

Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Hypertension 2002;39;603-608

DOI: 10.1161/hy0202.103295

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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Selective Gene Transfer to Key Cardiovascular Regions of the Brain: Comparison of Two Viral Vector Systems

Puspha Sinnayah, Timothy E. Lindley, Patrick D. Staber,
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Abstract—The systemic renin-angiotensin system (RAS) plays a critical role in cardiovascular (CV) homeostasis. All components of the RAS are also known to be produced cell-specifically within specific brain regions, although the role of the brain RAS relative to the systemic RAS has remained a puzzle due to the difficulty of dissecting these two systems. Selectively targeting these regions with genes that modify the RAS could help unravel this puzzle. We compared the ability of adenovirus (Ad) and lentivirus (feline immunodeficiency virus, FIV) vectors to mediate gene delivery in vivo to the supraoptic nucleus (SON) and subfornical organ (SFO), two important CV control regions known to express the various RAS genes. SON or SFO of adult C57BL/6 mice (n=37) were stereotaxically injected with replication-deficient recombinant Ad or FIV harboring a β -galactosidase (β -gal) reporter gene. At 1, 3, or 8 weeks post-injection, brain sections were processed for β -Gal activity, double immunofluorescence to verify cell-type specificity of viral transduction, or immunohistochemical detection of inflammatory mediators. Our results demonstrate that: (1) murine SFO and SON can be selectively targeted for gene transfer in vivo; (2) FIV mediated neuron-specific gene delivery, whereas Ad transduced both neuronal and glial cell types in SFO and SON; (3) Ad injected into the SON transduced neurons within the SFO through retrograde transport, whereas FIV did not; (4) β -gal activity remained stable for 3 weeks but then declined by 8 weeks with Ad, while minimal decline occurred with FIV; (5) FIV did not cause inflammatory responses, whereas infiltrate was detectable in Ad-injected SFO and SON. These vectors are potentially important tools for dissecting the cell- and site-specific components of the brain RAS and other important CV regulatory systems within this circuitry, and may have therapeutic applications for centrally mediated CV diseases. (*Hypertension*. 2002;39[part 2]:603-608.)

Key Words: renin-angiotensin system ■ brain ■ gene regulation

The importance of the classic systemic renin-angiotensin system (RAS) in cardiovascular (CV) and volume homeostasis is well established. However, the CV regulatory role of intrinsic tissue RAS, defined as tissue-based systems with the potential for local angiotensin II (Ang-II) production and action, remain unresolved because of the difficulty in experimentally dissecting tissue and systemic RAS. The brain RAS¹ has remained particularly puzzling, in part because of the direct interfacing of the brain and systemic RAS at circumventricular organs (devoid of a blood-brain-barrier), and an inability to manipulate the brain RAS cell and site selectively.

The RAS in the forebrain neural circuitry containing the subfornical organ (SFO)-supraoptic nucleus (SON) axis is one example of a system that is known to be critically involved in blood pressure and body fluid regulation, yet that remains poorly understood because of difficulties in dissecting it. The SFO, a circumventricular organ, is thought to couple blood-borne signals such as Ang-II with brain struc-

tures that trigger endocrine and autonomic reflexes designed to restore homeostasis.² The hypothalamic nucleus SON, containing magnocellular vasopressinergic neurosecretory cells, receives direct projections from neurons of the SFO.^{3,4} Stimulation of the SFO-SON pathway is considered to be important in the control of osmolality and blood pressure by affecting the release of vasopressin,^{5,6} although the precise mechanisms are not known. The RAS has been implicated as an important mediator within this axis,² and various RAS components are expressed cell-selectively within SFO and SON. For example, angiotensinogen is expressed in neurons and glial cells of the SFO and SON,^{7,8} whereas Ang-II receptors are expressed specifically within neurons in these regions.⁹⁻¹¹ However, the precise role of the RAS, as well as other signaling systems within this important CV control circuitry, remain to be elucidated.

Cell- and site-selective targeting of the SFO-SON axis with genes that modify the RAS and/or other signaling pathways could help unravel important mechanisms of CV regulation

Received September 24, 2001; first decision November 2, 2001; accepted November 21, 2001.

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both in health and disease. Replication-deficient recombinant viral vectors have become important tools for gene delivery to the central nervous system (CNS) and other tissues in recent years.^{12–14} Adenovirus (Ad) and lentivirus, such as the feline immunodeficiency virus (FIV), appear to be the most promising vehicles for localized gene transfer to the brain, although each exhibit unique properties. Ad vectors allow highly efficient (although transient) delivery of relatively large transgenes, and infect both neuronal and glial cell types.¹³ Ad is also known to be taken up by terminals and retrogradely transported to neuronal somata distant to the site of injection.^{15,16} In contrast, when the envelop protein of the feline immunodeficiency virus is replaced by the envelop protein from the vesicular stomatitis virus (VSV-G), this VSV-G pseudotyped FIV mediates gene transfer predominantly to neurons rather than to nonneuronal cells,¹⁷ and retrograde transport is limited to local, but not distant, neuronal somata.¹⁷ Additionally, since FIV integrates the transgene into the host genome, transgene expression is highly stable.¹⁸ The majority of studies comparing Ad, FIV, and other viral vectors for CNS gene transfer have been carried out *in vitro*^{14,19,20} or in cerebellum, cerebrum, or striatum *in vivo*.^{13,16–18,21,22} *In vivo* Ad- or FIV-mediated gene transfer to specific CV control regions has not been extensively explored.

We hypothesized that by taking advantage of the unique properties of each of these viral vectors to target gene transfer to appropriate populations of cells within the SFO-SON axis, we could address our long-range goal of dissecting the role of the brain RAS within this important CV control circuitry. The aims of the current study were (1) to establish the feasibility of selectively targeting murine SFO and SON for gene transfer *in vivo* and (2) to compare Ad and FIV transduction of SFO and SON with respect to cell-type specificity, stability, and local versus distant infectivity through retrograde transport.

Methods

Animals

Experiments were performed on adult (8 to 10 weeks old) C57BL/6 male and female mice. Mice were individually housed in a temperature-controlled, 12:12-hour light-dark cycle and had free access to standard mouse chow (LM-485; Teklad Premier Laboratory Diets) and water. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Preparation of Viral Vectors

Ad and FIV vectors encoding *E. coli* β -galactosidase (Ad β -gal and FIV β -gal) driven off the cytomegalovirus promoter were prepared in the Gene Transfer Vector Core Laboratory at The University of Iowa as described in detail previously.^{13,23} Briefly, the recombinant Ad vector is based on the human adenovirus serotype 5, from which the E1a and E1b replication genes have been deleted.²⁴ The FIV vector construct containing mutant *vif* and *orf2* sequences was cotransfected with a vesicular stomatitis glycoprotein G envelope protein-expressing plasmid.²³ FIV β -gal and Ad β -gal titers were $\approx 3 \times 10^8$ TU/mL and 1×10^9 pfu/mL, respectively.

Viral Injections Into SFO and SON

Mice were anesthetized with ketamine (90 mg/kg, IP) and acepromazine (1.8 mg/kg, IP), placed in a stereotaxic instrument (David Kopf Instruments) and the skull exposed by an incision. After leveling the

skull between lambda and bregma, a burr hole was drilled and a Hamilton syringe was inserted at coordinates (relative to bregma):²⁵ SFO (n=19): midline, 0.2 mm caudal, 2.9 mm ventral; SON (n=18): 3.1 mm either side of midline at 20° angles, 0.6 mm caudal, 5.0 mm ventral. These coordinates placed the injector just dorsal to each site, allowing selective injection without damage to the structure. Titer-matched vector stocks of FIV β -gal (SFO, n=8; SON, n=10) or Ad β -gal (SFO, n=11; SON, n=8) were injected in volumes of 200 nL for SFO and 300 nL for SON (bilaterally). Incisions were sutured and mice were kept warm until fully recovered from anesthesia, at which time they were returned to their home cages.

β -Gal Histochemistry and Double Immunofluorescence

At 1, 3, or 8 weeks after virus injections, mice were sacrificed and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in PBS. Brains were removed, post-fixed, saturated in 20% sucrose, and cryo-sectioned (coronal, 30 to 40 μ m). Some sections were processed for β -galactosidase activity using 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal, Boehringer Mannheim), counterstained with eosin, and analyzed by light microscopy as described.^{13,26} Separate brains were processed for double immunofluorescence for cell-specific detection of β -galactosidase (β -gal) protein. Sections were incubated with a rabbit anti- β -gal antibody (1:500, 5 Prime-3 Prime) combined with either mouse monoclonal anti-MAP-2 (1:500, Sternberger Monoclonal Antibodies) or mouse monoclonal anti-GFAP (1:1000, Chemicon) overnight at 4°C, followed by incubation in Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:200, 2hrs, Molecular Probes) and rhodamine conjugated goat anti-mouse antibody (1:200, 2hrs, Sigma). Mounted sections were analyzed by confocal laser microscopy (Zeiss LSM 510).

Immunohistochemical Analysis of Inflammatory Responses to Viral Injections

A subset of FIV β -gal-injected (n=6) and Ad β -gal-injected (n=4) mice were sacrificed at 1, 3, or 8 weeks postinjection, and brains were prepared as described above. Alternating serial sections were X-gal stained (see above) or incubated for 24 hours at 4°C with either rat anti-CD-3 (lymphocytes, 1:200, Novacastra) or anti-F4/80 (macrophages, 1:200, Serotec), followed by biotinylated goat anti-rat IgG, then streptavidin-horseradish peroxidase. Sections were stained with DAB and counterstained with hematoxylin.

Cell Counts

Numbers of β -gal-positive neuronal and glial cell fragments were scored in three to four corresponding serial sections each for SFO and SON (20 to 40 \times objective). Cell-type scoring was done both in X-gal-stained sections based on morphological analyses of distinct characteristics of neuronal and glial cell types, and in immunolabeled samples based on dual staining for β -gal and cell-specific markers.

Data Analysis

Cell counts were calculated as an average number of β -gal-positive fragments over three to four serial sections per animal, and data were expressed as mean \pm SEM for each site and time point. Data were analyzed by ANOVA followed by the Student's modified *t* test with Bonferroni correction for multiple comparisons between means using the modified error mean square term from the ANOVA.

Results

Titer-matched stocks of FIV β -gal or Ad β -gal were microinjected into the SFO or SON of adult mice, and transduction was evaluated with regard to localization, cell-type specificity, and stability over time. First, highly localized and site-specific transduction of SFO and SON was achieved with both viruses as determined by light microscopic visualization of X-gal staining (Figure 1) or confocal analysis of immunostaining for β -gal (Figure 2). With the injector placed just

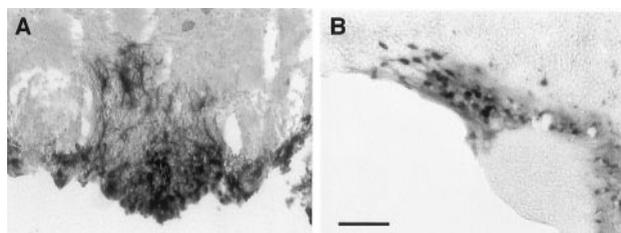


Figure 1. Representative photomicrographs of mouse brain sections stained for β -gal activity after stereotaxic microinjection of Ad- β Gal into SFO (A) or FIV- β Gal into SON (B) 3 weeks earlier. Magnification bar represents 25 μ m.

dorsal to either structure and with an observed spread of both viruses approximately 1 mm from the injector tip, both brain sites were effectively transduced throughout. It should be noted that both the annulus and core regions of the SFO²⁷ exhibited β -gal-positive staining. There was occasional transduction along the injector track itself, but otherwise spread of either virus at the injection site was limited to the target structure. β -gal expression was not observed in any peripheral tissues after viral injections into either SFO or SON. We achieved such site-specific targeting of these small brain regions approximately 70% and 50% of the time for SON and SFO, respectively.

Within these sites, there was differential transduction of neuronal and glial cell types with the two viral vectors. In

Ad β -gal-injected SFO or SON, transgene-positive cells showed neuronal, glial, and microglial morphologies, and when costained for immunofluorescence, expressed both neuronal (MAP-2) and glial (GFAP) marker proteins (Figure 2, top). In contrast, only cells with neuronal morphology were transduced by FIV- β -gal, with colocalization of β -gal being limited to MAP-2-immunoreactive cells (Figure 2, bottom). Numbers and types of cells in SFO and SON transduced by the two vectors are summarized in Figure 3. Both neuronal and glial cell types exhibited β -gal-positive staining in Ad β -gal-injected SFO and SON, with a larger proportion of glial cells transduced in the SFO, but approximately equal numbers of transgene-expressing neurons and glia in the SON (Figure 3A). In contrast, injection of FIV β -gal resulted in the exclusive transduction of neurons in both SFO and SON (Figure 3B), with a greater total number of neurons showing β -gal expression, especially in SFO, compared with Ad β -gal-injected sites.

There were also differences between the two viruses with regard to stability of transgene expression over time. The pattern and density of β -gal-positive cells were not significantly changed for as long as 2 months after FIV β -gal injection in either SFO or SON (Figure 3). In contrast, by 8 weeks following Ad β -gal administration to SFO or SON, transgene expression in both cell types was reduced by approximately 75% compared with that at 1 and 3 weeks

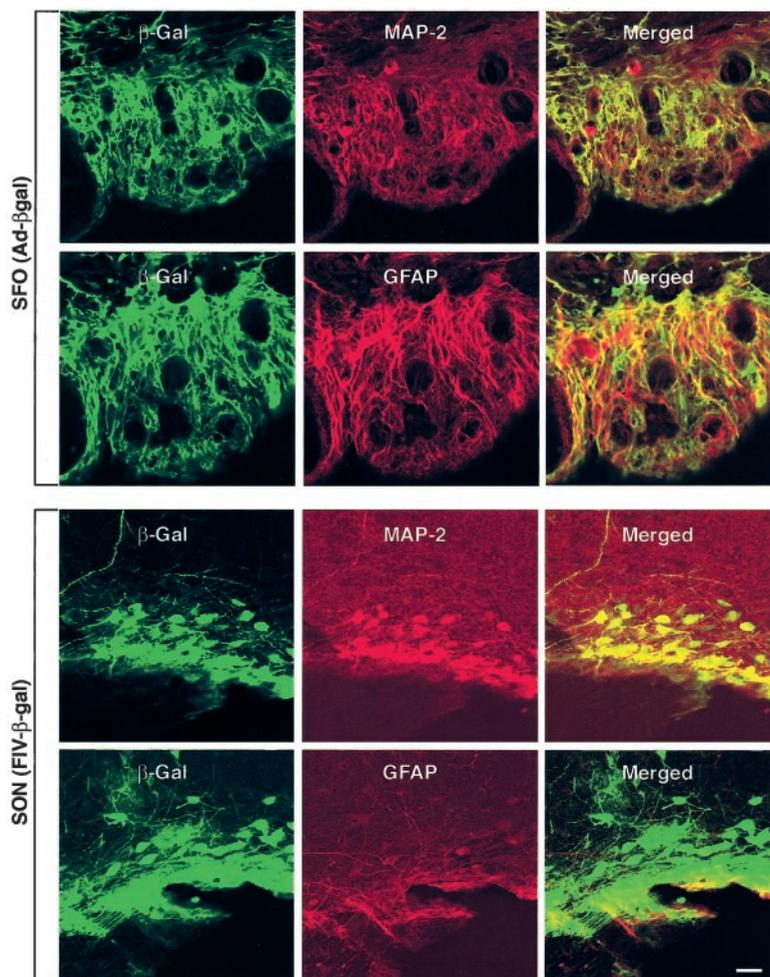


Figure 2. Representative confocal images of immunocytochemical staining of SFO transduced with Ad- β Gal (top) and SON transduced with FIV- β Gal (bottom) 3 weeks earlier. Coronal brain sections were dual stained for β -gal (green) and either neuronal (red nuclei) or glial (red processes) markers. Cell-specific double labeling is shown in merged (yellow) images. Magnification bar represents 100 μ m.

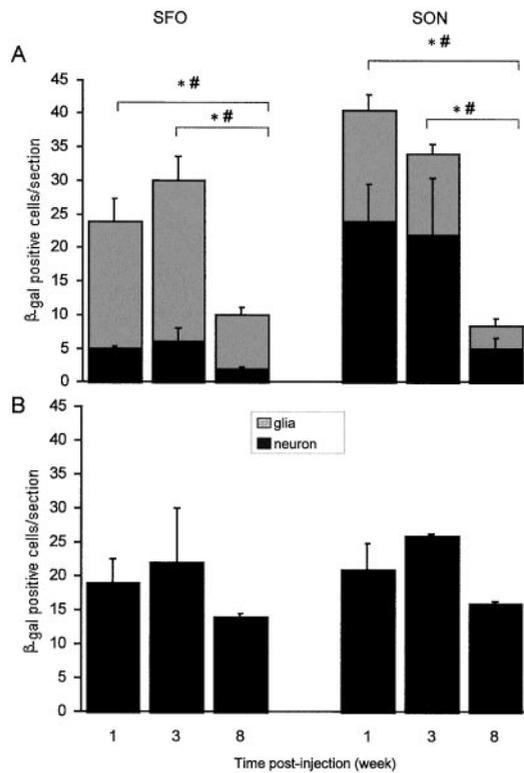


Figure 3. A summary of the numbers of β -gal-positive neuronal and glial cells (averaged over 3 to 4 sections per animal) at 1, 3, or 8 weeks after stereotaxic microinjection of Ad- (A) or FIV- β Gal (B) into the SFO or SON. Cell counts were made based on morphological analysis of X-gal-positive cells or dual immunostaining of β -gal and cell-specific markers. Data represent mean \pm SEM; * P <0.05 for neurons; # P <0.05 for glia.

postinjection. Numbers of β -gal-positive cells were comparable at the 1- and 3-week time points for Ad β -gal-infected SFO and SON. Invading lymphocytes and macrophages were detected in and immediately around the injector tract for both FIV β -gal- and saline-treated groups, which most likely represents the reaction to mechanical trauma from the injector itself. No further infiltrate was observed in FIV β -gal-injected SFO and SON at 1, 3, or 8 weeks, whereas CD-3- and F4/80-positive cells were detectable in these sites at the various time points following Ad- β -gal (data not shown). However, this was a relatively mild response that was not associated with obvious tissue trauma or changes in cell morphology in the injected sites.

Previous studies in other brain regions have shown that Ad can undergo retrograde transport to neuronal soma distant to the site of injection, whereas FIV transport is more limited.^{16,17} We examined both X-gal- and immunofluorescence-

processed sections for β -gal-positive cells in sites other than those directly injected with FIV β -gal or Ad β -gal. Figure 4 shows a representative example of a double-labeled SFO from an animal that had been injected with Ad β -gal into SON (bilaterally) 3 weeks earlier. β -gal was detected in the distant SFO, and was localized to cells with neuronal morphology. The complete lack of colocalization of β -gal with the glial marker protein GFAP suggests neuronal expression of the transgene in this site (Figure 4). Because SON receives direct short-loop projections from the SFO,⁴ these results suggest that Ad β -gal was able to undergo retrograde transport from terminals in the SON back to neuron somata in the SFO. No other sites contained retrograde labeled cells. Finally, only local transgene expression at the primary site of injection was observed with local FIV β -gal. We found no evidence of β -gal-positive staining in secondary regions because of either transport or diffusion.

Discussion

Classical studies have established that forebrain neural circuitry containing the SFO and SON plays a major role in CV regulation both in normal and pathophysiological states, yet the precise mechanisms and signaling molecules remain unknown. Powerful new experimental strategies such as somatic gene transfer via recombinant viral vectors offer potential avenues for dissecting these important systems. However, despite numerous reports comparing different viral vectors in CNS regions such as cerebellum,¹⁷ cerebrum,²⁸ and striatum^{12,21} comparatively little is known about these vectors as gene delivery systems for specific CV control nuclei. Using the SFO-SON axis as a model system, we compared Ad and FIV, 2 viral vectors that show particular promise for gene transfer to the CNS.^{13,29} We demonstrated that mouse SFO or SON could be selectively targeted for gene transfer in vivo using either vector, but that Ad and FIV differed with respect to cell-type specificity, stability of transgene expression, and transport to distant sites. FIV transduced exclusively neurons in the SFO and SON, whereas Ad targeted both neurons and glia within these sites with approximately equal affinity. Transgene expression was sustained over the long-term with FIV (at least 2 months), whereas Ad-mediated gene transfer was more transient. Finally, Ad was able to transduce the SON and undergo retrograde transport to target a subpopulation of SFO neurons for transgene expression, whereas FIV-mediated gene delivery remained highly localized to the primary site of injection.

To our knowledge, this is the first report of in vivo delivery of a transgene selectively to the SFO, either directly or indirectly, in any species. An earlier report demonstrated

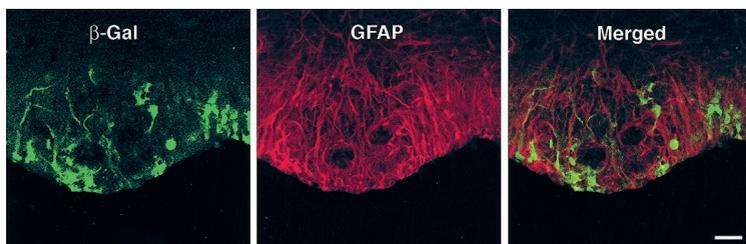


Figure 4. Representative confocal images of retrograde staining in the SFO 3 weeks after injection of Ad- β Gal into the SON (bilaterally). Coronal brain sections were double-immunolabeled for β -gal (green) and a glial (red processes) marker. The lack of β -gal-positive glial cells is shown in the merged image. Magnification bar represents 100 μ m.

Ad-mediated transduction of cultured rat SFO cells *in vitro*, but transgene expression was not detected in sections following *in vivo* administration of the virus.³⁰ The SFO is a complex CNS structure that is thought to provide an interface between blood pressure and volume regulatory signals circulating in the blood, and brain circuitry involved in homeostatic neurohumoral reflexes.² It has been implicated in normal CV regulation and in the pathogenesis of hypertension for many years,² although the underlying mechanisms are not known. There are several properties of the SFO that the unique features of the Ad and FIV vectors could potentially be brought to bear on in dissecting this pivotal structure. For example, the RAS, implicated as an important mediator in SFO's coupling of circulating signals with efferent pathways, is expressed cell-selectively in the SFO. Angiotensinogen, the only known substrate of Ang-II production, is expressed primarily in glial cells throughout the brain³¹ but is highly localized to neurons in a few select nuclei including the SFO.^{8,7} Ang-II receptors are expressed neuron-selectively within SFO, but other RAS components exist both in glial and neuronal cell types in this site.⁹ Indeed, a similar cell-specific expression of the various RAS components exists in the SON,^{32,33} although the significance is unknown. Our data showing neuron-exclusive transduction of SFO and SON with FIV, but equal targeting of glia and neurons in these sites with Ad, lead us to speculate that each of these vectors, harboring genes that modify or disrupt expression of particular RAS genes, could be useful in understanding the significance of the different RAS-containing cell types. Certainly, other signaling molecules in this axis could be similarly targeted.

Another poorly understood attribute of the SFO that could lend itself to investigation by Ad-mediated gene delivery is the relative significance of different subgroups of neurons within this structure that have specific efferent connections.²⁷ It is known that subpopulations of neurons in the outer zone or annulus of the SFO send projections to vasopressin-containing neurons of the hypothalamic paraventricular nucleus (PVN) and SON, to parvocellular neurons of the PVN, and to neurons of the median preoptic nucleus, all sites known to be involved in CV homeostasis.²⁷ Much less is known about the SFO neurons projecting from the core and caudal parts. Our lack of understanding of these various neuronal subgroups is due in part to the lack of investigative methods for selectively manipulating them. Findings in the current study and those of others¹⁴ suggest that, by capitalizing on the ability of Ad to be taken up by nerve terminals at the primary injection site and undergo retrograde transport to the cell soma, genes can be delivered to specific subpopulations of neurons that are difficult to approach directly. Our findings suggest that a subgroup of SFO neurons was targeted *in vivo* by retrograde transport of Ad β -gal from the SON, the first such report to our knowledge. A similar strategy has been used to successfully target genes back to neurons of the PVN and SON via pituitary injections of an Ad vector.³⁴ We are currently investigating the feasibility of inducing transgene expression in additional functionally unique subgroups of SFO neurons by selectively injecting Ad β -gal into its other sites of projection, eg, PVN and median preoptic nucleus.

Additionally, since it has been shown that higher Ad titers at the primary injection site transduce a greater number of cells at a distance in the target region,^{16,19} we are currently exploring different Ad titers in our studies. Defining a concentration that allows for efficient transport to distant neurons without undue inflammatory responses at the primary injection site is the goal.

In the SFO-SON axis, FIV appears to be the vector of choice for spatially restricting transduction to the site of injection without transgene delivery to a secondary region by retrograde transport or diffusion. We consistently showed highly localized transgene expression in FIV β -gal-injected SFO or SON, with no observed transport or spread of the virus, even with higher titers (data not shown). This may be a consequence of unique characteristics of these sites since Alisky et al reported retrograde transport of FIV to cerebellar nuclei outside of the primary injection site, albeit to nearby sites.¹⁷ Another feature of the FIV vector that may be a function of the particular brain region being transduced is the tropism of the virus for neurons. In both the SFO and SON, β -gal-positive staining was found exclusively in neurons, with no evidence of transgene expression in nonneuronal cells. Studies using FIV in other brain regions report up to 20% of transduced cells being nonneuronal.²² Additionally, FIV has been shown to efficiently transduce some populations of neurons but not others in the cerebellum.¹⁷ The mechanism underlying the tropism of the VSV-G pseudotyped FIV is incompletely understood, but differences in expression of the receptor for the virus may play a role in the regional differences in FIV-mediated transduction.²⁹

Consistent with other reports,¹³ our results indicate that there is a decline in numbers of β -gal-positive cells with Ad β -gal but not FIV β -gal over time. The stability of FIV-mediated gene transfer may in part be due to the well-known feature of lentiviruses to integrate the message into the host genome, whereas adenoviral DNA remains primarily episomal.^{35,18} Additional hypotheses for transient Ad-mediated gene transfer include a host immune response to infected cells, toxicity of the transgene product, or alterations in the level of transcription associated with the cytomegalovirus promoter over time.^{13,35}

The power of recombinant viral vectors is already being deployed to study central regulation of CV function. Several studies reporting intracerebroventricular administration of viral vectors encoding antisense genes in rats have provided further evidence of the importance of the RAS in blood pressure regulation.^{36,37} The long-term goal of our work is to capitalize on the unique properties of particular viral vectors to allow selective targeting of transgenes to specific populations of cells within key forebrain sites, including but not limited to the SFO-SON axis. For example, we are currently establishing the tools for generating brain site-selective RAS gene deletion using the Cre-loxP system.³⁸ In particular, we plan to utilize the regional and cell-type specificity of Ad and FIV vectors demonstrated herein to mediate selective delivery of Cre recombinase in mice with floxed genes.³⁸ We believe viral-mediated delivery of Cre and other transgenes will provide a powerful means for dissecting the molecular

mechanisms by which key brain regions regulate CV function in health and disease.

Acknowledgments

This work was supported by grants to R.L.D. from the American Heart Association (0030017N) and the National Institutes of Health (HL63887). The authors would like to thank Mr Jeremy Bonsol and Ms Molly Wise for excellent technical assistance, and Dr Stanley Perlman and Mr Ajai Dandekar for help with the immune response analyses.

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