

**Puspha Sinnayah, Timothy E. Lindley, Patrick D. Staber, Beverly L. Davidson,
Martin D. Cassell and Robin L. Davisson**

Physiol Genomics 18:25-32, 2004. First published Apr 6, 2004;
doi:10.1152/physiolgenomics.00048.2004

You might find this additional information useful...

This article cites 54 articles, 19 of which you can access free at:

<http://physiolgenomics.physiology.org/cgi/content/full/18/1/25#BIBL>

This article has been cited by 9 other HighWire hosted articles, the first 5 are:

Scavenging superoxide selectively in mouse forebrain is associated with improved cardiac function and survival following myocardial infarction

T. E. Lindley, D. W. Infanger, M. Rishniw, Y. Zhou, M. F. Doobay, R. V. Sharma and R. L. Davisson

Am J Physiol Regulatory Integrative Comp Physiol, January 1, 2009; 296 (1): R1-R8.

[Abstract] [Full Text] [PDF]

An Intracellular Renin-Angiotensin System in Neurons: Fact, Hypothesis, or Fantasy

J. L. Grobe, D. Xu and C. D. Sigmund

Physiology, August 1, 2008; 23 (4): 187-193.

[Abstract] [Full Text] [PDF]

Optimized adeno-associated virus 8 produces hepatocyte-specific Cre-mediated recombination without toxicity or affecting liver regeneration

K. J. Ho, C. E. Bass, A. H. K. Kroemer, C. Ma, E. Terwilliger and S. J. Karp

Am J Physiol Gastrointest Liver Physiol, August 1, 2008; 295 (2): G412-G419.

[Abstract] [Full Text] [PDF]

RNA interference shows interactions between mouse brainstem angiotensin AT1 receptors and angiotensin-converting enzyme 2

Z. Lin, Y. Chen, W. Zhang, A. F. Chen, S. Lin and M. Morris

Exp Physiol, May 1, 2008; 93 (5): 676-684.

[Abstract] [Full Text] [PDF]

Longitudinal noninvasive monitoring of transcription factor activation in cardiovascular regulatory nuclei using bioluminescence imaging

J. R. Peterson, D. W. Infanger, V. A. Braga, Y. Zhang, R. V. Sharma, J. F. Engelhardt and R. L. Davisson

Physiol Genomics, April 1, 2008; 33 (2): 292-299.

[Abstract] [Full Text] [PDF]

Updated information and services including high-resolution figures, can be found at:

<http://physiolgenomics.physiology.org/cgi/content/full/18/1/25>

Additional material and information about *Physiological Genomics* can be found at:

<http://www.the-aps.org/publications/pg>

This information is current as of June 10, 2009 .

Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain

Puspha Sinnayah,¹ Timothy E. Lindley,¹ Patrick D. Staber,²
Beverly L. Davidson,^{2,3} Martin D. Cassell,¹ and Robin L. Davisson¹

Departments of ¹Anatomy and Cell Biology, ²Internal Medicine, and ³Neurology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa 52242

Submitted 24 February 2004; accepted in final form 30 March 2004

Sinnayah, Puspha, Timothy E. Lindley, Patrick D. Staber, Beverly L. Davidson, Martin D. Cassell, and Robin L. Davisson. Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain. *Physiol Genomics* 18: 25–32, 2004. First published April 6, 2004; 10.1152/physiolgenomics.00048.2004.—The Cre/loxP system has shown promise for investigating genes involved in nervous system function and pathology, although its application for studying central neural regulation of cardiovascular function and disease has not been explored. Here, we report for the first time that recombination of loxP-flanked genes can be achieved in discrete cardiovascular regulatory nuclei of adult mouse brain using targeted delivery of adenovirus (Ad) or feline immunodeficiency virus (FIV) bearing Cre recombinase (Ad-Cre, FIV-Cre). Single stereotaxic microinjections of Ad-Cre or FIV-Cre into specific nuclei along the subfornical organ-hypothalamic-hypophysial and brain stem-parabrachial axes resulted in robust and highly localized gene deletion as early as 7 days and for as long as 3 wk in a reporter mouse model in which Cre recombinase activates β -galactosidase expression. An even greater selectivity in Cre-mediated gene deletion could be achieved in unique subpopulations of cells, such as vasopressin-synthesizing magnocellular neurons, by delivering Ad-Cre via retrograde transport. Moreover, Ad-Cre and FIV-Cre induced gene recombination in differential cell populations within these cardiovascular nuclei. FIV-Cre infection resulted in LacZ activation selectively in neurons, whereas both neuronal and glial cell types underwent gene recombination upon infection with Ad-Cre. These results establish the feasibility of using a combination of viral and Cre/loxP technologies to target specific cardiovascular nuclei in the brain for conditional gene modification and suggest the potential of this approach for determining the functional role of genes within these sites.

adenovirus; feline immunodeficiency virus; renin-angiotensin system; central nervous system; gene therapy; blood pressure; hypertension

THE ABILITY TO STUDY MECHANISMS underlying physiological and pathophysiological processes has been revolutionized by the development of methods that allow spatiotemporal control of gene deletion or expression in transgenic and knockout animals (14). The ability to interfere with the function of a single protein in a specific tissue or cell type allows unprecedented flexibility for exploring gene function in both health and disease. This is particularly true when studying complex systems that encompass multiple genes and/or widespread tissue expression patterns. The multigene renin-angiotensin system

(RAS) is one such example that has proved difficult to functionally dissect because of the interfacing of the classic systemic RAS and the individual tissue RASs located in the heart, kidneys, adrenals, vessels, and brain (13). Indeed, the brain RAS has remained especially puzzling, in part because of its complex regional and cellular gene expression patterns and the lack of tools for targeting it selectively (34).

One approach for inducing tightly restricted gene modification in mice is with the Cre/loxP system (42). Cre is a bacteriophage P1-derived DNA recombinase that catalyzes recombination between two appropriately oriented 34-bp recognition sequences termed loxP sites (47, 48). LoxP sites can be inserted into the mouse genome such that they flank important coding sequences of a particular gene of interest (gene “floxed”). Genes modified to contain loxP sites within introns function normally in the absence of Cre, but are rendered nonfunctional in the presence of Cre due to excision of the floxed segment (41, 42). Since the floxed gene is found in every cell of the mouse, the specificity of the gene excision arises from tissue- or cell-selective delivery of Cre recombinase. This can be accomplished through breeding of the model containing the floxed allele with a second transgenic mouse that expresses Cre only in the cells/tissue of interest through the use of a highly specific promoter (22). However, in cases where such promoters are not available, recombinant viral vectors provide an important alternative for selective delivery of Cre. Indeed, we and others have demonstrated the utility of adenovirus (46), adeno-associated virus (19), and herpes virus (5) to deliver Cre recombinase to the mouse *in vivo*.

The Cre/loxP system has increasingly shown promise for investigating genes involved in central nervous system (CNS) function. To date, most studies have relied on engineered transgenic models with Cre recombinase driven off brain region-selective promoters (49). However, for brain regions such as cardiovascular regulatory circuitries that do not yet have specific promoters identified (10, 20), this approach has been limited. Recent studies demonstrate that viral vectors provide a powerful alternative for delivering genes to brain nuclei involved in cardiovascular regulation (20, 45). For example, our findings showed that adenovirus (Ad) and feline immunodeficiency virus (FIV) can be used to efficiently and selectively target gene delivery in mice to regions such as the subfornical organ (SFO) and the supraoptic nucleus (SON), two brain nuclei implicated in the control of blood pressure and volume homeostasis (45). We also demonstrated that the two viruses differ markedly with regard to cell-type specificity and local vs. distant infectivity in these neural axes. For example, FIV targeted transgene expression selectively to neurons and in tissue only at the primary injection site. In contrast, Ad trans-

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

Address for reprint requests and other correspondence: R. L. Davisson, Dept. of Anatomy and Cell Biology, Univ. of Iowa Roy J. and Lucille A. Carver College of Medicine, 1-251 BSB, Iowa City, IA 52242 (E-mail: robin-davisson@uiowa.edu).

duced both neurons and glial cells and could be used to target distant subpopulations of neurons through retrograde transport (45). These studies suggested that by capitalizing on the unique properties of Ad and FIV for transgene delivery, neural mechanisms within central cardiovascular networks could be effectively dissected (45).

The objective of the current study was to establish the feasibility of utilizing these two recombinant viruses to deliver Cre recombinase and induce highly localized gene recombination in key cardiovascular networks, i.e., the SFO-hypothalamic-hypophysial and brain stem-parabrachial axes. The rationale for focusing on these circuits is twofold. First, both are strongly implicated in cardiovascular and volume homeostasis, although the mechanisms are poorly understood (10). Second, genes of the RAS are expressed in these neural networks, but the functional significance of this remains to be determined (11, 24, 25). Using a transgenic mouse model generated specifically as an *in vivo* reporter for Cre-triggered recombination ("CAG-CATZ"; Refs. 1, 4), we report here that virally delivered Cre can mediate efficient gene ablation in highly select populations of cells in these central cardiovascular regulatory sites. These studies suggest the potential of the Cre/loxP system for unraveling complex mechanisms of central neurocardiovascular regulation such as that mediated by the brain RAS.

METHODS

Animals. Experiments were performed in adult (8–10 wk) transgenic reporter mice ("CAG-CATZ") in which Cre recombinase activates β -galactosidase (β -gal) expression (kind gift of Dr. M. Schneider, Baylor College of Medicine). It has been shown previously that the Cre-dependent reporter transgene is expressed ubiquitously in CAG-CATZ mice, and the model has been characterized extensively for Cre-induced gene deletion at the DNA, RNA, and protein levels (1, 4, 36, 37, 39, 40). Mice were individually housed in a temperature-controlled, 12:12-h light-dark cycle and had free access to standard mouse diet (LM-485; Teklad Premier Laboratory Diets) and water. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Viral vectors. All Cre-encoding and control viral vectors were provided by the University of Iowa Gene Transfer Vector Core. The Ad vector encoding Cre recombinase (Ad-Cre) was generated and characterized previously as described (46). The Cre transgene is driven off the human cytomegalovirus (CMV) promoter, and the Ad backbone is based on dl309 and is of serotype 5 (18). The Ad vector without an inserted transgene was used as a control for the vector itself.

Second-generation FIV packaging and vector constructs used for these studies have been described previously (17). The FIV vector construct pVET_LCMVCre was obtained by subcloning the Cre expression cassette (46) into the pVET_LCmcs backbone (17). The vesicular stomatitis virus (VSV)-g envelope expression plasmid, pCMV-G, has been described (53). Pseudotyped FIV-Cre vector particles were obtained by transient transfection of human kidney 293T cells with FIV packaging (pCFIV Δ orf Δ vif), FIV vector (pVET_LCMVCre), and envelope pCMV-G constructs in a 2:3:1 weight ratio using standard calcium phosphate methods. Harvested supernatants were filtered and concentrated by centrifugation as described (6). Ad and FIV viral titers were evaluated as described previously (3, 6, 45, 55), and FIV-Cre and Ad-Cre titers were $\sim 1 \times 10^8$ TU/ml and $\sim 1 \times 10^{10}$ pfu/ml, respectively.

In vitro studies in primary neuronal cultures. The lamina terminalis from pups of the CAG-CATZ reporter mice (10 days old, 8 pups per culture) were dissected and cultured as previously described (54). Homogenous cell suspensions were plated in poly-L-lysine-coated

chamber slides containing prewarmed serum-enriched media, and the cells were allowed to recover overnight. Cultures were then washed and grown in serum-free media for 24 h before being inoculated with $\sim 1 \times 10^4$ pfu/ml per well of Ad-Cre or $\sim 2 \times 10^2$ TU/ml per well FIV-Cre diluted in culture medium. Vehicle and empty viral vector controls were also employed using the same conditions. Thirty hours later, cells were washed and fixed with 4% paraformaldehyde. Cells that had undergone Cre-mediated gene excision were identified using X-gal histochemistry to detect β -gal activity as described (45). Cultures were analyzed by light microscopy ($\times 40$ magnification), and neurons and nonneuronal cells were identified on the basis of their characteristic morphology as described (30). X-gal-positive cells were counted over four randomly selected fields within the middle of the coverslip from each of 4–6 wells. Studies were performed in triplicate for each of three separate CAG-CATZ litters.

In vivo gene transfer of Cre to cardiovascular nuclei in the brain. CAG-CATZ mice were anesthetized with ketamine (90 mg/kg ip) and acepromazine mix (1.8 mg/kg ip), then positioned in a stereotaxic frame (David Kopf Instruments), and the skull was exposed by an incision and leveled between lambda and bregma (45). Ad-Cre ($\sim 1 \times 10^{10}$ pfu/ml) or FIV-Cre ($\sim 1 \times 10^8$ TU/ml) was microinjected as described (45) into one of the following brain sites: lateral ventricles (LV; Ad-Cre, $n = 6$; FIV-Cre, $n = 4$), SFO (Ad-Cre, $n = 24$; FIV-Cre, $n = 14$), SON (Ad-Cre, $n = 9$; FIV-Cre, $n = 7$), extended lateral parabrachial nucleus (eLPB; FIV-Cre, $n = 4$), mesencephalic trigeminal nucleus (meV; FIV-Cre, $n = 3$), or neurohypophysis (NH, Ad-Cre, $n = 4$). Empty viral vectors or vehicle (3% sucrose in PBS) were injected as controls ($n = 7$). We have established previously that these viral concentrations do not induce cytotoxic or inflammatory effects *in vivo* (45). Brain coordinates and microinjection volumes have been established in previous studies (23, 45, 50) and are as follows (relative to bregma): LV (200 nl), 0.3 mm caudal, 1.0 mm from midline, 3.5 mm ventral; SFO (200 nl), midline, 0.1 mm caudal, 2.9 mm ventral; SON (200 nl), 3.1 mm either side of midline at 20° angles, 0.6 mm caudal, 5.0 mm ventral; eLPB (200 nl), 5.0 mm caudal, 1.1 from midline, 4.1 ventral; meV (200 nl), 5.0 mm caudal, 1.0 from midline and 4.1 ventral; and NH (500 nl), midline, 2.3 mm caudal, 7.2 mm ventral. With the exception of the LV, these coordinates place the injector just dorsal to each site, allowing selective injection without damage to the structure (45). Incisions were closed and mice were kept warm until full recovery from anesthesia before being returned to their home cages in the Animal Care Unit.

β -Galactosidase histochemistry and double immunofluorescence. At 1 or 3 wk after microinjection of Cre viruses, mice were anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB). Brains were removed, incubated in the same fixative for 2 h, and then transferred to 20% sucrose overnight for cryoprotection. Serial coronal sections (30 μ m) were cut on a freezing microtome and free-floated in PB. Some sections were processed for β -gal activity using X-gal (Boehringer Mannheim), counterstained with eosin, and analyzed by light microscopy as described (45). Corresponding serial sections were processed for double immunofluorescence to provide further confirmation of Cre-mediated gene deletion. Sections were incubated with rabbit anti- β -gal antibody (1:500, RBI) and Cre-specific mouse antibody (1:100, Covance) overnight at 4°C. These primary antibodies were detected using Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes) and Alexa Fluor 564-conjugated goat anti-mouse antibody (1:200, Molecular Probes), respectively. A separate subset of brains was processed for double immunofluorescence to determine cellular selectivity of Ad-Cre. Sections were incubated with a rabbit anti- β -gal antibody (1:500, RBI) combined with either mouse monoclonal anti-MAP-2 (1:500, Sternberger Monoclonal Antibodies) or mouse monoclonal anti-GFAP (1:1,000, Chemicon) overnight at 4°C, followed by incubation in Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Molecular Probes) and rhodamine-conjugated goat anti-mouse antibody (1:200, Sigma) for 2 h. For both immuno-

fluorescence series, sections were washed, mounted, and analyzed by confocal laser microscopy (Zeiss model LSM 510).

Statistical analysis. Cell count data are expressed as means \pm SE. Data were analyzed by the Student's modified *t*-test using Prism (GraphPad Software).

RESULTS

In initial studies to validate the function of the Ad-Cre and FIV-Cre vectors, *in vitro* experiments were performed in primary cells cultured from the lamina terminalis of CAG-CATZ pups. This region was chosen because of its rich population of cells important in cardiovascular regulation (16) and because it encompasses some of the regions that were studied *in vivo*. Furthermore, cultured lamina terminalis neurons are easily distinguished morphologically from nonneuronal cells by their characteristic spherical somata of 10–25 μ m in diameter (30). Both Ad-Cre and FIV-Cre induced robust LacZ activation in lamina terminalis cells of the CAG-CATZ reporter mouse (Fig. 1), demonstrating functional Cre-mediated recombination with either vector in this model system. However, the viral constructs differed with regard to cell type selectivity. Ad-Cre induced β -gal expression with equal efficiency in neuronal and nonneuronal cells (Fig. 1, A and C). Nearly all cells counted were X-gal positive, with approximately half of the positive cells characterized as neuronal ($48 \pm 3\%$) and the other half as nonneuronal ($49 \pm 4\%$, $P > 0.05$). In contrast, FIV-Cre induced gene recombination only in cells with neuronal morphology (Fig. 1, B and C). Almost no X-gal staining was detected in nonneuronal cells infected with FIV-Cre ($3 \pm 2\%$), resulting in cultures with approximately half of the cells ($51.9 \pm 2\%$) showing LacZ activation. It should be noted that no positive X-gal staining was observed in cultured cells treated with vehicle or control vectors (data not shown).

To extend these studies and demonstrate that *in vivo* deletion of genes can be accomplished in specific cardiovascular and volume regulatory centers of the brain, we targeted delivery of Ad-Cre and FIV-Cre to the SFO or SON by stereotaxic microinjection in CAG-CATZ reporter mice. Robust X-gal staining was observed as early as 1 wk and for as long as 3 wk (the longest time point in this study) in both of these sites with either Ad-Cre or FIV-Cre infection (Fig. 2A). Except for occasional X-gal staining along the injection track itself, Cre-triggered LacZ activation was restricted to the intended regions. Similar to our earlier gene transfer studies (45), our success rates for accurately targeting these small brain regions for Cre-mediated deletion were $\sim 75\%$ for the SFO and 50% for the SON. No X-gal staining was detected when control vectors or vehicle were injected (data not shown), suggesting that the expression of β -gal protein and the predicted recombination event were contingent on the delivery of Cre recombinase. To provide further corroboration of this, double immunohistochemistry was performed using anti- β -gal and anti-Cre antibodies in consecutive sections that had stained positive for X-gal. As shown in a representative example in Fig. 2B, there was colocalization of β -gal and Cre expression in the SFO of a CAG-CATZ mouse injected with Ad-Cre in this site. Taken together, these findings establish the feasibility of inducing selective gene deletion by direct microinjection of Cre viruses into these cardiovascular regulatory nuclei.

An alternative to using direct microinjection for targeting gene delivery to the SFO is suggested by our recent studies

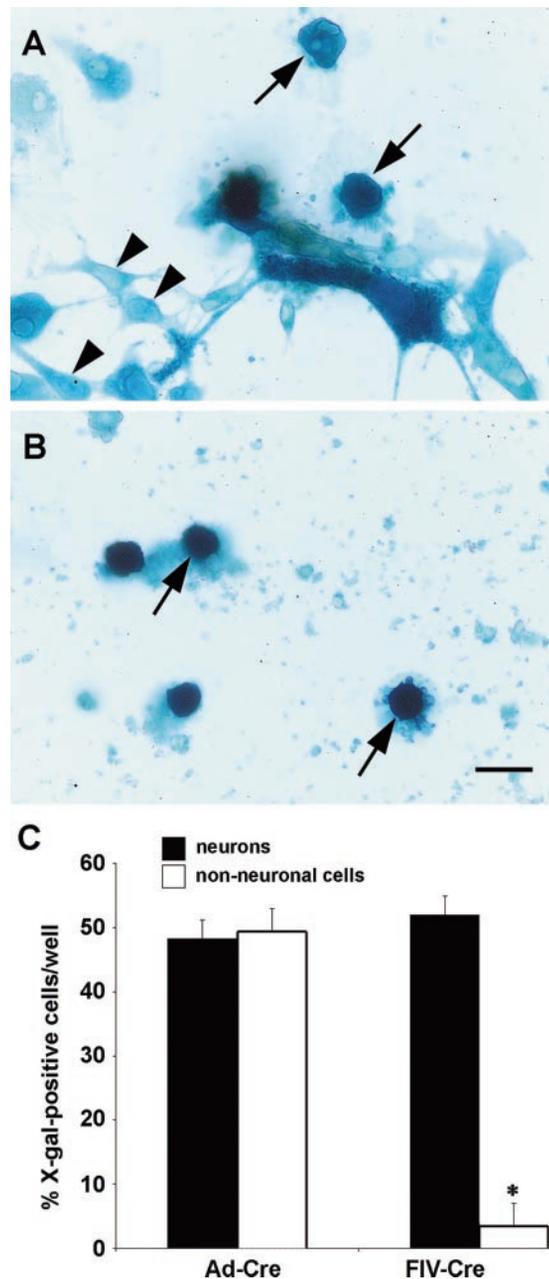


Fig. 1. Adenovirus (Ad) and feline immunodeficiency virus (FIV) bearing Cre recombinase (Ad-Cre and FIV-Cre, respectively) are functional *in vitro* and exhibit differential cellular selectivity. Cells derived from the lamina terminalis of CAG-CATZ reporter mice were cultured and incubated with Ad-Cre or FIV-Cre for 30 h prior to X-gal histochemistry. Ad-Cre induced LacZ activation in both neurons (arrows) and nonneuronal cells (arrowheads) (A), whereas FIV-Cre caused positive X-gal staining only in cells with neuronal morphology (arrows) (B). Percentage of X-gal-positive cells that were neuronal vs. non-neuronal following Ad-Cre or FIV-Cre treatment (C). Data represent the means \pm SE of percentage of X-gal-positive cells counted in 4 randomly selected fields from 4–6 wells cultured from 3 separate CAG-CATZ litters. Scale bar = 20 μ m. * $P < 0.01$ vs. neurons. CAG-CATZ, a transgenic mouse model generated specifically as an *in vivo* reporter for Cre-triggered recombination.

demonstrating that highly efficient transduction of this site can also be achieved with injection of viral vectors into the lateral ventricles (27, 54). This is due both to the bulging of this structure into the ventricular space, and to its lack of a blood-brain barrier (12). To determine whether this is a viable

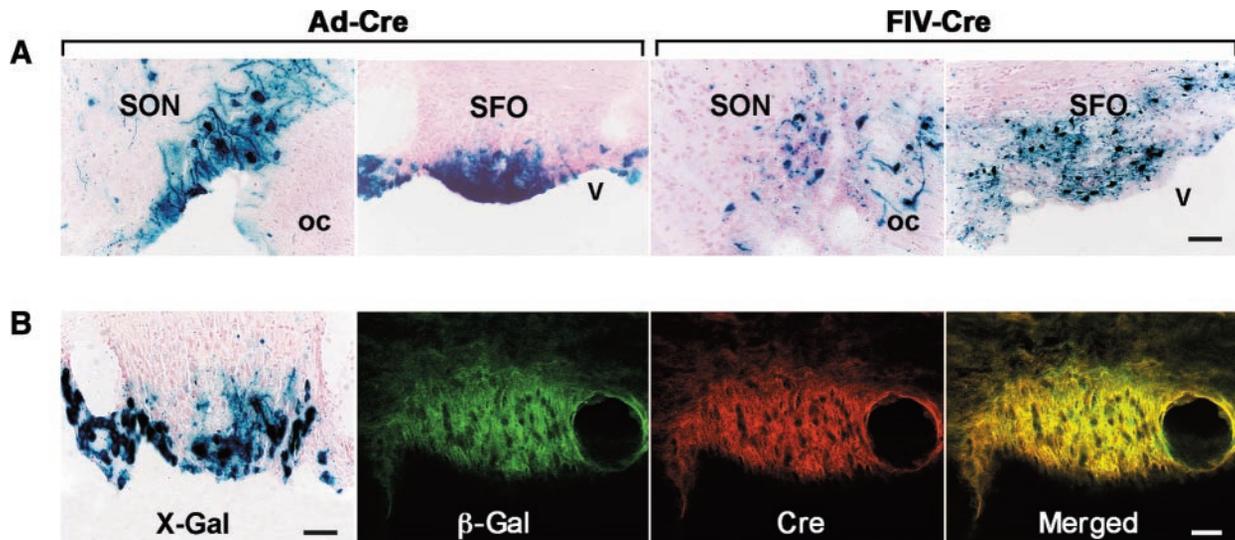


Fig. 2. Ad-Cre and FIV-Cre are functional *in vivo* and induce gene deletion in discrete cardiovascular control nuclei of the brain. *A*: representative photomicrographs showing β -galactosidase (β -gal) expression as detected by X-gal staining in the supraoptic nucleus (SON) and subfornical organ (SFO) at 1 and 3 wk, respectively, after direct stereotaxic microinjection of either Ad-Cre (*left*) or FIV-Cre (*right*) into these sites of CAG-CATZ mice. *B*: representative images of histochemical and immunohistochemical staining of the SFO from a CAG-CATZ mouse that was injected with Ad-Cre into this site 3 wk earlier. Consecutive coronal brain sections were processed for detection of LacZ activation (X-gal) or were dual stained using anti- β -gal (green) and anti-Cre (red) antibodies. Colocalization of β -gal and Cre in the SFO is shown in the merged (yellow) image. Scale bars = 100 μ m; oc, optic chiasm; v, ventricle.

approach for inducing Cre-mediated gene recombination in the SFO, CAG-CATZ mice were injected intracerebroventricularly with Cre viruses, and brains were analyzed for X-gal staining. As shown in the example in Fig. 3 using Ad-Cre, LacZ activity was limited to the periventricular tissue of the lateral and third ventricles, as well as the SFO. Consistent with our previous findings, the X-gal staining in the SFO was particularly prominent in the outer perimeter and lateral horns of the structure (27, 54). No other staining was detected outside of these areas, including mid- or hindbrain regions. The relatively simpler approach of intraventricular vs. site-directed injection of viruses is reflected in our 100% success rate in targeting the SFO for gene deletion using this injection strategy.

Our previous studies have shown that adenoviruses can be used to target gene delivery to secondary subpopulations of neurons along the SFO-hypothalamic axis through retrograde transport (45). In contrast, FIV-mediated gene transfer to this network was shown to be restricted to the sites of injection

(45). To determine whether these properties of the viruses can be extended to Cre-dependent gene recombination, brains were examined for X-gal staining in sites other than those directly injected with Ad-Cre or FIV-Cre. As seen in representative examples in Fig. 4, induction of LacZ activity was detected in a subpopulation of neurons within the outer perimeter and lateral horns of the SFO when Ad-Cre was injected downstream in the SON (Fig. 4A). No other sites that project to the SON showed positive X-gal staining. Similarly, injection of Ad-Cre into the NH resulted in positive X-gal staining in the upstream hypothalamic nuclei, paraventricular nucleus (PVN) and SON (Fig. 4B). In the PVN, the β -gal expression was localized to the area of the nucleus where the magnocellular neurons reside (25). No X-gal staining was detected outside of these secondary sites. Since both the SFO-SON and PVN/SON-NH pathways are characterized by direct short loop projections (31), these findings suggest that Ad-Cre was taken up and transported retrogradely to induce gene deletion in the

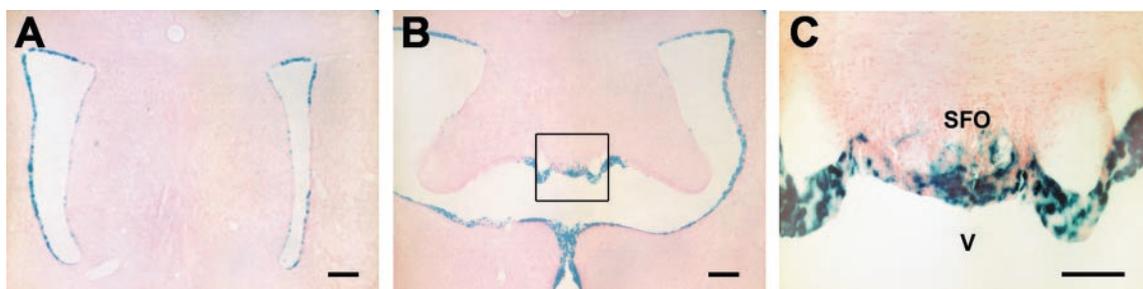


Fig. 3. Ad-Cre triggers gene deletion in the SFO when administered intracerebroventricularly. Representative images showing positive X-gal staining in coronal brain sections of a CAG-CATZ mouse that was injected with Ad-Cre into the lateral ventricles 1 wk earlier. LacZ activation was confined to tissue surrounding the ventricular system, particularly the ependymal layer (*A* and *B*). However, the SFO, a periventricular structure lacking a blood-brain barrier, also exhibited intense X-gal staining with this mode of Ad-Cre delivery (boxed area in *B* is magnified in *C*). Scale bars = 250 μ m in *A* and *B*, and 50 μ m in *C*.

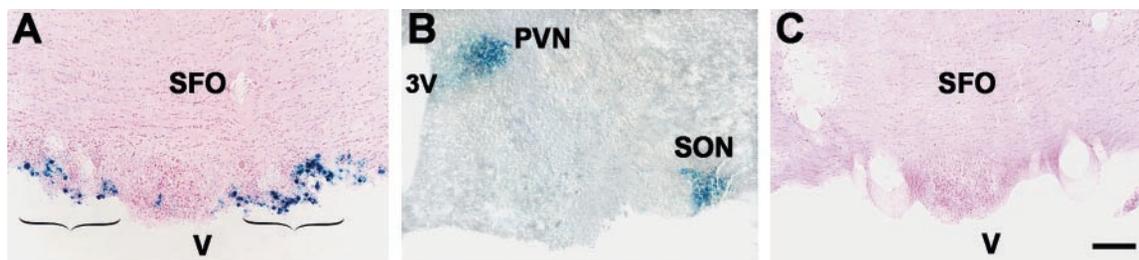


Fig. 4. Cre-mediated gene deletion can be induced in subpopulations of neurons in secondary sites by retrograde transport of Ad-Cre but not FIV-Cre. *A*: representative photomicrograph showing LacZ activation in the outer perimeter and lateral horns of the SFO 3 wk after Ad-Cre injection into the SON of a CAG-CATZ mouse. *B*: representative image showing positive X-gal staining in the PVN and SON of a CAG-CATZ mouse injected with Ad-Cre into the neurohypophysis 1 wk earlier. X-gal staining is localized to the area of the nucleus where the magnocellular neurons reside. *C*: representative photomicrograph showing negative X-gal staining in the SFO of a CAG-CATZ mouse injected with FIV-Cre into the SON 3 wk earlier. Sections shown in *A* and *C* were counter-stained, whereas the one shown in *B* was not. Scale bar = 100 μ m.

soma of neurons in these secondary targets. In contrast, Cre-mediated recombination remained localized to each of the respective primary injection sites when FIV-Cre was used. As seen in the example in Fig. 4C, no X-gal-positive staining was detected in the SFO (or any other sites) when FIV-Cre was injected into the SON.

Finally, the cellular selectivity of the two Cre-expressing viruses was assessed *in vivo*. Morphological analyses were employed initially, and the results for FIV-Cre were clear. As seen in examples in Fig. 5A in which the eIPB or meV nuclei were microinjected with FIV-Cre, X-gal staining was restricted to cells with neuronal morphology. In Ad-Cre-injected brains, morphological data were more difficult to discern due to multiple cell types staining positive for X-gal. As such, double-immunohistochemistry for β -gal and either neuronal (MAP-2, microtubule-associated protein-2) or glial (GFAP, glial fibrillary acidic protein) marker proteins was carried out. As shown in an example of Ad-Cre-injected SON (Fig. 5B), Cre-triggered LacZ activation was observed in both neuronal and glial cell types. Together, these results suggest that Cre/loxP recombination can be induced with differential cell selectivity in central cardiovascular regulatory sites using Ad-Cre and FIV-Cre. The differential tropism of these two Cre-expressing viruses is consistent with our recent studies using FIV and Ad reporter viruses *in vivo* (45), other reports investigating different brain regions (2, 9), and with the current *in vitro* results (see Fig. 1).

DISCUSSION

It is well known that central cardiovascular and body fluid homeostasis involves the coordinated activity of numerous brain nuclei made up of multiple cell types and molecules (16, 34). Understanding the molecular mechanisms governing these complex neural networks has posed a significant challenge due in part to a lack of tools for selectively dissecting the function of genes within these regions. The Cre/loxP system has shown substantial promise for investigating genes involved in nervous system function and pathology (19, 49). However, the application of this strategy to central cardiovascular circuits has been problematic in part due to the lack of promoters for generating transgenic mice expressing Cre selectively in these brain regions. Given the recent evidence that viral gene transfer is a powerful tool for manipulating gene expression in these neural pathways (20, 45), we sought to establish the feasibility

of using a combination of viral vector and Cre/loxP technologies to target specific cardiovascular nuclei in the brain for conditional gene modification.

Here, we report for the first time that recombination of loxP-flanked genes can be achieved in select cardiovascular regulatory nuclei of adult mouse brain using targeted delivery of viruses bearing Cre recombinase. Single stereotaxic microinjections of Ad-Cre or FIV-Cre into specific nuclei along the SFO-hypothalamic-hypophysial and brain stem-parabrachial axes resulted in robust and highly localized gene deletion as early as 7 days and for as long as 3 wk in CAG-CATZ Cre reporter mice. An even greater spatial selectivity in Cre-mediated gene deletion could be achieved in unique subpopulations of neurons within some of these regions by delivering Ad-Cre via retrograde transport. Moreover, whereas the CAG-CATZ reporter gene is functional in all cell types, Ad-Cre and FIV-Cre induced gene recombination in differential cell populations within these cardiovascular nuclei. FIV-Cre infection resulted in LacZ activation selectively in neurons, whereas both neuronal and glial cell types underwent gene recombination upon infection with Ad-Cre. Together, these results provide proof-of-principle that the Cre/loxP system can be used to induce conditional gene modification in discrete cardiovascular nuclei in the brain and suggest the potential of this approach for determining the functional role of genes within these sites.

The SFO-hypothalamic-hypophysial and brain stem-parabrachial axes are complex circuits encompassing a variety of nuclei in different brain regions. As such, we rationalized that they would serve as good model systems for testing the feasibility of this approach *in vivo*. The SFO, one of the circumventricular organs, is thought to couple blood-borne signaling molecules such as angiotensin II (ANG II) with a number of central neural networks involved in maintaining cardiovascular and fluid homeostasis. For example, it sends direct projections to magnocellular vasopressinergic cells of the PVN and SON, the axons of which in turn project to the NH where this neuropeptide is stored for release into the circulation (26, 31). The SFO also innervates parvocellular neurons of the PVN, which send efferent projections to sympathetic outflow centers in the brain stem and spinal cord (35). The parabrachial complex is implicated as a major relay center through which various neuroeffector nuclei receive sensory information related to blood pressure and volume status (15, 32), and the meV is involved in processing sensory information

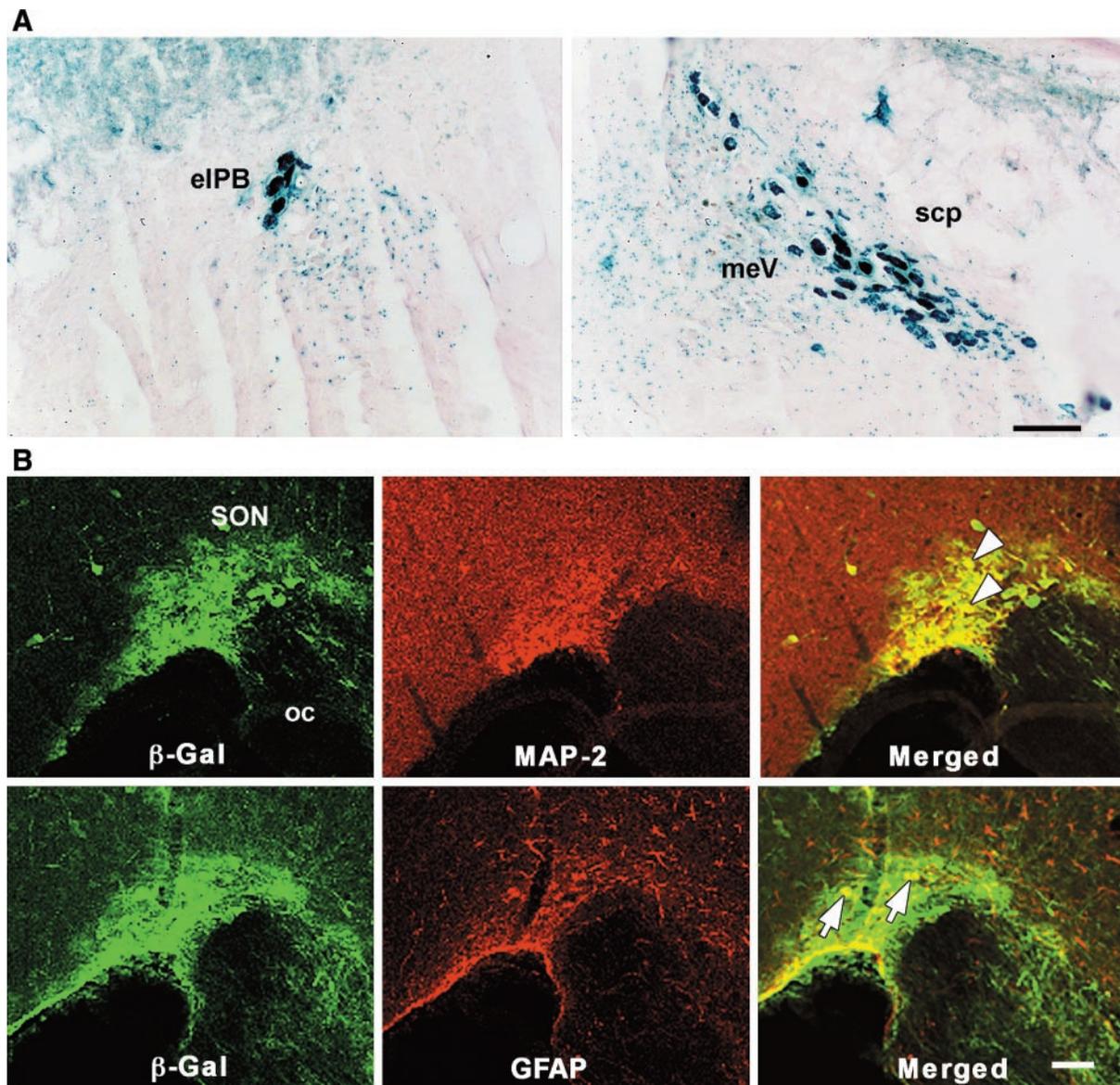


Fig. 5. Ad-Cre and FIV-Cre induce gene deletion differentially in neurons and glia within cardiovascular nuclei. *A*: representative photomicrographs of coronal brain sections from CAG-CATZ mice showing FIV-Cre-mediated LacZ activation selectively in cells with neuronal morphology in eIPB (*left*) and meV (*right*) 1 wk after injection into these sites. *B*: representative confocal images of immunocytochemical staining of the SON from a CAG-CATZ mouse 3 wk after Ad-Cre injection into this site. Coronal brain sections were dual stained for β -gal (green) and either neuronal (MAP-2, red nuclei) or glial (GFAP, red processes) markers. Cell-specific double-labeling is shown in merged (yellow) images, demonstrating that Ad-Cre-triggered LacZ activation is detected in both cell types. Scale bar = 100 μ m. eIPB, extended lateral parabrachial nucleus; meV, mesencephalic trigeminal nucleus; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2.

that may have implications for fluid intake (28). In addition, the meV has been suggested to receive projection fibers from the area postrema (29) and the dorsal raphe nucleus (8), two cardiovascular regulatory nuclei. However, despite evidence of important roles for each of these sites in cardiovascular and/or fluid balance, the precise pathways and molecular mechanisms involved remain poorly understood. Demonstration in this study that these nuclei can be selectively targeted for gene ablation using viral delivery of Cre suggests the potential of this approach for unraveling some of the complexities of these neural networks.

In addition to providing the means for inducing robust Cre expression in a spatially regulated manner, recombinant viral

vectors offer a number of other advantages for achieving Cre-mediated gene modification in neural networks such as these. The differential cellular selectivity of FIV-Cre and Ad-Cre could be brought to bear in investigating the function of genes that exhibit cell-specific expression. For example, much of the interest and controversy surrounding the brain RAS is related to the complex differential pattern of expression of RAS genes in neurons and glia in these cardiovascular nuclei. Angiotensinogen (AGT), the only known precursor for ANG II, is clearly expressed in glial cells in many of these brain regions (44). However, the recent identification of AGT in distinct populations of neurons in select regions has raised questions about the significance of these different cellular

sources of the substrate. Sites such as the SFO, SON, eIPB, and meV each have been reported to contain neuronal AGT, although the functional significance of this is unknown (11, 52). Similarly, ANG II receptors are expressed exclusively within neurons in sites such as the SFO and SON, whereas they are detected in both neurons and glia in other regions (24, 25). Given the findings in this study that neurons in these regions can be discretely targeted for gene deletion using FIV-Cre, the Cre/loxP approach could be useful in dissecting out the significance of the differential cellular selectivity of the RAS as well as other molecules. It should be noted that verification of normal cellular properties would be important in studies where functional genes are deleted. For example, Scammell et al. (43) demonstrated that except for the lack of any response to adenosine, neurons that had undergone Cre-mediated adenosine A₁ receptor deletion exhibited normal electrophysiological responses.

Another feature of virally mediated Cre expression that is unique and could lend itself to investigation of central cardiovascular mechanisms is the retrograde transportability of adenoviruses from axon terminals at the injection site to distant secondary regions (38). In this study, Cre-mediated gene recombination was demonstrated in the vasopressin-synthesizing magnocellular neurons of the PVN and SON when Ad-Cre was microinjected into the downstream NH. Similarly, the subset of neurons that reside in the outer annular region of the SFO were selectively targeted for gene deletion by injection of Ad-Cre into the SON. Since each of these subgroups of neurons is functionally unique, the ability to induce selective gene deletion in them would provide an important tool for determining underlying molecular pathways. For example, vasopressin plays a pivotal role in cardiovascular and fluid regulation through its actions as an antidiuretic hormone and vasopressor agent (16, 51), so the ability to selectively target gene deletion to the cells in the brain that synthesize this important neuropeptide would be very powerful. Likewise, the neurons in the outer perimeter and lateral horns of the SFO are thought to be important in mediating the effects of ANG II on water intake and vasopressin secretion (33), but they cannot be selectively manipulated by direct approaches. This strategy provides the opportunity to determine the role of specific genes in these and other specialized cell groups within central cardiovascular circuits. We are currently investigating the feasibility of inducing Cre-mediated gene deletion in parvocellular PVN neurons by injecting Ad-Cre into rostral ventrolateral medulla, a site that receives projections from this unique subgroup of PVN cells and is involved in regulating sympathetic nerve activity (35).

There are also a number of practical considerations that make using virally delivered Cre very attractive. First, it eliminates the need for time-consuming and resource-intensive breeding strategies that involve crossing Cre-expressing transgenic lines with mice engineered with loxP sites. Confounding problems associated with increased variation in the genetic background that come with crossing transgenic lines are also circumvented. However, perhaps the most important advantage is that the timing of the onset of gene recombination can be easily controlled. Although there are methods for achieving temporally regulated gene modification in the brain through the use of inducible promoters (21), the viral/Cre system offers a more straightforward approach for achieving similar goals. Since Cre-expressing viruses can be administered at any time

postnatally (within mouse size limitation), this not only allows control over the time of induction of gene recombination, it also provides the opportunity to collect preinjection baseline phenotype data for comparison to Cre-evoked changes within the same animal. Although in the current study we did not perform strict time-course experiments, i.e., 1 and 3 wk were the only time points examined, Cre-mediated gene recombination was shown to be stable for at least 3 wk. Although gene deletion may in fact be more long-lasting (19), even this amount of time would allow investigations of gene function in long-term neural regulation of cardiovascular function.

Central neural mechanisms play a key role in cardiovascular and volume homeostasis, and alterations in neurocardiovascular regulation have long been implicated in pathological states such as hypertension, heart failure, diabetes, and obesity (7). The demonstration in this study that loxP-modified genes within discrete cardiovascular nuclei can be targeted for Cre-mediated deletion suggests that this will be a powerful strategy for unraveling the physiological and pathophysiological mechanisms of central cardiovascular control.

ACKNOWLEDGMENTS

We thank Matthew Banford, Jeremy Bonsol, and Martha Kienzle for excellent technical assistance.

GRANTS

This work was supported by grants to R. L. Davisson from the American Heart Association (0030017N) and the National Institutes of Health (HL-14388 and HL-63887). P. Sinnayah is funded by a Postdoctoral Fellowship from the American Heart Association (0225723Z).

REFERENCES

1. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, and Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest* 100: 169–179, 1997.
2. Alisky JM, Hughes SM, Sauter SL, Jolly D, Dubensky TW Jr, Staber PD, Chiorini JA, and Davidson BL. Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors. *Neuroreport* 11: 2669–2673, 2000.
3. Anderson RD, Haskell RE, Xia H, Roessler BJ, and Davidson BL. A simple method for the rapid generation of recombinant adenovirus vectors. *Gene Ther* 7: 1034–1038, 2000.
4. Araki K, Araki M, Miyazaki J, and Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci USA* 92: 160–164, 1995.
5. Brooks AI, Cory-Slechta DA, and Federoff HJ. Gene-expression interaction alters the cholinergic septohippocampal pathway of mice. *Proc Natl Acad Sci USA* 97: 13378–13383, 2000.
6. Brooks AI, Stein CS, Hughes SM, Heth J, McCray PM Jr, Sauter SL, Johnston JC, Cory-Slechta DA, Federoff HJ, and Davidson BL. Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors. *Proc Natl Acad Sci USA* 99: 6216–6221, 2002.
7. Chupleau MW and Abboud FM. Neuro-cardiovascular regulation: from molecules to man. Introduction. *Ann NY Acad Sci* 940: xiii–xxii, 2001.
8. Copray JC, Liem RS, Ter Horst GJ, and van Willigen JD. Origin, distribution and morphology of serotonergic afferents to the mesencephalic trigeminal nucleus of the rat. *Neurosci Lett* 121: 97–101, 1991.
9. Davidson BL, Allen ED, Kozarsky KF, Wilson JM, and Roessler BJ. A model system for in vivo gene transfer into the central nervous system using an adenoviral vector. *Nat Genet* 3: 219–223, 1993.
10. Davisson RL. Physiological genomic analysis of the brain renin-angiotensin system. *Am J Physiol Regul Integr Comp Physiol* 285: R498–R511, 2003; 10.1152/ajpregu.00190.2003.
11. Davisson RL, Yang G, Beltz TG, Cassell MD, Johnson AK, and Sigmund CD. The brain renin-angiotensin system contributes to the

- hypertension in mice containing both the human renin and human angiotensinogen transgenes. *Circ Res* 83: 1047–1058, 1998.
12. **Dellmann HD.** Structure of the subfornical organ: a review. *Microsc Res Tech* 41: 85–97, 1998.
 13. **Ganong WF.** Circumventricular organs: definition and role in the regulation of endocrine and autonomic function. *Clin Exp Pharmacol Physiol* 27: 422–427, 2000.
 14. **Glueck SB and Dzau VJ.** Physiological genomics: implications in hypertension research. *Hypertension* 39: 310–315, 2002.
 15. **Herbert H, Moga MM, and Saper CB.** Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. *J Comp Neurol* 293: 540–580, 1990.
 16. **Johnson AK, Cunningham JT, and Thunhorst RL.** Integrative role of the lamina terminalis in the regulation of cardiovascular and body fluid homeostasis. *Clin Exp Pharmacol Physiol* 23: 183–191, 1996.
 17. **Johnston JC, Gasmi M, Lim LE, Elder JH, Yee JK, Jolly DJ, Campbell KP, Davidson BL, and Sauter SL.** Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J Virol* 73: 4991–5000, 1999.
 18. **Jones N and Shenk T.** An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc Natl Acad Sci USA* 76: 3665–3669, 1979.
 19. **Kaspar BK, Vissel B, Bengoechea T, Crone S, Randolph-Moore L, Muller R, Brandon EP, Schaffer D, Verma IM, Lee KF, Heinemann SF, and Gage FH.** Adeno-associated virus effectively mediates conditional gene modification in the brain. *Proc Natl Acad Sci USA* 99: 2320–2325, 2002.
 20. **Kasparov S and Paton JF.** Somatic gene transfer: implications for cardiovascular control. *Exp Physiol* 85: 747–755, 2000.
 21. **Kellendonk C, Troche F, Casanova E, Anlag K, Opherck C, and Schutz G.** Inducible site-specific recombination in the brain. *J Mol Biol* 285: 175–182, 1999.
 22. **Kilby NJ, Snaith MR, and Murray JA.** Site-specific recombinases: tools for genome engineering. *Trends Genet* 9: 413–421, 1993.
 23. **Lazartigues E, Dunlay SM, Loihl AK, Sinnayah P, Lang JA, Espelund JJ, Sigmund CD, and Davisson RL.** Brain-selective overexpression of angiotensin (AT1) receptors causes enhanced cardiovascular sensitivity in transgenic mice. *Circ Res* 90: 617–624, 2002.
 24. **Lenkei Z, Corvol P, and Llorens-Cortes C.** The angiotensin receptor subtype AT1A predominates in rat forebrain areas involved in blood pressure, body fluid homeostasis and neuroendocrine control. *Mol Brain Res* 30: 53–60, 1995.
 25. **Lenkei Z, Corvol P, and Llorens-Cortes C.** Comparative expression of vasopressin and angiotensin type-1 receptor mRNA in rat hypothalamic nuclei: a double in situ hybridization study. *Mol Brain Res* 34: 135–142, 1995.
 26. **Lind RW, Van Hoesen GW, and Johnson AK.** An HRP study of the connections of the subfornical organ of the rat. *J Comp Neurol* 210: 265–277, 1982.
 27. **Lindley TE, Doobay MF, Sharma RV, and Davisson RL.** Superoxide is involved in the central nervous system activation and sympathoexcitation of myocardial infarction-induced heart failure. *Circ Res* 94: 402–409, 2004.
 28. **Luo P and Dessem D.** Morphological evidence for recurrent jaw-muscle spindle afferent feedback within the mesencephalic trigeminal nucleus. *Brain Res* 710: 260–264, 1996.
 29. **Manni E, Lucchi ML, Filippi GM, and Bortolami R.** Area postrema and the mesencephalic trigeminal nucleus. *Exp Neurol* 77: 39–55, 1982.
 30. **Meyrelles SS, Sharma RV, Whiteis CA, Davidson BL, and Chappleau MW.** Adenovirus-mediated gene transfer to cultured nodose sensory neurons. *Mol Brain Res* 51: 33–41, 1997.
 31. **Miselis RR.** The efferent projections of the subfornical organ of the rat: a circumventricular organ within a neural network subserving water balance. *Brain Res* 230: 1–23, 1981.
 32. **Moga MM, Herbert H, Hurley KM, Yasui Y, Gray TS, and Saper CB.** Organization of cortical, basal forebrain, and hypothalamic afferents to the parabrachial nucleus in the rat. *J Comp Neurol* 295: 624–661, 1990.
 33. **Oldfield BJ, Badoer E, Hards DK, and McKinley MJ.** Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. *Neuroscience* 60: 255–262, 1994.
 34. **Phillips MI and Summers C.** Angiotensin II in central nervous system physiology. *Regul Pept* 78: 1–11, 1998.
 35. **Pyner S and Coote JH.** Identification of branching paraventricular neurons of the hypothalamus that project to the rostromedial lateral medulla and spinal cord. *Neuroscience* 100: 549–556, 2000.
 36. **Ray MK, Fagan SP, Moldovan S, DeMayo FJ, and Brunicardi FC.** Beta cell-specific ablation of target gene using Cre-loxP system in transgenic mice. *J Surg Res* 84: 199–203, 1999.
 37. **Ray MK, Fagan SP, Moldovan S, DeMayo FJ, and Brunicardi FC.** A mouse model for beta cell-specific ablation of target gene(s) using the Cre-loxP system. *Biochem Biophys Res Commun* 253: 65–69, 1998.
 38. **Ridoux V, Robert JJ, Zhang X, Perricaudet M, Mallet J, and Le Gal La Salle G.** Adenoviral vectors as functional retrograde neuronal tracers. *Brain Res* 648: 171–175, 1994.
 39. **Sakai K, Mitani K, and Miyazaki J.** Efficient regulation of gene expression by adenovirus vector-mediated delivery of the CRE recombinase. *Biochem Biophys Res Commun* 217: 393–401, 1995.
 40. **Sakai K and Miyazaki J.** A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun* 237: 318–324, 1997.
 41. **Sauer B.** Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol* 225: 890–900, 1993.
 42. **Sauer B and Henderson N.** Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 85: 5166–5170, 1988.
 43. **Scammell TE, Arrigoni E, Thompson MA, Ronan PJ, Saper CB, and Greene RW.** Focal deletion of the adenosine A1 receptor in adult mice using an adeno-associated viral vector. *J Neurosci* 23: 5762–5770, 2003.
 44. **Sernia C, Zeng T, Kerr D, and Wyse B.** Novel perspectives on pituitary and brain angiotensinogen. *Front Neuroendocrinol* 18: 174–208, 1997.
 45. **Sinnayah P, Lindley TE, Staber PD, Cassell MD, Davidson BL, and Davisson RL.** Selective gene transfer to key cardiovascular regions of the brain: comparison of two viral vector systems. *Hypertension* 39: 603–608, 2002.
 46. **Stec DE, Davisson RL, Haskell RE, Davidson BL, and Sigmund CD.** Efficient liver-specific deletion of a floxed human angiotensinogen transgene by adenoviral delivery of Cre recombinase in vivo. *J Biol Chem* 274: 21285–21290, 1999.
 47. **Sternberg N and Hamilton D.** Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* 150: 467–486, 1981.
 48. **Sternberg N, Hamilton D, and Hoess R.** Bacteriophage P1 site-specific recombination. II. Recombination between loxP and the bacterial chromosome. *J Mol Biol* 150: 487–507, 1981.
 49. **Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, and Tonegawa S.** Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87: 1317–1326, 1996.
 50. **Vasquez EC, Beltz TG, Meyrelles SS, and Johnson AK.** Adenovirus-mediated gene delivery to hypothalamic magnocellular neurons in mice. *Hypertension* 34: 756–761, 1999.
 51. **Wilkin LD, Mitchell LD, Ganten D, and Johnson AK.** The supraoptic nucleus: afferents from areas involved in control of body fluid homeostasis. *Neuroscience* 28: 573–584, 1989.
 52. **Yang G, Gray TS, Sigmund CD, and Cassell MD.** The angiotensinogen gene is expressed in both astrocytes and neurons in murine central nervous system. *Brain Res* 817: 123–131, 1999.
 53. **Yee JK, Miyanojima A, LaPorte P, Bouic K, Burns JC, and Friedmann T.** A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc Natl Acad Sci USA* 91: 9564–9568, 1994.
 54. **Zimmerman MC, Lazartigues E, Lang JA, Sinnayah P, Ahmad IM, Spitz DR, and Davisson RL.** Superoxide mediates the actions of angiotensin II in the central nervous system. *Circ Res* 91: 1038–1045, 2002.
 55. **Zwacka RM, Dudus L, Epperly MW, Greenberger JS, and Engelhardt JF.** Redox gene therapy protects human IB-3 lung epithelial cells against ionizing radiation-induced apoptosis. *Hum Gene Ther* 9: 1381–1386, 1998.