Central Neuronal Circuit Innervating the Rat Heart Defined by Transneuronal Transport of Pseudorabies Virus

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We defined the central circuit innervating various regions of the rat heart using a neurotropic herpesvirus as a transneuronal tracer. Location of viral antigens in the brain after cardiac injection of three strains of pseudorabies virus (PRV) provided insight into vagal preganglionic neurons and their connected interneurons. At short survival times, labeled vagal preganglionic neurons were localized in both the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DMV), and in an arc-like band through the reticular formation between the NA and the DMV. The amount of DMV labeling was dependent on viral strain. Similar distributions of labeled neurons were observed following either ganglionic, sinoatrial node, or ventricular injections. At intermediate survival times postcardiac injection, the virus replicated in vagal preganglionic neurons and was trans-synaptically transported to interneurons observed primarily in the NA regions and in an arc-like band through the reticular formation. Labeled neurons were also observed in ventral regions of the nucleus of the solitary tract (NTS). At longer survival times, labeled neurons were found in various regions of the NTS with the most abundant label dorsal and dorsomedial to the solitary tract. Abundant neuronal labeling was also found in the intermedialateral cell column, the raphe nuclei, the caudal and rostral ventral lateral medulla, the A5 region, the locus coeruleus, and the lateral and paraventricular hypothalamic nuclei. These data define the central circuits including the interneuronal connections that innervate various cardiac targets.

[Key words: cardiac control circuit, rat heart, vagus nerve, viral tracing, pseudorabies virus]

While an important role of the vagus nerve in the control of cardiac rhythm and rate has been established (reviewed by Levy and Martin, 1984), there are still significant unanswered questions concerning the central circuitry of the parasympathetic innervation of the heart and the physiological role of vagal regulation. In an effort to further understanding of vagal cardiac

regulation, our goal is to develop computational models of the baroreceptor vagal reflex. A particularly difficult problem has been the identification of specific interneurons in the reflex since most conventional tracers cannot cross synapses. Alpha herpesviruses, like pseudorabies virus (PRV), infect neurons and spread from neuron to neuron in functionally connected circuits (reviewed by Enquist, 1994). The present viral connection tracing study was performed in the context of our modeling program with the aim of identifying interneuronal connections of the reflex.

Anatomical tracing studies have localized the distribution of cardiac vagal preganglionic motoneurons (CVMs) with somewhat variable results (see Discussion) (Todo et al., 1977; Bennett et al., 1981; Nosaka et al., 1982; Cabot et al., 1991; Izzo et al., 1993). However, we have little information concerning the identification of interneurons innervating the CVMs, and how their activity is regulated. It has been reported that CVMs receive inhibitory inputs with the respiratory rhythm (McAllen and Spyer, 1978) and that they receive serotonergic inputs (Izzo et al., 1993), but the cell group(s) involved is not described. CVMs are intermixed with neurons involved in other visceral and somatosensory functions and their connections cannot be selectively labeled by central injections of tracers; moreover, conventional tracers injected at the heart do not cross synapses.

The distribution of baroreceptor afferent connections to the nucleus of the solitary tract (NTS) (Ciriello and Calaresu, 1980; Panneton and Loewy, 1980; reviewed by Kalia, 1987) and the location of cardiac afferent terminals within the NTS have been determined using conventional tracers (Escardo et al., 1991). Physiological studies have also determined the region of baroreceptor termination within the NTS (Lipski et al., 1975; Donoghue et al., 1982; Numao et al., 1985; reviewed by Kalia, 1987). Surprisingly, the activity of barosensitive NTS neurons is relatively irregular and low (Humphrey, 1967; Rogers et al., 1993) compared to pulse rhythmic baroreceptor activity (Abboud and Chapleau, 1988). Such studies may point to the presence of local interneuronal networks within the NTS or other interneuronal connections that inhibit NTS activity. McAllen and Spyer (1978) demonstrated that the interval between the onset of pulse synchronous activity of baroreceptor nerves and the discharges of antidromically identified CVMs in the NA could be as long as 100 msec, suggesting that there is at least one interposed synapse. However, the location of interneuronal connections between the second order barosensitive neurons and motoneurons has yet to be identified.

In the present study, cardiac injections of PRV defined syn-

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aptically linked cardiac motoneurons, interneuronal connections, and presumed second order NTS neurons. Experiments by several laboratories have established that PRV can be a reliable trans-synaptic retrograde tracer (Card et al., 1990, 1991, 1993; Strack and Loewy, 1990; Jansen et al., 1993; Rinaman et al., 1993) and, for the circuits examined, was transported in a selective and specific manner with no obvious spread to the extracellular space (Card et al., 1993; Rinaman et al., 1993). Furthermore, the ability of PRV to replicate in neurons provides automatic signal amplification as the virus passes from first to second to third order neurons. In this study, we expected that after cardiac injection of PRV virus particles would be transported to CVMs by retrograde mechanisms, spread to connected neurons by egress at synapses, and subsequently infect interneurons innervating CVMs. Because the virus spreads by cycles of replication in each neuron, the time course of viral spread provided insight into circuit organization, and allows a partial distinction between parasympathetic- and sympathetic-related central cardiac circuits and the identification of interneurons specifically projecting onto CVMs.

The goals of this study were to determine (1) the specific parasympathetic interneuronal groups innervating CVMs, (2) the neuronal circuitry involved in cardiac regulation, and (3) the CVMs related specifically to the cardiac ventricles and the SA node.

Our initial results on cardiac ventricular innervation have been reported in brief (Standish et al., 1994) and other cardiac viral tracing results have been reported in abstracts (Miselis et al., 1989; Escardo et al., 1992; Standish et al., 1993).

Materials and Methods

One hundred and three Sprague-Dawley and Wistar rats (175–240 gm) were anesthetized with a mixture of chloral hydrate (200 mg/kg) and pentobarbital (50 mg/kg) administered intraperitoneally. The animals remained in the Biosafety level 2 laboratory (BSL-2) and were maintained in a standardized environment throughout the course of the experiment (14 hr light and 10 hr dark). Food and water were freely available.

PRV strains. Three strains of PRV were used in this study: the Becker strain, the Bartha strain, and PRV-91. The Bartha strain contains a number of mutations that reduce its virulence. PRV-91, an isogenic deletion variant of the Becker strain, lacks the gene encoding glycoprotein gE (previously called gI) (Card et al., 1992). The gE gene is not required for viral replication in cell culture (Mettenleiter, 1991) but has been implicated in other important viral functions such as virulence, target cell recognition, and efficient viral release (Wittmann and Rziha, 1989; Mettenleiter, 1991). PRV-Bartha is also missing the gE gene.

Rationale for using three strains of virus. One of the useful attributes of neurotropic herpesviruses as transneuronal tracers is that a number of different strains and well characterized mutant viruses are available that have different degrees of pathogenicity or have altered tropisms (efficiency of infecting different cell types). For our analysis we chose three PRV strains that had different degrees of virulence. We have previously noted that PRV-Bartha and PRV-Becker have distinct patterns of neurotropism in the CNS after infection of the retina (Card et al., 1991), and that PRV-Bartha can infect only a subset of retinorecipient neurons infected by PRV-Becker. We have also noted that deleting the gE gene from PRV-Becker (PRV-91) gives rise to the restricted neurotropism phenotype of PRV-Bartha after retinal infection and also reduces the virulence (Card et al., 1991; Whealy et al., 1993). Since PRV-Bartha could not trace all retinorecipient regions after retinal injection, we wanted to insure we would not miss any cardiorecipient regions after heart injection; thus, we used PRV-Bartha, PRV-Becker, and its isogenic deletion mutant PRV-91 for our analysis.

Preparation of PRV. All strains of PRV were propagated and titered on porcine kidney fibroblasts (PK15 cells). The average titer of the PRV stocks was 1×10^8 plaque-forming units per milliliter. Aliquots of viral stocks were kept at -70°C until use. All experimental procedures in-

volving PRV were conducted in a Biosafety level 2 laboratory (BSL-2).

Cardiac viral injections. The chest and neck regions of anesthetized animals were scrubbed with Betadine. A tracheostomy was performed for subsequent mechanical ventilation. The heart was exposed following a midline bisection of the sternum using a rotary Dremel drill. Once through the sternum, the animal was placed on a ventilator (Harvard Apparatus, South Natick, MA). PRV was injected using a Hamilton (Hamilton Co., Reno, NA) syringe with an attached glass micropipette tip.

Cardiac injections of PRV were made into the ganglia around the heart, the sinoatrial node, and the ventricular muscle wall. To trace vagal cardiac innervation from the preganglionic neurons, the fat pads around the heart were injected with PRV at four sites around the great vessels, 2–4 μl per site. These sites were (1) between the superior vena cava and the aorta, (2) at the intersection of the precaval vein, the left ventricle, and pulmonary artery, (3) between the aorta, the pulmonary artery, and the heart, and (4) at the junction between the right atrium and the ventricle. These sites were chosen based on anatomical studies (Burkholder et al., 1992) as well from our previous CT-HRP study (Escardo et al., 1991), which showed the location of vagal preganglionic terminals. Approximately 2–4 μl of PRV was injected into each site, about $1\text{--}5 \times 10^5$ total plaque-forming units. In other experiments only the SA node was injected with 2–4 μl of virus. Lastly, in other experiments the cardiac ventricular muscle wall was injected (2–8 μl ; approximately $1\text{--}7 \times 10^5$ total plaque-forming units) with PRV at various locations, including the apex. Some of the rats received one injection of 2 μl of PRV at varying ventricular locations while other rats received multiple injections (up to three) with 2–3 μl of PRV. Following the cardiac injections, the sites were swabbed with sterile cotton applicators, then the sternum was sutured, followed by the muscle layer and the skin. The animal was taken off the respirator and, in most cases, immediately began breathing. The tracheal tube was removed and when needed gentle aspiration was applied at the tracheal opening to remove fluid. The trachea was sutured, followed by the muscle layer and skin. Rats that died after surgery or before their scheduled death were not included in the total number of experiments.

Spinalized preparation. In some experiments, heart surgery was followed by spinal cord transection (in 21 of the 87 rats) to eliminate the transport of virus along spinal pathways. However, there was a high mortality in this group and rats that died after surgery were not included in the total number of animals used. In the spinalization procedure, atropine (0.5 mg/kg) was administered via the tail vein. Next, a small incision was made along the dorsal midline and the muscle was cleared from the vertebra in the region of $\text{C}_6\text{--T}_7$. A small cut in the cartilage between the vertebra was made to expose the spinal cord. The spinal cord was completely transected with small scissors. The muscle layers were sutured, followed by the skin layer. The bladder was cannulated to allow for the drainage of urine in some rats while in other rats the bladder was manually expressed. The animal was given approximately 5 cc lactated Ringers subcutaneously on a daily basis thereafter.

Controls. It was essential to demonstrate the specificity of viral labeling after cardiac injection. We performed specific control experiments including unilateral vagotomies to demonstrate vagal transport, intentional contamination of the surgery field with virus for nonspecific spread experiments, intravenous injections to study possible hematogenous viral spread, and topical application of virus to nerves. These analyses are described in greater detail in the Results. We also performed double immunofluorescent labeling experiments to label both neurons and non-neuronal cells. Brain tissue from a rat infected with PRV-Bartha was reacted with monoclonal antibodies against astrocytic glial fibrillary acidic protein (GFAP) and in antiserum generated against PRV particles (Rb134). We determined that we can readily distinguish neurons and interneurons from astrocytes based on size and morphology.

Perfusion and histochemical localization. At varying survival times postinjection of PRV, the animals were killed by transcardial perfusion. Each animal was deeply anesthetized and transcardially perfused with 100 ml of 0.1 M phosphate buffer (pH 7.4) and then with 200–300 ml of paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974). This fixative had a final concentration of 4% paraformaldehyde in 0.1 M phosphate buffer, 0.075 M lysine, and 0.01 M sodium *m*-periodate. The brain and spinal cord tissues were removed and post-fixed with the same fixative for 1 hr. The tissue was then rinsed in 0.1

Table 1. Experimental animals and treatments

Procedure	Injection site	Number of rats for each viral strain and hour range postinjection		
		PRV-Ba	PRV-Be	PRV-91
Intact	Ganglionic	<i>n</i> = 21 (34–95)	<i>n</i> = 8 (29–49)	<i>n</i> = 5 (51–71.5)
	Ventricular	<i>n</i> = 7 (47–95)	<i>n</i> = 6 (26–55)	<i>n</i> = 6 (49–78.5)
	SA node	<i>n</i> = 3 (79.5–82)	<i>n</i> = 4 (42–45.5)	—
Spinalized	Ganglionic	<i>n</i> = 16 (70–127)	<i>n</i> = 5 (60–72)	—
Vagotomy (unilateral)	Ganglionic	<i>n</i> = 3 (75–88)	<i>n</i> = 5 (46–49)	—
Controls (see Results)		<i>n</i> = 2	<i>n</i> = 10	<i>n</i> = 2

All injections of pseudorabies virus were made with a Hamilton microliter syringe. PRV-Ba, PRV attenuated Bartha strain; PRV-Be, Becker strain; PRV-91, Becker strain deleted for gE gene.

M sodium phosphate buffer for 1 hr and then placed in 20% sucrose solution for at least 10 hr.

After the fixation process was complete, the brain and spinal cord were serially sectioned (40 μ m thickness) using a freezing microtome. The brain was cut in the transverse or sagittal plane and the spinal cord in the horizontal plane. Free-floating tissue sections were incubated in rabbit polyclonal antisera (Rb134) (Card et al., 1990). Rb134, generated against the entire virion, recognizes major viral membrane and capsid proteins. Rb134 was diluted to a final concentration of 1:2000 with 0.1 M sodium phosphate buffer containing 1% normal goat serum and 0.3% Triton X-100. After an approximately 48 hr antibody incubation at 4°C, the tissue was processed with the avidin-biotin immunoperoxidase procedure (ABC: Vectastain, Vector Laboratories, Burlingame, CA). Tissue sections were then mounted on gelatin-coated slides, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped with Permount (Fisher Scientific).

Data analysis. Cases were analyzed with the aid of computer-based mapping and graphics. PRV-infected motor cells were distinguished from trans-synaptically virally labeled interneurons by comparison of somatic and dendritic morphology and comparisons to the location of labeled neurons by CT-HRP cardiac injections (Escardo et al., 1991; A. Standish, L. W. Enquist, J. A. Escardo, and J. S. Schwaber, unpublished observations). The brain tissue from all animals was analyzed and a detailed quantitative analysis of PRV-labeled neurons was conducted on brainstem tissue from nine animals (at short survival times) which received injections of PRV-Bartha, PRV-91, and PRV-Becker into the cardiac ganglia.

Results

Cardiac injection of PRV (PRV-Bartha, PRV-Becker, and PRV-91) resulted in consistent patterns of neuronal labeling and proved a reliable method to define populations of neurons that innervate cardiac sites. At progressively longer survival periods different populations were labeled sequentially, suggesting a hierarchical series of neuronal connections. First, we present the results on cell groups labeled at early times after infection and then we present results at increasing lengths of time following injections of PRV into the cardiac ganglia. By such a time course analysis we aim to partially distinguish parasympathetic- from sympathetic-related central cardiac circuits, and thus enable the identification of interneurons specifically projecting onto CVMs. The rationale for this analysis is that the CVMs are directly infected while only postganglionic sympathetic neurons are infected by cardiac ganglionic injection. Transneuronal infection next affects central neurons which project to CVMs in the brainstem and sympathetic preganglionic neurons in the spinal cord. The sympathetic neuronal chain from the heart to the brain in-

volves at least two additional neurons. The additional time required for infection, replication, and trans-synaptic transfer in sympathetic neurons creates a time period in which the early infection of brainstem neurons is almost certainly of parasympathetic origin. In addition, other control experiments using vagotomy and spinal transection, described below, confirmed the vagal origin of labeled cell groups.

Several additional significant findings emerged from the selective injection of various nonganglionic cardiac sites, the use of different viral strains, and control experiments such as vagotomy and spinal transection. Table 1 shows the experimental procedures, number of animals, and hours postinjection for each treatment group. In order to highlight these findings, they are presented in separate sections below.

Central cell groups labeled by cardiac injections

At various postinjection times immunohistochemical analysis of brain and spinal cord tissue was performed to detect infected neurons following cardiac ganglionic injection of PRV. Each viral strain had a characteristic rate of spread (PRV-Becker > PRV-91 > PRV-Bartha). Since the cell to cell spread of PRV-Bartha strain was the slowest, these cases were the easiest group to segment by time. Thus, the findings in the section below are based on PRV-Bartha experiments. In these cases, the pattern of results seemed distinct at “early,” approximately 47–60 hr postinjection; “intermediate,” approximately 65–75 hr postinjection; and “late,” approximately 80–95 hr postinjection. Over five animals were in each time category. Table 2 shows the location of labeled neurons in the brain after PRV-Bartha injection into the cardiac ganglia at various time points. The regions listed in this table illustrate the areas of significant neuronal labeling, and six sections from three different cases were examined and analyzed.

Early labeling. Brainstem neurons initially infected following cardiac ganglionic injections appear to be solely due to viral transport along the vagus nerve. After cardiac injection of PRV-Bartha, viral antigens were found in neurons in both caudal and rostral NA regions, and in a few neurons in the DMV. These neurons must be CVMs because they were identical in distribution and morphology to CVMs backfilled from cardiac ganglionic injections with conventional tracers that do not cross synapses, such as CT-HRP (Escardo et al., 1991; Izzo et al.,

Table 2. Immunoreactive neurons in the rat brain at early, intermediate, and late survival periods following PRV-Bartha injection into the cardiac ganglia

	"Early"	"Inter"	"Late"
Medulla			
Nucleus ambiguus	+	++	+++
Reticular arc	+	+	++
Dorsal motor nucleus of vagus	—	—	—
Nucleus tractus solitarius			
Commissural	0	+	+++
Medial/intermediate	0	++	+++
Dorso/dorsolateral	0	+	+++
Interstitial	0	+	+++
Ventral/ventrolateral	0	++	+++
Area postrema	0	+	++
Rostral ventrolateral medulla	0	++	+++
Raphe obscurus	0	++	+++
Raphe pallidus	0	++	+++
Raphe magnus	0	+	+++
Parapyramidal	0	++	+++
Lateral paragigantocellularis	0	+	+++
Pons			
Locus coeruleus	0	+	+++
Subcoeruleus	0	—	+++
Kolliker–Fuse nucleus/A7	0	—	+++
A5	0	++	+++
Parabrachial nucleus	0	—	+++
Mesencephalon			
Central gray	0	—	+++
Diencephalon			
Paraventricular nucleus	0	+	+++
Dorsal hypothalamic area	0	—	+++
Lateral hypothalamic area	0	—	+++

Not all cell groups are included in this table. 0, zero cells in case. —, 1–5 cells in case. +, 1–4 cells/section. ++, 5–10 cells/section. +++, >10 cells/section. Averaged from six sections.

1993; Standish, Enquist, Escardo, Schwaber, unpublished observations), and depend on an intact vagus nerve. Also consistent with the results using conventional connection tracers, these neurons were located (typically bilaterally) in the NA both at the level of the area postrema and in more rostral regions of the NA, extending 2 mm rostral to the obex. Labeled neurons were also found in an arc-like pattern in the lateral reticular region between the DMV and the NA primarily caudal to the obex. Neurons in the NA and the lateral reticular arc were relatively large multipolar cells (ranging from 20–30 μm) with prominent dendrites. DMV neurons were smaller (10–15 μm) and usually fusiform in shape. The absolute and relative number of neurons located in the DMV varied with the viral strain, as discussed below. Figure 1*B* shows typical early neuronal labeling of CVMs in the NA following cardiac ganglionic injection of the attenuated Bartha strain of PRV.

Intermediate labeling. At intermediate survival times more preganglionic neurons were labeled. In sections caudal to the obex, labeled neurons were found scattered in regions of the NA and the reticular arc (Fig. 1*C*). At these time points new populations of infected neurons appeared which presumably were trans-synaptically infected interneurons that project onto CVMs. These presumed interneurons were dissimilar to NA CVMs in

size and shape in that they were typically round or spindle shaped, 10–15 μm in diameter, with relatively few and relatively short dendrites. The arrows in Figure 1*F* point to interneurons in the NA region at the level of the area postrema. Infected interneurons were principally in the caudal ventrolateral medullary region, closely adjacent and intermixed with the NA motor neurons, but were also distributed within the lateral reticular arc and in the NTS. Labeled neurons were also found in the external division of the NA rostral to the obex (Fig. 1*E*) and some labeled neurons also typically appeared in the raphe pallidus and raphe obscurus. In addition, labeled neurons were located in the gigantocellular reticular nucleus and the lateral paragigantocellularis nucleus (termed parapyramidal and lateral parapyramidal nucleus), with a few scattered in the dorsal paragigantocellular nucleus. There were also infected neurons found along the superficial ventral medullary surface, in particular near the exit of the hypoglossal nerve and slightly more lateral.

Labeled neurons within the NTS (Fig. 1*C*) were located in both intermediate and ventral subdivisions of the nucleus at the level of the area postrema. Labeled neurons were absent or more rarely observed in the dorsal/dorsomedial and commissural subdivisions associated with cardiorespiratory afferents and functions (Fig. 1*D*). At the level of and about 1 mm rostral to the obex, infected neurons were present in the ventral and ventromedial regions of the NTS. At these intermediate time points labeled neurons were sometimes present in the area postrema.

Rostral to the medulla oblongata, at pontine levels, there were labeled neurons located in the A5 catecholaminergic nuclei and few to none in the A7 and A6 (locus coeruleus) catecholaminergic nuclei. Infected neurons were occasionally located in the parabrachial nuclei. Labeled neurons were present in regions of the dorsal, lateral, and ventral paraventricular hypothalamic nuclei. In addition, some preganglionic neurons of the thoracic spinal cord were labeled.

Late labeling. At late survival times virally labeled neurons were abundant in the medulla oblongata. In addition to abundant motoneuronal labeling, several NTS regions were heavily labeled (Fig. 2*B*). Many of these NTS-labeled neurons were concentrated as a distinct population in interstitial, dorsomedial, and commissural "cardiorespiratory" subdivisions (Fig. 2*C*; Paton et al., 1991). Also, labeled neurons were located within the intermediate, ventral, and ventrolateral regions of the NTS. At more rostral levels, the medial and ventral regions of the NTS were labeled. Both caudal and rostral to the obex, neurons were very heavily labeled and distributed in very distinct patterns according to caudal/rostral position relative to the obex. A distinct arc-like pattern of PRV-Bartha-infected neurons was observed in caudal sections with labeled neurons in the NA, caudal ventrolateral medulla (cVLM), throughout the lateral reticular arc, the NTS, and in the area postrema, particularly on the midline (Fig. 2*A*). This arc-shaped distribution of labeled neurons extended approximately from the level of the decussation of the pyramids to the obex rostrally. A quite distinct pattern of label was seen in more rostral sections, and this pattern extended from approximately 400 μm rostral to the obex to the caudal pole of the facial nucleus. The pattern was primarily in the ventral medulla and distributed in the shape of an inverted Y, with labeled neurons in the raphe pallidus, parapyramidal nuclei (gigantocellularis reticular nucleus and the lateral paragigantocellularis nucleus). Labeled neurons in the vicinity of the rVLM form a distinct cell group adjacent to labeled NA neurons (Fig. 2*D*).

Figure 1. *A*, Drawing of a transverse section through the caudal medulla oblongata. *B*, Labeled cardiac vagal motoneurons in the caudal NA at 53 hr postinjection of PRV-Bartha. *C*, Seventy-two hours postcardiac injection of PRV-Bartha, neurons were labeled in the NA and in reticular arc with few located in the NTS. *D*, Labeled neurons in ventral regions of NTS at 72 hr postcardiac injection. Note the lack of labeled neurons in the region of DMV indicated by dashed circle. *E*, Labeled NA neurons rostral to the obex. *F*, Two intermingled labeled NA neurons. Arrow points to small interneuron. Scale bar for *B*, *C*, *E*, 250 μ m; for *D*, 100 μ m; for *F*, 50 μ m.

Figure 2*E* shows virally infected raphe pallidus neurons. Infected neurons found along the superficial ventral medullary surface were similar in number to that observed at intermediate survival times. Labeled neurons were found scattered through the medial longitudinal fasciculus and a few infected neurons located in the parvocellular reticular nucleus, α . A few labeled neurons were located in the external cuneate nucleus rostral to the obex. Preganglionic neurons of the thoracic spinal cord were also heavily labeled (Fig. 2*F*).

At pontine levels, infected neurons were found in the A5, A6 (locus coeruleus), and subcoeruleus catecholaminergic nuclei. This label was much more abundant compared to intermediate time points. In addition, there were labeled neurons in Kolliker-Fuse nucleus/A7 region and in the lateral parabrachial nucleus.

At mesencephalic levels some infected neurons were located in the dorsal and lateral portions of the periaqueductal gray.

In diencephalic regions, the paraventricular hypothalamic regions were heavily labeled. The labeled regions include: the medial, posterior, and anterior parvocellular, the dorsomedial cap, and the ventral paraventricular hypothalamic nucleus (Fig. 3*A–D*). There were also labeled neurons in the dorsal and lateral hypothalamic nucleus. Figure 3*B* shows labeled neurons in the medial parvocellular and posterior region of the paraventricular nucleus. Figure 3, *C* and *D* shows neurons in the dorsal and ventral paraventricular hypothalamic nucleus are well labeled with a relative absence of labeled neurons between these two populations (in the medial parvocellular region) and an absence of labeled neurons in the lateral magnocellular region.

Figure 2. *A*, Caudal to the obex, neurons were heavily labeled in the NA, reticular arc, cVLM, raphe nuclei, and NTS at 87 hr postinjection of PRV-Bartha. *B*, Transneuronal labeling in the NTS region with an absence of labeled neurons in the DMV, indicated by the dashed circle. *C*, Abundant label dorsomedial to the tractus solitarius, *ts*. *D*, Rostral to the obex neurons were labeled in the NA, rVLM, and raphe nuclei. *E*, Infected neurons in the raphe pallidus. *F*, Labeled neurons in the intermediolateral cell column. Scale bar for *A*, *D*, 250 μ m; for *B*, *F*, 100 μ m; for *C*, *E*, 50 μ m.

Few to no labeled neurons were detected in telencephalic structures of the visceral neuraxis. Some labeled neurons were found in the central nucleus of the amygdala, lateral bed nucleus of the stria terminalis, and in the median preoptic nucleus in a few late survival cases.

Selective labeling of the brainstem by different viral strains

The work presented above used the slow-spreading PRV-Bartha strain, which enabled better assignment of potential interneurons in the cardiac circuit. Since PRV-Bartha previously showed restricted neurotropism in the eye model (Card et al., 1991), we also injected the parental strain, PRV-Becker, into various cardiac sites and detected immunoreactive neurons in the brain. CVMs in the medulla were infected by PRV-Becker at ± 2 mm of the obex as well as interneurons within the vis-

ceral neuraxis. Cardiac injection of PRV-Becker consistently resulted in the typical pattern of central labeling seen with PRV-Bartha, with two major exceptions: (1) the pattern of early, intermediate, and late labeling appeared on an accelerated time scale; and (2) there were differences in the absolute and relative amounts of labeling of the DMV cardiomotor population. PRV-Becker injection resulted in substantial neuronal labeling within both the NA and DMV cardiomotor populations, whereas in contrast PRV-Bartha infected relatively few DMV neurons. While both strains labeled similar numbers of NA neurons, in PRV-Becker cases up to 70% of the total cardiomotor neurons labeled were located in the DMV. Cardiac injection of both PRV strains infected DMV neurons at intermediate levels of the nucleus within ± 1 mm of the obex. Both viral strains labeled typical DMV spindle-shaped neurons ranging

Figure 3. *A*, Drawing of a section through the diencephalon. *B*, Transneuronal labeling in the paraventricular nucleus 87 hr postinjection of PRV-Bartha. *C* and *D*, Transneuronal labeling of the dorsal and ventral parvocellular nucleus. Scale bar for *B*, 250 μ m; for *C*, 100 μ m; for *D*, 50 μ m.

from 15–25 μ m with dendrites usually extending mediolaterally as observed in transverse sections. PRV-Becker and PRV-Bartha infected similar distributions of apparent motoneurons in the external formation of the NA and the reticular arc, which tended to be larger (20–30 μ m) with multipolar dendrites. Figure 4 shows neuronal labeling following cardiac injection of PRV-Becker. As seen in Figure 4*A*, labeled neurons were first observed simultaneously in both vagal motor nuclei at 46 hr postinjection. The arrows in Figure 4*A* show immunoreactive glia cells which were associated with infected vagal axons. Such non-neuronal cell labeling appears only in close proximity to infected neurons. Rinaman et al. (1993) demonstrated that some astrocytes and microglial cells in the vicinity of infected neurons become infected with PRV-Becker. They suggest that these non-neuronal cells are likely to play a role in containing viral infection so that nonspecific infection of adjacent neurons does not occur. Our studies are consistent with this hypothesis, since even at the latest survival times we never see focal spread of viral antigen in the vagal motor nuclei, as would be expected from nonspecific release of virus. At 47.5 hr postinjection of PRV-Becker, both the DMV (Fig. 4*B*) and NA (Fig. 4*C*) were heavily labeled. It was not immediately obvious whether the labeled neurons were localized within the DMV in such a way as to form rostrocaudal columns, but many labeled neurons were located in the periphery of the nucleus, for example along dorsal or lateral boundaries. Forty-eight hours postinjection labeled neurons were found in the NA and in the rVLM (Fig. 4*D*). Note the lack of labeled neurons in the compact region of the NA.

In order to determine the cause of such differences, further experiments were performed with a mutant of PRV-Becker,

PRV-91. PRV-91 is missing only one glycoprotein gene gE and this glycoprotein deletion is one of several mutations in PRV-Bartha. Card and colleagues (1992) have found that the restricted neurotropism of PRV-Bartha in the rat retina could be partially mimicked by deleting the gE gene from PRV-Becker. Therefore, we determined if PRV-91 would recapitulate the restricted neurotropism exhibited by PRV-Bartha after cardiac injection. The results obtained from cardiac injection of PRV-91 were clear: the pattern of brain labeling was now similar to the results from injection with PRV-Bartha. At early survival times a significant population of NA neurons were labeled, with relatively few infected DMV neurons. Thus, removal of a single gene from PRV-Becker resulted in markedly reduced ability to infect DMV neurons after cardiac injection. PRV-91 also infected neurons in the lateral reticular arc. Table 3 shows the percentage of labeled motoneurons in the NA, DMV, and lateral reticular arc following cardiac injection of PRV-Bartha, PRV-Becker, and PRV-91. Figure 5*A* shows a caudal section with labeled neurons in the NA, reticular arc, and NTS 68 hr postcardiac ganglionic injection of PRV-91. Similar to the PRV-Bartha cases, at intermediate survival times (60–72 hr) distinct rostral/caudal distributions were observed following injection with PRV-91. Neurons in the NTS, especially dorsomedial to the tractus, were well labeled, as shown in Figure 5*B*. Figure 5*C* shows infected neurons in the NTS region, with highest density of labeled neurons around the tractus. Figure 5*D* shows a section rostral to the obex with labeled neurons in the NA and NTS regions. At the longer survival times, labeled neurons were observed in similar regions as obtained from injection of PRV-Bartha.

Figure 4. Neuronal labeling 46 hr following cardiac injection of PRV-Becker. *A*, Infected neurons were located in the NA, reticular arc, and DMV. Arrows point to immunoreactive glia cells associated with infected vagal axons. *B*, Labeled neurons in the DMV. *C*, Infected neurons in the rostral NA; note the lack of label in the compact region. *D*, Labeled neurons in the rostral NA and rVLM. *cNA*, compact division of the nucleus ambiguus. Scale bar for *A*, 250 μ m; for *B*, 90 μ m; for *C*, 50 μ m; for *D*, 100 μ m.

Selective injections into the sinoatrial node and the cardiac ventricle

In some cases PRV injections were made selectively into the sinoatrial nodal region ($n = 5$) to examine specific innervation of this cardiac region. Figure 6*A,B* shows the distribution of labeled neurons following injection of PRV-Becker and PRV-

Bartha into the sinoatrial nodal region, similar to the distribution of neuronal labeling following ganglionic injection. When PRV-Becker was injected into the sinoatrial node region, infected neurons were found in both DMV and NA, whereas injection of PRV-Bartha infected NA neurons but more rarely infected DMV neurons. The interneuronal populations of labeled neurons, that is, in the NTS, cVLM, rVLM and raphe nuclei, were comparable to labeled neurons observed following ganglionic injections.

We next determined whether the cardiac ventricles receive vagal innervation and, if so, whether it originates from distinct central circuits as compared to the atria. PRV was directly injected into the ventricular muscle wall ($n = 6$ for PRV-Becker, $n = 6$ for PRV-91, $n = 7$ for PRV-Bartha). Following injection of PRV-Bartha and PRV-91, infected neurons were primarily located in the NA, and following injection of PRV-Becker, labeled neurons were found in both the DMV and NA. Labeled neurons were also found in the lateral reticular arc and at longer survival times in the NTS. Even when just the myocardium at the ventricular apex was injected with 2 μ l of PRV, a reduced number but a similar distribution of labeled neurons was observed. Comparisons among the different viral strains following cardiac injection into the ganglia, the left ventricle muscle wall, and ventricular apex are shown by computer-aided maps of la-

Table 3. Average number of retrogradely labeled vagal preganglionic motoneurons from 16 sections through approximately 1 mm caudal to obex, at obex, and 1 mm rostral to obex

	NA	DMV	LRA
PRV-Ba	13.3 $\pm$ 1.17 (74%)	1.4 $\pm$ 0.26 (8%)	3.3 $\pm$ 0.35 (18%)
PRV-Be	8.9 $\pm$ 0.6 (27%)	22.5 $\pm$ 1.7 (69%)	1.14 $\pm$ 0.18 (4%)
PRV-91	3.4 $\pm$ 0.4 (70%)	1.1 $\pm$ 0.29 (22%)	0.4 $\pm$ 0.08 (8%)

Neurons were located in the nucleus ambiguus (NA), dorsal motor nucleus of the vagus (DMV), and lateral reticular arc (LRA). Data was taken from nine rats following cardiac injection, three injected with PRV-Bartha (PRV-Ba), three with PRV-Becker (PRV-Be), and three with PRV-91.

Figure 5. *A*, Neuronal labeling in caudal NA, reticular arc, and NTS 68 hr after cardiac injection of PRV-91. *B*, Labeled neurons in the NTS with an absence of label in the DMV. *C*, Heavily infected neurons around and dorsal to the tractus. *D*, Rostral to the obex, labeled neurons in the NA, NTS, and raphe nuclei. Scale bar for *A*, *D*, 250 μ m; for *B*, 100 μ m; for *C*, 50 μ m.

beled neurons within brain sections (Fig. 7). While no significant differences were observed in the distributions of preganglionic labeling or interneuronal populations infected from ganglionic versus ventricular injections, the latter route of infection was slower and appeared to take about 10–15 hr longer for comparable infection. This slower rate of infection was consistent with there being an interposed synapse between the myocardium and the preganglionic neurons. If such a synapse existed, CT-HRP ventricular injection should not label brainstem neurons. We did this control experiment and confirmed that no brainstem label was observed (data not shown). Only ganglionic injection of CT-HRP resulted in labeled neurons in the brainstem (Escardo et al., 1991; Standish, Enquist, Escardo, and Schwader, unpublished observations).

Controls: vagotomy, spinal transection

In order to demonstrate that the infection of the DMV and NA by PRV-Becker depended on an intact vagus nerve, we severed one vagus nerve after cardiac injection of PRV-Becker and examined the brainstem for viral antigens. Figure 8 shows that after cardiac injection of PRV-Becker there were no infected neurons on the vagotomized side of the brain while there were a substantial number of infected DMV neurons on the contralateral side. On the vagotomized side of the brain there was an absence of label in the NA, lateral reticular arc, and the DMV, while these regions were well labeled on the intact side. In ad-

dition, there were few labeled NTS neurons on the vagotomized side of the brain.

In an effort to label only the parasympathetic components of the circuitry involved in cardiac control at late survival times, rats were spinalized at the C₈–T₁ level after cardiac injections of PRV. At the time points when we had previously observed significant viral infection in intact animals, we observed little to no infection in animals with spinal transections. Infected neurons did not appear in the brain of spinalized rats until over 110 hr postinjection for PRV-Bartha and 60 hr postinjection for PRV-Becker. This time was significantly longer than found with intact rats. The amount of neuronal label was also greatly reduced. This departure from the usually extremely predictable results of viral cardiac injection may be due to some interaction of high spinal transection and open-chest surgery (i.e., spinal shock or trauma) that influences viral infection/transport/replication in neurons. Figure 9*A–C* shows neuronal labeling at 127 hr postcardiac injection of PRV-Bartha in spinal rats. At a long survival time a few labeled neurons were present in the ventral and interstitial NTS (Fig. 9*B*), and only some dorsal to the tractus. Nevertheless, the limited results to date are consistent with our results in intact preparations. This issue will have to be the focus of separate studies designed to examine which mechanisms are involved in delayed infection/transport/replication of PRV in spinal animals.

Figure 6. Distribution of labeled neurons after injection of PRV into the SA nodal region. *A*, Labeled DMV neurons after injection of PRV-Becker. *B*, Neuronal labeling in the NTS and not in the DMV after injection of PRV-Bartha. Scale bar for *A*, 125 μ m; for *B*, 100 μ m.

Controls for nonspecific uptake and transport of virus

We purposefully contaminated the surgical field by injecting PRV into the chest cavity ($n = 3$), topically onto the vagus nerve ($n = 2$), onto a cut nerve ($n = 4$), into the lungs ($n = 2$), or into the blood ($n = 3$). In each case, no brainstem neuronal labeling was found. While topical application of PRV onto the vagus nerve did not result in brainstem infection, direct injection ($n = 3$) of PRV into the vagus nerve did result in very extensive neuronal labeling in both the NA and DMV. Direct cervical vagal injection with either PRV-Bartha or PRV-91 significantly infected neurons in the DMV, whereas cardiac injection with the same virus did not (data not shown).

Discussion

We used PRV as a trans-synaptic tracer to define the central neuronal circuitry that innervates the heart and specific targets including the sinoatrial node and the cardiac ventricle. At early time points following injection of the heart, only CVMs were infected. At intermediate survival times, PRV infected interneurons that project onto the CVMs. At late survival time points, additional neurons were infected that delineate cell groups implicated in central cardiac control circuits, some of which might be third order neurons with respect to CVMs and some of which might be labeled via a sympathetic route of infection. Among these cell groups are those of the cardiorespiratory (interstitial, commissural, and dorsomedial) NTS. Thus, based on the time course of PRV infection following injection into the heart, we were able to make a reasonable determination of the location of preganglionic neurons, interneurons, and presumed cardiorespiratory NTS neurons.

Central connections to CVMs

Taking advantage of the time course of viral spread, at intermediate survival times we were able to identify interneurons which are presumed to largely innervate CVMs. These interneurons were predominantly located within the lower brainstem including the NA regions, caudal and ventral medulla, the lateral reticular arc, and extending to the ventro/ventrolateral NTS. Neurons projecting to CVMs, which distribute in an arc through the lateral reticular field, delineate a brain region strongly associated with autonomic function (reviewed by Loewy and Spyer, 1990). It is particularly interesting that several ventral NTS regions are well labeled at this intermediate time point, while the more dorsal regions which receive direct cardiovascular sensory input are not. Labeled cell groups also included A5, locus coeruleus, and raphe cell groups which may provide catecholaminergic and serotonergic inputs to CVMs. CVMs in the NA have 5-HT-immunoreactive boutons (Izzo et al., 1993), and retrograde tracer injected into the NA labeled medullary raphe neurons (Smith et al., 1989). Our study indicates that the labeled raphe neurons were likely transneuronally labeled from the CVMs. Microinjection of HRP into cardioinhibitory regions of the NA have shown major projections from the rostral NA region, with other projections from the NTS, medial and lateral parabrachial nuclei, and the Kolliker-Fuse nucleus (Stuesse and

Figure 7. Computer-based mapping of the distribution of labeled neurons after cardiac injection of PRV. Maps are representative of the number of neurons observed in four sections located approximately 1 mm caudal to the obex. *A*, Ganglionic injection of PRV-Bartha-infected neurons in the NA (Δ), reticular arc (*), and sparsely in the DMV ($\circ$). *B*, Distribution of labeled neurons after injection of PRV-Bartha into the left ventricle. *C*, Two microliters of PRV-Bartha injected into the apex of the ventricle. *D*, Distribution of labeled neurons after ganglionic injection of PRV-Becker. *E*, Distribution of labeled neurons after ventricular injection of PRV-Becker. *F*, Distribution of labeled neurons after injection of 2 μ l of PRV-Becker into the apex of the ventricle.

Figure 8. Labeled neurons from a unilaterally vagotomized animal after injection of PRV-Becker. The vagotomized side of the brain was not infected, while on the intact side DMV neurons were well labeled. Scale bar 100 μ m.

Fish, 1984). However, HRP can be taken up by terminals on non-CVMs; thus, results from their study are not cardiac specific. In our viral tracing studies, at intermediate survival times labeled neurons are not significantly present in these areas, sug-

gesting that these regions may primarily connect to VLM interneurons, not to CVMs.

Labeled neurons were observed in the intermediolateral cell column at intermediate survival times with increased neuronal

Figure 9. Labeled neurons in spinally transected animals. *A–C*, Infected neurons in the NA 127 hr after cardiac injection of PRV-Bartha. *D*, Few labeled neurons located in the NTS region. Scale bar for *A*, 250 μ m; for *B*, 50 μ m; for *C*, 25 μ m; for *D*, 85 μ m.

labeling at later times. It is well established that the reflex fall in sympathetic nerve activity and blood pressure results from inhibition of reticulospinal neurons in the rVLM which tonically excite sympathetic preganglionic neurons in the intermediolateral nucleus. Many areas in the brain project to cardiac sympathetic preganglionic neurons including the raphe nuclei, paraventricular nucleus, A5 and A6 catecholaminergic cell groups, and rVLM (reviewed by Coote, 1988). At later survival times we found labeled neurons in the rVLM as well as in the raphe nuclei, paraventricular nucleus, and paraventricular nucleus. At longer survival times, it becomes increasingly difficult to interpret if such neurons are involved in the modulation of sympathetic or parasympathetic preganglionic neurons since both spinal and vagal routes of viral entry may have occurred. Interneurons afferent to CVMs are localized in the same cell groups as spinally projecting neurons. It appears parasympathetic and sympathetic cardiac interneurons are intermixed. In this context, the rVLM and cVLM are of particular interest. These regions are also identified with respiratory function (reviewed by Richter and Spyer, 1990), and the interneuronal cell groups may serve important integrative roles in the coordination of cardiovascular and cardiorespiratory regulation.

Labeled neurons were also found on the ventral medullary surface, a region implicated in central chemoreceptor activity (Lioy et al., 1981). Activation of this area can alter blood pressure and respiration (Lioy et al., 1981; Lawing et al., 1987), and this region has also been implicated to play a role in some fatal hypoxic syndromes. Inputs to the NA from this area would be important in the coordination of cardiorespiratory function.

Connections from the NTS

The NTS region is highly integrative, receiving inputs from many visceral structures. Specific regions of the nucleus receive distinct types of visceral inputs. Baroreceptor inputs innervate a distinct region dorso/dorsomedial relative to the tractus, which also receives cardiac as well as respiratory inputs and is thus termed the cardiorespiratory NTS (crNTS; Paton et al., 1991). This study estimates the connectivity within and to the NTS region by time series analysis after retrograde transport from infected interneurons and CVMs. At intermediate survival times following cardiac injection of PRV, a few NTS neurons were labeled, some in ventral regions, some in the interstitial division, and a few dorsomedial relative to the solitary tract. At even longer survival times, neurons in the dorsal/dorsomedial NTS were well labeled following injection of PRV. The majority of NTS neurons were labeled only at the longer survival times used, suggesting the possible interposition of another interneuron between second order NTS neurons and the cardiomotor output neurons. There are direct connections from intermediate and ventral NTS to the rVLM and regions where CVMs are located (Ross et al., 1985; reviewed by Dampney, 1994). Direct connections from the crNTS to the VLM may primarily end on interneurons rather than directly on CVMs. The labeled neurons observed in the more ventral NTS regions at intermediate survival times also suggest a plausible location for NTS interneurons afferent to CVMs. Some of these NTS neurons, especially the more ventrally located neurons, may be involved in respiratory modulation of CVMs (McAllen and Spyer, 1978). We also observed a distinctly labeled cell group within the NTS, ventral and medial to the tractus, approximately 0.8–1.5 mm rostral to the obex. To our knowledge this cell group has not been specifically implicated in cardiovascular regulation, and these neu-

rons are labeled at intermediate time points, which suggests a possible direct connection to CVMs.

Neurons in the area postrema, particularly on the midline, were also labeled at longer survival times, implying a role of this circumventricular organ in cardiovascular function. Nonspecific neuronal labeling by hematogenous spread was ruled out since no brainstem or area postrema neuronal labeling was observed after direct intravenous injection. These neurons most likely were infected by retrograde transport from NTS neurons. Efferent pathways from the area postrema to NTS have been shown by several investigators (van der Kooy and Koda, 1983; Shapiro and Miselis, 1985). Circulating hormones and peptides easily cross at the area postrema and activation of these neurons appears important for cardiovascular regulation.

Location of CVMs and PRV strain differences

Our variable results showing viral strain-dependent differences in the extent of DMV contribution to the CVM population are relevant to long standing controversies in the literature. Various studies have shown that CVMs are located (1) primarily in the NA (McAllen and Spyer, 1976; Stuesse, 1982; Cabot et al., 1991; Izzo et al., 1993), (2) mostly in the DMV (Calaresu and Cottle, 1965; Todo et al., 1977), or (3) in both the DMV and NA (Ciriello and Calaresu, 1980; Geis and Wurster, 1980; Nosaka et al., 1982; Ter Horst et al., 1993). Some of this inconsistency has been attributed to species variations, but only a few studies have examined cardiac innervation in rats (Nosaka et al., 1982; Stuesse, 1982; Izzo et al., 1993). Previous work in our laboratory using CT-HRP as a tracer showed labeling of CVMs in the NA with few in the DMV following injection of the rat cardiac ganglia (Escardo et al., 1991). Reviews suggest that the NA is the primary location of CVMs (reviewed by Loewy and Spyer, 1990) and the DMV projects primarily to subdiaphragmatic structures (reviewed by Hopkins, 1987). Our results do not significantly alter the conclusion that many DMV neurons project to subdiaphragmatic structures since the total CVM population suggested by our results totals less than 100 cells, <2.5% of the approximately 4000 vagal motor neurons (Rogers et al., 1990). The earliest retrogradely labeled neurons observed from cardiac injection of PRV-Becker were simultaneously located in the DMV and in the NA. PRV-Bartha and PRV-91 labeled only a few neurons in the DMV, but all three viral strains showed extensive labeling of the same regions of the NA. We have considered the possibility of nonspecific DMV labeling by PRV-Becker, which is discussed in the next section, but at present our results indicate a significant innervation of the heart by both the NA and DMV.

Specificity of PRV as transneuronal marker

PRV has proven to be very useful in delineating many autonomic circuits. Both PRV-Bartha (Strack et al., 1989; Card et al., 1990, 1991, 1993; Jansen et al., 1993; Rinaman et al., 1993) and PRV-Becker (Card et al., 1990, 1991, 1993; Rinaman et al., 1993) have been shown to be specific transneuronal tracers. Although cardiac injection of PRV-Becker produced increased DMV labeling, the neuronal labeling appears to be specific for cardiac circuits based on numerous control studies that show point-to-point specific label. In our study, all three PRV strains effectively labeled only functionally related neurons involved in cardiac control and none of the strains showed signs of nonspecific spread to unrelated circuits. Transecting the vagus nerve after cardiac injection of PRV-Becker demonstrated that the ob-

served label was due to viral transport along the vagus nerve. The different results obtained by PRV-Becker cannot be explained by viral spread axon to axon within the vagus nerve since a significantly different distribution of labeled neurons is found from injection into other vagally innervated organs, that is, the stomach or esophagus (Card et al., 1990; Standish, Enquist, Escardo, Schwaber, unpublished observations). For example, PRV-Becker injections directly adjacent to the esophageal/stomach border showed no signs of spread from one area to the other, as demonstrated by lack of labeling in the NA when the stomach was injected and lack of DMV labeling when the esophagus was injected (Card et al., 1990). There was no evidence of any axon–axon spread within the nerve and no non-specific central diffusion of virus from even the most severely infected cells. Both electron and light microscopic analysis demonstrated that the central release of virus was restricted by astrocytes and other non-neuronal cells responding to the local infection. Further, PRV-Becker activated local brain inflammatory responses greater than that from PRV-Bartha (Card et al., 1990; Rinaman et al., 1993).

While PRV-Bartha is an excellent tracer of neural circuitry because it has significantly reduced pathogenicity in rats and labels neurons exceedingly well, it contains a number of characterized and uncharacterized mutations that affect virulence, spread, virus stability, and tropism (Wittman and Rziha, 1989; Enquist, 1994). Card and coworkers have noted that after infection of the retina, PRV-Bartha infects only a subset of retinorecipient centers in the rat brain. This restricted neurotropism was shown to be due to the lack of the gE gene. We included PRV-91 in our studies since it is identical to PRV-Becker except that it has a defined deletion of the gE gene (Card et al., 1992). Unlike the parental PRV-Becker strain, PRV-91 was significantly impaired in its ability to infect DMV neurons after cardiac injection. This demonstrates that the more extensive labeling of the DMV by PRV-Becker is likely to be specific and dependent on the function of the glycoprotein gE. PRV glycoprotein gE is found on the virus envelope and in membranes of infected cells, and clearly plays an important role in infection and spread of PRV in certain classes of neurons. Our results open the question of the role of gE in distinguishing between DMV and NA neurons infected from cardiac injections.

Our results suggest there may be two classes of cardiomotor neurons that can be infected by PRV: one requiring the gE gene on the virus and one that does not. This observation may provide some support for the concept that the vagal cardiac outflow is in two separate functional channels. A two-component influence, fast and slow-long-lasting, of the vagus on the heart has been shown (Brown and Eccles, 1936) and these effects are attributable to myelinated (B-fiber) versus unmyelinated (C-fiber) vagal efferents (McAllen and Spyer, 1976; Ford and McWilliam, 1986). Studies have suggested that neurons in the NA and lateral reticular arc have myelinated axons while those in the DMV have unmyelinated axons (Bennett et al., 1981; Nosaka et al., 1982). Geis and Wurster (1981) demonstrated that stimulation of the DMV resulted in a reduced force of contraction with unchanged heart rate, while stimulation of the NA mediated a decrease in heart rate and increased ventricular contractility. Since axons from NA neurons pass very close to the DMV, electrical stimulation of neurons in the DMV could have activated fibers of passage. Nonetheless, the distinctions revealed in DMV and NA cardiomotor neurons by selective viral infection may be correlated with differences in functional outflows.

Innervation of specific cardiac targets

Our results demonstrate that the cardiac ventricles receive significant vagal innervation from CVMs distributed within the NA and DMV. Our results provide an anatomical substrate for the controversial but potentially important inotropic function of vagal cardiac innervation, and thus support the physiological results of Levy (reviewed by Levy and Martin, 1984). Other anatomical studies using conventional tracers would not be expected to label CVMs since cardiac postganglionic neurons have not been found in the ventricles (Loffelholz and Pappano, 1985). This prediction was supported by our own negative findings following injection of CT-HRP into the ventricles. The single study reporting positive results using this approach (Stuesse, 1982) used free HRP, which is well known to have no diffusion barriers when injected peripherally. Consistent with our findings, Ter Horst et al. (1993) observed neuronal labeling after injection of PRV-Bartha into the left ventricle made through an incision in the diaphragm. However, in contrast to our findings, they found only a limited number of animals that exhibited a pattern considered specific for the left ventricle, while other cases were excluded because of suspected overinfection (Ter Horst et al., 1993). In our study all cases appeared specific and were included in data analyses. We exposed the heart by a midline bisection of the sternum and perhaps specificity differences may be attributed to different methods used to gain access to the heart.

We found that ganglionic, sinoatrial nodal, and ventricular injections of PRV resulted in similar patterns and distributions of labeled neurons with only differences in the time course of infection. This may indicate that neurons projecting to the ventricles participate in similar control circuits, and/or that there is a great degree of convergence onto postganglionic targets. By using multiple viruses carrying distinct immunological or enzymatic reporter genes, it might be possible to distinguish whether the same or different pools of CNS neurons project to different cardiac targets. Such experiments are in progress.

Connections to other brain regions

Although our focus is on medullary circuitry, there was also significant neuronal label in the paraventricular hypothalamic neurons, in the dorsal, lateral, and ventral parvocellular nuclei. These results are consistent with known projections to the NTS (Calaresu and Ciriello, 1980; Luiten et al., 1985), raphe nuclei, parabrachial nucleus, locus coeruleus, and preganglionic neurons in the spinal cord (Saper et al., 1976; Luiten et al., 1985). Other forebrain structures of the visceral neuraxis were not well labeled, which may indicate a direct projection of paraventricular hypothalamic neurons to CVMs. Similarly, significant label was observed in paraventricular neurons but not in other forebrain structures after cardiac injection of PRV-Bartha (Ter Horst et al., 1993). It has been well established that noradrenergic and adrenergic pathways such as from the A1, A2, and C1 groups of neurons project to the paraventricular nucleus (reviewed by Spyer and Loewy, 1990). Projections from areas such as the amygdala, insular cortex, and bed nucleus of the stria terminalis to lower brainstem regions involved in cardiovascular function have been reported (reviewed by Schwaber, 1986). In the present study, the lack of neuronal label in these structures suggests their influences on cardiovascular function are likely mediated by interneurons, possibly via integrative inputs to relay neurons in the NTS. It is quite possible that the survival times have not been long enough to observe label in these areas.

References

- Abboud FM, Chapleau MW (1988) Effects of pulse frequency on single unit baroreceptor activity during sine-wave and natural pulses in dogs. *J Physiol (Lond)* 401:295–308.
- Bennett JA, Kidd C, Latif AB, McWilliam PN (1981) A horseradish peroxidase study of vagal motoneurons with axons in cardiac and pulmonary branches of the cat and dog. *Q J Exp Physiol* 66:145–154.
- Brown GL, Eccles JC (1934) The action of a single vagal volley on the rhythm of the heart rate. *J Physiol (Lond)* 612:211–239.
- Cabot JB, Carroll J, Bogan N (1991) Localization of cardiac parasympathetic preganglionic neurons in the medulla oblongata of pigeon, *Columba livia*: a study using fragment C of tetanus toxin. *Brain Res* 544:162–168.
- Calaresu FR, Cottle MK (1965) Origin of cardiomotor fibres in the dorsal nucleus of the vagus in the cat: a histological study. *J Physiol (Lond)* 176:252–260.
- Card JP, Rinaman L, Schwaber J, Miselis RR, Whealy ME, Robbins AK, Enquist LW (1990) Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. *J Neurosci* 10:1974–1984.
- Card JP, Whealy ME, Robbins AK, Moore RY, Enquist LW (1991) Two alpha-herpesvirus strains are transported differentially in the rodent visual system. *Neuron* 6:957–969.
- Card JP, Whealy ME, Robbins AK, Enquist LW (1992) Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. *J Virol* 66:3032–3041.
- Card JP, Rinaman L, Lynn RB, Lee BH, Meade RP, Miselis RR, Enquist LW (1993) Pseudorabies virus infection of the rat central nervous system: ultrastructural characterization of viral replication, transport and pathogenesis. *J Neurosci* 13:2515–2539.
- Ciriello J, Calaresu RF (1980) Distribution of vagal cardioinhibitory neurons in the medulla of the cat. *Am J Physiol* 238:R57–R64.
- Coote JH (1988) The organization of cardiovascular neurons in the spinal cord. *Rev Physiol Biochem Pharmacol* 110:148–285.
- Dampney RAL (1994) Projections from the NTS to the ventrolateral medulla: role in vasomotor control. In: *Nucleus of the solitary tract* (Barraco IRA, ed), pp 119–133. CRC Press.
- Donogue S, Garcia M, Jordan D, Spyer KM (1982) Identification and brainstem projections of aortic baroreceptor afferent neurons in nodose ganglia of cats and rabbits. 322:337–352.
- Enquist LW (1994) Infection of the mammalian nervous system by pseudorabies virus (PRV). *Semin Virol*, in press.
- Escardo JA, Schwaber JS, Paton JFR, Miselis RR (1991) Rostro-caudal topography of cardiac vagal innervation in the rat. *Soc Neurosci Abstr* 17:993.
- Escardo JA, Enquist LW, Whealy M, Schwaber JS (1992) Use of pseudorabies virus in defining the connective circuit of the medullary baroreceptor vagal reflex in the rat. *Soc Neurosci Abstr* 18:1188.
- Ford TW, McWilliam PN (1986) The effects of electrical stimulation of myelinated and non-myelinated vagal fibres on heart rate in the rabbit. *J Physiol (Lond)* 380:341–347.
- Geis GS, Wurster RD (1980) Cardiac responses during stimulation of the dorsal motor nucleus and nucleus ambiguus in the cat. *Circ Res* 46:606–611.
- Geis GS, Kozelka JW, Wurster RD (1981) Organization and reflex control of vagal cardiomotor neurons. *J Auton Nerv Syst* 3:437–450.
- Hopkins DA (1987) The dorsal motor nucleus of the vagus nerve and the nucleus ambiguus: structure and connections. In: *Cardiogenic reflexes* (Hainsworth R, McWilliams PN, Mary DASG, eds), pp 185–204. London: Oxford UP.
- Humphrey DR (1967) Neuronal activity in the medulla oblongata of cat evoked by stimulation of the carotid sinus nerve. In: *Baroreceptors and hypertension* (Kozdi P, ed), pp 131–167. New York: Pergamon.
- Izzo PN, Deuchars J, Spyer KM (1993) Localization of cardiac vagal preganglionic motoneurons in the rat: immunocytochemical evidence of synaptic inputs containing 5-hydroxytryptamine. *J Comp Neurol* 327:572–583.
- Jansen ASP, Farwell DG, Loewy AD (1993) Specificity of pseudorabies virus as a retrograde marker of sympathetic preganglionic neurons: implications for transneuronal labeling studies. *Brain Res* 617:103–112.
- Kalia M (1987) Anatomy and neurochemistry of the nucleus of the tractus solitarius. In: *Cardiogenic reflexes* (Hainsworth R, McWilliams PN, Mary DASG, eds), pp 171–184. London: Oxford UP.
- Lawing WL, Millhorn DE, Bayliss DA, Dean JB, Trzebski A (1987) Excitatory and inhibitory effects on respiration of L-glutamate microinjected superficially into the ventral aspects of the medulla oblongata. *Brain Res* 435:322–326.
- Levy M, Martin P (1984) Parasympathetic control of the heart. In: *Nervous control of cardiovascular function* (Randall WC, ed), pp 68–94. London: Oxford UP.
- Lioy F, Hanna BD, Polosa C (1981) Cardiovascular control by medullary surface chemoreceptors. *J Auton Nerv Syst* 3:1–7.
- Lipski J, McAllen RM, Spyer KM (1975) The sinus nerve and baroreceptor input to the medulla of the cat. *J Physiol (Lond)* 251:61–78.
- Loewy AD, Spyer KM (1990) Vagal preganglionic neurons. In: *Central regulation of autonomic function* (Loewy AD, Spyer KM, eds), pp 68–87. London: Oxford UP.
- Loffelholz K, Pappano AJ (1985) The parasympathetic junction of the heart. *Pharmacol Rev* 37:1–24.
- Luiten PGM, terHorst GJ, Karst H, Steffens AB (1985) The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord. *Brain Res* 329:374–378.
- McAllen RM, Spyer KM (1976) The location of cardiac vagal preganglionic motoneurons in the medulla of the cat. *J Physiol (Lond)* 258:187–204.
- McAllen RM, Spyer KM (1978) The baroreceptor input to cardiac vagal motoneurons. *J Physiol (Lond)* 282:365–374.
- McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative. *J Histochem Cytochem* 22:1077–1083.
- Mettenleiter TC (1991) Molecular biology of pseudorabies (Aujeszky's disease) virus. *Comp Immunol Microbiol Infect Dis* 14:151–163.
- Miselis RR, Rogers WT, Schwaber JS, Spyer KM (1989) Proceedings of the Physiology Society. *J Physiol (Lond)* 416:63P.
- Nosaka S, Yasunaga K, Tamai S (1982) Vagal cardiac preganglionic neurons: distribution, cell types and reflex discharges. *Am J Physiol* 243:R92–R98.
- Numao Y, Saito M, Terui N, Kumada M (1985) The aortic nerve-sympathetic reflex in the rat. *J Auton Nerv Syst* 13:65–79.
- Panneton WM, Loewy AD (1980) Projections of the carotid sinus nerve to the nucleus of the solitary tract in the rat. *Brain Res* 191:239–244.
- Paton JFR, Rogers WT, Schwaber JS (1991) Tonically rhythmic neurons within a cardiorespiratory region of the nucleus tractus solitarius of the rat. *J Neurophysiol* 66:824–838.
- Richter DW, Spyer KM (1990) Cardiorespiratory control. In: *Central regulation of autonomic function* (Loewy AD, Spyer KM, eds), pp 189–207. London: Oxford UP.
- Rinaman L, Card JP, Enquist LW (1993) Spatiotemporal responses of astrocytes, ramified microglia, and brain macrophages to central neuronal infection with pseudorabies virus. *J Neurosci* 13:685–702.
- Rogers RE, Paton JFR, Schwaber JS (1993) Firing response patterns of neurons in the nucleus of the solitary tract to changes in arterial pressure in the rat. *Am J Physiol*.
- Rogers WT, Moser AR, Schwaber JS (1990) Digital brain atlas for anatomical data comparison and integration. In: *Three dimensional neuroimaging* (Toga AW, ed), pp 123–148. New York: Raven.
- Ross CA, Ruggiero DA, Reis DJ (1985) Projections from the nucleus tractus solitarius to the rostral ventrolateral medulla. *J Comp Neurol* 242:511–534.
- Saper CB, Loewy AD, Swanson LW, Cowan WM (1976) Direct hypothalamic-autonomic connections. *Brain Res* 117:305–312.
- Schwaber JS (1986) Neuroanatomical substrates of cardiovascular and emotional-autonomic regulation. In: *Central and peripheral mechanisms of cardiovascular regulation*, pp 353–384.
- Shapiro RE, Miselis RR (1985) The central neural connections of the area postrema of the rat. *J Comp Neurol* 234:344–364.
- Smith JC, Morrison DE, Ellenberger HH, Otto MR, Feldman JL (1989) Brainstem projections to the major respiratory neuron populations in the medulla of the cat. *J Comp Neurol* 28:69–96.
- Standish A, Enquist LW, Schwaber JS (1993) Localization of vagal cardiac ventricular medullary circuits as defined by pseudorabies virus in the rat. *Soc Neurosci Abstr* 19:316.
- Standish A, Enquist LW, Schwaber JS (1994) Innervation of the heart and its central medullary origin defined by viral tracing. *Science* 263:232–234.
- Strack AM, Sawyer WB, Platt KB, Loewy AD (1989) CNS cell groups

- regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. *Brain Res* 491:274–296.
- Stuesse SL (1982) Origins of cardiac vagal preganglionic fibers: a retrograde transport study. *Brain Res* 236:15–25.
- Stuesse SL, Fish SE (1984) Projections to the cardioinhibitory region of the nucleus ambiguus of rat. *J Comp Neurol* 229:271–278.
- Ter Horst GJ, Van den Brink A, Homminga SA, Rakhorst G, Mettenleiter TC, De Jongste MJL, Lie KI, Korf J (1993) Transneuronal viral labelling of rat heart left ventricle controlling pathways. *NeuroReport* 4:1307–1310.
- Todo K, Yamamoto T, Satomi H, Ise H, Takatama H, Takahashi K (1977) Origins of vagal preganglionic fibers to the sino-atrial and atrio-ventricular node regions in the cat heart as studied by the horseradish peroxidase method. *Brain Res* 130:545–550.
- van der Kooy D, Koda LY (1983) Organization of the projections of a circumventricular organ: the area postrema in the rat. *J Comp Neurol* 219:328–338.
- Whealy ME, Card JP, Robbins AK, Dubin JR, Rziha HJ, Enquist LW (1993) Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. *J Virol* 67:3786–3797.
- Wittmann G, Rziha H-J (1989) Aujeszky's disease (pseudorabies) in pigs. In: *Herpesvirus disease of cattle, horses and pigs* (Wittman G, ed), pp 230–325. Boston: Kluwer.