Published in final edited form as:

Mol Cell Neurosci. 2014 May ; 60: 72–80. doi:10.1016/j.mcn.2014.04.004.

Intraneural convection enhanced delivery of AAVrh20 for targeting primary sensory neurons

Josef Pleticha, Christian Jeng-Singh, Rahaf Rezek, Manal Zaibak, and Andreas S. Beutler*

Departments of Anesthesiology and Oncology, Mayo Clinic, Rochester, MN, USA

Abstract

Gene therapy using adeno-associated virus (AAV) is an attractive strategy to treat disorders of the peripheral nervous system (PNS), such as chronic pain or peripheral neuropathies. Although intrathecal (IT) administration of AAV has been the standard in the field for targeting the PNS, it lacks anatomical specificity and results in wide rostro-caudal distribution of the vector. An alternative approach is to deliver AAV directly to the peripheral nerve axon. The present study employed convection-enhanced delivery (CED) of a novel AAV serotype, AAVrh20, expressing enhanced green fluorescent protein (EGFP) into rat sciatic nerve investigating its efficacy, anatomical selectivity, and safety, compared to the IT route. Intraneural CED resulted in transduction confined to the ipsilateral L4 and L5 DRG while IT administration led to promiscuous DRG transduction encompassing the entire lumbar region bilaterally. The transduction rate for intraneural AAV administration was similar to IT delivery (24% for L4 and 31.5% for L5 DRG versus 50% for L4 and 19.5% for L5 DRG). Use of hyperosmotic diluent did not further improve the transduction efficiency. AAVrh20 was superior to reference serotypes previously described to be most active for each route. Intraneural CED of AAV was associated with transient allodynia that resolved spontaneously. These findings establish intraneural CED as an alternative to IT administration for AAV mediated gene transfer to the PNS and, based on a reference rodent model, suggest AAVrh20 as a superior serotype for targeting the PNS.

Keywords

Adeno-associated virus rh20; peripheral nervous system; convection-enhanced delivery; intraneural; intrathecal; dorsal root ganglion

Introduction

Gene transfer to the peripheral nervous system (PNS) is an appealing approach to treat disorders such as chronic pain or peripheral neuropathies (Federici and Boulis 2007; Beutler

© 2014 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: Mayo Clinic, Rochester, 200 First Street SW, Rochester, MN, 55905, USA, Tel.: +1-507-774-1873, beutler.andreas@mayo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2010; Mason et al. 2011; Wolfe et al. 2012). Adeno-associated virus (AAV) represents an attractive vector system thanks to its widespread use in other gene therapy settings, favorable safety profile, and clear translational potential (Ashtari et al. 2011; Nathwani et al. 2011; Mittermeyer et al. 2012). We have previously shown that intrathecal (IT) administration of self-complementary AAV8 results in a robust and anatomically widespread transduction of sensory neurons within the dorsal root ganglia (DRG)—a finding subsequently confirmed by other groups (Towne et al. 2009; Vulchanova et al. 2010; Snyder et al. 2011; Jacques et al. 2012)—and that expression of analgesic genes through this paradigm has a therapeutic effect in a rat model of chronic pain (Storek et al. 2008).

AAVrh20 is a simian AAV serotype recently isolated by PCR-based screening of host genomes using primers specific to conserved regions of the cap gene (Gao et al. 2004; Gao et al. 2005; Asokan et al. 2012). AAV serotypes often differ in their ability to transduce various target cells of the central nervous system (CNS), whereby some of the newer types were found to be superior to the previously known serotypes (Lawlor et al. 2009; Sondhi et al. 2012). When AAVrh20 was injected into the rat striatum by Lower et al. (2009), it transduced the highest number of brain cells out of a panel of AAV serotypes that included AAV8. The superior performance of AAVrh20 in the brain suggested that it might also be advantageous for other nervous system applications including the PNS, where AAVrh20 has not been tested.

While IT administration has been the most widely used approach for targeting the PNS with AAV, it may be associated with limitations for certain types of therapeutic genes, particularly when anatomically confined expression in DRG neurons is desired. Our own studies and those of several other laboratories found that IT administration of AAV 1, 5, 6, or 8 led to selective targeting of DRG neurons, i.e., cell type specific; these studies were restricted to rodents (Storek et al. 2006; Storek et al. 2008; Towne et al. 2009; Vulchanova et al. 2010; Jacques et al. 2012). However, others found transduction of additional targets such as anterior horn motor neurons while focusing their studies on other serotypes or testing in large animals models (Snyder et al. 2011; Federici et al. 2011; Samaranch et al. 2013). In those studies, promiscuous transduction was especially evident when the most potent vectors such as AAV9 were tested. To achieve an anatomically restricted gene expression in the serotypes using vectors with a ubiquitous tropism, an alternative and more selective route of delivery could be considered. For applications in the PNS, a direct injection of AAV into a peripheral nerve has been studied (Xu et al. 2003; Hollis et al. 2008; Towne et al. 2009; Homs et al. 2011). The intraneural injection directly targets the axons of primary sensory or motor neurons, and the vector reaches the cell soma by means of retrograde axonal transport (Kaspar et al. 2003; Hadaczek et al. 2004; Cearley et al. 2008). Efficacy of intraneural AAV administration has also been shown to be serotype-dependent, with AAV1 demonstrating high transduction rates within both motor and sensory systems (Hollis et al. 2008; Homs et al. 2011).

Convection-enhanced delivery (CED) has been utilized in the central and peripheral nervous system to optimize vector distribution within the target tissue (Lonser et al. 1998; Ratliff and Oldfield 2001; Chen et al. 2011). CED uses bulk flow as opposed to simple diffusion to deliver macromolecules into a solid tissue. Needles of various designs have been used for

CED of various therapeutics, including AAV, by Bankiewicz et al. (Hadaczek et al. 2006; Yin et al. 2010; Rosenbluth et al. 2011) as well as other groups (Bobo et al. 1994; White et al. 2011). The stepped needle design consisting of a sharp transition from the wider needle shaft to a narrow tip was found to optimize the volume of distribution of the injectate, to allow higher flow rates, and to prevent reflux of the injectate along the needle path.

Use of hyperosmotic diluent is another potential approach to increase the efficacy of intraneural gene transfer. Osmotic stress induced directly by a hyperosmotic injectate has been demonstrated to open the perineural membranes and to increase the axonal uptake of agents in various pharmacological applications (Antonijevic et al. 1995; Wolf et al. 2011; Kreimer et al. 2012). In the context of AAV, systemic pre-treatment with mannitol has been proposed to enhance IT delivery of AAV to the PNS neurons (Vulchanova et al. 2010) and facilitate transduction of brain parenchyma following intracerebroventricular administration (Mastakov et al. 2002; Fu et al. 2003). However, direct effect of hyperosmotic diluent on transduction efficacy of AAV has not been evaluated.

In the present study, we tested the ability of AAVrh20 to target the primary sensory neurons following IT delivery in rats and established gene transfer characteristics for this route. Next, we tested and optimized the intraneural route delivering the same vector by CED to the sciatic nerve.

Materials and methods

Stepped needle and injection apparatus

The stepped needle was made by welding a 52 mm, 33G tip (OD: 0.008"; ID: 0.0035; wall thickness: 0.004") inside a 40 mm, 26G shaft (OD: 0.01825"; ID: 0.01224"; wall thickness: 0.03"). The tip projected from the shaft 2 mm distally, forming the step needed for effective CED, and 10 mm proximally. The proximal end of the needle was inserted into PE08 polyethylene tubing (BB31695-PE/08, Scientific Commodities, Lake Havasu City, AZ, USA), which was in fitted over a 33G Hamilton needle (7762-06, Hamilton Company, Reno, NV, USA). The needle was connected to 100 ul Hamilton syringe (7637-01, Hamilton Company, Reno, NV, USA). A syringe pump (Fusion 100 Touch Syringe pump, Chemyx, Stafford, TX, USA) was used to deliver the injectate at a defined flow rate and volume.

Agarose gel injection

The custom-made stepped needle was compared to non-stepped needles of two gauges, 26G and 33G. Ultrapure agarose (16500-100, Life Technologies, Carlsbad, CA, USA) solution (0.5% w/v in phosphate buffered saline, PBS) was used. Upon cooling down to room temperature, a tested needle was inserted 20 mm into the agarose gel and 10 µl of 0.4% Evans Blue dye (Sigma-Aldrich, St. Louis, MO, USA) was injected at flow rates between 0.5 and 8 µl/min. The needle was left in place for additional 3 min after the injection had been completed. The needle was attached to a stereotactic frame throughout the injection to prevent irregularities in its position.

Vector preparation

Self-complementary variants of AAV expressing the EGFP reporter gene under control of cytomegalovirus (CMV) promoter/enhancer and rabbit β -globulin (rBG) polyA sequence were used and the vector was packaged in AAV1, AAV8, or AAVrh20 capsid. The vector was produced at the Penn Vector Core (University of Pennsylvania, Philadelphia, PA, USA) by triple transfection of HEK293 cells and iodixanol gradient centrifugation of concentrated culture supernatant as described previously (Lock et al. 2010).

Animals

Male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 250–300 g were used throughout this study. All procedures were approved by Institutional Animal Care and Use Committee.

Peripheral nerve injection

Rats were put under general anesthesia with 2 % isoflurane (Terrell; Piramal Healthcare, Bethlehem, PA, USA). Animals were placed in prone position and the limbs were fixed on the operation table. A 1.5 cm long vertical incision was applied along the rat's right femur. Under a surgical microscope (Universal S3, Carl Zeiss, Jena, Germany) the hind limb muscles were retracted and the sciatic nerve exposed. The injection needle was advanced into the sciatic nerve just proximal of the trifurcation. 15 μ l of injectate was administered at a controlled flow rate of 2 μ l/min using the same infusion pump as for the agarose gel injection. The injectate contained 1.2×10^{10} genome copies (GC) of either self-complementary AAV1 or self-complementary AAVrh20, and each serotype was suspended in either PBS or 0.5 M sorbitol solution in PBS. A total of $n=30$ rats was used; $n=6$ receiving AAV1 in PBS; $n=6$ receiving AAV1 in sorbitol in PBS; $n=6$ receiving AAVrh20 in PBS; $n=6$ receiving AAVrh20 in sorbitol in PBS; $n=3$ receiving PBS only; and $n=3$ receiving sorbitol in PBS only. The latter two groups served as negative controls for gene transfer studies. After the injection was completed, the needle was left in place for additional 3 min.

Spinal nerve ligation

The procedure was performed exactly as described by Chung, Kim, & Chung (2004). Briefly, under anesthesia, the spinal nerve injury was produced by ligating and transecting the right L5 spinal nerve distal to the L5 DRG. After incision of the skin, the right paraspinal muscles were separated from the spinous processes at the L5–S1 levels. The right L6 transverse process was resected with a small rongeur to visualize the L4 and L5 spinal nerves. The L5 spinal nerve was isolated, tightly ligated, and transected. In sham-operated control animals, the right L5 spinal nerve was exposed as described, but not ligated or transected. The paraspinal muscles were approximated with surgical sutures, and the skin was closed with 9-mm wound clips. $N=3$ rats per either the SNL or sham group were used.

IT catheterization in rats

The IT catheterization was performed as described originally by Yaksh and Rudy (1976) and used previously by us (Storek et al. 2008). Briefly, under anesthesia, the atlanto-occipital membrane was exposed, and a 2-mm-long incision was made, cutting the dura and

arachnoid membrane. A 9-cm-long catheter made of PE08 polyethylene tubing was inserted through the cisterna magna into the IT space and advanced to the caudal end of the spinal cord. 3.9×10^{10} GC of either self-complementary AAVrh20 or self-complementary AAV8 suspended in 30 μ l of PBS were injected over 3 mins followed by 10 μ l of PBS flush and the catheter withdrawn 5 minutes after the injection was completed. The neck muscles were then approximated with surgical sutures, and the skin was closed with 9-mm wound clips. N=3 animals per serotype were used, with additional n=3 receiving an equal volume of PBS and serving as negative controls for gene transfer studies.

Von Frey testing

Mechanical allodynia was quantified with the von Frey test (Chaplan et al. 1994). Briefly, rats were placed on a wire mesh, allowing access to the paws. Spontaneous rat behavior was observed, with special attention to standing postures, walking, and resting position. Graded von Frey filaments (Stoelting, Wood Dale, IL, USA) were applied at the midplantar area of the right and left hind paws and depressed slowly until they bent. The filaments were tested in order of increasing logarithmic stiffness (0.41, 0.69, 1.2, 1.48, 2.04, 3.63, 5.5, 8.51, 11.75, and 15.14 g), with each applied three times. The first filament in the series that evoked three reflexive withdrawals from the three applications was designated the threshold. N=3 rats from each experimental group undergoing intraneural CED (including the vehicle-only controls) were randomly selected for behavioral testing. The SNL and sham animals served as positive and negative controls, respectively. The experimenter was blinded towards the treatment group throughout the duration of the study.

Euthanasia and tissue harvest

Rats were sacrificed 4 weeks after vector administration and transcardially perfused with heparinized PBS for 3 min, followed by 4% paraformaldehyde in PBS for 9 min. Spinal cord and DRGs were completely excised and postfixed in 4% paraformaldehyde in PBS for 24 hours. Tissue was cryoprotected in 30% sucrose, snap-frozen, and cut with a cryostat (CM3050 S, Leica Biosystems, Nussloch, Germany) at a slice thickness of either 16 μ m (for native EGFP fluorescence detection) or 8 μ m (for immunohistochemistry staining). Biodistribution samples (frontal cortex, liver, and spleen) were snap-frozen and stored at -80°C until further analysis.

Immunohistochemistry

The slides were desiccated overnight and post-fixed in 16% paraformaldehyde in PBS. The slides were then incubated in citrate buffer (21545; Millipore, Temecula, CA, USA) at 95°C for antigen retrieval and blocked with 0.6% Triton X in 10% normal goat serum. The slides were then incubated overnight with primary antibodies. Mouse monoclonal anti-NeuN antibodies (MAB377; Millipore, Temecula, CA, USA) diluted 1:50 were used. Next, the slides were incubated for two hours with goat anti-mouse, Alexa 594 conjugated secondary antibodies (A11032; Life Technologies, Grand Island, NY, USA; diluted 1:100) and mounted with a cover slip. The slides were washed with PBS in between all steps. Controls lacking either the primary or secondary antibodies were used to ensure specificity of the staining protocol.

Fluorescent microscopy

All slides were mounted using 4',6-diamidino-2-phenylindole (DAPI) containing mounting media (H-1500, Vector Laboratories, Vector Laboratories, Burlingame, CA, USA) and the fluorescent markers were visualized by laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany). For the quantification of transduction, native EGFP fluorescence was detected in λ -stack mode and the specific EGFP signal distinguished from a non-specific tissue autofluorescence by linear unmixing as reported in Storek et al. (2008). The sensitivity of this method was cross-validated by comparing it to a standard immunohistochemistry staining protocol (data not shown). For the phenotyping of transduced cells, Alexa 594 signal labeling the NeuN positive cells was visualized in the λ -stack mode and compared to the EGFP signal of an adjacent section.

Quantification of transduction

Unbiased quantification of transduction rate was adopted from Jacques et al. (2012) and performed by a blinded experimenter. Every 5th section throughout the tissue block was collected and the median section was used for cell counting. Briefly, the DAPI staining was used to count the total number of cells per slide and then the unmixed EGFP signal was applied to determine the % transduced (Fig. 1). The diameters of all cells with large round dim nuclei (satellite cells showing bright bean-shaped nuclei were not counted) were marked, recorded, and used to create a mask. The mask was then transferred over a binary version of the EGFP image. Positive areas (EGFP+) overlapping with the diameter markings were then automatically counted as positive (transduced) cell.

Quantitative polymerase chain reaction (qPCR) for vector biodistribution

DNA from the peripheral tissue samples was extracted by DNAeasy Blood and Tissue Kit (69506, Qiagen Sciences, Germantown, MA, USA). qPCR based on the SYBR Green detection system (0470756001, Roche Diagnostics, Mannheim, Germany) was used and copies of vector genomes were normalized to the copies of host diploid genomes. EGFP specific primers (forward, 5'-GAGCAAAGACCCCAACGAGA-3'; reverse, 5'-GTCCATGCCGAGAGTGATCC-3') were used for detection of the vector target sequence. Rat GAPDH primers (forward, 5'-CCGTTGTCCCAATCTGTTCT-3'; reverse, 5'-TGTGAGGGAGATGCTCAGTG-3') were used as a reference for the relative quantification. The amplification was performed using LightCycler 480 (Roche Diagnostics, Mannheim, Germany).

Results

AAVrh20 compared to the reference serotype AAV8 in the IT paradigm

IT delivery of AAVrh20 resulted in a robust transduction of the primary sensory neurons. Examination of DRG revealed that sensory neurons were transduced in all lumbar segments (Fig. 2A). DRG transduction rates for AAVrh20 were higher compared to those for AAV8, 50% vs. 9.6% for L4, and 19.5% vs. 9.6% for L5, respectively. Despite inter-individual variation, AAVrh20 yielded transduction rates as high as 95% in some cases, suggesting extraordinary potency of this serotype to target the primary sensory neurons. For both

serotypes, the transduction rates were highest in the DRG closest to the injection site (tip of IT catheter) and decreased gradually with greater distance, whereby gene expression was robust throughout all lumbar segments and absent in cervical regions.

Examination of the spinal cord demonstrated transduction of the dorsal roots, dorsal root entry zones and posterior columns. These structures correspond to the centripetal dendrites of the DRG neurons projecting to the dorsal horns or ascending cranially towards the brainstem as the *fasciculus gracilis* (Fig. 2B). The levels of gene expression in the spinal cord appeared to correspond to the DRG transduction rates at the same spinal levels. Motor neurons (or any other neurons located within the spinal cord) were not transduced by either serotype.

Intraneural injection of AAVrh20 compared to the reference serotype AAV1

Superior performance of the stepped needle design (Fig. 3A) used for CED was confirmed by a comparison with regular needles in agarose gel. Use of the stepped needle resulted in a spherical and homogenous distribution of the dye around the needle tip for flow rates up to 2 $\mu\text{l}/\text{min}$ and only limited reflux was found for flow rates up to 8 $\mu\text{l}/\text{min}$. Use of regular, blunt needles of either 26 or 33 G (gauges of the outer and inner tubing used for constructing the stepped needle, respectively) led to a notable reflux of the dye along the injection path even for the flow rate as low as 0.5 $\mu\text{l}/\text{min}$. (Fig. 3B).

Next, AAV1 or AAVrh20 was injected into the rat sciatic nerve. Fig. 4A and Fig. 4B show proportions of transduced neurons in L4 and L5 DRG for both serotypes. Administration of AAVrh20 led to significantly higher transduction rates compared to AAV1, 24% vs. 7.9% for L4 and 31.5% vs. 8.1% for L5, respectively. The gene expression was limited to the L4 and L5 ganglia ipsilateral to the injected nerve for both AAV1 and AAVrh20. There was no transduction seen in contralateral DRG of the same segments; neither was there any evidence of transduction at higher or lower anatomical segments. This finding indicates that AAV transduction remained restricted to the neurons that had been in primary contact with the transducing agent and that no trans-synaptic spread or spillage into the CSF had occurred. The spinal cord transduction was restricted to the dorsal root entry zones and the posterior columns of L4 and L5 spinal segment ipsilateral to the injected nerve (Fig. 4C). No motor neuron transduction was observed.

Effect of hyperosmotic diluent

To investigate whether osmotic stress affects the efficacy of gene transfer by intraneural injection, sorbitol was added to the vector diluent at a final concentration of 0.5M. For both AAV serotypes tested, the addition of sorbitol did not enhance the level of gene expression in either DRG or spinal cord (Fig. 4B). Instead, sorbitol showed a trend towards a decrease in the DRG transduction rates compared to the animals given AAVrh20 suspended in an isotonic buffer. Levels of gene expression within the spinal cord closely followed the pattern found in the DRG. The lack of a sorbitol effect was also supported by the results for the reference vector, for which iso-osmotic and hyperosmotic diluent performed similarly.

Cellular tropism and systemic distribution of AAVrh20 and the reference vectors

Immunohistochemical staining of the transduced DRG (Fig. 5A) using a neuronal marker NeuN showed co-labeling of EGFP and NeuN for all serotypes. The transduction pattern within the DRG did not appear to be affected by the route of delivery or type of diluent for any of the AAV serotypes used. Systemic distribution of the vectors delivered both by intraneural CED or IT was characterized by qPCR (Fig. 5B). Intraneural CED of AAVrh20 or AAV1 in PBS led to only minimal systemic spillage, with less 0.05 vector copies per host diploid genome found in either the liver or spleen. Use of 0.5 M sorbitol as a vector diluent, however, resulted in up to 10-fold increase in systemic counts of AAV genomes. No detectable vector genomes were found in the brain, confirming the anatomical specificity of the intraneural CED. IT administration of either AAVrh20 or AAV8, in contrast, led to higher distribution of both serotypes to the liver and spleen and also to a wider rostro-caudal distribution along the neuraxis, as evidenced by presence of vector genomes in the brain. The vector biodistribution was consistent within each experimental group and we observed no trend towards high systemic distribution in animals with lower DRG transduction rates, suggesting that a systemic spillage of the vector may not explain the observed inter-individual variation of the DRG transduction rates.

Transient allodynia following sciatic nerve injection

To assess functional consequences of the sciatic nerve exposure and of the intraparenchymal insertion of the needle, rats injected with either the vector or the vehicle were tested for tactile allodynia in the hind paw plantar region using von Frey filaments. We found transient allodynia in the paw ipsilateral to the injected nerve. The allodynia was present in animals receiving the vector and in animals receiving either type of the vehicle control and reversed back to normal within 4 weeks following the procedure in all rats. In contrast, positive controls that underwent spinal nerve ligation (SNL) of the L5 spinal nerve demonstrated stable allodynia with no signs of improvement during the tested period (Fig. 6). Sham controls, undergoing exposure of the sciatic nerve without intraneural insertion of the needle, showed no evidence of allodynia. No allodynia was found in the hind paws contralateral to the side of the procedure.

Discussion

The present study established intraneural CED of AAV as a highly effective method for gene transfer to the PNS. A stepped needle design was chosen to deliver the vector to the target tissue. The stepped needle design was found to result in superior distribution of the injectate when tested in agarose gel – a modeling method previously demonstrated to reliably predict performance of CED needles *in vivo* (Krauze et al. 2005; Yin et al. 2010). While the current study does not provide direct comparison of the stepped- versus a regular, non-stepped, needle *in vivo*, extensive testing in agarose gel performed by us as well as multiple studies investigating CED into various tissue types performed by others (Bobo et al. 1994; Lonser et al. 1998; Yin et al. 2010; French et al. 2010) support the choice of the stepped needle for intraneural CED. When intraneural CED was compared to IT administration, the intraneural route resulted in equally high transduction rates while improving the anatomical selectivity of vector targeting. By eliminating the distribution of

the vector within the CSF, the transduction was confined to the DRG of the injected peripheral nerve with no evidence of gene expression in the neighboring or contralateral DRG. In addition, hyperosmotic diluent did not further improve the transduction efficiency of AAV for the intraneural route. No long-term functional consequences of intraneural insertion of the needle were observed, suggesting there was no permanent nerve damage associated with the procedure.

AAVrh20 was superior to AAV8 and AAV1 - reference serotypes previously described to be most active for targeting the PNS by IT or intraneural delivery, respectively (Storek et al. 2008; Hollis et al. 2008; Jacques et al. 2012). AAVrh20 was found to transduce neuronal cells within the DRG and its intraneural CED led to only minimal off-target distribution. The present study was performed in the rat suggesting that the results will likely apply to other rodent models. The relative efficacy of AAVrh20 may need to be confirmed in other species, especially large animal models, before more general conclusions crossing species barriers can be drawn (Samaranch et al. 2012; Gray et al. 2013; Samaranch et al. 2013).

Clinical translation of intraneural CED may be a realistic future goal supported by converging developments in the fields of gene therapy and clinical medicine. AAV is gaining increasing acceptance as the leading clinical vector based on its efficacy and safety in patient studies in various organ systems (Nathwani et al. 2011; Bowles et al. 2012; Mittermeyer et al. 2012; Leone et al. 2012). CED has been used in a recent AAV clinical trial targeting the central nervous system suggesting that its use in the PNS would be acceptable in the scope of a clinical trial (Eberling et al., 2008). Ultra-sound guidance or nerve stimulation techniques are widely used in clinical neurology and would provide a non-invasive platform for CED to peripheral nerves in patients (Tsui and Suresh 2010; Gadsden et al. 2010). Experiments in a large animal model may therefore be warranted as the next step to assess the viability of the approach presented in this study towards translation into a clinical trial in patients with disorders of the PNS such as intractable pain.

Acknowledgments

The study was supported by NIH grant R01NS063022 and funds from the Schulze Family Foundation.

References

- Antonijevec I, Mousa SA, Schäfer M, Stein C. Perineurial defect and peripheral opioid analgesia in inflammation. *J Neurosci.* 1995; 15:165–172. [PubMed: 7823127]
- Ashtari M, Cyckowski LL, Monroe JF, et al. The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J Clin Invest.* 2011; 121:2160–2168. [PubMed: 21606598]
- Asokan A, Schaffer DV, Samulski RJ. The AAV vector toolkit: poised at the clinical crossroads. *Mol Ther.* 2012; 20:699–708. [PubMed: 22273577]
- Beutler AS. AAV provides an alternative for gene therapy of the peripheral sensory nervous system. *Mol Ther.* 2010; 18:670–673. [PubMed: 20357781]
- Bobo RH, Laske DW, Akbasak a, et al. Convection-enhanced delivery of macromolecules in the brain. *Proc Natl Acad Sci U S A.* 1994; 91:2076–2080. [PubMed: 8134351]
- Bowles DE, McPhee SWJ, Li C, et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Mol Ther.* 2012; 20:443–455. [PubMed: 22068425]

- Cearley CN, Vandenberghe LH, Parente MK, et al. Expanded repertoire of AAV vector serotypes mediate unique patterns of transduction in mouse brain. *Mol Ther.* 2008; 16:1710–1718. [PubMed: 18714307]
- Chaplan SR, Bach FW, Pogrel JW, et al. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods.* 1994; 53:55–63. [PubMed: 7990513]
- Chen X, Astarly GW, Mareci TH, Sarntinoranont M. In vivo contrast-enhanced MR imaging of direct infusion into rat peripheral nerves. *Ann Biomed Eng.* 2011; 39:2823–2834. [PubMed: 21809145]
- Chung JM, Kim HK, Chung K. Segmental spinal nerve ligation model of neuropathic pain. *Methods Mol Med.* 2004; 99:35–45. [PubMed: 15131327]
- Eberling JL, Jagust WJ, Christine CW, et al. Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology.* 2008; 70:1980–1983. [PubMed: 18401019]
- Federici T, Boulis N. Gene therapy for peripheral nervous system diseases. *Curr Gene Ther.* 2007; 7:239–248. [PubMed: 17969557]
- Federici T, Taub JS, Baum GR, et al. Robust spinal motor neuron transduction following intrathecal delivery of AAV9 in pigs. *Gene Ther.* 2011:1–8.
- French JT, Goins B, Saenz M, et al. Interventional therapy of head and neck cancer with lipid nanoparticle-carried rhenium 186 radionuclide. *J Vasc Interv Radiol.* 2010; 21:1271–1279. [PubMed: 20478719]
- Fu H, Muenzer J, Samulski RJ, et al. Self-complementary adeno-associated virus serotype 2 vector: global distribution and broad dispersion of AAV-mediated transgene expression in mouse brain. *Mol Ther.* 2003; 8:911–917. [PubMed: 14664793]
- Gadsden J, McCally C, Hadzic A. Monitoring during peripheral nerve blockade. *Curr Opin Anaesthesiol.* 2010; 23:656–661. [PubMed: 20689413]
- Gao G, Vandenberghe LH, Alvira MR, et al. Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol.* 2004; 78:6381–6388. [PubMed: 15163731]
- Gao G, Vandenberghe LH, Wilson JM. New recombinant serotypes of AAV vectors. *Curr Gene Ther.* 2005; 5:285–297. [PubMed: 15975006]
- Gray SJ, Nagabhushan Kalburgi S, McCown TJ, Jude Samulski R. Global CNS gene delivery and evasion of anti-AAVneutralizing antibodies by intrathecal AAV administration in non-human primates. *Gene Ther.* 2013:1–10.
- Hadaczek P, Kohutnicka M, Krauze MT, et al. Convection-enhanced delivery of adeno-associated virus type 2 (AAV2) into the striatum and transport of AAV2 within monkey brain. *Hum Gene Ther.* 2006; 17:291–302. [PubMed: 16544978]
- Hadaczek P, Mirek H, Bringas J, et al. Basic fibroblast growth factor enhances transduction, distribution, and axonal transport of adeno-associated virus type 2 vector in rat brain. *Hum Gene Ther.* 2004; 15:469–479. [PubMed: 15144577]
- Hollis ER, Kadota K, Hirsch M, et al. Efficient retrograde neuronal transduction utilizing self-complementary AAV1. *Mol Ther.* 2008; 16:296–301. [PubMed: 18223548]
- Homs J, Ariza L, Pagès G, et al. Schwann cell targeting via intrasciatic injection of AAV8 as gene therapy strategy for peripheral nerve regeneration. *Gene Ther.* 2011; 18:622–630. [PubMed: 21326330]
- Jacques SJ, Ahmed Z, Forbes A, et al. AAV8(gfp) preferentially targets large diameter dorsal root ganglion neurones after both intra-dorsal root ganglion and intrathecal injection. *Mol Cell Neurosci.* 2012; 49:464–474. [PubMed: 22425560]
- Kaspar BK, Lladó J, Sherkat N, et al. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science.* 2003; 301:839–842. [PubMed: 12907804]
- Krauze MT, Saito R, Noble C, et al. Reflux-free cannula for convection-enhanced high-speed delivery of therapeutic agents. *J Neurosurg.* 2005; 103:923–929. [PubMed: 16304999]
- Kreimer T, Kiser R, Reader A, et al. Anesthetic Efficacy of Combinations of 0.5 mol/L Mannitol and Lidocaine with Epinephrine for Inferior Alveolar Nerve Blocks in Patients with Symptomatic Irreversible Pulpitis. *J Endod.* 2012; 38:598–603. [PubMed: 22515886]
- Lawlor Pa; Bland, RJ.; Mouravlev, A., et al. Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol Ther.* 2009; 17:1692–1702. [PubMed: 19638961]

- Leone P, Shera D, McPhee SWJ, et al. Long-term follow-up after gene therapy for canavan disease. *Sci Transl Med*. 2012; 4:165ra163.
- Lock M, Alvira M, Vandenberghe LH, et al. Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale. *Hum Gene Ther*. 2010; 21:1259–1271. [PubMed: 20497038]
- Lonser RR, Weil RJ, Morrison PF, et al. Direct convective delivery of macromolecules to peripheral nerves. *J Neurosurg*. 1998; 89:610–615. [PubMed: 9761055]
- Malkmus SA, Yaksh TL. Intrathecal catheterization and drug delivery in the rat. *Methods Mol Med*. 2004; 99:109–121. [PubMed: 15131333]
- Mason MRJ, Tannemaat MR, Malessy MJA, Verhaagen J. Gene therapy for the peripheral nervous system: a strategy to repair the injured nerve? *Curr Gene Ther*. 2011; 11:75–89. [PubMed: 21291360]
- Mastakov MY, Baer K, Symes CW, et al. Immunological aspects of recombinant adeno-associated virus delivery to the mammalian brain. *J Virol*. 2002; 76:8446–8454. [PubMed: 12134047]
- Mittermeyer G, Christine CW, Rosenbluth KH, et al. Long-term evaluation of a phase I study of AADC gene therapy for Parkinson's disease. *Hum Gene Ther*. 2012; 23:377–381. [PubMed: 22424171]
- Muramatsu S, Fujimoto K, Kato S, et al. A phase I study of aromatic L-amino acid decarboxylase gene therapy for Parkinson's disease. *Mol Ther*. 2010; 18:1731–1735. [PubMed: 20606642]
- Nathwani AC, Tuddenham EGD, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia. *BN Engl J Med*. 2011; 365:2357–2365.
- Ratliff JK, Oldfield EH. Convection-enhanced delivery in intact and lesioned peripheral nerve. *J Neurosurg*. 2001; 95:1001–1011. [PubMed: 11765815]
- Rosenbluth KH, Luz M, Mohr E, et al. Design of an in-dwelling cannula for convection-enhanced delivery. *J Neurosci Methods*. 2011; 196:118–123. [PubMed: 21187114]
- Samaranch L, Salegio Ea, San Sebastian W, et al. Adeno-Associated Virus Serotype 9 Transduction in the Central Nervous System of Nonhuman Primates. *Hum Gene Ther*. 2012; 389:382–389. [PubMed: 22201473]
- Samaranch L, Salegio EA, San Sebastian W, et al. Strong cortical and spinal cord transduction after AAV7 and AAV9 delivery into the CSF of non-human primates. *Hum Gene Ther*. 2013:1–21. [PubMed: 23339283]
- Snyder BR, Gray SJ, Quach ET, et al. Comparison of adeno-associated viral vector serotypes for spinal cord and motor neuron gene delivery. *Hum Gene Ther*. 2011; 22:1129–1135. [PubMed: 21443428]
- Sondhi D, Johnson L, Purpura K, et al. Long-term expression and safety of administration of AAVrh.10hCLN2 to the brain of rats and nonhuman primates for the treatment of late infantile neuronal ceroid lipofuscinosis. *Hum Gene Ther Methods*. 2012; 23:324–335. [PubMed: 23131032]
- Storek B, Harder NM, Banck MS, et al. Intrathecal long-term gene expression by self-complementary adeno-associated virus type 1 suitable for chronic pain studies in rats. *Mol Pain*. 2006; 2:4. [PubMed: 16445862]
- Storek B, Reinhardt M, Wang C, et al. Sensory neuron targeting by self-complementary AAV8 via lumbar puncture for chronic pain. *Proc Natl Acad Sci U S A*. 2008; 105:1055–1060. [PubMed: 18215993]
- Towne C, Pertin M, Beggah AT, et al. Recombinant adeno-associated virus serotype 6 (rAAV2/6)-mediated gene transfer to nociceptive neurons through different routes of delivery. *Mol Pain*. 2009; 5:52. [PubMed: 19737386]
- Tsui B, Suresh S. Ultrasound imaging for regional anesthesia in infants, children, and adolescents: a review of current literature and its application in the practice of extremity and trunk blocks. *Anesthesiology*. 2010; 112:473–492. [PubMed: 20068455]
- Vulchanova L, Schuster DJ, Belur LR, et al. Differential adeno-associated virus mediated gene transfer to sensory neurons following intrathecal delivery by direct lumbar puncture. *Mol Pain*. 2010; 6:31. [PubMed: 20509925]

- White E, Bienemann A, Malone J, et al. An evaluation of the relationships between catheter design and tissue mechanics in achieving high-flow convection-enhanced delivery. *J Neurosci Methods*. 2011; 199:87–97. [PubMed: 21549753]
- Wolf R, Reader A, Drum M, et al. Anesthetic efficacy of combinations of 0.5 m mannitol and lidocaine with epinephrine in inferior alveolar nerve blocks: a prospective randomized, single-blind study. *Anesth Prog*. 2011; 58:157–165. [PubMed: 22168805]
- Wolfe D, Mata M, Fink DJ. Targeted drug delivery to the peripheral nervous system using gene therapy. *Neurosci Lett*. 2012; 527:85–89. [PubMed: 22565023]
- Xu Y, Gu Y, Wu P, et al. Efficiencies of transgene expression in nociceptive neurons through different routes of delivery of adeno-associated viral vectors. *Hum Gene Ther*. 2003; 14:897–906. [PubMed: 12828860]
- Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. *Physiol Behav*. 1976; 17:1031–1036. [PubMed: 14677603]
- Yin D, Forsayeth J, Bankiewicz KS. Optimized cannula design and placement for convection-enhanced delivery in rat striatum. *J Neurosci Methods*. 2010; 187:46–51. [PubMed: 20026357]

Figure 1. Quantification of the DRG transduction rates by unbiased cell counting

All slides were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and captured in both blue channel and λ -stack mode to visualize the DAPI and native EGFP fluorescence, respectively. Diameters of all cells with large round dim nuclei (satellite cells showing bright bean-shaped nuclei were not counted) in the DAPI channel were marked, recorded, and used to create a mask (left). Next, the mask was transferred over a binary version of the EGFP image (right). Positive areas (EGFP+) overlapping with the diameter markings were then automatically counted as positive (transduced) cells. The % cell transduced was established as a ratio of the positive cells to the total number cells counted in the DAPI channel.

Figure 2. AAVrh20 matching properties of the reference serotype for the intrathecal (IT) route
The activity of AAVrh20 was compared with AAV8, one of the most active serotypes for dorsal root ganglia (DRG) transduction in the IT paradigm. For both serotypes, 3.9×10^{10} genome copies (GC) of the vector, suspended in 30 μ l of injectate, were administered IT in adult Brown Norway rats.

A. Rostro-caudal distribution of DRG transduction. High transduction rates were found in lumbar segments where the tip of the IT catheter was situated. Transduction rates were highest at the L4 and L5 levels. Transduction remained anatomically confined to the lumbar region; no evidence of gene expression was observed in either thoracic or cervical regions. Transduced cells displayed the morphology of primary sensory neurons, with large cell bodies and occasionally seen pseudounipolar axons (arrowheads). Expression of the EGFP marker was visualized by laser scanning microscopy. The samples were scanned in λ -stack mode and the EGFP-specific fluorescence (green) distinguished from non-specific

background (red) by linear unmixing. Error bars: mean $\pm$ standard error of the mean, scale bars: 200 μ m.

B. Transduction of the lumbar spinal cord. Gene expression was found in the posterior columns of the spinal cord at the anatomical level corresponding to the DRG transduction (left). The morphology of the transduced structures was consistent with that of the centripetal axons of the primary sensory neurons as they enter the spinal cord at the dorsal root entry zone and continue rostrally as the *fasciculus gracilis*. The transduction of the *fasciculus gracilis* extended to the spinal segments rostral to the administration level (right). Analysis of the ventral horns revealed transduction of the axons (*arrowhead*); no transduction of motor neurons (*arrow*) was observed. Specific EGFP fluorescence: green, non-specific background fluorescence: red. Scale bars: 200 μ m.

Figure 3. Convection-enhanced delivery (CED) in agarose gel demonstrating superior performance of the stepped needle

A. Stepped needle design used for intraneural CED. A 52 mm stepped-needle was constructed by welding together 26G and 36G stainless steel tubings, forming a sharp transition between the larger shaft and the smaller tip (hence “stepped needle”) at its proximal ending. For comparison are also shown both the outer (26 G) and inner (33G) tubing used modeling of CED in agarose gel.

B. Modeling of CED in agarose gel. Performance of the stepped needle was compared to that of 26 G and 33 G conventional needles by administration of Evans blue dye into agarose gel. Spherical intraparenchymal distribution of injectate was achieved with the stepped needle design for flow rates $\geq 2 \mu\text{l}/\text{min}$. Both conventional-tip needles (with gauges equivalent to the inner and outer tubing used for constructing the stepped needle) by comparison led to tracking of injectate along the injection path for all examined flow rates.

Figure 4. Intraneural administration of AAVrh20 demonstrating high DRG transduction and anatomical selectivity

1.2×10^{10} GC of AAVrh20 were administered into the sciatic nerve by convection-enhanced delivery (CED). Transduction of the DRG and spinal cord was examined for both iso-osmolar and hyperosmolar diluents and compared with AAV1, the most active serotypes for DRG transduction in the intraneural paradigm.

A. Gene expression in the DRG. DRG transduction was limited to the L4 and L5 ganglia for both serotypes; no positive cells were found in the neighboring ganglia. While AAVrh20

resulted in almost complete transduction in some cases, the transduction for AAV1 was markedly lower.

B. DRG transduction rates. AAVrh20 delivered in the iso-osmotic diluent, phosphate buffered saline (PBS), resulted in robust DRG transduction. AAV1 led to significantly lower transduction rates compared to AAVrh20. Use of the hyperosmotic diluent, sorbitol (0.5M) in PBS, did not further enhance the high transduction rates achieved by AAVrh20. Data are shown as boxplots with whiskers encompassing 1.5 multiples of the interquartile ranges. The groups were compared by two-tailed Student t-test using JMP 9.0.3 Software.

C. Anatomically selective transduction of the spinal cord. Intraneural injection of AAVrh20 resulted in gene expression limited to the dorsal columns of the lumbar spinal cord ipsilateral to the injected nerve (section at the L5 level is shown). No transduction was found on the contralateral side or within the motor neurons.

D. Sciatic nerve transduction. The nerve fibers showed strong gene expression along its entire length for both serotypes.

Specific EGFP fluorescence: green, non-specific background fluorescence: red. Scale bars: 200 μ m.

Figure 5. Intraneural CED of AAVrh20 targeting neurons and glia with minimal off-target distribution

A. Cellular tropism. AAVrh20 selectively targeted DRG neurons, as evidenced by comparison of NeuN (red) and EGFP (green) immunohistochemical staining of adjacent DRG sections where all EGFP positive cells colabeled with NeuN (yellow). Identical pattern of transduction was observed for AAV1 and AAV8. Scale bars: 100 μ m.

B. Systemic distribution. Intraneural CED of AAVrh20 and AAV1 suspended in PBS led to only minimal distribution of the vector to the liver or spleen. Addition of 0.5 M sorbitol to the vehicle resulted in up to 10-fold increase in the AAVrh20 genomes found in the liver and spleen. No vector genomes were detected in the brain for any vehicle / serotype combination. IT administration of AAVrh20 or AAV8 resulted in higher systemic distribution of the vector compared to the intraneural route, with the vector found in liver, spleen, and brain. Primers specific to the EGFP insert were used and the detection threshold of the assay (0.00297 genome copies / host diploid genome) was determined as a 95% confidence interval of the values obtained in independent negative controls.

Figure 6. Intraneural convection-enhanced delivery resulted in transient allodynia

To assess the nerve injury associated with the surgical exposure of the nerve and the intraneural injection, the animals were tested weekly for allodynia using the von Frey filaments. Transient allodynia could be detected at post-operative week 1 but returned to the baseline within the 4-week survival period. Positive control animals that had undergone spinal nerve ligation (SNL) showed stable allodynia with no trends towards improvement during the testing period.