viral genomes (vg)/cell. At DIV8 or DIV12 cultures were fixed and visualized after immunofluorescent amplification of GFP signal and immunolabeling neurons (NeuN) or astrocytes (GFAP). Serotypes 2 and 9 exhibited minimal GFP signal at the two highest MOIs tested on both DIV8 (Fig. 1) and DIV12 (Fig. 2) and showed 5–10% colocalization with either GFAP-immunoreactive (IR) or NeuN-IR cells. Serotype 5 resulted in GFAP-IR and non-NeuN-IR cell expression of GFP at DIV8 (Fig. 1). At DIV12, serotype 5 continued to show colocalization to predominantly GFAP-IR and non-NeuN-IR cells and the expression of GFAP appeared reduced (Fig. 2). On DIV8 at each MOI, serotypes 1 and 6 exhibited the highest intensity of GFP signal compared to all other serotypes which were primarily colocalized to GFAP-IR cells and non-NeuN-IR cells, but NeuN-IR cells expressing low GFP signal were present at the two highest MOIs (Fig. 1a, d). Serotypes 7 and 8 produced similar pattern of GFP expression as serotypes 1 and 6 but less efficiently i.e. serotypes 1 and 6 at 2×10^4 was similar to 7 and 8 at 2×10^5 (Figs. 1 and 2). On DIV8 at higher MOIs, serotypes 1 and 6 produced GFP+ cells that were highly vacuolized suggesting a cytopathic effect (Fig. 1p). At DIV12, the GFP expression in cells transduced with serotypes 1, 6, 7 and 8 showed a high degree of colocalization with NeuN-IR and a loss of colocalization with GFAP-IR (Fig. 2a, b).

Effects of AAV serotypes on immunoreactivity of cell specific markers

To determine whether the observed changes in the GFP expression patterns from 2 to 6 days post-transduction were due to loss of GFAP-IR, the cultures were examined at 2 and 6 days after transduction for the presence of neurons (NeuN immunoreactive cells) or astrocytes (GFAP immunoreactivity). At 2 days post-transduction, there was no significant difference in the number of NeuN-IR cells between non-transduced cells and all serotypes tested at all MOIs (data not shown). Similarly, at 6 days post-transduction there was no change in NeuN+ cell counts for all serotypes except AAV6 which exhibited a significant decrease ($p<0.05$, One-way ANOVA–Dunnett's) compared to non-transduced cells (Fig. 2a). At 6 days post-transduction, cultures treated with serotypes 1, 6, 7 and 8 exhibit GFP+ cells that mainly colocalize with NeuN-IR cells at the higher MOIs, however AAV5 shows minimal colocalization with NeuN (Fig. 2a panels).

Fig. 2. Loss of cell specific marker immunoreactivity in response to AAV serotypes. (a) NeuN or (b) GFAP immunoreactivity of primary cortical cultures transduced with AAV serotypes on DIV6 and immunostained 6 days after transduction. Graphs (left) show the average number of (A) NeuN+ cells per field or (b) GFAP immunoreactivity per field in the indicated AAV-treated group as percentage of the untreated group (dashed line) for different MOIs. Panels (right) are representative fields of (a) NeuN+ (red) or (b) GFAP-IR (red) and (a, b) GFP+ (green) cells for MOI of 2×10^3, 2×10^4 and 2×10^5. #$p<0.05$ versus control by One-way ANOVA, Dunnett's post-hoc test;. Scale bar=100 um.


a)
MOI = 2x10^3
MOI = 2x10^4
MOI = 2x10^5
NeuN+ cells per field (% untreated)
AAV1
AAV2
AAV5
AAV6
AAV7
AAV8
AAV9
100
20
0
#
2
3
4
5
2 x Log [MOI]

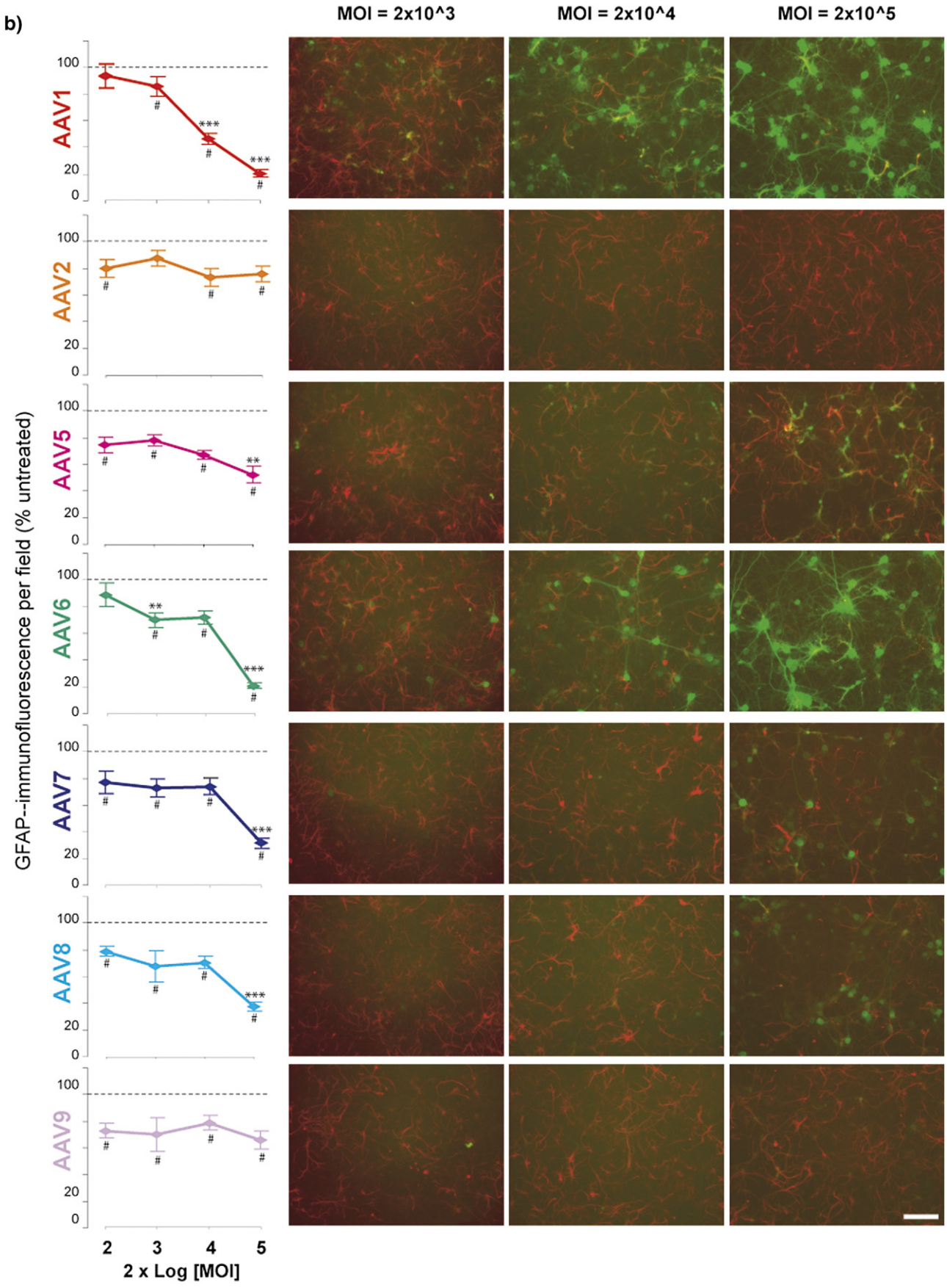


Fig. 2 (*continued*).

Two days after transduction, the GFAP-IR showed no significant difference among the serotypes and controls (data not shown). At 6 days post-transduction, all serotypes showed slight loss of GFAP-IR (15–20%) compared to controls at nearly all MOIs (Fig. 2b). A MOI-dependent decrease in GFAP-IR was observed at 6 days post-transduction for AAV1 and AAV6 (Fig. 2b). At the highest MOI (2×10^5), serotypes 5, 7 and 8 exhibited a further decrease in GFAP-IR compared to the next lowest MOI, 2×10^4 (Fig. 2b). As observed at 2 days post-transduction, AAV5 colocalized to GFAP-IR cells and minimal expression was detected in NeuN-IR cells.(Fig. 2b).

Acute toxicity of AAV serotypes on primary cortical cultures

The acute toxicity associated with transduction by the various serotypes was next examined as a function of MOI at 2 days post-transduction. Primary cortical cultures were transduced on DIV6 with various serotypes of dsAAVGFP at MOIs of 2×10^2, 2×10^3, 2×10^4 and 2×10^5 vg/cell. At 2 days post-transduction, overall cell viability was assessed by lactate dehydrogenase (LDH) release assay on the media and the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) on cells. All serotypes tested except 2 and 9 exhibited a MOI-dependent decline in cell viability (Fig. 3a, b). At a MOI of 2×10^4, MTS levels were significantly less in cultures treated with serotypes 1, 5, 6 and 7 compared to uninfected controls ($p<0.05$, One-way-ANOVA–Dunnett's; Fig. 4a) Similarly, at a MOI of 2×10^4, LDH release was significantly increased by serotype 1 ($p<0.001$, ANOVA–SNK), 5 and 6 ($p<0.05$, ANOVA–SNK; Fig. 4c). Unlike the MTS assay results at MOI of 2×10^4 (Fig. 4a) serotype 7 did not show significant increase in toxicity over control by LDH assay (Fig. 4c). Overall, serotype 1 exhibited the highest toxicity at all MOIs. Serotypes 5 through 8 had less, but significant toxicity compared to AAV1 and serotypes 2 and 9 had minimal toxic effects.

Mutating GFP partially attenuates toxicity of AAV serotypes on primary cultures

To examine whether the observed toxicity was related to toxic effects of GFP, a mutation was successfully introduced into the GFP gene creating a frameshift after the 10th amino acid of GFP that resulted in a protein (ΔGFP) that was 5 amino acids longer than GFP but only homologous at the first 10 amino acid. Primary cultures were transduced with the different serotypes of dsAAVΔGFP at four MOIs. For all MOIs tested, there was no detectable fluorescence from the dsAAVΔGFP. Two days after transduction, the overall viability was assessed by LDH release assay on the media and MTS assay on cells. At 2×10^4 MOI, dsAAVΔGFP serotypes 1, 5, 6, and 7 exhibited significant decrease (approximately 10%, $p<0.05$, One-way ANOVA, Dunnett's post-hoc), in viability by MTS compared to untreated cells (Fig. 4a). However, there was no significant difference compared to the serotype equivalent of dsAAVGFP at the same MOI (Fig. 4a). Compared to untreated cells, no significant change in LDH release was observed at 2×10^4 MOI for all serotypes of dsAAVΔGFP, however, dsAAVGFP showed significant increase in LDH levels for serotypes 1, 5 and 6 (Fig. 4c). At the highest MOI (2×10^5), dsAAVΔGFP exhibited significantly less toxicity ($p<0.05$, *t*-test) than AAVGFP for serotypes 1, 5, and 6 by both LDH and MTS assays. For serotypes 7 and 8, both dsAAVGFP and dsAAVΔGFP caused a significant decrease in cell viability compared to untreated cells by both LDH and MTS assays ($p<0.05$, One-way ANOVA, Dunnett's post-hoc), but there was no difference between dsAAVGFP and dsAAVΔGFP by Student *t*-test (Fig. 4b, d). Serotypes 2 and 9 of either dsAAVGFP or dsAAVΔGFP produced no significant change in cell viability at 2×10^4 MOI by MTS and LDH assay (Fig. 4a, c) or 2×10^5 by LDH assay (Fig. 4d). There was a significant decrease in viability compared to untreated cells by MTS assay at 2×10^5 MOI for serotypes 2 and 9 of dsAAVGFP ($p<0.05$, One-way ANOVA, Dunnett's

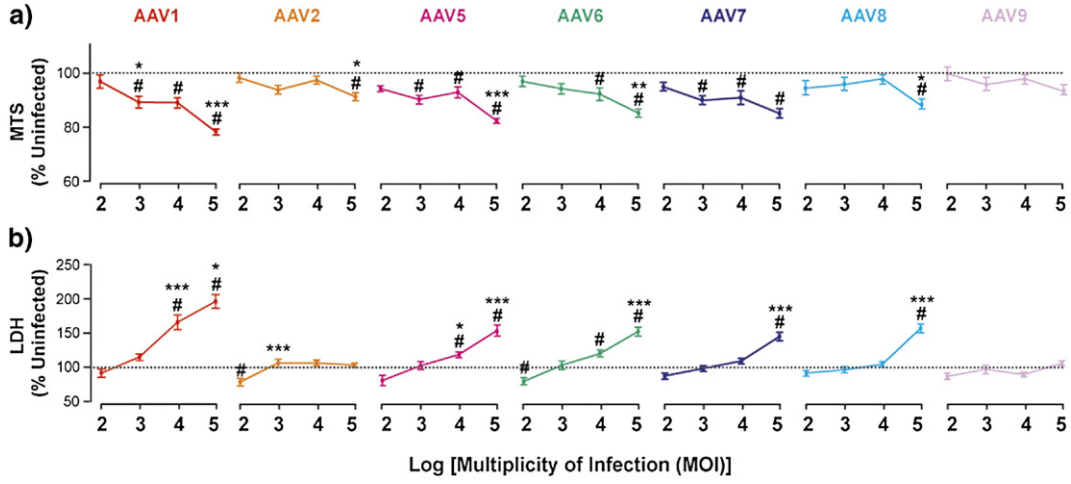


Fig. 3. Toxicity of AAV serotypes on primary cortical cultures. Primary cortical cultures (DIV6) were transduced with different serotypes of dsAAVGFP and assayed 2 days later for viability. MTS assay (a) and LDH assay (b) of primary cortical cells and media, respectively. Serotype comparison of MTS (a) and LDH (b) at MOI of 2×10^4. #$p<0.05$ versus control by One-way ANOVA, Dunnett's post-hoc test; *$p<0.05$, **$p<0.01$ and ***$p<0.001$ versus adjacent lower MOI by One-way ANOVA, SNK post-hoc test.

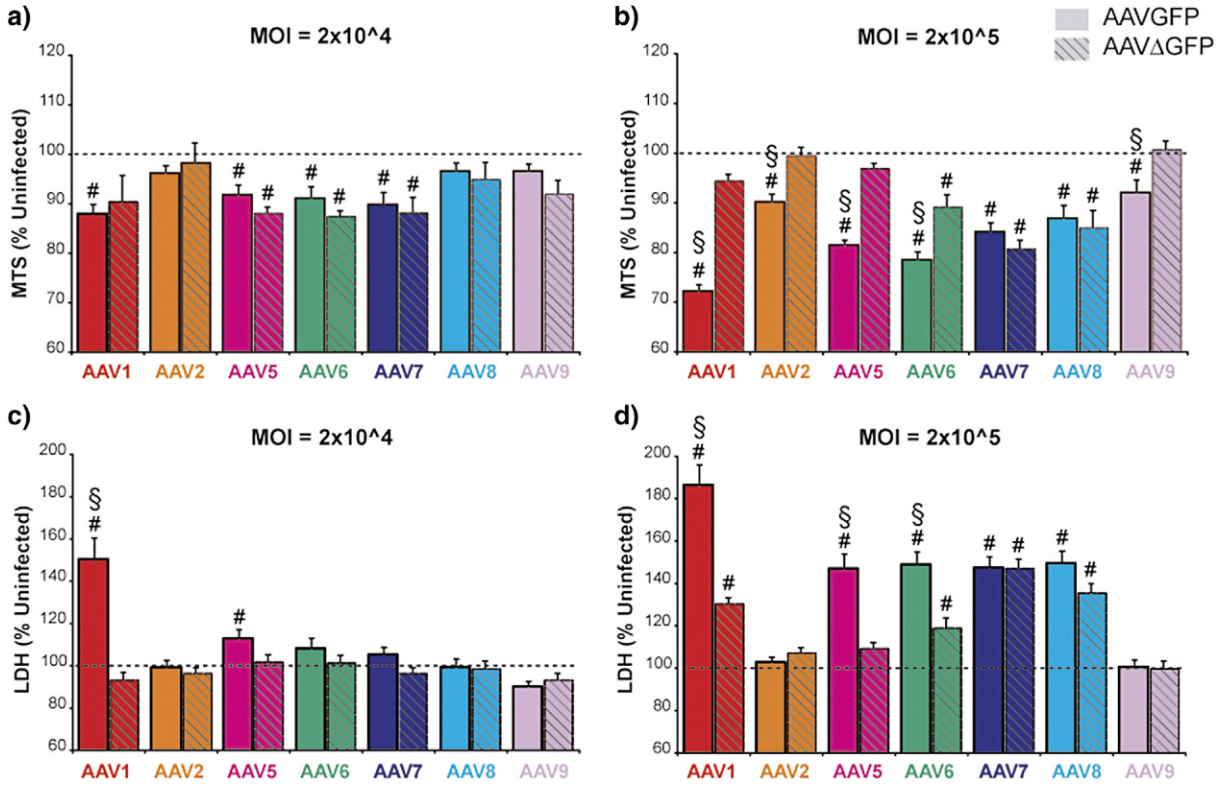


Fig. 4. Effects of mutant GFP transgene on cellular toxicity. Primary cortical cultures (DIV6) were transduced with different serotypes of dsAAVGFP (solid bars) or dsAAVΔGFP (hatched bars) at a MOI of 2×10^4 (a,c) and 2×10^5 (b,d). Two days after transduction, cell viability was measured by MTS assay (a,b) and LDH assay (c,d). # $p<0.05$ versus control by One-way ANOVA, Dunnett's post-hoc test § $p<0.05$ Student *t*-test versus dsAAVΔGFP of same serotype.

post-hoc), but dsAAVΔGFP was significantly different from dsAAVGFP of the same serotype ($p<0.05$, Student *t*-test; Fig. 4b).

Discussion

In this study the tropism and toxicity of AAV serotypes 1, 2, 5, 6, 7, 8 and 9 on primary cortical cultures derived from rat embryos were examined. A double-stranded rAAV vector was used to reduce the delay from viral exposure to transgene expression by removing the requirement of the second-strand DNA synthesis (Wang et al., 2003). In primary cortical cultures transduced with dsAAVGFP, GFP expression could be detected as early as 8 h after transduction using immunofluorescence to amplify GFP fluorescent signal and at 2 days without amplification (data not shown). Using GFP fluorescence as an index of tropism, at 2 days post-transduction there was a MOI-dependent increase in GFP expression for all serotypes except 2 and 9. The use of GFP transgene expression for comparing the tropism(s) of different serotypes of rAAVs has been described (Burger et al., 2004; Cearley and Wolfe, 2006; Paterna et al., 2004; Taymans et al., 2007). When examined 2 days post-transduction, the tropism (GFP expression) for all serotypes was predominantly glia, however, by 6 days post-transduction, the GFP expression was largely in NeuN-IR cells at the higher MOIs. Previous studies have shown that AAV2 or AAV5 containing the CMV promoter produced strong expression in glia compared to neurons, but exchanging the CMV promoter for the neuronal synapsin promoter restricted the GFP expression to neurons (Kugler et al., 2003; Shevtsova et al., 2005). In the current study, we examined the relative differences among serotypes of GFP expression by the CMV promoter and observed primarily glial expression initially which changes to neuronal over time with the exception of serotype 5.

The toxicity of the serotypes was characterized at 2 days post-transduction using MTS and LDH assays which showed a MOI-related toxicity for all serotypes except 2 and 9. Previous studies have indicated that GFP overexpression has been linked to neuronal toxicity (Detrait et al., 2002; Klein et al., 2006; Liu et al., 1999). We found that, at the highest MOIs used for serotypes 1 and 5 through 8, GFP expression was correlated with LDH ($R=.0.936$; $p=0.034$). There is an inverse correlation between GFP expression and MTS ($R=0.8324$; $p=0.069$). To address whether the overexpression of GFP was contributing to the overall toxicity, a frameshift mutation in the GFP gene of the dsAAV vector was made to create a mutant protein of similar size but only the first 10 amino acids of GFP. At the highest MOI of 2×10^5, dsAAVΔGFP significantly reduced overall toxicity for serotypes 1, 5 and 6, but did not affect the toxicity of 7 or 8. At the next lowest MOI of 2×10^4, dsAAVΔGFP exhibited no toxicity by LDH assay but similar toxicity to dsAAVGFP. Together, the overexpression of GFP at the highest MOI is a large contributor to the observed toxicity, but the differential effects

the mutant GFP among serotypes suggests that other mechanisms of toxicity remain possibly mediated via viral vector protein (s)–cell interactions. Although cells treated with AAV1 or AAV2 empty capsids with equivalent capsid proteins to those seen with MOIs used above did not alter MTS or GFAP-IR compared to untreated cells (data not shown). These data suggest that serotype 1 toxicity is primarily due to the toxic effects of GFP, but additional studies are needed to identify the toxic effects observed with all serotypes tested.

The toxicity at the higher MOIs, particularly with serotypes 1 and 6, corresponds to an eventual loss of GFAP-IR. The GFP expression in cells transduced with highest MOI of AAV1 or AAV6 for 2 days reveals the presence of large intracellular vacuoles in GFAP-IR cells. The vacuole may result from excessive endocytosis and accumulation of virion in multiple endosomal pathways (Ding et al., 2005). Although the vacuoles are likely clear predictors of cell death based on their early presence in GFAP-IR cells and subsequent loss of GFAP-IR cells, the exact mechanism by which they are formed and whether they contribute to the cell death is not known.

When compared to the phylogenetic analysis of the capsid viral protein 1 (VP1) for each serotype (Fig. 5), the intensity of expression, cellular tropism and toxicity observations are consistent with the relative capsid protein homologies. For example, the pairs 1/6, 7/8 and 2/9 had similar characteristics in tropism, expression and toxicity and each shows closest homology to each other. In fact, the homologies of AAV1 and AAV6 capsid proteins are nearly identical (Gao et al., 2002) and show similar tropism and toxicity on the cortical cultures in the current study. The toxicity of both serotypes 7 and 8 where unaffected by mutating GFP and these two serotypes are closely related. The parallels of serotype tropism, toxicity and capsid homology imply that the observed differences in the serotypes are a reflection of the capsid–cell interactions and are consistent with observations that single amino acid changes can alter tropism of AAV vectors (Wu et al., 2006). Cellular receptors have been identified for serotypes 1 and 6 (sailic acid (Chen et al., 2005; Seiler et al., 2006)), serotype 2 (heparan sulfate proteoglycans (Summerford and Samulski, 1998)), fibroblast growth factor 1 (Qing et al., 1999), integrin (Summerford et al., 1999), hepatocyte growth factor receptor (Kashiwakura et al., 2005) and laminin receptor (Akache et al., 2006), serotype 5 (platelet derived growth factor receptor (Di Pasquale et al., 2003)), serotypes 8 and 9 (laminin (Akache et al., 2006)). Many of these receptors are capable of signaling upon activation by a ligand. AAV2 has been shown to promote pro-death pathways in the absence of transgene expression (Duverger et al., 2002). Future studies examining the capsid–receptor interactions for the various serotypes may provide a better understanding of how cell signaling and viability is altered by the process of vector attachment and entry into the cell.

Based on the observations of this study, AAV vectors can be toxic to cultured neurons and glia; therefore, a MOI at or below 2×10^4 vg/cell is recommended to create minimal toxicity to glia and observable expression in both glia and neurons. This recommended MOI is comparable to the MOIs (10^3 to 10^5) in other *in vitro* studies using AAV-mediated gene transfer to neurons (Cao et al., 2004; Doroudchi et al., 2005; Gong et al., 2004; Kells et al., 2007; Kugler et al., 2003; Shevtsova et al., 2005). To achieve further specificity and physiologically relevant expression, the use of cell specific promoters will be needed. AAV vectors have been generated to provide cell-restricted gene expression by virtue of a cellular promoter (Kugler et al., 2003; Shevtsova et al., 2005). By incorporating cellular promoters into select pseudotyped AAV vectors and transducing cells at MOIs of minimal toxicity, the physiological or therapeutic levels of gene product may be achieved *in vitro*.

Materials and methods

Primary cortical cultures

Cells were isolated from E15 embryos collected from timed-pregnant Sprague–Dawley rats as described previously with modifications (Cox et al., 2004) and in accordance with approved procedures by the NIH Animal Care and Usage Committee. Specifically, brain cortices from E15 embryos were pooled and digested 20 min in prewarmed (37 °C) 1 ml/embryo of 0.05% trypsin–EDTA (0.2% (Invitrogen)). Cortices were triturated and diluted in plating media [Neurobasal media (Invitrogen), 2% heat-inactivated fetal bovine serum (Sigma-Aldrich, Milwaukee, WI), 2% B27 supplement (Invitrogen), 200 mM L-glutamine and 25 mM L-glutamate] added at approximately 2 ml per embryo. Viability was assessed by trypan blue staining (Invitrogen) and cells were plated at 3×10^4 viable cells/well in 0.2 ml plating media into 96 well plates coated with 0.15–0.2% polyethyleneimine in 150 mM sodium borate, pH 8.5 (Sigma-Aldrich). Plated cells were placed in a 37°C humidified incubator with

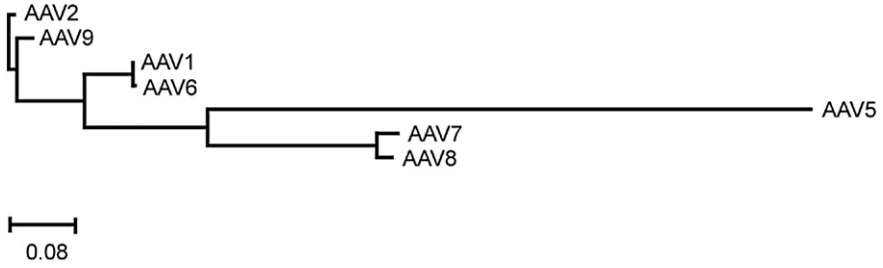


Fig. 5. Phylogram of capsid proteins for AAV serotypes 1, 2 and 5 through 9. Tree rooted to AAV2. Units represent frequency of changes per 100 residues.

5.5% CO2. Cells were fed by 50% media exchange on the 4th day *in vitro* (DIV4) with feed media (plating media without serum or glutamate).

Plasmids

The construction of pdsAAVGFP has been described previously (Wang et al., 2003). To generate pdsAAVΔGFP, pdsAAVGFP was digested with BseRI and T4 polymerase to remove the 3′ overhang of 2 bases. This created a frameshift mutation at amino acid 11 of GFP and an overall protein of 243 amino acids (compared to 238 aa of GFP). For the serotypes 1, 2, 5, 6, 7, 8 and 9 the respective plasmids were used in transfections described below: pXR1 (aka pXX12; (Rabinowitz et al., 2002)), pXX2 (Xiao et al., 1998), pXR5 (Rabinowitz et al., 2002), pAAV2/6 (Rutledge et al., 1998), pAAV7 (Gao et al., 2002), pAAV8 (Gao et al., 2002), and pAAV9 (Gao et al., 2004). Plasmids used for packaging AAV were generously provided by Dr. Xiao Xiao (UNC, Chapel Hill, NC).

AAV packaging

All vectors were prepared by triple transfection method as described (Xiao et al., 1998). Specifically, HEK293 cells are grown in 293 media [DMEM-HG (Invitrogen)] containing 5% bovine growth serum, (BGS; HyClone, Logan, UT) and 1% penicillin–streptomycin in 20×150 mm dishes until 80–90% confluent. For one 150 mm dish, 25 μg pHelper (Stratagene, La Jolla, CA, USA), 18 μg of pdsAAVGFP or pdsAAVΔGFP, and 7 μg of rep/cap plasmid which varies with serotype (see above) were added to 2 ml of 0.25 M $CaCl_2$. The solution was gently vortexed while a 2× buffer (280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4) was slowly dripped into the solution. After 15–17 h, transfection media were replaced with 293 media. Forty eight hours post-transfection, the cells and media were harvested and centrifuged for 5 min at 800 ×g, 4 °C. The pellet was resuspended in 1 ml of 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$ (resuspension buffer) per 150 mm dish. The resuspended cells were frozen and stored at −80 °C until purification.

AAV purification and titering

All serotypes of AAV vectors were purified by CsCl ultracentrifugation based on established methods (Xiao et al., 1998) with modifications. Cell pellets were freeze–thawed 3 times, vortexing after each thaw. Benzonase was then added (50 units/ml of cell solution; Sigma-Aldrich) for 1 h at 37 °C with occasional mixing. Cell debris was centrifuged for 20 min at 2450 ×g, 4 °C. Supernatant was adjusted to 30 ml using resuspension buffer followed by 10 ml 2.5 M NaCl and 10 ml 40% PEG 8000 and incubated at 4 °C for 18–20 h. The solution was centrifuged for 20 min at 2450 ×g, 4 °C and the pellet was resuspended in 20 ml of 50 mM HEPES, 150 mM NaCl, 20 mM EDTA, pH 8.0 and 1% Sarcosyl. The solution was transferred to 36 ml ultracentrifuge tube and under laid consecutively with 1.3 g/ml and 1.5 g/ml CsCl in PBS. The CsCl interfaces were marked and mineral oil was used for balancing. The tubes were centrifuged (Discovery 100SE; Sorvall, Newton, CT) in a Surespin 630/36 rotor (Sorvall) at 166,900 ×g 10 °C for 20–24 h. Approximately 8 ml was removed using an 18 g needle inserted 2 mm below the 1.5–1.3 g/ml CsCl interface. The solution was transferred to a 17 ml ultracentrifuge tube, under laid with 2 ml of 1.5 g/ml CsCl, balanced and centrifuged for 24–48 h at 110,000 ×g, 10 °C. Fractions were collected via an 18 g needle inserted below the 1.5 g/ml interface. Using the refractive index, fractions with a Brix % between 21.0 and 28.0% were pooled and dialyzed using a 10,000 MWCO dialysis cassette (Pierce, Rockford, IL, USA) PBS containing 0.5 mM $MgCl_2$ with three exchanges over 25–30 h. The equilibrated virus was aliquoted, frozen, and stored at −80 °C. Viral titers were determined using Real-time quantitative PCR with "TaqMan™" chemistry and analyzed by Opticon2 thermalcycler (Biorad, Hercules, CA, USA). Titers were calculated as viral genomes based on a standard curve of pdsAAVGFP plasmid linearized with MluI. The concentration of the linearized plasmid was determined using a ND-1000 spectrophotometer (Nanodrop, Wilminton, DE, USA) and visually checked on agarose gel containing a Low Mass DNA ladder (Invitrogen). The linearized plasmid was diluted in PBS to obtain concentrations ranging from 1×10^{-1} to 10^{-5} vg/ml equivalents. A viral aliquot was thawed, sonicated for 10 s and serially diluted from 1:1000 to 1:10,000 in PBS. Standards and 10^{-3} and 10^{-4} dilutions (triplicates) were assayed using Taqman Master mix (Applied Biosystems, Foster City, CA, USA) with eGFP primers and probe (Biosearch Technologies, Inc. Novato, CA, USA) under the following reaction conditions: 95 °C, 5 min; 94 °C, 20 s and 60 °C, 1 min, 41 times. Primer and probe sequences: 5′ AGCAAAGACCCCAACG AGAA 3′(fwd), 5′ FAM-CGCGATCACAT GGTCCTGCTGG-BH1 3′ (probe), and 5′GGCGGCGGTCACGAACT'3′(rev).

AAV transduction of primary cortical cultures

Cultures were transduced on DIV6 using each rAAV serotype normalized to 2×10^{12} vg/ml and serially diluted in the PBS containing 0.5 mM MgCl to obtain 2×10^{11}, 2×10^{10}, and 2×10^{9} viral genomes (vg)/ml. Five microliters of media was added to each well of a 96 well plate containing approximately 50,000 cells in 100 μl. Plates were gently mixed and returned to 37 °C incubator for 2 h followed by a replacement of 100 μl of fresh feed media. For cultures harvested at DIV12, cells were fed by 50% media exchange on DIV8 and DIV11 using feed media.

LDH and MTS assays

For LDH assay, media were collected from DIV8 cultures and assayed using the Cytotoxicity Detection Kit (LDH; Roche Applied Sciences, Indianapolis, IN, USA) according to manufacturer's recommendations (Koh and Choi, 1987). For MTS assay, 100 μl of media was removed from each well on DIV8 for LDH then 20 μl of MTS reagent from the CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega, Madison, WI) was added to each well. Plates were returned to the 37 °C incubator for 1 h then the A490 nm was read using a Biotek EL_x 808iu spectrophotometer (BioTek Instruments).

Immunofluorescent labeling and image analysis

At indicated time (DIV8 or DIV12), cells were fixed in 200 µl of 4% paraformaldehyde in PBS for 1 h at room temperature, washed and stored in PBS at 4 °C. For immunofluorescent labeling cells were permeabilized 15 min in room temperature in PBS containing 0.1% Triton X-100 (Sigma) and 0.2% bovine serum albumin (BSA; Sigma). Cells were incubated in PBS containing 0.1% Triton X-100, 2% BSA, and 5% goat serum (Sigma) for 1 h. Primary antibodies to GFAP (mouse anti-GFAP; #MAB360; Millipore, Billerica, MA) or NeuN (mouse anti-NeuN; #MAB377; Millipore) were diluted 1:500, and/or GFP (chicken anti-GFP; AB16901; Millipore) was diluted 1:5000 in PBS containing 0.1% Triton X-100 and 5% goat serum. Cells were incubated in primary antibodies overnight at 4 °C with gentle shaking. After 3 × 5 min washes in PBS, secondary antibodies (Alexa fluor 568 goat anti-mouse and/or Alexa fluor 488 goat anti-chicken; Invitrogen) were diluted 1:500 in PBS containing 0.1% Triton X-100 and 5% goat serum and added to cells for 1 h at room temperature. After 2 × 5 min washes in PBS, 0.001% DAPI (Invitrogen) in PBS was added 10 min at room temperature. Cells were washed 2 × 5 min and stored away from light at 4 °C until imaging.

Immunolabelled cells were imaged with a Nikon Eclipse TE2000-E inverted microscope (Nikon, Melville, NY) using a Spot RT slider camera (Diagnostic Instruments, Sterling Heights, MI) and MetaMorph v6.2 software (Molecular Devices, Sunnyvale, CA). Briefly, the microscope stage was programmed to move to the center of each well for manual focusing. Once focused, the stage moved to 4 locations per well and acquired images using UV filter (DAPI), FITC filter (GFP and Alexa 488) and rhodamine (Alexa 568). Exposure times were kept constant for each filter. Immunoreactive pixel densities (GFAP) or cell counts (NeuN or DAPI) were made using the integrated morphometry feature of Metamorph.

Phylogenetic analysis of AAV capsid-encoding genes

A phylogenetic tree was generated using Discovery Studio Gene v1.5 (Accelyrs, San Diego, CA) by first aligning with ClustalW analysis of the translated capsid region of AAV1 (Accession# AF063497, nts2223-4433), AAV2 (AF043303, nts2203-4410), AAV5 (AF085716, nts2207-4381), AAV6 (AF028704, nts 2208-4418), AAV7 (AF513851, nts 2122-4435), AAV8 (AF513852, nts 2121-4437) and AAV9 (AY530629). Phylogenetic reconstruction of the AAV capsids was generated using neighbor-joining method in best tree mode with distances calculated as uncorrected "*p*" with gaps distributed proportionally. The resulting phylogenetic tree was rooted to AAV2.

Statistics

All experiments were conducted independently 3–6 times using $n=6–18$ wells (MTS and LDH) or 2 times using $n=3–9$ wells (immunolabeling). Statistical analyses were performed with One-way ANOVA and either post-hoc Student–Newman–Keuls test or Dunnett's. Significance was inferred at $p<0.05$. Data are presented as mean +/− S.E.M.

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