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Intravascular Injections of Adenoassociated Viral Vector Serotypes rh10 and PHP.B Transduce Murine Sciatic Nerve Axons

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Abstract

Adenoassociated viral vectors provide a safe and robust method for expression of transgenes in nondividing cells such as neurons. Intravenous injections of these vectors provide a means of transducing motoneurons of peripheral nerves. Previous research has demonstrated that serotypes 1, rh10 and PHP.B can transduce motor neuron cell bodies in the spinal cord, but has not quantified expression in the peripheral nerve axon. Axonal labeling is crucial for optogenetic stimulation and detection of action potentials in peripheral nerve. Therefore, in this study, serotypes 1, PHP.B, and rh10 were tested for their ability to label axons of the murine sciatic and tibial nerve following intravenous injection. Serotype rh10 elicits expression in 10% of acetylcholine transferase positive axons of the sciatic nerve in immuno histochemically-stained sections. Serotype rh10 transduces a variety of axon diameters from <1–12 microns, while PHP.B transduces larger axons of diameter (4–16 microns). Expression was not seen with serotype 1. These results show the potential of serotypes PHP.B and rh10 delivery of transgenic products to axons of the peripheral nerve.

Keywords

Adenoassociated viral vector; motor neuron; peripheral nerve; green fluorescent protein; intravascular injection; mouse

Correspondence should be addressed to H.E.A. (hans.anderson@ucdenver.edu). Work was conducted in Aurora, Colorado, USA. . Author Contributions

H.E.A. conceptualized, wrote manuscript, analyzed, wrote software, and performed the investigation. K.L.S. assisted with the methodology, and provided resources. J.H.C. and K.L.S. supervised this work. J.H.C. and R.F.W. provided project administration. R.F.W. acquired funding for this work. All authors edited the manuscript.

*John H. Caldwell and Richard F. Weir jointly supervised this work

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Introduction

Limb loss affects 1.6 Million Americans, significantly impairing the quality of life for those afflicted. Prosthetic limbs can restore a level of function to these individuals. Current invasive methods for interfacing with the nervous system suffer from limitations in number and specificity of input and output channels, nerve damage from penetrating electrodes and scarring that limits functionality over time.[1,2] The deployment of optogenetic proteins in peripheral nerves could form the basis of an optical neural interface. This less invasive approach could alleviate some of these problems, and provide a robust, long term and specific solution of peripheral neural interrogation and stimulation.[3]

In order to create an optical peripheral nerve interface, optogenetic proteins must be delivered to peripheral nerve axons. Adenoassociated viral vectors (AAVs) provide a safe, effective and relatively easy means of gene delivery to the central nervous system.[4] AAVs been deployed to treat Parkinson's Disease, Alzheimer's Disease, amyotrophic lateral sclerosis, Pompe Disease, spinal muscular atrophy,[5] and diabetic neuropathy,[6] and have been used in the regeneration of damaged nerves.[7] AAVs have also been used to deliver optogenetic actuators[8,9] and reporters.[10,11]

The primary determinants of AAV transduction efficiency are: route of administration, viral serotype (pseudoserotype), transgene promoter, and viral titer. A self-complementary DNA payload increases efficiency 10- to 100-fold,[12] but reduces genetic payload from 4.5kb to 2.2kb.

In targeting motor neurons of the periphery, intranerve,[6,13] intramuscular,[8,14,15] intravenous,[16–19] intrathecal,[6,16,20] intraperitoneal,[17] and intraparenchymal[20] injections have been used. Intravenous (IV) and intramuscular injections are of particular interest for use in the a peripheral neural interface because they are less invasive than spinal injections, and more straightforward than intranerve injections. Following intramuscular injections, expression is limited to axons innervating that muscle,[8] thereby reducing off-target labeling. However, with IV injections, a broader populations of axons can be accessed, including those once innervating now-absent muscles. Following IV injection, AAV1,[18,19] and AAVrh10,[18,21] along with the two engineered capsids, AAV-B1[22] and AAV-PHP.B[23] have transduced motor neurons innervating the periphery.

Promoter selection also improves targeting. The cytomegalovirus (CMV) promoter is a ubiquitous promoter capable of robust expression in many cell types, including motoneurons.[24] Neuronal promoters, such as synapsin and calmodulin dependent protein kinase II (CaMKII), have also been used to transduce motoneurons, with less off-target expression.[25–27]

The final key element for efficient motoneuron expression is the viral titer. The number of viral particles required for successful transduction is heavily dependent upon the serotype, route of administration and the promoter, with higher titers generally helping to overcome poor efficacy, at added expense.[28,29]

Through the careful selection of these parameters, an optimal AAV serotype could be determined to deliver optogenetic reporters to axons of the peripheral nerve, where they may serve in an optical neural interface. Previous literature has established labeling of sciatic nerve axons, with AAV1,[28] and AAVrh10, [6,30] but none of these studies provide quantitative values of extent of labeled motor axons, an important metric for understanding the utility of an optogenetically based read-out interface. Other studies have looked only at the motor neuron soma, but as 80% of protein expression can be limited to the soma, [25] and the axon itself may play a role in translation, [31] it is important to investigate the axons themselves. Given that an optogenetic neural interface would be deployed in an adult, the relevance of neonatal studies (e.g. [18,30]), where differences in the immune system and blood-brain barrier increase transduction is somewhat limited. Thus, to better determine which serotype to pick for an optogenetic peripheral nerve interface, we have investigated IV injections in adult mice of AAV serotypes rh10 and PHP.B using CMV promoter, and AAV1 using either the CMV or CaMKII promoter, for their efficacy in labeling motor neuron axons in sciatic and tibial nerves.

Materials and Methods

Materials

Adenoassociated viral vectors of serotypes 1 (AAV1.CMV.PI.eGFP.WPRE.bGH, AAV1.CamKII0.4.eGFP.WPRE.rBG), and rh10 (AAVrh10.CMV.PI.eGFP.WPRE.bGH) were acquired from the University of Pennsylvania Vector Core (Philadelphia, PA). AAVPHP.B (CMV.EGFP.WPRE.SV40pA) was obtained from Packgene (Worcester, MA). All vectors employed in this study used single-stranded DNA.

Primary antibodies were rat anti-mouse Laminin-1 1:250 (R&D systems; Minneapolis, MN), chicken anti-GFP 1:1000 (Aves Labs; Tigard, OR), rabbit anti-ChAT 1:750 (Fisher Scientific; Lafayette, CO). Secondary antibodies used included: donkey anti-rat 405 1:500 (Jackson ImmunoResearch Laboratories; West Grove, PA), donkey anti-chicken 488 1:500 (Life Technologies; Carlsbad, CA), and donkey anti-rabbit 568 1:500 (Life Technologies; Carlsbad, CA). Determination of the number of animals required for the injection groups was done using the resource equation, as no known study has compared these serotypes quantitatively in peripheral axons.[32,33]

Animal Procedures

All animal procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee with accreditation by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal procedures were carried out under the relevant guidelines.

Eleven-week-old, female C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME) were injected into the tail vein with 2×10^{11} viral genomes (vg) of viral vector in normal saline. An additional set of animals was injected with saline only (sham group). Five mice were used for each serotype-promoter combination.

Tissue Processing and Fixation

Five weeks post injection, animals were sacrificed using isoflurane followed by cervical dislocation. Sciatic and tibial nerves were removed and placed in phosphate buffered saline (PBS). Sciatic nerves were then fixed in 4% paraformaldehyde for 15 minutes at 4°C, rinsed three times for five minutes in PBS, and cryoprotected overnight in 20% sucrose and overnight in 30% sucrose, all at 4°C. Nerves were frozen in Optimal Cutting Temperature compound (OCT, Tissue-Tek) using dry ice and stored at -80°C until cryosectioning.

Immunohistochemistry

Cryo-transverse sections of sciatic nerve (10 µm thickness) were collected on Superfrost Plus microscope slides (Fisher Scientific). Slides containing sections were heated at 37°C for 15 minutes, rinsed with PBS twice for 10 minutes, then blocked with 5% donkey serum in PBS with 0.3% Triton X100 for one hour. Slides were incubated with primary antibodies in this same buffer overnight at 20°C. Slides were rinsed three times for five minutes with PBS; then secondary antibody was added in this same buffer for two hours. Slides were rinsed again three times for five minutes in PBS and coverslipped using Prolong Gold as a mountant.

Imaging

Imaging was performed using an inverted spinning disk microscope (Intelligent Imaging Innovations; Denver, CO) equipped with multiple laser lines including ones at 405, 488 and 561nm and a 16-bit EMCCD camera. Sections were imaged using a 40x air objective (NA 0.75). Unfixed tibial nerves in 1x PBS were placed on #1 coverslips and imaged using a 10x air objective (NA 0.3), a 20x air objective (NA 0.75), or the 40x air objective (NA 0.75).

Image Processing

Montages were assembled using Slidebook 6.0 (Intelligent Imaging Innovations; Denver, CO), then exported to ImageJ (National Institutes of Health; Rockville, MD). Using a custom macro, based on the “Nucleus Counter” plugin, expression in motoneurons was quantified as a fraction: GFP⁺ and ChAT⁺ colabeled axons divided by the total number of ChAT⁺ axons.[34] The false positive rate for detection of axons in sham sections was set to <1%. Axon diameters of unfixed tibial nerve axons were measured in Slidebook 6.0 using the line tool.

Statistical Analysis

MATLAB (Mathworks; Natick, MA) was used for statistical tests. One-way Analysis of Variance tests were used when more than two quantities were being compared, with significance defined as $p < 0.05$, unless an Anderson-Darling test determined that data was non-normal. If one or more of the data sets were non-normal (as determined by an Anderson-Darling test), a Kruskal-Wallis Nonparametric Analysis of Variance was used. When ANOVA tests rejected the null hypothesis, multiple comparisons were performed using the Tukey-Kramer method. For comparisons of two quantities, a Student's t-test was used; if data was non-normal, a Wilcoxon Rank Sum test was used. A difference was considered significant if $p < 0.05$.

Results

Intravascular Injections of AAVrh10 and AAVPHP.B Transduce Peripheral Nerve Axons

Both AAVrh10 and AAVPHP.B, expressing GFP under the control of the CMV promoter, transduced axons of the tibial nerve following IV injection at titers of 2×10^{11} vg (Figure 1). Liver expression was consistently present in all AAVrh10 and AAVPHP.B injected mice (Table 1; Supplementary Figure 1). AAVPHP.B and AAVrh10 (Figure 1) transduced different axonal populations, based on axonal diameter (Wilcoxon Rank-Sum, $p=2.59 \times 10^{-13}$). Immunohistochemical labeled sections showed GFP⁺ axons (Figure 2). Quantitative analysis (ANOVA $p=7.10 \times 10^{-8}$) of the fraction of ChAT⁺ GFP⁺ axons showed a statistically significant difference with sham injected mice only for AAVrh10 (Figure 2; $p=4.47 \times 10^{-6}$).

Neither AAV1-CMV-GFP nor AAV1-CaMKII-GFP Transduce Axons of the Peripheral Nerve Following Intravascular Injection

Neither a CaMKII promoter driving GFP expression in AAV1 nor a CMV promoter driving GFP expression in an AAV1 construct labeled motor axons of the sciatic nerve (Supplementary Figure 2), nor was liver expression seen, with the exception of one mouse (Table 1; Supplementary Figure 1). Expression was also not seen in immunohistochemically stained sections (Figure 1). For AAV1-CMV-GFP (fraction ChAT⁺ GFP⁺ (mean $\pm$ SEM): 0.00482 ± 0.00178), AAV1-CaMKII-GFP (0.00115 ± 0.000699) and sham injected mice (0.00742 ± 0.00264), the ANOVA was not significant ($p=0.0546$).

Discussion

GFP expression in peripheral axons was seen with intravascular injections of AAVrh10 and AAVPHP.B. Quantification of the fraction of GFP⁺ ChAT⁺ axons, demonstrated that AAVrh10 had the best performance, with a statistically higher number of labeled axons than sham injected, AAV1, or AAVPHP.B injected mice. AAVrh10 also showed expression in a variety of smaller axon diameters, indicating its potential utility in labeling axons innervating smaller motor units, and type III and IV sensory fibers. The CMV promoter showed transduction of ChAT⁺ axons with AAVrh10, agreeing with previous reports of CMV promoter expression in motor neurons.[20,24,35,36] Because CMV induces expression in a broad range of tissues, and AAVrh10 has liver tropism, off target liver expression was anticipated.[21] Ultimately, in a peripheral nerve interface, such off-target expression should be reduced as much as possible to prevent unwanted side effects. Our attempts to minimize expression in the liver and other tissues using the CaMKII promoter and AAV1 were not successful. Other potential promoters for restriction of expression to neurons include the Synapsin promoter, which has had moderate success in motor neurons, [26] although expression is not as robust as with CMV.[25]

Unexpectedly, AAV1 did not transduce axons at a detectable level either ex vivo or using immune localization in fixed nerves following IV injection. Furthermore, liver expression was only apparent in one animal. This lack of transduction could be due to the low viral titers used in this study. While AAV1 has previously been shown to transduce liver cells with titers of 8.3×10^9 or 10^{10} vg,[19,37] transduction of spinal cord only occurred at titers of

4×10¹² vg in adult[21] and neonatal mice.[18] It is possible that the lower titer of 2×10¹¹ vg is insufficient for widespread expression or that expression is limited to the soma and is below detectable levels in axons.[25] Nonetheless, AAVrh10 may be the better vector for transduction of peripheral axons following IV injection because of its already substantial transduction profile at 2×10¹¹ vg. Further testing of AAV1 at a higher titer is not likely to prove more efficient, given its limited transduction at 2×10¹¹ vg.

AAVPHP.B was designed with the intent of improving transduction of CNS neurons following IV injection with AAV.[23] It had been demonstrated that AAVPHP.B transduces spinal cord ChAT⁺ motor neurons at 94–96% when injected IV at a titer of 2×10¹² vg with the cytomegalovirus-chicken beta actin hybrid (CAG) promoter.[23] It is possible then, at a higher titer, AAVPHP.B also would also express in the motor axons of the periphery. At a titer of 2×10¹¹ vg, we only saw labeling of large diameter axons ex vivo and did not see labeling of ChAT⁺ axons, suggesting that AAVPHP.B preferentially labels nonmotor axons (e.g. sensory axons) of the sciatic nerve at this titer. A future study of different axonal populations, such as sensory neurons, could better identify which population AAVPHP.B is labeling.

In order to create a functional neural interface, only a fraction of the axons need to be labeled, although at a high enough level that can be detected without antibody amplification. Preferably, these labeled axons would be distributed among those innervating each muscle, and would contain a range of axon diameters so that the graded neural commands to those specific muscles could be detected. In fact, labeling of all fibers in the nerve may result in excessive background noise. For example, AAVrh10's labeling ratio of ~10% is probably sufficient to ensure error on par with the error in integrated force of the first dorsal interosseous.[38] Additional studies with increased titers or enhanced promoters may increase the labeling ratio.

Our study has indicated the capability of AAVrh10 in labeling ChAT⁺ axons of the peripheral nerve following IV administration. AAVPHP.B could also be of use in the labeling of non-ChAT⁺ axons of the peripheral nerve. These serotypes are strong candidates for further study for labeling of peripheral nerve axons, which may further the development of targeted gene therapies and refine the expression of optogenetic proteins for an optical peripheral nerve interface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AAV	Adenoassociated viral vector
CMV	Cytomegalovirus
CaMKII	Camodulin Kinase II
PBS	Phosphate Buffered Saline
ChAT	Choline acetyltransferase
GFP	Green Fluorescent Protein
Vg	Vector Genomes

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Highlights:

- Adenoassociated viral vector serotypes 1, rh10 and PHP.B are intervenously injected into mice
- AAVrh10 labels axons of ChAT⁺ motoneurons with green fluorescent protein in sciatic nerves
- AAVPHP.B labels non-ChAT⁺ axons of the sciatic nerve with GFP
- AAV1 using the CMV or CaMKII does not label axons of the sciatic nerve at a titer of 2×10^{11} vg

Figure 1.

Representative images of GFP labeled axons in murine tibial nerve. (A) Following IV AAVrh10-CMV-GFP injection. (B) Following IV AAVPHPB-CMV-GFP injection. (A, B) Spinning disk (488 excitation). Scale bars represent 25 μm. (C, D) Diameters of tibial nerve axons expressing GFP after IV injection with AAVrh10 (C) or AAVPHPB (D).

Figure 2.

Immunohistochemical staining of transverse sciatic nerve sections. Each section was stained with primary antibodies against Laminin (blue), GFP (green), and ChAT (red). (A) Saline injected. (B) AAV1-CaMKII-GFP. (C) AAV1-CMV-GFP. (D) AAVrh10-CMV-GFP. (E) AAVPHP.B. (F) Fraction of ChAT⁺ axons labeled with GFP following injection of AAV with CMV promoter. *** = P<0.001. Scale bars are

Table 1.

Ex vivo expression in tibial nerve and liver following IV injection of AAV. AAV: Adenoassociated Virus; CMV: Cytomegalovirus; CaMKII: Camodulin Kinase II.

Serotype (n=5 animals/group)	Promoter	Nerve Expression	Liver Expression
AAV1	CMV	0/5	1/5
AAV1	CaMKII	0/5	0/5
AAVPHP.B	CMV	5/5	5/5
AAVrh10	CMV	5/5	5/5