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Targeting Neurons of Rat Nucleus Tractus Solitarii with the Gene Transfer Vector Adeno-Associated Virus Type 2 to Up-Regulate Neuronal Nitric Oxide Synthase

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Abstract

Adeno-associated virus (AAV) has distinct advantages over other viral vectors in delivering genes of interest to the brain. AAV mainly transfects neurons, produces no toxicity or inflammatory responses, and yields long-term transgene expression. In this study, we first tested the hypothesis that AAV serotype 2 (AAV2) selectively transfects neurons but not glial cells in the nucleus tractus solitarii (NTS) by examining expression of the reporter gene, enhanced green fluorescent protein (eGFP), in the rat NTS after unilateral microinjection of AAV2eGFP into NTS. Expression of eGFP was observed in 1–2 cells in the NTS 1 day after injection. The number of transduced cells and the intensity of eGFP fluorescence increased from day 1 to day 28 and decreased on day 60. The majority ($92.9 \pm 7.0\%$) of eGFP expressing NTS cells contained immunoreactivity for the neuronal marker, protein gene product 9.5, but not that for the glial marker, glial fibrillary acidic protein. We observed eGFP expressing neurons and fibers in the nodose ganglia (NG) both ipsilateral and contralateral to the injection. In addition, eGFP expressing fibers were present in both ipsilateral and contralateral nucleus ambiguus (NA), caudal ventrolateral medulla (CVLM) and rostral ventrolateral medulla (RVLM). Having established that AAV2 was able to transduce a gene into NTS neurons, we constructed AAV2 vectors that contained cDNA for neuronal nitric oxide synthase (nNOS) and examined nNOS expression in the rat NTS after injection of this vector into the area. Results from RT-PCR, Western analysis, and immunofluorescent histochemistry indicated that nNOS expression was elevated in rat NTS that had been injected with AAV2nNOS vectors. Therefore, we conclude that AAV2 is an effective viral vector in chronically transducing NTS neurons and that AAV2nNOS can be used as a specific gene transfer tool to study the role of nNOS in CNS neurons.

Keywords

Adeno-associated virus; Enhanced green fluorescent protein; Gene transfer; Immunofluorescent histochemistry; Neuronal nitric oxide synthase; Nucleus tractus solitarii; RT-PCR; Western blot

Introduction

As the primary site of termination of visceral afferent inputs, the nucleus tractus solitarius (NTS), located in the dorsomedial medulla oblongata, is involved in central regulation of gastrointestinal, cardiovascular, and ventilatory functions (Kalia and Sullivan 1982; Spyer et al. 1984; Nattie 1999; Broussard and Altschuler 2000; Boscan et al. 2002). Mediation of these physiological functions relies on signal transduction governed by numerous potential neurotransmitters and modulators that are present in the NTS (Lawrence and Jarrott 1996; Bradley et al. 1996; Nattie 1999; Broussard and Altschuler 2000; Lin 2009). In recent years, in addition to pharmacological manipulation and neuroanatomical analyses, gene transfer using replication-deficient recombinant viral vectors to up- or down-regulate neurotransmitters has been used to delineate the role of some of the putative neurotransmitters in the NTS (Sakai et al. 2000; Hirooka et al. 2003; Chen et al. 2006). For example, adenovirus has been used as a vector to carry the DNA sequence coding interference RNA for angiotensin receptor type 1 into the NTS and thus to down-regulate angiotension receptors. Doing so caused a decrease in blood pressure with no effect on heart rate in mouse (Chen et al. 2006). In contrast, injection into the NTS of adenovirus vector encoding endothelial nitric oxide synthase (eNOS) increased production of nitric oxide (NO) and led to decreased heart rate and blood pressure in rat (Sakai et al. 2000; Hirooka et al. 2003).

Another class of viral vector, adeno-associated virus (AAV), a non-pathogenic parvovirus, appears to be a highly promising tool for gene transfer into the brain (Van Vliet et al. 2008). Like adenovirus, AAV vectors are capable of transducing non-dividing cells and leading to efficient and stable transgene expression in the brain (Xiao et al. 1997a). However, AAV vectors possess several unique and advantageous characteristics. While adenovirus vectors often elicit a strong immune response (Yang et al. 1996; Xiao et al. 1997b) and may have cellular toxicity (Durham et al. 1996), AAV vectors have been shown to be not immunogenic and not toxic to the host tissue (Xiao et al. 1997a; Bueler 1999). In addition, in vivo gene transduction by AAV vectors lasts for a long time with some reports indicating continued transduction for months (Kaplitt et al. 1996; Xiao et al. 1996) or even a year or more (Gasmi et al. 2007; Belur et al. 2008). In contrast, adenovirus gene expression may diminish 1 month after injection (Boulis et al. 1999). Furthermore, some types of AAV vectors have been found to preferentially transfect neurons in some areas of the brain (Nomoto et al. 2003) while adenovirus may preferentially transfect non-neuronal cells in the CNS (Allen et al. 2006). What type of cell a vector transfects may be important in dissecting how a neurotransmitter system works when that vector is used to up-regulate or down-regulate a neurotransmitter in a specific group of cells. Among AAV serotypes, AAV serotype 2 (AAV2) vector has been studied extensively and has been shown to transduce neurons exclusively (Nomoto et al. 2003). This means that AAV2 would be a powerful tool in delineating the role of certain neurotransmitters such as neuronal nitric oxide synthase (nNOS), which is only found in neurons in the NTS (Lin and Talman 2005a; Lin et al. 2007), as opposed to endogenous eNOS, which is found in non-neuronal cells in the same region (Lin et al. 2007). In that both of the NOS isoenzymes are capable of producing NO and the origin of the NO could affect different physiological effects, any alteration of synthesis would optimally be directed toward the cell type that naturally synthesizes the NO involved in those effects.

AAV2 has been used as a tool for gene transfer in several regions of the brain (Gasmi et al. 2007; Yang et al. 2009). Although AAV2 was used in the rat NTS in one publication to knockdown the expression of leptin receptors (Hayes et al. 2010), it is not known what type of NTS cells are transduced and the time course of AAV2 transduction in the NTS. Therefore, the purposes of the current study were to test the hypothesis that AAV2

transduces signals in neurons, in preference to glia, in the rat NTS and to study the time course of AAV2 transduction in the NTS by using AAV2 vectors carrying the gene for enhanced green fluorescent protein (eGFP). We also examined the nodose ganglion (NG) for evidence of transduction after injection of AAV2 vector into the NTS to determine if there is uptake and retrograde transport of the vector from the NTS to the NG, the site of sensory afferent neurons that send arterial baroreceptor, chemoreceptor, and cardiopulmonary afferent axonal projections to the NTS (Dampney 1994). Furthermore, we examined the nucleus ambiguus (NA), rostral ventrolateral medulla (RVLM), and caudal ventrolateral medulla (CVLM) to determine if the AAV2 vector may undergo anterograde transport from the NTS. Finally, we constructed an AAV2 vector that encoded cDNA for nNOS (AAV2nNOS) and tested the hypothesis that nNOS expression in the rat NTS can be up-regulated after injection of this vector to this area. We examined mRNA and protein levels of nNOS in the rat NTS with real time RT-PCR, Western blot analysis, and immunofluorescent histochemistry.

Methods

Viral Vectors

AAV2 vector encoding enhanced green fluorescent protein (AAV2eGFP) driven by cytomegalovirus (CMV) promoter was generated and titered by the Gene Transfer Vector Core of the University of Iowa using a baculovirus system according to methods described previously (Urabe et al. 2002). The titer of vectors used in the current study was 9.68×10^{13} viral genomes/ml. The vectors were stored at -80°C in 20 mM Tris-HCl (pH 8.0) containing 250 mM NaCl. They were dialyzed against phosphate buffered saline (PBS, pH 7.4) at 4°C for 15 min immediately before use.

Rat nNOS cDNA, a gift from Dr. David S. Bredt (Bredt et al. 1991), was cloned into a modified rAAV2 packaging plasmid pFBGR (Gene Transfer Vector Core, University of Iowa, USA) with a CMV promoter. AAV2 vectors encoding nNOS cDNA (AAV2nNOS) were then prepared by a triple baculovirus infection in SF-9 insect cells by The Gene Transfer Vector Core of The University of Iowa using similar methods described above. The titer of the AAV2nNOS vector was 1.12×10^{13} viral genomes/ml. The vectors were stored at -80°C and dialyzed against PBS immediately before use as described above.

Cell Culture

Human embryonic kidney cells (HEK293), which do not naturally express nNOS, were used to examine the efficiency of recombinant AAV plasmid that contained nNOS cDNA (AAVp-nNOS). HEK293 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in six-well plate in Dulbecco's modified Eagles medium and supplements with heat-inactivated fetal bovine serum and antibiotics. They were incubated with AAVp-nNOS for 48 h before they were harvested for real time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis.

Animals and Injections

All procedures conformed to standards established in the *Guide for Care and Use of Laboratory Animals* (National Academy Press, Washington, DC 1996). The Institutional Animal Care and Use Committees of the University of Iowa and Department of Veterans Affairs Medical Center, Iowa City reviewed and approved all protocols. Both institutions are AAALAC accredited. All efforts were made to minimize the number of animals used and to avoid their experiencing pain or distress.

Adult male Sprague–Dawley rats (275–340 g) were anesthetized with isoflurane (5% induction and 1.5–2.0% maintenance) delivered in 100% O₂ (2 l/min) by a nasal mask. The dorsal surface of the brain stem was exposed as previously described (Talman 1989), and a glass micropipette filled with viral vectors was stereotactically placed (0.4 mm rostral to the calamus scriptorius, 0.5 mm from the midline, and 0.5 mm below the surface of the brain stem) unilaterally into the dorsolateral and medial subnuclear regions of the NTS at the level of the area postrema. Injections (individual increments of 25–50 nl to a combined total of 200 nl) were made over 15 min and the pipette was left in place for 15 additional minutes to limit efflux of injectate from the pipette track. Surgical wounds were closed, hemostasis assured, the animal treated with buprenorphine (0.05 mg/kg), and anesthesia stopped. After recovery from anesthesia, the animal was returned to the animal care facility until it was later brought to the laboratory to be euthanized. For the time course study of AAV2eGFP in the NTS, we euthanized rats under deep pentobarbital (50 mg/kg) anesthesia 1, 2, 4, 7, 14, 28, and 60 days after the injection ($n = 2$ for 1 and 2 days, $n = 3$ for all the other time points). For fluorescent immunohistochemical evaluation of nNOS expression in the NTS, rats ($n = 4$) were euthanized 2 weeks after the injection.

Procedures for euthanasia and perfusion fixation of tissues have been described in our earlier publications (Lin and Talman 2005a; 2006; Lin et al. 2007). The brain was removed, postfixed in 4% paraformaldehyde for 2 h and then cryoprotected for 2 days in 30% sucrose in PBS at 4°C. Frozen 20 µm coronal sections were cut with a cryostat and processed for double-label immunofluorescent staining of a glial marker, glial fibrillary acidic protein (GFAP), and a neuronal marker, protein gene product 9.5 (PGP 9.5), as described below. In some cases, sections were stained for PGP9.5 or nNOS alone. As previously described (Lin et al. 1998), we removed the ipsilateral and contralateral NG removed after perfusing the rat. The NG were then postfixed for 1 h, transferred to 30% sucrose overnight, cut, mounted to slides and processed for immunofluorescent staining.

For real time RT-PCR and Western blot analysis of nNOS expression, rats were bilaterally injected with AAV2nNOS (200 nl, containing 2.24×10^9 viral genomes) in the NTS as described above and were euthanized with an overdose of pentobarbital (150 mg/kg) 2 weeks later ($n = 10$ for AAV2nNOS injection, $n = 9$ for control). The NTS was micropunched out with a stainless tubing (inner diameter of 0.038 inch or 0.96 mm) from consecutive 150 µm frozen cryostat sections. The punches were stored at –20°C until they were used for Western blot analysis or placed in cold RNeasy Lysis Buffer (Qiagen Inc., Valencia, CA, USA) overnight and then stored at –20°C for real time RT-PCR. Tissue from each rat was analyzed separately.

Immunofluorescent Staining

Procedures similar to those described in our previous publications (Lin and Talman 2005a; 2006; Lin et al. 2007) were used for immunofluorescent staining of brain stem and NG sections. For double-label immunofluorescent staining for PGP9.5 and GFAP, brain stem sections were incubated in a mixture of mouse anti-GFAP antibody (1:100, Sigma, USA) and rabbit anti-PGP9.5 (1:200, Chemicon, USA) in 10% donkey normal serum for 24 h in a humid chamber at 25°C. After being washed with PBS, sections were incubated with Cy5-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch Labs, USA) and rhodamine red X (RRX)-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch Labs.) in PBS for 20–24 h at 4°C. The primary and secondary antibodies for GFAP were omitted when we performed fluorescent immunostaining of PGP9.5 alone. For immunofluorescent staining of nNOS alone, sheep anti-nNOS antibody (1:1000, from Dr. Piers C. Emson) (Herbison et al. 1996) was used as the primary antibody and Cy2-conjugated donkey anti-sheep IgG (1:200, Jackson ImmunoResearch Labs) as the secondary antibody.

Stained sections were washed and mounted with Prolong Gold Antifade Reagents (Invitrogen-Molecular Probes, USA). Negative controls consisted of tissue processed (a) in the absence of primary antibodies or (b) after addition of only one of the primary antibodies. In addition, we have used both PGP9.5 and GFAP antibodies in our previous publications with satisfactory results (Lin et al. 2007, 2010). We also have used sheep anti-nNOS antibody in a number of previous publications (Lin and Talman 2005a; Lin et al. 2004, 2007). Stained brain stem sections and NG sections were examined with a Zeiss LSM 510 or LSM 710 confocal microscope as described below.

Confocal Laser Scanning Microscopy

As we previously described (Lin and Talman 2006; Lin et al. 2007), we analyzed labeled sections with a Zeiss LSM 510 or a Zeiss LSM 710 confocal laser scanning microscope and scanned specimens sequentially with one to three channels to separate labels. For optimal visualization of the relationship between eGFP-, RRX-, Cy2-, and Cy5-labeled elements, images from three channels were assigned the pseudocolor green (for eGFP or Cy2), red (for RRX) and blue (for Cy5) respectively, and then were superimposed. Confocal images were obtained and processed with software provided with the Zeiss LSM 510 or Zeiss LSM 710. Adobe Photoshop image editing software (Adobe Photoshop CS2) was used to examine if a structure had single labeling or double labeling by switching between channels on the monitor. Another image editing program, Microsoft PowerPoint (2003), was used to create montages. Contrast and brightness of images were the only variables we adjusted digitally.

Cell Counts

Cell counts were performed in time course studies of AAV2eGFP injections. We counted stained cells in images of 3–4 selected sections at comparable levels of the brain stem (within 200 μ m of the center of the injection site) from each rat. We avoided analysis of sequential sections so that sections used for counting were at least 20 μ m (the thickness of the sections) apart. Therefore, we minimized the possibility that the same stained neurons would be counted twice. For multiple-label sections, labeled cells in each section were counted three times, first using the green channel for the number of eGFP expressing cells, then the red channel for PGP9.5 positive cells, then the blue channel for GFAP positive neurons, and finally the combined channel for multiple-stained structures. The percentage of single- to double-labeled cells in each section was calculated after each section had been counted. Percentages of single- to double-labeled cells were expressed as mean $\pm$ standard deviation (SD).

Real Time RT-PCR

Real time quantitative RT-PCR was used to assess expression of nNOS in rat NTS after transduction with AAV2nNOS. Following extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), RNA of NTS was prepared using the RNeasy Mini kit (Qiagen, CA, USA). RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) with an OD260/OD280 ratio of greater than 1.9. Reverse transcription was performed according to methods described in an earlier publication (Chu et al. 2002) using 300 ng RNA for each sample. We performed real time RT-PCR in 96-well plate with identical amount of reverse transcription product using nNOS TagMan[®] Expression Assays and eNOS TagMan[®] Expression Assays purchased from Applied Biosystems (Carlsbad, CA, USA). These kits contain probes and primers for real time RT-PCR for nNOS and eNOS. RT-PCR for β actin (Rat ACTB Endogenous Control, Applied Biosystems) was used as an endogenous reference control and was performed in the same well as nNOS or eNOS. FAM fluorophore was used for nNOS or eNOS, VIC[®] fluorophore was used for β actin. Expression levels of nNOS or eNOS were first normalized by β actin level,

and relative expression levels were then obtained using the $\Delta\Delta C_t$ method (Wakisaka et al. 2010).

Western Blot Analysis

Procedures like those in our previous publication (Lin and Talman 2005b) were used for Western blot analysis of nNOS. In brief, we homogenized HEK293 cells or NTS tissue in homogenization buffer containing 2% sodium dodecyl sulfate (SDS). After centrifugation, protein concentration of the supernate was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Homogenates containing 10 μ g protein were separated alongside Bio-Rad Precision Plus Proteins Standards (Bio-Rad Laboratories) by 7.5% SDS-polyacrylamide gel electrophoresis (Ready Gel, Bio-Rad Laboratories) using the Mini Protein II System (Bio-Rad Laboratories) according to Laemmli (1970). The separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories) using the Mini Trans-Blot Cell (Bio-Rad Laboratories). The blot was blocked in 10% milk in PBS and then incubated with sheep anti-nNOS (1:20,000) antibody, described above for fluorescent immunostaining, at 4°C for 24 h. After a thorough wash, the blot was incubated with horseradish peroxidase-conjugated anti-sheep antibody (1:10,000, Jackson ImmunoResearch Lab.) at 25°C for 4 h. Protein bands were visualized with ECL PlusTM Western Blotting Reagents (GE Healthcare/Amersham Biosciences, South San Francisco, CA, USA) and exposed to X-ray films.

We used glyceraldehyde-3-phosphate (GAPDH) as an internal control for Western analysis of rat NTS. Goat anti-GAPDH antibody (1:20,000) was purchased from Gen-Script (Piscataway, NJ, USA).

Results

AAV2eGFP Injection

AAV2eGFP in NTS—We noted expression of eGFP, the marker protein of AAV2 gene transfer in this study, in the NTS after the vectors had been injected into the area (Fig. 1b, d). We observed one or two eGFP expressing cells in the injected NTS 1 day after AAV2eGFP injection. The number of transfected cells in the NTS increased from day 1 through day 28 after the injection of AAV2eGFP (Fig. 2, top panel). These eGFP expressing cells were seen throughout the rostral-caudal length of the NTS from day 7 (Fig. 3) through day 28. Although we did not analyze it quantitatively, we noted that the intensity of eGFP fluorescence in cell bodies also appeared to increase over time. In addition, the density of eGFP expressing fibers also appeared to increase from day 1 to day 28. On day 28, the density of eGFP expressing fibers in the NTS became so great that it was often hard to clearly identify the boundary of individual cell bodies. On day 60, there was a decrease in the number of eGFP expressing cells in the NTS and their staining intensity (Fig. 2, top panel).

We also observed eGFP expressing cells and fibers in the contralateral NTS. One or two cells that weakly expressed eGFP were noted 2 days after the injection. The expression of eGFP in cells and fibers in the contralateral NTS became more evident on day 7 (Fig. 1a, c). The number of eGFP expressing cells in the contralateral NTS also increased over time from day 2 to day 28. However, the number of eGFP expressing cells in the contralateral NTS was always fewer than in the injected side for the same survival time period (Fig. 2, top panel). Similar to observations in the injected NTS, the intensity of eGFP fluorescence in cell bodies also increased from day 2 to day 28. The density of eGFP expressing fibers in the contra-lateral NTS also increased from day 2 to day 28. On day 60, there were fewer eGFP

expressing cell in the NTS (Fig. 2, top panel) and a lower density of fibers that expressed eGFP.

Some neurons and fibers in adjacent structures, such as the area postrema (AP), dorsal motor nucleus of vagus (DMV), and gracilis nucleus (Gr), also were observed to express eGFP on day 2 and to become more abundant over time (Fig. 1b, d), reaching a maximum by day 28, and then decreasing by day 60.

Type of Cells Transduced—Double-label immunofluorescent staining showed that $92.9 \pm 7.0\%$ (1,553 cells were counted) of NTS cells expressing eGFP after AAV2eGFP had been injected were positive for PGP9.5-IR but none contained for GFAP-IR (Fig. 4). Furthermore, the majority of eGFP expressing cells in the contralateral NTS were positive for PGP9.5-IR and negative for GFAP-IR.

The majority of eGFP expressing cells in the AP, DMV, and Gr, also stained for PGP9.5-IR but not for GFAP-IR. For example, $92.2 \pm 4.6\%$ (619 cells were counted) of cells in the DMV were positive for PGP9.5-IR and none was positive for GFAP-IR.

AAV2eGFP in NG—We observed a few eGFP expressing cells and fibers in the ipsilateral and contralateral NG on day 4 after AAV2eGFP had been injected into the NTS. The number of eGFP expressing cells in both the ipsilateral and contralateral NG increased from day 4 to day 28, and decreased on day 60 (Fig. 2, bottom panel). Similar to what was observed in the NTS, the majority ($94.6 \pm 1.8\%$ of 1,872 cells counted) of cells expressing eGFP also contained PGP9.6-IR (Fig. 5a, b) while none contained GFAP-IR.

AAV2eGFP in NA, CVLM, RVLM, and Other Brain Areas—Fibers expressing eGFP were seen in both the ipsilateral and contralateral NA on day 4 after the injection of AAV2eGFP into the NTS. The density of eGFP expressing fibers also appeared to increase from day 4 to day 28. Some eGFP expressing fibers surrounded NA neurons with close apposition to cell bodies (Fig. 5c, d) of these neurons on day 14, day 28, and day 60. NA neuronal cell bodies rarely contained eGFP; however, we observed occasional low intensity eGFP fluorescence in some NA neurons on day 14, day 28, and day 60. In addition to the NA, we also noted eGFP expressing fibers in the both the ipsilateral and contralateral CVLM and RVLM on day 7 after injection. The density of eGFP expressing fiber also appeared to increase from day 7 to day 28 and decreased on day 60. Some eGFP expressing fibers were seen surrounding neurons in the CVLM (Fig. 5) and RVLM and were sometimes in close apposition to cell bodies of these neurons on day 14, day 28, and day 60. However, we did not see eGFP expressing neurons in either CVLM or RVLM at any time.

We also observed eGFP expressing fibers in other brain regions where NTS projects. For example, eGFP expressing fibers were present in the ipsilateral and contralateral parabrachial nucleus, lateral hypothalamus, paraventricular nucleus, substantia nigra, midbrain periaqueductal gray, and dorsomedial thalamus on day 14 after injection and persisted on day 60. We did not see eGFP expressing neurons in any of these areas at any time.

AAV2nNOS Injection

As a first step, we used HEK cells to test if AAV2 plasmid that carries nNOS cDNA can induce HEK cells to express nNOS. Figure 6 shows that HEK cells (left panel) expressed high level of nNOS protein after they were incubated with AAVp-nNOS. In contrast, untreated HEK cells did not express nNOS (Fig. 6). Having established that AAVp-nNOS induced nNOS expression in cell culture, we then prepared AAV2nNOS and injected the vector into rat NTS to determine if nNOS expression in the NTS is up-regulated. Results

from real time RT-PCR demonstrated that 2 weeks after introduction of AAV2nNOS, mRNA levels in the NTS of the injected rats increased 11.4 ± 2.3 fold ($P < 0.005$), when compared to that in un-injected control rats (Fig. 7, left panel). As an additional control, we demonstrated that mRNA levels of eNOS were not altered (Fig. 7, right panel).

Western blot analysis demonstrated increased expression of nNOS protein after injection of AAV2nNOS into the NTS (Right panel of Fig. 6) when compared to that in un-injected control rats. Such increased nNOS protein in NTS was consistent with results from immunofluorescent staining of nNOS (Fig. 8), which showed increased nNOSIR in both fibers and cell bodies of NTS neurons. The DMV, which is adjacent to the NTS, also appeared to have increased nNOS-IR (Fig. 8), and some hypoglossal cells, which normally do not express nNOS, expressed nNOS-IR after transduction (Fig. 8).

Discussion

This study makes the following four contributions. (1) It demonstrates that AAV2eGFP microinjected into the rat NTS transduces cells to express eGFP. This transduction reached its peak on day 28 and lasted for at least 60 days. (2) It demonstrates that the majority of transduced NTS cells were neurons, as indicated by their containing IR of the neuronal marker, PGP9.5. (3) It demonstrates that AAV2 vectors were retrogradely transported from the NTS to the NG and anterogradely transported from the NTS to the NA, CVLM, and RVLM. There was no evidence of trans-synaptic transport of the vector at those sites. (4) It demonstrates that AAV2 vectors containing nNOS cDNA increased mRNA and protein levels of nNOS in the NTS.

Our observation that AAV2 vectors were capable of transducing non-mitotic neurons in the NTS is consistent with that of other studies in other areas of the brain (Gasmi et al. 2007; Belur et al. 2008). Among different serotypes of AAV vectors, AAV2 is the most widely used in neurological gene therapy in humans, largely because of its ability to transduce neural cells in rodent and primate brain (Nomoto et al. 2003; Palomeque et al. 2007; Markakis et al. 2010). The mechanism for AAV2's tropism for neurons most likely involves the unique structure of its capsids, which may bind to specific membrane-associated receptors in neurons (Michelfelder and Trepel 2009; Mason et al. 2010). This selectivity makes AAV2 an excellent vector for gene transduction in NTS neurons when studies are aimed at proteins that are typically found in neurons. For example, AAV2 would target the source of the endogenous enzyme when one seeks to study the role of nNOS within the NTS where the enzyme is found exclusively in neurons (Lin and Talman 2005a; Lin et al. 2007). AAV2 is, then, one of a group of good viral vectors for this purpose. For example, neuronal tropism is also achieved with the feline immunodeficiency virus vector in the rat NTS (Lin et al. 2010).

Another advantage of AAV2 as a gene transfer tool is that its transduction is long-lasting. Our results show that AAV2eGFP expression in the NTS lasted for at least 2 months, the longest time point in our study. Previous studies in CNS and PNS have demonstrated that gene transduction of AAV2 usually lasts for more than 6 months and may persist for more than a year (Gasmi et al. 2007; Belur et al. 2008; Hadaczek et al. 2010). In contrast, adenovirus gene expression may diminish 1 month after injection (Boulis et al. 1999) and lentivirus gene expression may be limited to 3 months (Naldini et al. 1996). The reason that gene expression of AAV vectors lasts longer has been attributed to there being no viral coding sequence in the AAV vector DNA. There is, therefore, no expression of viral protein to induce an immune reaction by the host (McCown 2005). Though chronic transduction is a valuable feature of AAV, it has been suggested that chronicity may vary in different brain regions (McCown et al. 1996). Furthermore, the choice of promotor may significantly

influence both pattern and longevity of gene expression in the CNS because diminution in gene expression may result from promotor suppression rather than loss of transduced cells (Klein et al. 1998; McCown 2005). Our AAV2eGFP vector contained a CMV promotor, which is one of the strongest constitutively active virus-derived transcription elements (Nomoto et al. 2003). Longer-lasting gene expression may be achieved by using an endogenous promotor, such as neuron specific enolase, as suggested by some investigators (Klein et al. 1998). Compared to adenovirus vectors, which are highly efficient (although transient) and provide high level of expression of transgene (Sinnayah et al. 2002; Lonergan et al. 2005), AAV2 vectors sometimes do not offer high levels of expression of transgene in tissues other than brain and muscle (Sanlioglu et al. 2001; Zhong et al. 2008). This problem has been attributed to the rate-limiting steps of AAV transduction, in which the single-stranded AAV viral DNA failed to be converted to the transcriptionally active double-stranded form efficiently (Sanlioglu et al. 2001; Zhong et al. 2008). Furthermore, high levels of adenovirus transduction are often observed within 1 week, high levels of AAV transduction are not noted until 2–3 weeks after introduction of the vectors.

After AAV2eGFP was injected into the NTS, we observed numerous eGFP expressing neurons and fibers in the NG both ipsilateral and contralateral to the injection. This observation indicates that AAV2eGFP was taken up by terminals in the NTS and retrogradely transported to the NG. The observation that AAV2 vectors are capable of undergoing retrograde transport in the nervous system has been reported in several studies (Kaspar et al. 2002; Johnston et al. 2009). In contrast, some investigators did not observe retrograde transport of AAV2 even when they studied similar areas of the brain (Chamberlin et al. 1998; Burger et al. 2004; Hollis et al. 2008). The reason for the discrepancy between these studies is not clear (Burger et al. 2004; Hollis et al. 2008). Of note, AAV2 is not the only AAV serotype that can be retrogradely transported. Serotypes AAV1 and AAV5 may similarly undergo retrograde transport in the nervous system (Burger et al. 2004; Hollis et al. 2008).

We also observed anterograde transport of AAV2eGFP (or eGFP protein) from the NTS to the NA, CVLM, and RVLM. This finding was consistent with a report that showed anterograde transport of AAV2 vector when it was injected into the putamen of Rhesus monkey (Johnston et al. 2009). Again anterograde transport is not a unique property of AAV2 but may also occur with AAV1 and AAV9 (Cearley and Wolfe 2007). Indeed, it has been suggested that AAV vectors may be used as tract tracers that may even be superior to biotinylated dextran amines and Phaseolus vulgaris leucoagglutinin because AAV vectors produce a clearly defined injection site and are not taken up by all type of cells (Chamberlin et al. 1998). Since AAV vectors can be transported long distances and may last for a long time, it has been considered a particularly good anterograde tracer for long axonal pathways (Chamberlin et al. 1998). Our finding some neurons that express eGFP in the NA is most likely due to the suggested bidirectional connection between the NTS and some neurons of the NA (Hayakawa et al. 1997) and not because AAV2 was transported trans-synaptically. Other than the NA, we observed only eGFP expressing fibers, and not neurons, at other sites that receive projections from NTS. Therefore, it is not likely that AAV2 underwent trans-synaptic transport from the NTS to the NA. Supporting this suggestion, we found no reports that indicate AAV2 may undergo trans-synaptic transport. On the other hand, one study suggests that AAV1 and AAV9 may be transported anterogradely, released and taken up by target cells (Cearley and Wolfe 2007).

Since AAV vector may transduce neurons and is capable of retrograde axonal transport, AAV2 may be a vector of choice when seeking to deliver gene to specific neurons that are not easily directly approachable. For example, injection of AAV2 vectors into muscle or peripheral nerve to allow retrograde transport to motor neurons of spinal cord may provide a

noninvasive approach for treating amyotrophic lateral sclerosis (Kaspar et al. 2002). In the NTS, AAV2 vector may be useful also as a retrograde tracer and an anterograde tracer, by itself or combined with other methods, to study areas that project to this nucleus. In contrast to AAV2, feline immunodeficiency virus vector, which does not undergo any type of transport could be the vector of choice when targeting neurons at the site of delivery (Lin et al. 2010). In the latter case, physiological changes as a result of the vector introduction could be more reliably attributed to effects on those local neurons.

Finally, and most importantly, we demonstrated that AAV2 vectors were capable of carrying nNOS cDNA and up-regulating it in the rat NTS by real time RT-PCR, Western blot analysis and immunofluorescent staining. Our studies were done 2 weeks after the injection of AAV2n-NOS vectors. Based on our studies of AAV2eGFP, higher levels of up-regulation would be expected at 3 weeks. The elevated levels of nNOS found with RT-PCR and Western blot analysis were most likely produced from NTS neurons, the target of the vector. AAV2nNOS vector thus can be used as a specific gene transfer tool to delineate the role of nNOS in CNS neurons without the complication of transfecting glial cells or other type of cells. However, interpretation of results from such studies should take into consideration that this vector may undergo anterograde and retrograde transport.

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Abbreviations

AAV	Adeno-associated virus
AAV2	Adeno-associated virus type 2
AAV2eGFP	Adeno-associated virus type 2 encoding enhanced green fluorescent protein
AAV2nNOS	Adeno-associated virus type 2 encoding cDNA of nNOS
AP	Area postrema
CMV promotor	Cytomegalovirus promotor
CVLM	Caudal ventrolateral medulla
DMV	Dorsal motor nucleus of vagus
eGFP	Enhanced green fluorescent protein
eNOS	Endothelial nitric oxide synthase
GAPDH	Glyceraldehyde-3phosphate
Gr	Gracilus nucleus
GFAP	Glial fibrillary acidic protein
NA	Nucleus ambiguus
NG	Nodose ganglion
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
RT-PCR	Reverse transcription polymerase chain reaction

RVLM	Rostral ventrolateral medulla
PGP9.5	Protein gene product 9.5
NTS	Nucleus tractus solitarii
PBS	Phosphate buffered saline
RRX	Rhodamine red X
Tr	Tractus solitarius

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Fig. 1.

Pseudo-colored confocal images of the rat NTS at the injection level 7 days after AAV2eGFP had been injected unilaterally into this nucleus, which then was subjected to immunofluorescent staining for PGP9.5 (**c, d**) to show the structure of the NTS. Numerous cells and fibers expressing eGFP (*green*), the fluorescent protein, are observed in the injected side (**b, d**). Transfected cells in the adjacent areas, such as the dorsal motor nucleus of vagus (DMV), area postrema (AP), and gracilus nucleus (Gr) are also noted. A few cells and fibers expressing eGFP are observed in the contralateral NTS (**a, c**). Panels **c** and **d** are merged confocal images of eGFP and PGP9.5-IR of the same section as **a** and **b**, respectively. *Tr* tractus solitarius. Scale bar = 100 μ m

Fig. 2.

Time course of eGFP expression in NTS (*upper panel*) and NG (*lower panel*) after unilateral injection of AAV2eGFP into the NTS. The number of eGFP expressing cells (average per section) increased from day 1 to day 28, and then decreased on day 60 in both the injected and contralateral NTS. The number of eGFP expressing cells first appeared on day 4, increased until day 28, and then decreased on day 60 in both ipsilateral and contralateral NG

Fig. 3.

Confocal images of rostral-caudal levels of the NTS 7 days after AAV2eGFP had been injected into this nucleus. Neurons and fibers expressing eGFP are seen throughout the length of NTS. **a** Rostral level at Bregma -12.7 mm, **b** intermediate level at Bregma -13.3 mm, **c** subpostremal level at Bregma -13.7 mm, **d** caudal level at Bregma -14.2 mm. *4V* 4th ventricle, *Cu* cuneate nucleus, *DMV* dorsal motor neuron of vagus, *Gr* gracilus nucleus, *Tr* tractus solitarius. Scale bar = $100\ \mu\text{m}$

Fig. 4.

Confocal images of rat NTS 7 days after injection of AAV2eGFP into the nucleus, which was then subjected to double immunofluorescent staining for PGP9.5 and GFAP. Panel **d** is a merged confocal image of panels **a** (eGFP, green), **b** (PGP9.5, red) and **c** (GFAP, blue). White arrows in **a**, **b** and **d** indicate representative cells that are positive for both eGFP and PGP9.5-IR and thus appear yellow in the merged image in panel **d**. Note that the majority of AAV2eGFP transduced NTS cells also contain PGP9.5-IR, and none of them contains GFAP-IR. Empty arrows in **c** and **d** indicate cells and processes that contain only GFAP-IR. Scale bar = 20 μ m

Fig. 5.

Confocal images with immunofluorescent stain for PGP9.5 of the ipsilateral NG, NA and CVLM 14 days after unilateral injection of AAV2eGFP into the NTS. Numerous cells and fibers expressing eGFP are seen in the NG, as shown in **a**. Panel **b**, a merged confocal image of the same section as panel **a**, shows both eGFP (green) and PGP9.5-IR (red). Arrows in **a** and **b** indicate NG neurons that are double-labeled for eGFP and PGP9.5-IR and appear yellow. Numerous eGFP expressing fibers are noted in the NA (**c**) and CVLM (**e**). Panels **d** and **f**, merged confocal images of the same sections **b** and **c**, respectively, show both eGFP expression and PGP9.5-IR. Fibers expressing eGFP in the NA and CVLM often surround and are in close apposition to cell bodies (arrows in **d** and **f**) that do not express eGFP but are positive for PGP9.5-IR. Scale bar = 100 μ m in **a–b**, 50 μ m in **c–f**

Fig. 6.

Representative Western blots showing increased expression of nNOS protein in HEK cells (*left panel*) and rat NTS (*right panel*) after transduction with AAVp-nNOS plasmid or AAV2nNOS, respectively. HEK cells that were not treated with AAVp-nNOS and rat NTS that were not injected with AAV2nNOS served as control. There was no change in GAPDH expression in rat NTS after transduction (not shown). All lanes were loaded with 10 μ g of protein. Positions of molecular weight markers are indicated on the left side of each panel

Fig. 7.

Real time RT-PCR results showing injection of AAV2nNOS significantly (*left panel*, $**P < 0.005$) increased mRNA levels of nNOS in the NTS of rats ($n = 5$), as compared with that of un-injected control rats ($n = 4$) NTS. Data were normalized by β actin expression. Analyses of the same group of rats showed that levels of eNOS mRNA were not changed (*right panel*). Values were the average of 2 sets of real time RT-PCR, with nearly identical results for each NTS sample

Fig. 8. Confocal images showing increased nNOS-IR in the NTS after AAV2nNOS injection into the area (panel **b**) as compared with that in the un-injected control rat (**a**). This increase was also observed in adjacent areas such as the dorsal motor nucleus of vagus (DMV) and hypoglossal nucleus (XII) in AAV2nNOS injected rats (panel **d**) as compared with that of the control rat (**c**). Scale bar = 50 μ m