Published in final edited form as:

J Neurosci Methods. 2014 September 30; 0: 189–192. doi:10.1016/j.jneumeth.2014.07.005.

Comparison of the efficacy of five adeno-associated virus vectors for transducing dorsal raphe nucleus cells in the mouse

Melanie Vincent¹, Guangping Gao², and Lauren Jacobson^{1,3}

¹Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, NY, 12208, USA

²Gene Therapy Center and Vector Core, University of Massachusetts Medical School, Worcester, MA, 01605 USA

Abstract

Background—Delivery of genes to various brain regions can be accomplished using serotype 2 of the adeno-associated virus (AAV). Pseudotype AAV2 vectors, composed of the AAV2 genome packaged in the capsid of an alternative serotype, have increased efficiency of viral transduction. Transduction of pseudotype AAV2 vectors depends on cell type, brain region and stage of development. The dorsal raphe nucleus (DRN) and median raphe provides the majority of serotonin to forebrain regions and are implicated in the pathology and treatment of depression and anxiety. Viral vector technology in combination with stereotaxic surgery in mice provides a means to differentiate gene function in the DRN compared to the median raphe nucleus.

New Method—Since AAV transduction efficiency has not yet been characterized for the DRN, we tested if AAV2 pseudotypes are more efficient than a standard serotype (AAV2/2) in transducing DRN cells in adult male mice on a C57BL/6 background.

Results—Although transduction did not differ significantly among vectors by 15 days post-injection, pseudotype AAV2/9 and AAV2/rh.10 vectors achieved significantly greater transduction of the DRN than did AAV2/2 and AAV2/1 vectors by 30 days post-injection. Pseudotypes AAV2/1 and AAV2/5 tended, although not significantly, to transduce DRN cells more efficiently than did AAV2/2.

Comparison with Existing Methods—At the same titer, all pseudotype AAV tested tended to transduce the DRN more efficiently than standard AAV2/2 serotype at 30 days post-injection.

Conclusions—Our results support the use of pseudotype AAV2/9 and AAV2/rh.10 for studying gene deletion or overexpression in the DRN.

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³To whom correspondence should be addressed: Lauren Jacobson, Ph. D., Center for Neuropharmacology and Neuroscience, Albany Medical College, Mail Code 136, Albany, NY, 12208, Tel. (518)-262-4993, FAX (518)-262-5799, jacobsl@mail.amc.edu.

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The authors have no conflicts of interest to report.

Keywords

Adeno-associated virus; pseudotype; transduction; dorsal raphe nucleus

1. INTRODUCTION

Of the nine raphe nuclei, the dorsal raphe nucleus (**DRN**) and median raphe nucleus produce the majority of serotonin to the brain via six ascending projections. Serotonin affects many physiological functions including sleep, appetite, circadian rhythm, autonomic function, fear, and mood, and has been implicated in the pathology and treatment of depression and anxiety disorders (10). Because both the DRN and the median raphe innervate brain structures implicated in depression and anxiety such as the prefrontal cortex, amygdala, and hippocampus (6, 11, 14), it is unclear how or if either of these regions contributes to depression and anxiety-related symptoms.

The DRN projects to additional forebrain, mid-brain, and brainstem regions not innervated by the median raphe (6) that may serve to distinguish the roles of the DRN and median raphe. For example, the DRN sends additional serotonergic forebrain projections to the nucleus accumbens (14), which is thought to be involved in the anhedonia (loss of pleasure) often observed in depression (11). The DRN also innervates the hypothalamus (14), which may be involved in the altered sleeping patterns and weight fluctuations observed in depressed patients (11). The DRN brainstem projections have been shown to control panic and sympathetic nervous system responses, which may be relevant to anxiety disorders (6). These additional projection targets suggest that the DRN may be involved in a wider range of functions relevant to depression and anxiety than the median raphe. The use of virally transduced gene expression in the mouse DRN will provide a means of rapid gene deletion *in vivo* to differentiate DRN-specific functions from those of the median raphe and other raphe nuclei.

Recombinant adeno-associated virus (rAAV) was selected for this study because rAAV efficiently transduce non-dividing cells such as neurons (9). rAAV also elicit lower immunogenic responses compared to other viral vectors (2). In addition, unlike retrovirus or lentivirus vectors, rAAV do not incorporate into the host genome (3), which could lead to mutations that compromise the effects of gene manipulation. Furthermore, since rAAV do not incorporate into the genome, this viral vector also provides a safer option for studying gene deletion or expression compared to other viruses such as retrovirus or lentivirus. Additional advantages of using rAAV vectors include the ability to achieve both region-specific and relatively rapid gene deletion in weeks compared to transgenic mouse models, which may take months to achieve complete gene deletion (2). Since serotonin facilitates neuronal division, cell migration, and synaptogenesis during development (5), compensatory effects from prolonged gene deletion could be significant (2). Therefore, rAAV vectors offer a relatively safe, rapid approach for region-specific and temporally-defined gene manipulation.

The AAV capsid proteins determine the ability of the AAV virus to enter a target cell and consequently transduce, or express, the gene of interest (3). To increase rAAV transduction

efficiency, pseudotype rAAVs have been established in which the AAV2 genome is packaged into the capsid of another AAV serotype (8). For example, AAV2/9 denotes that the AAV2 genome carrying the transgene was packaged in an AAV9 capsid.

We used stereotaxic surgery to inject different rAAV vectors into the DRN of mice to determine if pseudotypes of AAV2 are more efficient than the parent serotype (AAV2/2) in transducing DRN cells. The titer, volume, and promoter for the fluorescent tag were the same among all viral vectors, allowing the extent of fluorescent marker expression to be used to determine the transduction efficiency of the different AAV serotypes in the DRN (7). To determine the time point with maximal viral transduction, mice were sacrificed at 15 and 30 days post-injection to collect the brains for immunofluorescence imaging.

2. MATERIALS AND METHODS

2.1 Animals

All animal use was approved by the Institutional Animal Care and Use Committee of Albany Medical College and followed the standards of the NIH *Guide for the Care and Use of Animals* (Institute of Laboratory Animal Resources, 1996). Experiments used male mice on a pure C57BL/6J background with a floxed glucocorticoid receptor gene from our colony (13). Mice were housed on a 12 hour light/ 12 hour dark cycle (lights on at 7:00 a.m.) with ad libitum access to rodent chow.

2.2 Stereotaxic Injection

Mice were injected with recombinant AAV2 vectors expressing the fluorescent marker, enhanced green fluorescent protein (eGFP) obtained from the Gene Therapy Center and Vector Core of the University of Massachusetts Medical School (Worcester, MA). All mice were 2–3 months old at the time of surgery. Mice were anesthetized with 125 mg/kg ketamine and 12.5mg/kg xylazine, ip. Anesthetized mice were placed in a Kopf model 922 tapered mouse ear bars and mounted in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, California). The scalp was anesthetized with bupivacaine (Abbott Laboratories, Abbott Park, Illinois). The skull was then exposed, and bregma and lambda were visualized with a dissecting microscope (PZMIII-AAC; World Precision Instruments, Sarasota, FL). A digitizer (Stoelting, Wood Dale, IL) attached to the micromanipulator of the stereotaxic apparatus was used to locate coordinates from bregma or lambda. Burr holes were drilled in the skull using a Dremel drill equipped with a 0.75 mm carbide bit (Stoelting). 500nl of virus was injected at 10 nl/sec through 33 ga. stainless steel beveled tubing (Vita Needle, Needham, MA) attached by PE 20 tubing (Fisher, Pittsburgh, PA) to a 2 µl Hamilton Syringe (Fisher). rAAV virus stocks were diluted on the day of surgery to 6×10^{12} genomic copies/ml with sterile 1X Dulbecco's phosphate buffered saline (Cellgro, Manassas, VA). For each injection, we administered 500nl of AAV at a titer of 6×10^{12} genomic counts/ml into the DRN using the following coordinates from the bregma: anteroposterior, -4.5 mm; mediolateral, 0.0 mm; dorsoventral, -3.5 mm. The injector was left in place 9 min before being withdrawn at 0.1 mm/10 sec to permit diffusion of the virus and to minimize backflow of the virus after needle retraction. After the scalp incision was sutured, 1 mg/kg indomethacin ip (Alexis Biochemicals, San Diego, CA) in 10ml/kg of

sterile-filtered 0.1M sodium carbonate was provided for analgesia. In addition, 0.001M indomethacin (Sigma Aldrich, St. Louis, MO) solution in 0.1M sodium carbonate (Fischer) was provided as a separate drinking fluid for 3 days after surgery. Mice were individually housed after surgery. Mice were sacrificed 15 and 30 days after surgery to collect the brains for immunohistochemistry (see below).

2.3 Recombinant adeno-associated virus vectors

Recombinant adeno-associated vectors (rAAV) expressing eGFP under the control of the a promoter containing the cytomegalovirus immediate –early (CMV IE) enhancer and 260 bp (nucleotides –1261 to –1,001) of the chicken β -actin 6 promoter were packaged and provided by the Gene Therapy Center and Vector Core of the University of Massachusetts Medical School, Worcester, MA (4). The AAV2 genome was packaged into AAV capsids from AAV1, AAV5, AAV9, and AAVrh.10, generating recombinant pseudotypes that will hereafter be referred to as AAV2/1, AAV2/5, AAV2/9, and AAV2/rh.10.

2.4 Immunocytochemical analysis of GFP expression

Mice were given lethal injections of sodium pentobarbital (100 mg/kg, ip) and perfused intracardially via a peristaltic pump (Harvard Apparatus, Holliston, Massachusetts) with 0.9% saline and then 4% paraformaldehyde. Brains were stored after perfusion fixation in 4% paraformaldehyde overnight at 4°C and then cryoprotected in 30% sucrose at 4°C (13). Within two weeks of cryoprotection, coronal sections (25 μ m) were cut using a cryostat (Leica, Buffalo Grove, IL), and distributed sequentially over the wells of a 6-well tissue culture plate (Corning Incorporated Life Sciences, Lowell, MA). Free-floating sections were stored in cryoprotectant solution (50% 0.05M, Tris buffered saline (pH 7.5), 30% ethylene glycol, 20% glycerol) in the 6-well tissue culture plate at –20°C.

Coronal brain sections were washed in Tris-buffered saline (TBS) with Triton (TBS-T) (0.01M Tris pH 7.5, 0.15 NaCl, 0.3% Triton-X-100), blocked for 30 min at room temperature with 3% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS (0.01M Tris buffered saline, 0.15 NaCl), and incubated overnight at 4°C in chicken anti-GFP (1:500, Invitrogen, cat. no. A-10262). On day 2, sections were washed in TBS-T buffer, incubated for 1 hour at room temperature in Alexa-488 goat anti-chicken in TBS (1:400, Invitrogen), washed in TBS-T buffer. The sections were mounted on slides and coverslipped with N-propyl-gallate (Sigma Aldrich, St. Louis, MO).

2.5 Image Analysis

Viral transduction efficiency was assessed by GFP immunofluorescence in the widest extent of the DRN surrounding the injection track (bregma –4.36 to –4.72). Missed injections were defined as those in which there was a lack of DRN GFP immunofluorescence in all DRN sections examined, *combined with* evidence of a clogged injector (injectate was noted during surgery as not moving smoothly with the syringe plunger). The ratio of successful to total number of injections were as follows: AAV2/2, 3/3 (15 d) and 3/3 (30d); AAV2/1, 5/7 (15 d) and 5/6 (30 d); AAV2/5, 4/8 (15 d) and 5/7 (30d); AAV2/9, 3/5 (15 d) and 3/5 (30 d); AAV2/rh.10, 3/6 (15 d) and 4/10 (30 d). All images were captured at 50 $\times$ magnification on an Olympus BX50 fluorescence microscope (Center Valley, PA) using the same exposure

settings. Based on the anatomical designations described in Abrams et al. (1), a total of 12 rostrocaudal sections representative of every 100 μm from -4.12 to -5.32 mm bregma were examined for GFP immunofluorescence. GFP immunofluorescence was analyzed semiquantitatively using ImageJ software (<http://imagej.nih.gov/ij/>) at the widest extent of the DRN, surrounding the injection site (bregma -4.36 to -4.72 mm). Density readings were taken from images after thresholding to subtract background and conversion to grayscale. Density data were analyzed by analysis of variance for the effects of virus and time post-injection, with post-hoc comparisons by Fisher's Least Protected Significant Difference (Statview 5.0, SAS Institute, Cary, NC).

3. RESULTS

Transduction efficiency was determined at 15 and 30 days post-injection (Figure 1). There was no significant effect of time or of virus $\times$ time interaction on the results of semi-quantitative densitometric analysis of GFP immunofluorescence, so the two time points were analyzed separately. At 15 days after injection, transduction by AAV2/2 was very low, and AAV2/1 and AAV2/9 appeared to transduce the DRN more efficiently than the other vectors tested (Figure 1, 15d). However, semi-quantitative analysis of the density of GFP immunoreactivity at 15 days (Figure 2, 15 d) did not reveal any significant main effect of virus on GFP expression.

By 30 days post-injection, there was a significant main effect of virus on GFP immunofluorescence in the DRN ($F_{4,19} = 3.068$; $P = 0.0494$). Transduction by AAV2/2 was still minimal (Figures 1 and 2, 30 d). Transduction by AAV2/1 tended, although not significantly, to be higher than that of AAV2/2 at 30 days. Levels of GFP expression transduced by AAV2/1 also tended to be lower at 30 compared to 15 days (Figures 1 and 2), although there was no significant effect of time to allow this difference to be tested statistically. Transduction by AAV2/5 was similar to that of AAV2/1 and not statistically different from any of the other vectors, although it tended to be lower than that of AAV 2/9 ($P = 0.077$; Figure 2, 30 d). GFP immunofluorescence in the DRN was significantly greater after injection of AAV2/9-GFP or AAV2/rh.10-GFP than after injection of AAV 2/2- or AAV 2/1-GFP (Figures 1 and 2, 30 d).

4. DISCUSSION

This study compares the transduction efficiency and temporal expression of the standard serotype and four pseudotype AAV2 vectors in the DRN of adult mice. We observed differential transduction efficiencies by 30 days post-injection, as determined by the area of virally-transduced GFP expression. Maximal transduction also required at least 30 days after injection. These observations will help facilitate the design and selection of AAV vectors for gene transfer in the DRN, an important structure implicated in the pathology and treatment of depression and anxiety disorders (6).

The standard AAV2/2 serotype exhibited minimal transduction at both time points compared to the pseudotypes tested. These results are consistent with prior findings that pseudotype AAV exhibit increased transduction efficiency compared to the standard serotype in other

brain regions (2, 12, 15). AAV2/9 and AAV2/rh.10 showed the highest viral transduction of DRN cells at 30 days post-injection, achieving significantly higher levels of transduction than AAV2/1 and AAV 2/2, and in the case of AAV2/9, marginally higher levels than AAV2/5. These results agree with reports that AAV2/9 or AAV2/rh.10 are superior to AAV 2/2 or AAV 2/1 for transducing striatum (15). AAV2/9 and AAV2/rh.10 have also been found to transduce amygdala and substantia nigra more efficiently than AAV2/5 (7, 12). However, in these studies, AAV2/1 exhibited similar transduction efficiency to that of AAV2/9, reinforcing the likelihood that pseudotype efficacy depends on cell- or region-specific factors.

Pseudotype AAV2/1 exhibited transduction of DRN cells at 15 days that was similar to that of AAV2/9. However, unlike transduction by AAV2/9, transduction by AAV2/1 did not increase further by 30 days. This limited transduction may still be useful for expression of genes, such as Cre recombinase, that have irreversible effects on gene expression. Pseudotype AAV2/5 showed only slight viral transduction at both 15 and 30 days post-injection and is unlikely to be useful for viral transduction of DRN cells.

It is possible that injection accuracy limited our ability to detect more or earlier differences in transduction efficiency among the rAAV pseudotypes tested. Without a means to verify the precise volume and location of the injection, it cannot be excluded that variations in GFP expression resulted from inconsistent virus delivery rather than differences in transduction efficiency. Nevertheless, the consistently low levels of transduction achieved with AAV2/2, the relatively similar levels of transduction by AAV2/1 and AAV2/5 across time, and the significantly greater transduction accomplished with more than one pseudotype at 30 days make it unlikely that results are due only to random effects of injection placement.

Taken together, we have shown that AAV pseudotypes AAV2/9 and AAV2/rh.10 efficiently transduce DRN cells 30 days post-injection. The parent AAV2/2 serotype achieved negligible levels of transduction at 15 and 30 day post-injection and is not recommended for viral transduction of DRN cells.

Acknowledgments

The author would like to thank Dr. Rifat Hussain for her assistance in the initial experiments and Julia Nalwalk for her expertise in the injection techniques used in this paper. This study was supported by RO1MH080394 from the National Institute of Mental Health to LJ.

Abbreviations

DRN	Dorsal raphe nucleus
eGFP	enhanced green fluorescent protein
rAAV	recombinant adeno-associated virus
TBS	Tris buffered saline
TBS-T	Tris-buffered saline with 0.3% Triton-X-100

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Figure 1.

Pseudotype adeno-associated virus (AAV) transduction in the DRN. GFP expression 15 days (top row) and 30 days (bottom row) after a single 500nl (6×10^{12} genomic counts/ml) midline injection of the standard AAV2/2 serotype or each virus pseudotype AAV into the DRN (see Methods for details and coordinates). The dashed lines outline the DRN, whereas dotted lines outline the aqueduct. The white scale bar in the lower right-hand panel represents 500 μ m.

Figure 2.
Results of semi-quantitative analysis of GFP immunofluorescence transduced by the standard serotype AAV2/2 (2/2), and by the AAV2/1, AAV2/5, AAV2/9, and AAV2/rh.10 pseudotypes (2/1, 2/5, 2/9, and 2/rh.10) at 15 (left) and 30 days (right) after DRN injection. Group *N* are shown within the data bars. * $P < 0.05$ vs. AAV2/2; #, $P < 0.05$ vs. AAV2/1.