Supporting Information

Card et al. 10.1073/pnas.1015033108

SI Materials and Methods

"Brainbow" Strain of Pseudorabies Virus. As noted above, PRV is a swine α-herpesvirus with a broad host range that is widely used for viral transneuronal tracing. Several reviews have provided detailed descriptions of the mechanisms that underlie PRV invasion, replication, and cell-to-cell transmission in the nervous system (e.g., ref. 1). These issues, as they relate to the current experiment, are summarized below.

The PRV DNA genome consists of unique long (U_L) and unique short (U_S) regions, with the U_S region bracketed by internal and terminal repeat sequences. The majority of tracing studies employing PRV have used the attenuated Bartha strain (2) or recombinants derived from PRV-Bartha. A deletion in the U_S segment of the Bartha genome is responsible for the reduced virulence and restricted, retrograde-only transport phenotype of the virus. The most commonly used PRV-Bartha recombinants contain transgenes inserted into the *gG* locus of the viral genome, and this is also true of PRV-263. PRV-263 was constructed from PRV-152, a PRV-Bartha recombinant that contains an EGFP reporter gene in the *gG* locus under the control of the strong immediate early CMV promoter.

The structure of the pBrainbow1.0L cassette (3) inserted into the PRV-263 genome is illustrated in Fig. 1*A*. The cassette contains three reporter genes in sequence, with stop cassettes present before the mCerulean and EYFP genes. Thus, dTomato is the default reporter in the absence of Cre. Canonical paired loxP sequences and mutated lox2272 sequences are arranged in the construct such that Cre-mediated recombination at like sequences (e.g., loxP:loxP) removes one of the other paired sequences (e.g., lox2272), thereby preventing subsequent Cre-mediated recombination of the Brainbow cassette (Fig. 1*A*). It is important to emphasize that Cre only mediates recombination of the Brainbow cassette at paired loxP or lox2272 sites and only one such recombination event per genome is possible. The site of Cre-mediated recombination also determines the reporter gene that will be expressed by the altered genome. Recombination at the lox2272 sites eliminates the dTomato gene and liberates mCerulean expression, and recombination at canonical loxP sites removes both the dTomato and mCerulean genes, thereby permitting expression of the EYFP reporter. Finally, more than one genome can be expressed in an infected cell and it is therefore possible for more than one fluorophor to be expressed by the same neuron (4).

Conditional Neuronal Expression from the Brainbow Cassette in Vitro. We first demonstrated that Cre-promoted recombination is efficient and fluorophor expression is robust after PRV-263 infection of primary cultures of superior cervical ganglia (SCG) neurons. Embryonic SCG neurons were grown in compartmentalized chambers (5, 6) and fluorophor profiles of live infected SCG neurons were determined. We demonstrated that spread of infection from PRV-263–infected, non–Cre-expressing PK15 cells to cultured SCG neurons resulted in exclusive default expression of the red dTomato fluorescent reporter (Fig. 1*B*). In contrast, spread of infection from Cre-expressing PK15 cells to SCG neurons enabled the expression of cyan and yellow reporters (Fig. 1 *C* and *D*). Interestingly, each neuron exhibited a unique fluorescent profile, indicating that only a limited number of viral genomes are expressed in each cell, a finding consistent with data obtained for PRV-263 spread in nonneuronal cells (4). Thus, reporter expression from the PRV-263 genome is stable in the absence of Cre (red only) and cyan or yellow reporter expression arises only from PRV-263 replication in Cre-expressing cells.

Targeted Neuronal Expression of Cre. We previously demonstrated that restricted transgene expression can be achieved in catecholamine neurons using a replication incompetent HIV-1–based lentivirus vector expressing EGFP under the control of a synthetic DβH promoter (7). The synthetic promoter contains eight copies of a *cis* regulatory element (PRS) that is activated by the homeodomain transcription factors Phox2a and Phox2b (8). Analysis of transgene expression from the PRSX8 vector in the rostroventrolateral medulla (RVLM), a brainstem nucleus that contains C1 catecholamine neurons important for the regulation of arterial blood pressure, demonstrated that EGFP reporter expression is restricted to Phox2a containing catecholamine neurons (7, 9).

In the present analysis, the lentivirus vector was modified to carry the Cre gene between the PRSX8 promoter and the EGFP reporter (Fig. S1). Vesicular stomatitis virus-G was used in packaging the vector and viral particles were produced according to previously published procedures (10). Specificity of Cre expression in RVLM from the modified lentivirus vector was examined in 13 adult male animals. The novel vector (100 nL) was injected into the RVLM using published procedures (7) and EGFP reporter expression was analyzed 7 ($n = 3$), 15 ($n = 2$), 22 to 25 ($n = 3$), 46 ($n = 2$), and 58 to 64 ($n = 3$) d later. Dual-labeling immunofluorescence localization of EGFP and tyrosine hydroxylase revealed transgene expression is restricted to RVLM catecholamine neurons (1,201 neurons; 97.5% colocalization) (Figs. S1 *B–D*). The few neurons that were $EGFP^+$ but did not colocalize tyrosine hydroxylase were observed at the rostral pole of the RVLM, which overlaps the retrotrapazoid nucleus (RTN); transgene expression in the RTN is caused by the presence of the Phox2b transcription factor in RTN neurons (11). The data demonstrate targeted transgene expression that is robust by 7 d after vector injection and stable through at least 64 d. It is also important to emphasize that analysis of the rostrocaudal extent of the brain at a frequency of 210 μm in these experiments demonstrated that vector transgene expression only occurred at the site of injection. Accordingly, even if the vector is retrogradely transported to catecholamine neurons projecting to the RVLM, it does not result in transgene expression. Collectively, these observations establish phenotypically defined and regionally specific Cre expression in an important component of the preautonomic network, the RVLM, responsible for the maintenance of cardiovascular homeostasis.

Animal Model for Proof-of-Principle Experiments. The central circuits infected by transneuronal passage of PRV from the kidney are well characterized (12–16) and the sympathetic innervation of the kidney is one of the primary means through which the brain exerts regulatory control over arterial blood pressure (17). In our experimental design, the left RVLM of each animal was first injected with the Cre-expressing lentivirus vector and PRV-263 was injected into the left kidney 7 d later. A significant attribute of this model is the ability to achieve a functionally defined and projection-specific transneuronal infection of brain circuitry by peripheral inoculation. Our prior analysis of the renal preautonomic neural network included retrograde transneuronal infection of circuits by injection of PRV-152 (the parent for PRV-263) into the parenchyma of the kidney (12). Those data provide a solid reference for evaluating the invasiveness of PRV-263. In addition, the

role of the RVLM in the control of arterial pressure is well studied, particularly with respect to the neurochemistry of the nucleus, knowledge of the cell groups that influence its activity, and the phenotypic characterization of C1 catecholamine neurons that contribute to descending reticulospinal projections to thoracic spinal cord (18). With respect to the C1 cell group, it is well established that not all C1 neurons contribute to the reticulospinal projection to thoracic spinal cord. Additionally, studies of reticulospinal projections from the RVLM have demonstrated that non-C1 glutamatergic neurons also contribute to this pathway for regulation of sympathetic outflow (19, 20). Although cell groups that project to the RVLM have been identified using classic tracers, their synaptic targets in the RVLM have not been fully defined. Thus, the ability to restrict Cre-mediated recombination of the PRV-263 genome to reticulospinal C1 neurons provides a rigorous test of the method to define connections to phenotypically defined and projection-specific populations of neurons, and also provide unique insights into the organization of the renal preautonomic circuitry.

Animals. Adult male Sprague-Dawley rats (Charles River), weighing 220 to 260 g at the outset of the experiment, were used in this study. Photoperiod (12-h light; light on at 0700) and temperature (22–25 °C) were standardized and animals had free access to food and water throughout the experiment. The University of Pittsburgh Institutional Animal Care and Use Committee, Recombinant DNA Committee, and the Department of Environmental Health and Safety approved all in vivo experiments. Regulatory bodies at Princeton University approved the in vitro experiments.

Pseudorabies Virus Strains. PRV-263 was constructed from PRV-152, a PRV-Bartha recombinant that contains an EGFP reporter gene in the *gG* locus of the viral genome. The EGFP reporter is expressed under the control of the strong immediate early CMV promoter. Details regarding the construction of PRV-152 have been published (21–23). Homologous recombination was used to substitute the pBrainbow 1.0L cassette developed by Lichtman and colleagues (24) for the EGFP cassette of PRV-152; pBrainbow 1.0L was linearized and cotransfected into PK15 porcine kidney epithelial cells with PRV-152 DNA (25). The recombinants were plaque-purified on PK15 cells. The titer of PRV-152 was 1.0×10^9 pfu/mL. The titer of PRV-263 was 3.8×10^8 pfu/mL.

Lentivirus Vector. An HIV-1 based lentivirus vector expressing Cre and EGFP under the control of a synthetic DβH promoter (PRSX8) was constructed using previously published procedures (26). The vector was identical in all respects to the parental vector used in a previous study (27), with the single exception that the Cre gene was inserted between the PRSX8 promoter and the EGFP reporter (Fig. 1). The synthetic promoter contains eight copies of a *cis* regulatory element (PRSX8) that is activated by the homeodomain transcription factors Phox2a and Phox2b (28). The expression of both of these transcription factors in the RVLM is restricted to C1 catecholamine neurons (29–31).

In Vitro Characterization of Cre-Mediated Recombination of PRV-263. SCG were grown in a compartmentalized neuronal culture system (32). The culture system consists of three compartments (soma, methocel, and neurite) isolated from one another by a tripartite Teflon ring that permits axonal penetration but eliminates diffusion of virus between the soma and neurite chambers. Rat SCG neurons isolated from day 15.5 to 16.5 embryos were plated into the soma chamber and treated with 1 μM of antimitotic drug cytosine β-D-arabinofuranoside to prevent the growth of non-neuronal cells. The SCGs were grown for 21 d when axonal growth into the neurite chamber was robust. Pig kidney (PK15) cells infected with PRV-263 for 3 h at a multiplicity of infection of 10 were then plated onto axons in the neurite chamber. One set of experiments used PK15 cells that do not express Cre and the other used Cre-expressing PK15 cells (33). We conducted three replications of each experiment.

In Vivo Characterization of PRV-Invasiveness. The ability of PRV-263 to spread from the periphery to the PNS and CNS was established in 23 rats. Animals were deeply anesthetized with isoflurane, the left kidney was exposed via laparotomy, and 2 μL of virus (0.5 μL injected into four sites) was injected into the kidney parenchyma, as described previously (12). Animals were anesthetized and perfused transcardially with buffered aldehyde solutions 4, 5, 6, and 7 d following virus injection. The brain and spinal cord was collected from each animal; the sympathetic chain ganglia were dissected from four of the animals at 4- and 5-d survival. The brain and spinal cord were postfixed, cryoprotected, and sectioned using a freezing microtome according to previously published procedures (9). Coronal sections of brain (35 μm per section) at a frequency of 210 μm and horizontal sections of spinal cord (40 μm per section) at a frequency of 160 μm were processed for immunoperoxidase localization of infected neurons using a rabbit polyclonal antiserum generated against acetone-inactivated PRV (34), biotinylated affinity-purified donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories), and Vectastain Elite reagents (Vector Laboratories). Adjacent sections were mounted on gelatin-coated slides for analysis of reporter-gene expression using dark-field fluorescence microscopy. Details of all of these procedures have been published (35). Sympathetic chain ganglia were mounted on gelatin-coated slides, air-dried, and coverslipped using Vectastain Hard-Set mounting media (Vector Laboratories).

In Vivo Characterization of PRV-263 Invasiveness in Dual-Injection Paradigms. Eleven animals were included in this analysis. Each animal received an injection of 100 nL of the lentivirus vector into the left RVLM, followed 7 d later by injection of PRV-263 into the left kidney. The procedures used for injection of lentivirus vector and PRV-263 duplicated those used when each of these reagents was injected alone. Animals were perfused and processed for analysis of viral invasiveness and Cre-mediated recombination 4 ($n = 4$) and 5 ($n = 7$) d after injection of PRV-263. Coronal sections (35 μm per section) at a 210-μm frequency through the rostrocaudal extent of the brain were processed for immunoperoxidase localization of infected neurons, as described above. Adjacent sections of the brain and horizontal sections of the spinal cord (40 μm per section) at a frequency of 160 μm were mounted on gelatin-coated slides, coverslipped using Vectastain Hard-Set media, and analyzed for reporter gene expression using an Olympus BX51 photomicroscope equipped for epifluorescence illumination and appropriate filters for visualization of dTomato, EYFP, and mCerulean.

The distribution of infected neurons identified in immunoperoxidase localization was mapped in 48 coronal sections that thoroughly sampled the renal preautonomic network (Fig. S2). Equivalent sections of each coronal plane of analysis were identified for each case and the distribution of infected neurons was mapped using StereoInvestigator software (version 8; MicroBrightfield, Inc.). The maps defined the distribution of infected neurons and also provided quantitative measures of the numbers of infected neurons at each level, as well as within each cell group (e.g., the RVLM) within a coronal plane (Fig. 4). These maps served as a reference for the fluorescence analysis of reporter-gene expression. Regions containing neurons exhibiting one or more fluorophor were photographed using the 20× objective. Images were analyzed using Adobe Photoshop software (CS4) to determine the fluorophor profile of neurons in each region of analysis.

1. Pomeranz LE, Reynolds AE, Hengartner CJ (2005) Molecular biology of pseudorabies virus: Impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev* 69: 462–500.
2. Bartha A (1961) Experimental reduction of virulence of Aujeszky's disease virus. *Magy Allatorv Lapja* 16:42–45.
3. Livet J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450:56–62.
4. Kobiler O, Lipman Y, Therkelsen K, Daubechies I, Enquist LW (2010) Herpesviruses carrying a Brainbow cassette reveal replication and expression of limited numbers of incoming genomes. *Nat Commun* 1:146.
5. Ch'ng TH, Enquist LW (2006) An in vitro system to study trans-neuronal spread of pseudorabies virus infection. *Vet Microbiol* 113:193–197.
6. Ch'ng TH, Enquist LW (2005) Neuron-to-cell spread of pseudorabies virus in a compartmentalized neuronal culture system. *J Virol* 79:10875–10889.
7. Card JP, et al. (2006) Efferent projections of rat rostroventrolateral medulla C1 catecholamine neurons: Implications for the central control of cardiovascular regulation. *J Comp Neurol* 499:840–859.
8. Hwang D-Y, Carlezon WA, Isacson O, Kim K-S (2001) A high-efficiency synthetic promotoer that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther* 12:1731–1740.
9. Card JP, Lois J, & Sved AF (2010) Distribution and phenotype of Phox2acontaining neurons in the adult Sprague-Dawley rat. *J Comp Neurol* 518:2202–2220.
10. Coleman JE, et al. (2003) Efficient large-scale production and concentration of HIV-1 based lentiviral vectors for use in vivo. *Physiol Genomics* 12:221–228.
11. Kang BJ, et al. (2007) Central nervous system distribution of the transcription factor Phox2b in the adult rat. *J Comp Neurol* 503:627–641.
12. Cano G, Card JP, Sved AF (2004) Dual viral transneuronal tracing of central autonomic circuits involved in the innervation of the two kidneys in the rat. *J Comp Neurol* 471: 462–481.
13. Schramm LP, Strack AM, Platt KB, Loewy AD (1993) Peripheral and central pathways regulating the kidney: a study using pseudorabies virus. *Brain Res* 616: 251–262.
14. Tang X, Neckel ND, Schramm LP (2004) Spinal interneurons infected by renal injection of pseudorabies virus in the rat. *Brain Res* 1004:1–7.
15. Weiss ML, Chowdhury SI (1998) The renal afferent pathways in the rat: a pseudorabies virus study. *Brain Res* 812:227–241.
16. Sly DJ, Colvill L, McKinley MJ, Oldfield BJ (1999) Identification of neural projections from the forebrain to the kidney, using the virus pseudorabies. *J Auton Nerv Syst* 77: 73–82.
17. DiBona GF (2005) Physiology in perspective: The wisdom of the body. Neural control of the kidney. *Am J Physiol* 289:633–641.
18. Guyenet PG (2006) The sympathetic control of blood pressure. *Nat Rev Neurosci* 7: 335–346.
19. Tucker DC, Saper CB, Ruggiero DA, Reis DJ (1987) Organization of central adrenergic pathways. I. Relationships of ventrolateral medullary projections to the hypothalamus and spinal cord. *J Comp Neurol* 259:591–603.
20. Haselton JR, Guyenet PG (1990) Ascending collaterals of medullary barosensitive neurons and C1 cells in rats. *Am J Physiol* 258:1051–1063.
21. Demmin GL, Clase AC, Randall JA, Enquist LW, Banfield BW (2001) Insertions in the gG gene of pseudorabies virus reduces expression of the upstream Us3 protein and inhibit cell-to-cell spread of virus infection. *J Virol* 75:10856–10869.
22. Billig I, Foris JM, Enquist LW, Card JP, Yates BJ (2000) Definition of neuronal circuitry controlling the activity of phrenic and abdominal motoneurons in the ferret using recombinant strains of pseudorabies virus. *J Neurosci* 20:7446–7454.
23. Smith BN, et al. (2000) Pseudorabies virus expressing enhanced green fluorescent protein: A tool for in vitro electrophysiological analysis of transsynaptically labeled neurons in identified central nervous system circuits. *Proc Natl Acad Sci USA* 97: 9264–9269.
24. Livet J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450:56–62.
25. Kobiler O, Lipman Y, Therkelsen K, Daubechies I, Enquist LW (2010) Herpesviruses carrying a Brainbow cassette reveal replication and expression of limited numbers of incoming genomes. *Nat. Commun.* 1:146.
26. Coleman JE, et al. (2003) Efficient large-scale production and concentration of HIV-1 based lentiviral vectors for use in vivo. *Physiological Genomics* 12:221–228.
27. Card JP, et al. (2006) Efferent projections of rat rostroventrolateral medulla C1 catecholamine neurons: Implications for the central control of cardiovascular regulation. *J Comp Neurol* 499:840–859.
28. Hwang D-Y, Carlezon WA Jr., Isacson O, Kim K-S (2001) A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther* 12:1731–1740.
29. Card JP, Lois J, Sved AF (2010) Distribution and phenotype of Phox2a-containing neurons in the adult sprague-dawley rat. *J Comp Neurol* 518:2202–2220.
30. Kang BJ, et al. (2007) Central nervous system distribution of the transcription factor Phox2b in the adult rat. *J Comp Neurol* 503:627–641.
31. Tiveron M-C, Hirsch M-R, Brunet J-F (1996) The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. *J Neurosci* 16:7649–7660.
32. Curanovic D, Ch'ng TH, Szpara ML, Enquist LW (2009) Compartmented neuron cultures for directional infection by alpha herpesviruses. *Curr Protoc Cell Biol* 43:26.4.1–26.4.23.
33. DeFalco J, et al. (2001) Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. *Science* 291:2608–2613.
34. Card JP, et al. (1990) Neurotropic properties of pseudorabies virus: Uptake and transneuronal passage in the rat central nervous system. *J Neurosci* 10:1974–1994.
35. Card JP, Enquist LW (1999) *Transneuronal circuit analysis with pseudorabies viruses* (John Wiley & Sons, San Diego), pp 1.5.1–1.5.28.

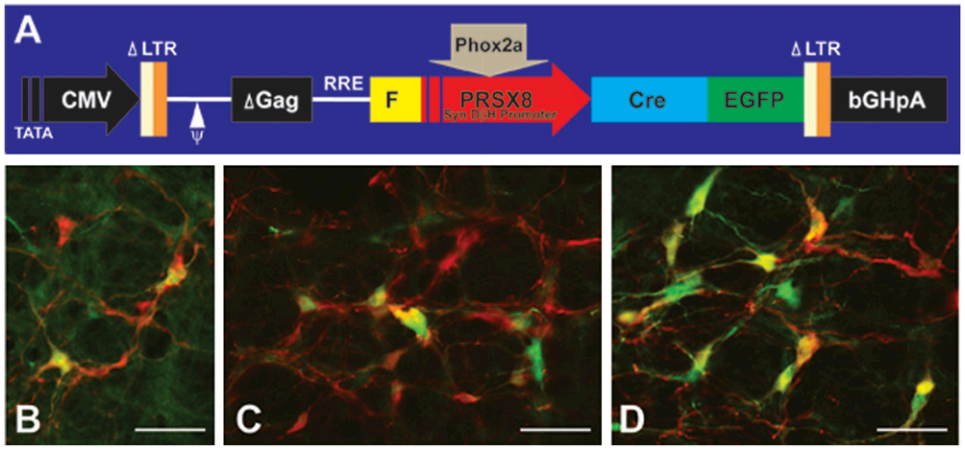

Fig. S1. The cassette carried by the lentivirus vector (*A*) and photomicrographs documenting the specificity of transgene expression in rostroventrolateral medulla (RVLM) C1 (*B–D*) neurons are illustrated. Transgene expression is controlled by a synthetic dopamine-β-hydroxylase (DβH) promoter activated by transcription factors Phox2a and Phox2b (*A*). In the RVLM, these transcription factors are restricted to C1 catecholamine neurons. (*B–D*) EGFP reporter expression (green) colocalized with tyrosine hydroxylase (red) in animals killed 7 (*A*), 25 (*B*), and 64 (*C*) d after injection of vector into the RVLM. Quantitative analysis demonstrated that 97.5% of 1,201 $EGFP^+$ RVLM neurons colocalized with tyrosine hydroxylase. (Scale bars, 50 μm.)

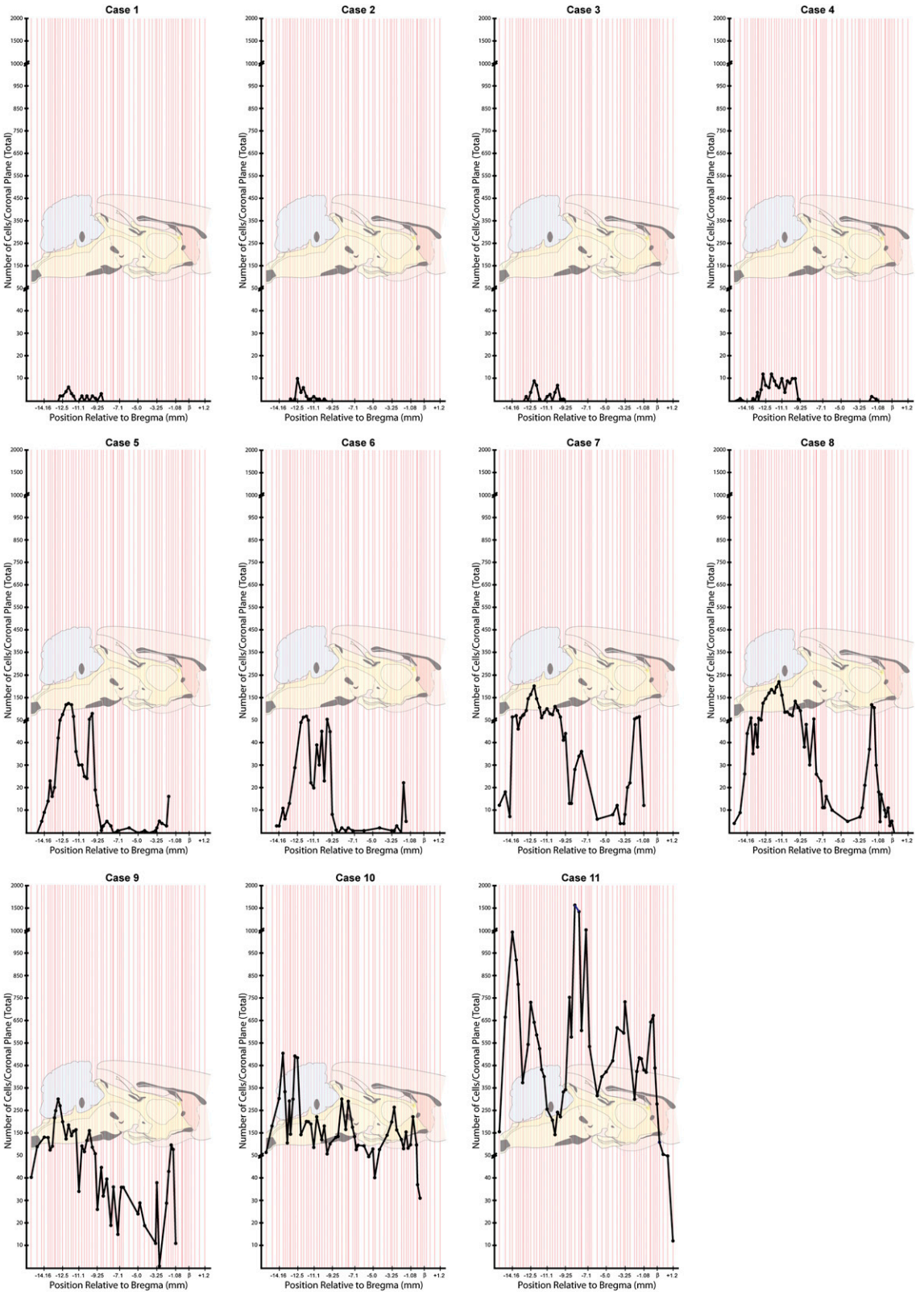

Fig. S2. The temporal progression of PRV-263 through renal preautonomic circuitry in the cases included in the dual injection paradigm (lentivirus into the RVLM and PRV-263 into the kidney) is illustrated. The quantitative analysis was derived from immunoperoxidase localization of viral antigen using a rabbit polyclonal antibody (Rb133) generated against acetone-inactivated virus, and therefore reflects the total number of infected neurons per section in each case. The localizations were conducted as a foundation for the immunofluorescence analysis of Cre-mediated recombination and transneuronal passage of PRV-263 from C1 neurons in the RVLM. The *x* axis defines the position of the 48 coronal sections included in the analysis (pale red vertical lines) relative to Bregma (β). The *y* axis indicates the number of neurons counted in each of the coronal planes. The sagittal schematic diagram included in each graph defines the position

Legend continued on following page

of each coronal plane with respect to the rostrocaudal axis of the rat brain. Cases are organized according to the extent of infection of renal preautonomic circuitry (e.g., case 1 exhibits the least infection and case 11 exhibits the most extensive infection). The analysis demonstrated that PRV-263 invades renal preautonomic circuitry with temporal kinetics equivalent to that documented for the parental virus (PRV-152) in prior analysis of this circuitry. The fluorescence analysis that established the fluorophor profiles of infected neurons documented in the main text was derived from analysis of adjacent sections from the same animals.

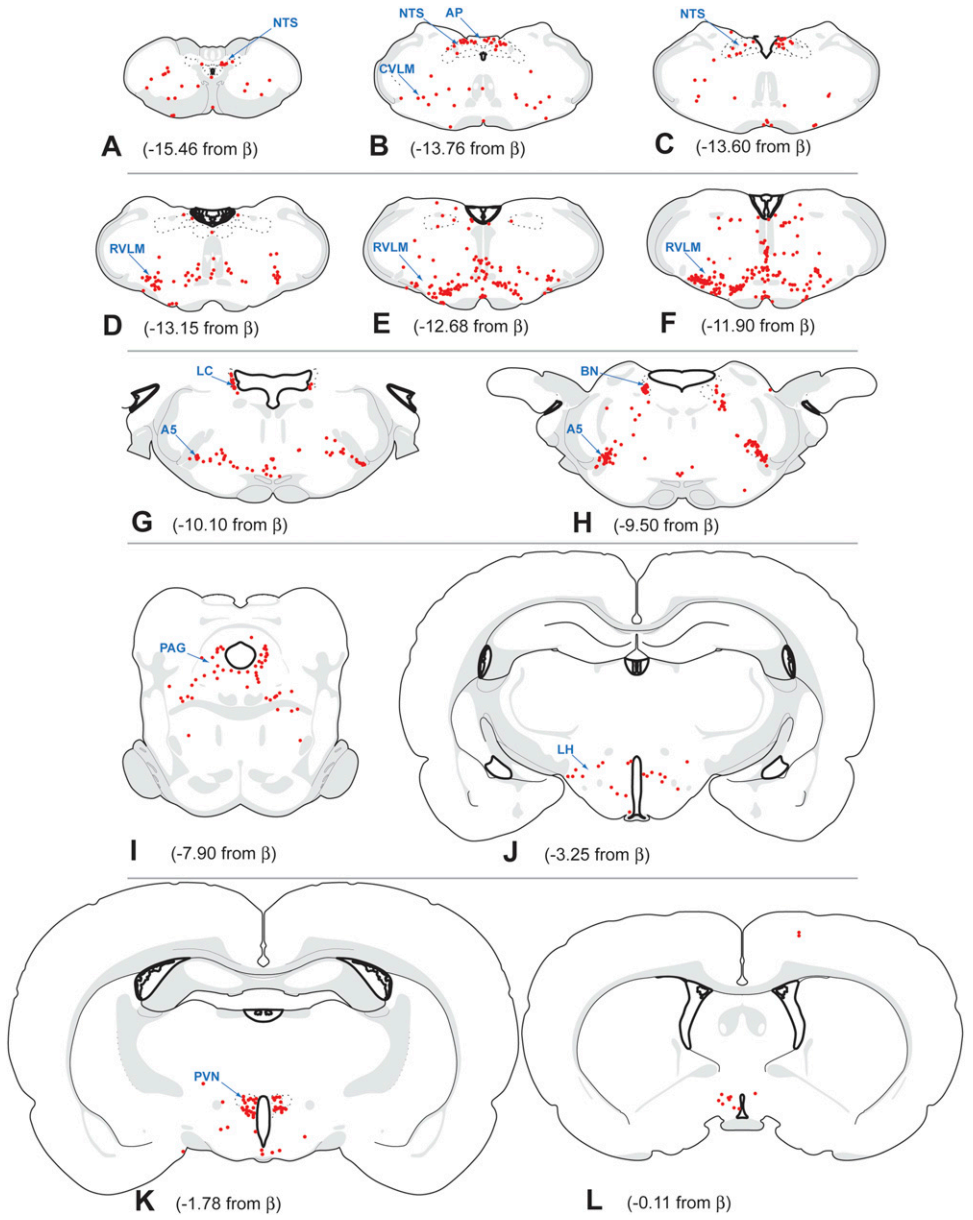

Fig. S3. The distribution of neurons detected with a rabbit polyclonal antiserum (Rb133) specific for viral antigens are illustrated for Case 8 (Fig. S2). (*A–L*) Twelve of the 48 coronal planes (Fig. S2) that systematically mapped the distribution of neurons in the renal preautonomic network are shown. Individual maps were acquired using StereoInvestigator image analysis software (v7; MicroBrightfield) and then transferred to comparable templates from *Brain Maps: Structure of the Rat Brain* (1). Each red dot represents a single infected neuron displaying PRV immunoreactivity. The distribution of infected neurons is equivalent to previously published analyses of retrograde transneuronal passage of PRV-152 from the kidney. AP, area postrema; NTS, nucleus tractus solitaius; PAG, periaqueductal gray; PVN, paraventricular nucleus.

1. Swanson LW (1998) *Brain Maps: Structure of the Rat Brain* (Elsevier, Amsterdam), 2nd Ed.

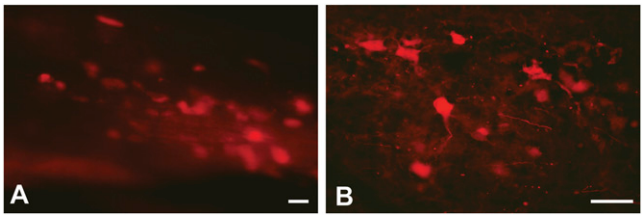

Fig. S4. Restricted expression of the dTomato reporter gene from the Brainbow cassette in the sympathetic ganglia (*A*) and the thoracic spinal cord (*B*) is illustrated. Injection of PRV-263 into the left kidney of each animal led to predictable first-order infection of neurons in lower sympathetic chain ganglia (viewed in a whole mount of the ganglia in *A* and retrograde transneuronal infection of synaptically linked sympathetic preganglionic neurons in the intermediolateral cell column (IML) of the thoracic spinal cord (*B*). Retrograde transneuronal infection of RVLM C1 neurons expressing Cre in these animals resulted in Cre-mediated recombination of the PRV-263 genome and elimination of the dTomato reporter gene, enabling expression of the EYFP and mCerulean reporters. However, infected chain ganglion neurons and IML neurons only expressed the dTomato reporter gene. (Scale bars, 50 μm.)

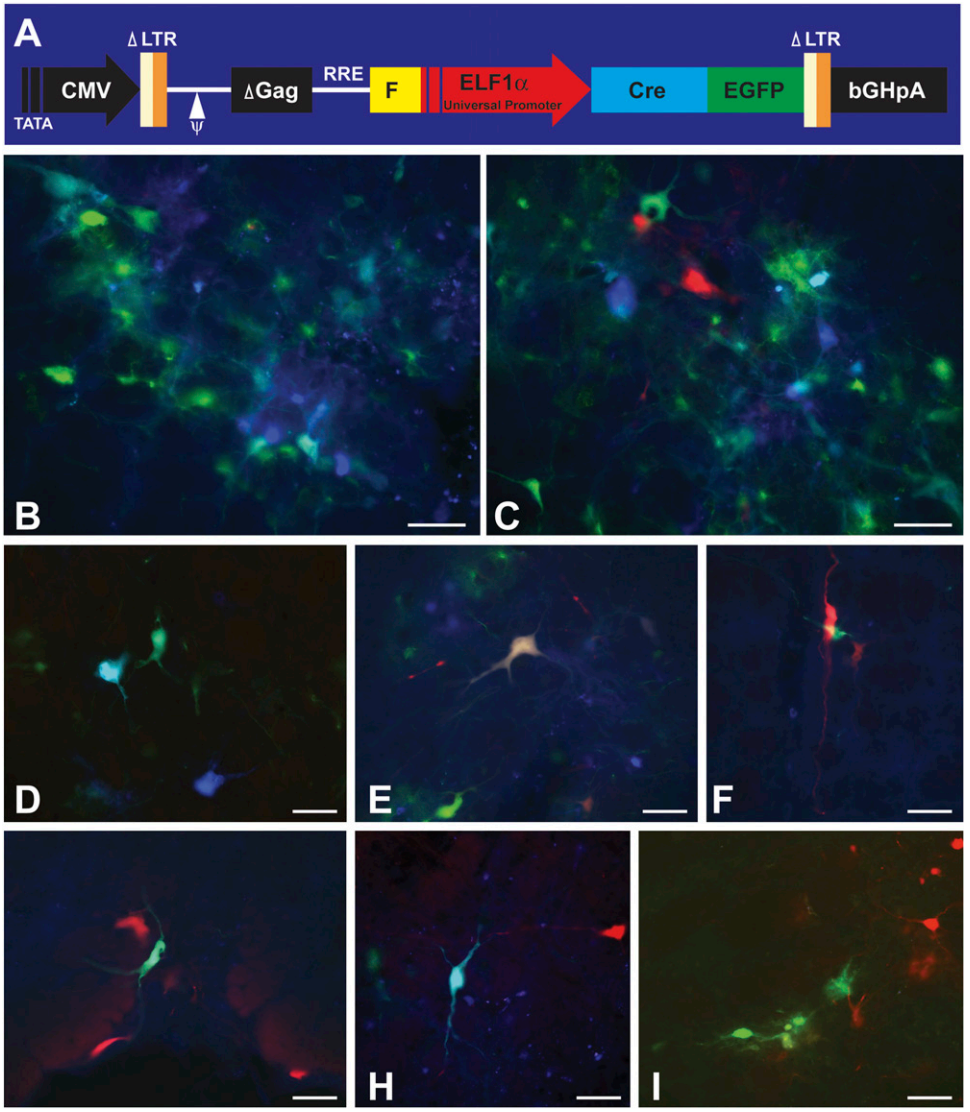

Fig. S5. The results of preliminary experiments using the same experimental model from the main text but substituting a lentivirus vector carrying the Cre gene under the control of the universal elongation factor 1α (ELF1α) promoter are illustrated. The cassette carried by the lentivirus vector differs only in the promoter driving Cre expression (*A*). Five days following injection of PRV-263 into the kidney, essentially all neurons in the rostral portion of the RVLM injected with the ELF1α vector demonstrate Cre-mediated recombination of the PRV-263 genome (*B* and *C*). This finding is in marked contrast to the restricted distribution of cyan and yellow reporters in animals in which Cre expression was controlled by a synthetic DβH reporter (see main text). (*D* and *E*) Illustration of the spread of infection to the caudal portion of the RVLM in the same animal is also marked by almost exclusive expression of the cyan and yellow reporters, consistent with RVLM circuit organization. Retrograde transneuronal passage of virus from RVLM in this animal demonstrated that the neurons expressing cyan and yellow reporters were a subset of neurons in the raphe obscurus (*F*), raphe pallidus (*G*), VMM (*H*), and nucleus of solitary tract (*I*). These data demonstrate the utility of the experimental approach for other neural systems. In this case, universal expression of Cre permits the use of PRV-263 to identify connections to all neurons within a node of the preautonomic network rather than to a phenotypically distinct population, as shown in the main text. (Scale bars, 50 μm.)

Table S1. Advantages of PRV263 over Ba2001

	Ba2001	PRV263
Replication in absence of Cre	No replication in nonmitotic cells; does not grow to high titers in standard cell lines.	Replicates well; grows to high titer in standard cell lines.
Genome stability	Selection pressure for faster growing revertants.	No selection pressure.
Injection site	Only replicates in and spreads from Cre-expressing neurons.	Replicates and marks all neurons with one fluorophor, but uniquely marks those that project to Cre-expressing neurons with distinct fluorophors.