Efficient Targeted Transgene Delivery to Injured Lower Motor Neurons

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Abstract

Peripheral nerve injuries yield devastating consequences, and surgical repair outcomes remain suboptimal. Novel therapeutic strategies such as gene therapy could improve peripheral nerve regeneration. Though adeno-associated virus (AAV) vectors have delivered transgenes to intact peripheral neurons, transduction of transected neurons relevant to management of peripheral nerve injuries has not been reported. Herein, in vivo transduction efficiency of axotomized murine facial neurons using four AAV capsids packaging a fluorescent reporter transgene, tdTomato, is characterized. Proximal stumps of transected facial nerve branches in C57Bl/6J mice were immersed in AAV solutions. Four weeks later, facial motor nuclei were volume-imaged via whole-mount two-photon excitation microscopy, and machine learning-based image segmentation quantified the proportion of transgene expressing neurons. We observed remarkable retrograde transduction efficiency with AAV-PHP.S and AAV-F, with expression levels sufficient to detect intrinsic tdTomato fluorescence. This study confirms successful in vivo retrograde transgene delivery to transected peripheral neurons, an approach that carries potential as a research tool and future therapeutic strategy.

Introduction

Peripheral nerve injuries number 600,000 yearly in the United States and carry devastating consequences [1–3]. As peripheral nerves can regenerate, surgery may be employed to restore critical sensory and motor functions [4, 5]. Suboptimal outcomes following surgical nerve repair are common and associated with increasing patient age, time to repair, and distance over which axons must regenerate [6–14]. Evidence suggests nerve regeneration is hampered by progressive downregulation of pro-regenerative transcription factors within axotomized neurons and Schwann cells following injury [15, 16]. Following nerve transection, neurons upregulate regeneration-associated genes (RAGs) while downregulating production of neurotransmitters [17–23]. Loss of the pro-regenerative phenotype is associated with insufficient axonal regeneration following injury [15, 16]. Gene therapy may prolong the peripheral neuronal RAG program following injury, and carries potential to enhance surgical repair outcomes [24–28]. However, virus vector-mediated transduction of transected peripheral neurons relevant to surgical nerve repair has heretofore not been reported.

Viral-mediated gene delivery to the peripheral nervous system was first reported by Geller and Breakefield, wherein cultured peripheral neurons were transduced using a modified herpes simplex virus 1 (HSV-1) vector to express beta-galactosidase [29]. Retrograde in vivo gene delivery to murine peripheral neurons was later described by Dobson et al. via intramuscular (IM) injection of a recombinant HSV-1 vector [30]. Subsequently, lentivirus, adenovirus, and adeno-associated virus (AAV) vectors have been employed for in vivo transduction of intact peripheral nerves via intravenous, intrathecal, peritoneal, intramuscular, or intraneural injection [31] [32–36]. AAVs are optimal vectors owing to their relatively low immunogenicity, lack of pathogenicity, wide range of infectivity, and capacity to establish long-term transgene expression in non-dividing cells such as neurons [37].
AAV vectors have demonstrated potential for treatment of neurodegenerative diseases [38]. FDA-approval has already been granted for two AAV-based gene therapies, one for treatment of lower motor neuron disease (onasemnogene abeparvovec for spinal muscular atrophy) [39–41]. Animal models suggest gene therapy may improve peripheral nerve regeneration and functional outcomes [28, 42–44]. Clinical translation of gene therapy for nerve repair, though, requires preclinical evidence of targeted transduction of transected axons relevant to peripheral nerve injuries. Prior work has established intraneural or intramuscular injection of specific AAV vector serotypes may yield targeted transduction of intact peripheral neurons via long-distance retrograde transport [45, 46]. Targeted transduction of transected peripheral neurons using AAV vectors has not been previously reported. Nerve transection prevents internalization of AAV vectors at the neuromuscular junction (NMJ), alters axonal transport machinery, and changes nuclear transcription processes [47–52]. Previous research has demonstrated axotomy decreases alphaherpesvirus retrograde trafficking, possibly indicating competition for axonal transport machinery between virions and local damage signals [53].

Herein, a murine model is employed to characterize in vivo transduction efficiency of axotomized murine facial neurons using four AAV capsids. AAV6 and AAV9 were studied as these vectors have demonstrated capacity for peripheral neuron transduction [54–56]. However, of capsids studied, AAV9 capsid variants, AAV-F and AAV-PHP.S, yielded the highest transduction efficiencies, quantified by whole-mount two-photon excitation microscopy (2PEM) and machine-learning based image segmentation.

**Results**

When Fluoro-Gold™ (FG) was delivered via the conduit reservoir technique to the proximal stump of transected buccal facial nerve branches in control C57Bl/6J mice, 786.5 (SD 96.5) neuronal cell bodies were labeled. Titer- (5.9x10^{11} viral genomes VGs/mL) and dose-matched (2.95x10^{9} VGs) solutions of AAV6, AAV9, AAV-F, and AAV-PHPS carrying the pAAV-CAG-tdTomato (tdT) expression cassette were delivered to the proximal stump of axotomized buccal branch neurons (Supplementary Figs. S1 and S2). Neuronal transduction efficiency was evaluated by whole-mount 2PEM imaging of murine brainstem, four weeks after AAV delivery and one week after subsequent retrograde labeling with FG. Machine-learning based image segmentation and automated cell body counts were used to quantify transduction efficiency. AAV-PHPS resulted in the greatest number of neurons expressing tdT in the facial nucleus (Figs. 1 and 2, Supplementary Figs. S3 and S4). AAV-PHPS delivery resulted in 33.6% (SD 5.77%) tdT expression in facial nerve buccal subnuclei, a significantly greater transduction efficiency compared to the other serotypes (Figs. 1d and 2, P < 0.01 for AAV-6 and AAV-9, P < .05 for AAV-F). AAV-F resulted in 18.7% (SD 6.95%) transduction efficiency (Figs. 1c and 2, P < 0.05 AAV6, P = 0.109 AAV9). AAV-6 and AAV-9 achieved transduction efficiencies of 2.8% (SD 2.2%) and 6.6% (SD 3.8%), respectively. When comparing expression of tdT in FG-labeled cell bodies, AAV-PHPS resulted in 79.8% (SD 1.29%) expression, AAV-F produced 56.0% (SD 8.9%) expression, AAV9 led to 15.6% (SD 7.6%) expression, and AAV6 resulted in 6.6% (SD 4.7%) expression (Fig. 3, Supplementary Fig. S4). There was no significant difference in FG-labeling between vector groups, and between vector groups and control buccal subnuclei.
labeled with FG three weeks after nerve transection (Supplementary Fig. S5). Cell bodies transduced by AAV-PHP.S were slightly but significantly larger than cells labeled by FG alone (Supplementary Fig. S6).

To secondarily confirm our data demonstrating successful vector retrograde transport and facial motoneuron transduction, confocal microscopy immunofluorescence (IF) using antibodies against tdT and motor neurons was performed on coronal sections of brainstem containing the facial nucleus. Each brainstem from facial nerves exposed to vector had neuronal cell bodies immunoreactive for anti-red fluorescent protein (RFP) (Supplementary Fig. S7). Double-labeled sections expressed tdT in choline acetyltransferase (ChAT) immunoreactive motoneurons (Fig. 4), with robust expression only in the ipsilateral facial nucleus.

**Discussion**

Though targeted delivery of AAVs to adult rodent intact peripheral motoneurons has been described, transduction efficiencies are only 1–7% [34, 35, 55, 57]. Clinically-relevant local delivery of AAV vectors to transected peripheral nerves has not been reported. Herein, we demonstrate successful *in vivo* retrograde transduction of transected murine facial nerves using AAV vectors. Exposure of nerve to a relatively low dose of AAV, 2.95x10^9 VGs (5.9x10^{11} VGs/mL), of AAV-F and AAV-PHP.S transduced 18.7% (SD 6.95%) and 33.6% (SD 5.8%) of buccal facial motoneurons, respectively. This surprising, high efficiency tdTomato expression was limited to buccal branch facial motor subnuclei, indicating trans-synaptic spread of AAV is unlikely (Supplementary Fig. S8). Transduction efficiencies of the two naturally-occurring serotypes tested, AAV6 and AAV9, were far lower than the two engineered capsid variants.

Hollis et al. evaluated retrograde transduction efficiencies of AAV1-6 delivering a self-complementary (sc) genome in rats. Extensor carpi intramuscular injections of 1.05x10^9 VGs of scAAV1 transduced 4.1% of cervical motoneurons, and intrasciatic injections of 2.0x10^8 VGs of scAAV1 transduced 7.5% of lumbar motoneurons [35]. Another group transduced 1.1% of lumbar motoneurons after intramuscular injection of 1.0x10^{10} VGs of single stranded (ss) AAV2 into murine quadriceps muscles [34]. Towne et al. delivered 2.6E5 transducing units (TUs) of AAV6 to mice via intrasciatic injection and transduced 28.1% of dorsal root ganglia sensory neurons; negligible motor neuron transduction was achieved [55]. This present report confirms poor tropism of AAV6 for murine motor neurons [55]. Findings herein correspond to prior work demonstrating enhanced CNS and PNS transduction following systemic delivery of AAV-F and AAV-PHP.S vectors, respectively [32, 33].

Prior research examined transduction efficiencies of intact facial motoneurons via peripheral injection of various viral vectors. Stern et al. injected adenovirus (10^9 GCs) vectors into intact murine facial nerves near the stylomastoid foramen, followed by transection and repeat injection of viral solution to the proximal facial nerve stump; they reported transduction efficiency of 40–70% of facial motoneurons [42]. However, they injected intact nerves in close proximity to the facial nucleus, minimizing distance of retrograde transport [58–60]. Stern et al. also determined total facial motoneurons using retrograde labeling three weeks after nerve transection; this technique may underestimate total motoneurons
exposed to virus as proximal stump axons degenerate after injury, decreasing axon count exposed to retrograde tracer [61–63]. In the present study, FG labeled 310 (SD 70) cell bodies when delivered three weeks after buccal branch transection, while 786.5 (SD 97) cell bodies were labeled when buccal motoneurons were exposed to FG immediately after transection. Control mice in which FG was delivered three weeks following nerve transection without virus delivery had 249 (SD 132) cell bodies labeled (Supplementary Fig S5). In contrast to prior work, the present study delivered AAV vectors to transected buccal branch motoneurons more than 2 cm from the facial nucleus, ensuring all transgene expression was due to long-distance retrograde vector transport [64].

Previously, AAV vectors have been employed for transgene delivery to intact facial motoneurons. Schuster et al. injected 3.3x10^{11} VGs of AAV9 packaged with GFP intrathecally in mice and characterized “consistent abundant” GFP-immunoreactive neurons throughout dorsal root ganglia and the CNS, including the facial motor nucleus [36]. Tail vein injection of 2.0x10^{11} VGs of AAV6 in mice transduced 3–5% of spinal, hypoglossal, and facial motoneurons [65]. When 3x10^{6} TUs of AAV2 were stereotactically injected into rat brainstems, transgene was delivered to facial motoneurons, although transduction efficiency was not quantified [66].

While promising, systemic and intrathecal vector administration do not allow for restricted gene delivery to the facial nucleus, and requires higher doses risking toxicity [67–69]. Though intramuscular injection allows for targeted gene delivery, this strategy is not translatable to peripheral nerve transection injuries where the NMJ is not intact. In contrast, the present study reports high retrograde transduction efficiency when transected proximal nerve stumps were exposed to AAV-PHP.S and AAV-F, providing critical proof-of-concept that targeted gene delivery to transected peripheral nerves is possible.

We employed a novel high-throughput murine model to quantify transduction efficiency. Prior research relies on delivering vector to intact nerve or NMJ, performing a subsequent secondary labeling study with a retrograde tracer, and then sectioning tissue for immunohistochemistry (IHC) or IF [35, 42]. Alternatively, groups have co-stained for a viral transgene and neuronal marker such as ChAT to quantify transduction using IHC [34, 55]. Herein, we delivered pAAV-CAG-tdTomato and, later, FG for co-labeling of facial motoneurons. These fluorophores are readily separable by 2PEM excitation wavelength; tdTomato has 97% maximal excitation at 1040 nm and minimal emission at 830 nm excitation, whereas FG has 20% maximal tested excitation at 830 nm with minimal excitation at 1040 nm (Fig. 3) [70, 71] [72]. Performing 2PEM using a dual-output laser at 830 nm and 1045 nm minimized spectral overlap between tdTomato and FG. Further, 1045 nm excitation of tdTomato minimized lipofuscin excitation (peak 2PEM excitation 770 nm, peak emission 650 nm), a primary cause of CNS autofluorescence [73, 74]. Simple immersion of brainstems in refractive-index matching solution permitted deep imaging of specimens to depths over 350 nm, avoiding the need for tissue sectioning while enabling volumetric analysis of facial motor nuclei.

Machine-learning based image segmentation was employed to quantify the proportion of transgene-expressing neurons. The software employed permits automated cell body counts, as well as volume and surface area measurements of segmented cell bodies (Supplementary Fig. S6). Neuronal cell bodies
transduced by AAV-F and AAV-PHP.S had greater surface areas and volumes compared to those transduced by AAV-6 and AAV-9. The reason for this difference is currently unclear. Neurons exposed to AAV-F and AAV-PHP.S had preserved cell body architecture and dendrites, while soma of neurons transduced by AAV6 and AAV9 were frequently rounded and vacuolated with loss of dendrite processes (Supplementary Fig. S9). These morphological changes have been associated with neuronal toxicity and degeneration [75–79]. Though neurons exposed to FG have decreased long-term survival, this study’s interval of 6 days between FG delivery and tissue harvest prevented neuronal loss secondary to FG toxicity [80].

While the mechanism by which AAV-PHP.S and AAV-F mediate efficient retrograde transduction of transected facial motoneurons remains to be determined, it is evident from our study they have higher efficiency than the parental capsid from which they were derived, AAV9. Both AAV-PHP.S and AAV-F have a unique 7-mer sequence, QAVRTSL and FVVGQSY, respectively, inserted after amino acid 588 in AAV9 VP1 which leads to surface display of ~ 50 copies of the peptide on the VRVIII loop at the three-fold axis of symmetry [32, 33]. Interestingly, both capsids were identified after an in vivo selection with AAV9 peptide display libraries after systemic injection in mice. While the mechanism for AAV-mediated transduction is a myriad of steps, the observation that both capsids facilitate higher transduction of transected motor neurons compared to AAV9 gives a place to start investigations. One can compare entry and trafficking along axons of fluorescently-labeled capsids (engineered capsids vs AAV9), to gain insight into the improved efficiency.

In the current study, axotomized facial motoneurons were exposed to virus immediately after nerve transection. Future studies will evaluate delayed AAV vector delivery to transected motoneurons, as delays of only a few days between nerve crush injury and exposure to adenovirus vector yields decreased transduction efficiency [81]. Lastly, while we demonstrate efficient and robust transduction of facial motor neurons using this approach, it will be important to test therapeutic transgene candidates such as glial derived neurotrophic factor in order to advance this technology towards clinical application [28, 43].

In conclusion, this study reports efficient in vivo retrograde transgene delivery to transected lower motor neurons using AAV-F and AAV-PHP.S capsids. This model can be used to test therapeutic strategies to improve peripheral nerve regeneration, as well as a tool to specifically probe the biology of facial motor neuron responses to axonal injury.

**Materials And Methods**

**AAV vector production, purification, and titration.** For all transgene expression studies with AAV vectors we used the AAV expression plasmid, pAAV-CAG-tdTomato (codon diversified), which was a gift from Edward Boyden (Addgene plasmid # 59462; http://n2t.net/addgene:59462). This plasmid contains AAV inverted terminal repeats (ITRs) flanking the tdTomato expression cassette which consists of: a chicken β-actin (CAG) promoter, Kozak sequence, tdTomato cDNA, woodchuck hepatitis virus post transcriptional regulatory element (WPRE), and an SV40 poly A signal sequence. We packaged this AAV transgene
expression cassette into the following capsids: (1) AAV-F (AAV-F in pAR-9 was a gift from Casey Maguire, Addgene plasmid # 166921; http://n2t.net/addgene:166921); (2) AAV9 (pAR9 plasmid obtained from the Massachusetts General Hospital virus vector core); and (3) AAV6 (plasmid pAAV-RC6, Cell Biolabs, Inc, San Diego, CA) [32]. AAV production of AAV-F, AAV9, and AAV6 was performed as previously described [82]. Briefly, 293T cells were triple transfected (calcium phosphate method) with (1) AAV rep/cap plasmid (2) an adenovirus helper plasmid, pAdΔF6, and (3) pAAV-CAG-tdTomato. Cell lysates were harvested 68–72 hours post transfection and purified by ultracentrifugation of an iodixanol density gradient. Iodixanol was removed and buffer exchanged to phosphate buffered saline (PBS) using Zeba desalting columns, 7 kDa molecular weight cutoff (MWCO; Thermo). Vector was concentrated from 4 ml to approximately 1 ml using 2 ml Amicon Ultra 100 kDa MWCO ultrafiltration devices. We obtained PHPS vector packaging the same AAV-CAG-tdTomato cassette from Addgene (Catalog # 59462-PHPS, Viviana Gradinaru laboratory) [33]. Addgene also uses iodixanol gradient purification. Vector titers in VG/ml were determined by Taqman qPCR in an ABI Fast 7500 Real-time PCR system (Applied Biosystems) using probes and primers to the AAV ITRs and an AAV plasmid standard curve. We titered all four vectors (AAV-F, PHPS, AAV9, and AAV6) on the same qPCR run to ensure accuracy of the head-to-head comparison experiments. Vectors were pipetted into single-use aliquots and stored at -80°C until use.

Conduit reservoir delivery of AAV-CAG-tdTomato and Fluoro-Gold to Transected Facial Nerves. Twenty-four adult C57 mice (7–9 weeks, 12 female) were used for vector and Fluoro-Gold™ (FG) (Fluorochrome LLC, Denver, Colorado) delivery. All animal surgeries were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with approval by the Massachusetts Eye and Ear Animal Care Committee (ACC Protocol # 16-006, IRBNet ID 884247). This study was carried out in compliance with the ARRIVE guidelines.

Procedures were performed under isoflurane anesthesia, 1–3% maintenance dosing in 1 L/min O₂. Buprenorphine (0.05 mg/kg subcutaneously) and meloxicam (1.0 mg/kg subcutaneously) were administered prior to skin incision. A 1 cm infra-auricular incision was made on the left, and skin-muscle flaps were elevated. The exorbital lacrimal gland was retracted to expose the buccal branch of the facial nerve. This branch was meticulously dissected circumferentially using the operating microscope at 25x magnification. Care was taken to avoid crush injury. The nerve was transected just proximal to the distal pes and the proximal stump then immersed in a pipette tip containing 5 µL of titer-matched vector solution (n = 4 mice/vector group, 2 males and 2 females per vector group) (5.9x10^{11} VG/mL) for 10 minutes (Supplementary Figs. S1 and S2a). In four control mice, the proximal nerve stump was dipped in 5 uL of 2% FG (Fluorochrome, Denver, CO, w/v in distilled water) for 10 minutes. In a second control group of three mice, the nerve was transected but no virus or dye was delivered. In one experiment, high-dose AAV-PHP.S (1.72x10^{13} GC/mL) carrying the pAAV-CAG-tdTomato transgene expression cassette was delivered to one mouse nerve. After nerve transection, the proximal stump was placed overlying the masseteric fascia, separated from its distal stump. The wound bed was irrigated with saline and closed in a single layer using 4 – 0 absorbable suture (Polysyn, Sharpoint, Westwood, MA). Animals recovered
from general anesthesia and returned to their cages. Postoperative Meloxicam was given for 72 hours post-procedure.

Three weeks after AAV delivery, the 16 vector-treated mice (n = 4 mice/vector x 4 vectors) and three mice who had undergone prior nerve transection without delivery of virus, underwent isoflurane anesthesia at the above dosing with the same analgesics. A 1 cm infra-auricular incision was made on the left and the previous buccal branch transection was identified. The nerve was transected just proximal to the neuroma and the proximal stump immersed in 5 µL of 2% FG (Supplementary Fig. S9b). The high-dose AAV-PHP.S mouse underwent facial nerve main trunk transection with proximal stump immersion in 5 µL of 2% FG. The wound bed was irrigated with saline and closed in a single layer using 4–0 absorbable suture (Polysyn). Animals were recovered from general anesthesia. Postoperative Meloxicam was given for 72 hours post-procedure.

**Tissue harvest.** Six days following FG delivery, animals underwent CO$_2$ euthanasia and cardiac perfusion using 2% phosphate-buffered paraformaldehyde fixative (PFA) solution. Animal heads were placed in 2% PFA overnight, then underwent brainstem harvest at the level of the facial nucleus. The intracranial facial nerve was used as a landmark for facial motor nucleus identification. Brainstems were placed in PBS in a light-tight container and stored at 4°C for seven days prior to whole mount imaging.

**Immunofluorescence staining of brain stems.**

One animal from each treatment group underwent CO$_2$ euthanasia followed by cardiac perfusion using 4% PFA solution. Animal heads were placed in 4% PFA for 48 hours prior to brainstem harvest and overnight cryoprotection in 30% sucrose solution. Brainstems were then embedded in O.C.T. media (Tissue-Tek) and cryosectioned in the coronal plane at 40 µm. Floating sections were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes at room temperature and blocked with 5% normal chicken serum (NCS, Abcam) in PBS for 1 hour at room temperature. Primary antibodies were incubated overnight (1:200 dilution factor) at 4°C in 1.5% NCS PBS. Primary antibodies used for this study were: rabbit anti-red fluorescent protein (RFP, Code: 600-401-379, Rockland Antibodies,); goat anti-ChAT (cat#AB144P EMD Millipore, Burlington, USA).

After washing sections, fluorophore-conjugated secondary antibodies were incubated in 1.5% NCS in PBS at 1:1000 dilution. The secondary antibody for anti-RFP primary was chicken anti-rabbit Alexa Fluor 594 (Thermo, Cat A-21442) and the secondary antibody for anti-ChAT primary was chicken anti-goat Alexa Fluor 488 (Thermo, Cat A-21467). After washing in PBS, nuclei were labeled with a 1:10,000 dilution of 4’,6-diamidino-2-phenylindole (DAPI). Sections were mounted onto slides with a fine brush, dried for 2 hours, and cover slipped with Dako fluorescent mounting medium (Agilent).

**Imaging of whole mount brainstems and immunofluorescent tissue sections.**

For IF imaging, sections were mounted with immersion oil (Code 1261, Cargille Laboratories, Cedar Grove, NJ) and imaged at multiple focal planes using a 40x objective (0.24mm WD, HC PL APO 40x/1.3 Oil CS2,
Leica) on a Leica DM 6000 CS confocal microscope. For Alexa Fluor 488 visualization, an Argon excitation laser was used and emitted fluorescence between 500–550 nm was collected. For Alexa Fluor 594 visualization, a Diode-pumped solid state (DPSS) 561 laser was used for excitation and emitted fluorescence between 600–650 nm was captured.

2PEM imaging was performed on a commercial multiphoton microscope (TrimScope II, LaVision Biotech) powered by a dual-output femtosecond laser (Insight X3, SpectraPhysics) at 830 nm and 1045 nm. Images were acquired with a set of galvanometer mirrors and piezo XYZ-stage for large-field volumetric imaging. Commercial image-analysis software (Bitplane Imaris 9.2; Oxford Instruments, Zurich, Switzerland) was used for stitching of tile scan 2PEM images. Optical clearing was achieved using a glycerol-immersion objective lens (CLr Plan-Neofluar 20x, Carl Zeiss) and refractive index matching solution (EasyIndex, LifeCanvas Technologies, Cambridge, MA). Fluorescent signal was spectrally filtered, a combination of short-pass and long-pass filters were used for collecting below 495 nm in the FG channel and above 560 nm in the tdTomato channel.

Facial motor neuron cell body counts were quantified from original 3D data sets using commercial machine learning software (Aivia v9.5, Leica Microsystems, Bellevue, WA). A random forest pixel classifier was trained by painting examples of cell body signal and background signal [83]. This classifier was used to generate a signal channel in Aivia’s 3D object mesh recipe console to highlight and segment neuronal cell bodies. The object meshes were generated in a region of interest and adjusted in an iterative manner, during which morphological smoothing was performed, minimum object radius and edge intensity defined, and holes filled until a satisfactory result was obtained. The segmentation parameters were then applied to the entire volume to generate a channel highlighting the pixels comprising cell bodies and automated cell counts were performed. This process was performed independently for FG-labeled and tdT-expressing cell bodies. The segmentation parameters were kept consistent for all reconstructions and all analyses were blinded to vector.

**Statistical analyses**

All statistical analyses were performed using SPSS (IBM SPSS Statistics 27). Levene’s test was used to verify homogeneity of variances. Normality of data was confirmed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. One-way AVOVA followed by Bonferroni post hoc analysis was performed to compare cell bodies expressing tdT, as well as FG-labeling of neuronal soma. One-way ANOVA with Games-Howell post hoc analysis was used to compare surface area and volume of segmented cell bodies. Confidence level was assessed at 95% (P < 0.05).

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**Declarations**

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**Author contributions**

M.Q.M performed the surgical experiments, assisted with imaging, assisted with data analysis, conceived the study, and wrote the manuscript. I.C.H. performed the imaging and critically reviewed the manuscript. S.M assisted with surgical experiments, assisted with data analysis, and critically reviewed the manuscript. J.N. produced, purified, and titered the AAV-F, AAV9, and AAV6 vectors used in this study. C.N. assisted with immunofluorescence. C.A.M. prepared the vectors, conceived the study, performed immunofluorescence, and critically reviewed the manuscript. N.J. conceived the study and critically reviewed the manuscript.
Competing interests:

CAM has a financial interest in Sphere Gene Therapeutics, Inc., Chameleon Biosciences, Inc., and Skylark Bio, Inc., companies developing gene therapy platforms. CAM has a filed patent application surrounding the AAV-F capsid and agreements between MGH and biotechnology companies has resulted in monetary payments. CAM’s interests were reviewed and are managed by MGH and Mass General Brigham in accordance with their conflict of interest policies. NJ declares holding shares of Moderna stock. MQM, ICH, SM, JN, and CN declare no competing interests.

Figures

Figure 1

AAV vectors mediate retrograde transduction of facial buccal subnuclei after dipping transected facial nerve into vector in solution. Panels a-d demonstrate buccal facial motor subnuclei segmentation
obtained from deep two-photon excitation microscopy imaging. Representative images of transduced facial motoneurons from mice with transected facial nerve exposed to AAV6 (panel a), AAV9 (panel b), AAVF (panel c), and AAV-PHP.S (panel d), all encoding a tdTomato expression cassette. Automated cell body counts demonstrated 27 (AAV6), 38 (AAV9), 170 (AAVF), and 289 (PHP.S) transduced (tdTomato+) cell bodies. Each color represents a segmented cell body. Segmentation performed using commercial software (Aivia v9.8, Leica Microsystems, Bellevue, WA). Scale bar 50 μm.

Figure 2

Facial motoneuron retrograde transduction with different AAV capsids after dipping transected facial nerve in vector solution. Boxplot demonstrating facial motoneuron transduction by different AAV capsids delivering a tdTomato expression cassette, calculated as percentage of total facial buccal subnuclei (determined by Fluoro-GoldTM labeling in control mice). AAV-PHP.S transduced significantly more cell bodies than the other vectors. * indicates P<.01 relative to AAV6 and AAV9. ** indicates P<.05 relative to AAV-F. # indicates P<.05 relative to AAV6. All P values calculated using ANOVA with Bonferroni correction.
Figure 3

Efficient section-free quantification of facial motoneuron transduction in buccal subnucleus after dipping AAV vector in transected facial nerve. Panels a-e demonstrate quantification of facial motoneuron cell body transduction with AAV-PHP.S-CAG-tdTomato after transected facial nerve dipping. Panels a-c are three-dimensional reconstructions of two-photon x-y-z tile scans. Fluoro-Gold™ and tdTomato were separated based on excitation wavelength. Panels d and e are corresponding neuron cell body
segmentations, each color represents a segmented cell body. Automated counts recorded 289 cell bodies co-labeled with tdTomato and Fluoro-Gold and 368 cell bodies labeled with Fluoro-Gold. Segmentation performed using using commercial software (Aivia v9.8, Leica Microsystems, Bellevue, WA). Scale bar 50 μm.

Figure 4

Immunofluorescence staining of mouse brainstem transduced with AAV-F-CAG-tdTomato delivered by transected facial nerve dipping. Panels a-d show 40x confocal microscopy images of anti-ChAT (panels a and c) and anti-RFP (panels b and d) staining of 40 μm thick mouse brainstem sections through the facial nucleus. Transected facial nerve was dipped into a solution of AAV-F carrying the expression cassette, AAV-CAG-tdTomato. Panels a and b show treatment facial nucleus with c and d demonstrating the contralateral control side. Arrowhead points to transduced cell body. Arrow points to cell body with anti-ChAT labeling but not anti-RFP positive. Scale bar 40 μm.

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