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Dendritic morphology of cardiac related medullary neurons defined by circuit-specific infection by a recombinant pseudorabies virus expressing β-galactosidase

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The transneuronal herpesvirus tracer, pseudorabies virus (PRV) was used to determine the dendritic architecture of cardiac-related neurons. We constructed a derivative of the Bartha strain of PRV called PRV-BaBlu, that carries the lacZ gene of E. coli. Expression of β-galactosidase by this recombinant virus enabled us to define the dendritic morphology of motoneurons and interneurons that innervate the heart. β-galactosidase antigen filled dendritic processes that were clearly revealed by antibodies to β-galactosidase. In contrast, the standard enzymatic reaction for detection of β-galactosidase activity stained the cell soma well, but was inferior for labeling dendrites. Following PRV-BaBlu cardiac injection, infected neurons were clearly defined and labeled dendrites could be traced for long distances, sometimes greater than 800 μm from the cell body. Labeled dendrites of cardiomotor neurons primarily located in the nucleus ambiguus (NA) were extensive and sometimes intertwined with dendrites from other labeled motoneurons. Dendrites of labeled neurons in the dorsal motor nucleus of the vagus (DMV) typically extended in the mediolateral direction in the transverse plane. Transynaptically labeled interneurons interposed between the cardiorespiratory region of the nucleus tractus solitarius (NTS) and the NA were primarily located in the NA region and the reticular arc, the area between the DMV and NA. These interneurons had long dendrites extending along the reticular arc in the transverse plane. The dendritic arborizations of infected cardiac-related neurons in the NTS were variable in extent. We conclude that antibody detection of β-galactosidase expressed by PRV-BaBlu after infection of neural cardiac circuits provides a superior method to define the dendrites and dendritic fields of cardiac-related motoneurons and interneurons.

Keywords: dendritic morphology; dendrites; rat heart; cardiac circuit; viral tracing; pseudorabies virus; β-galactosidase

Introduction

The present paper focuses on a new application of a genetically engineered viral tracer and detection method to understand the dendritic organization of cardiac-related neurons. In a previous study we have used pseudorabies virus (PRV, a swine neurotropic herpesvirus) to define the central circuitry that controls the heart (Standish *et al*, 1994, 1995). It has been established that PRV infects and spreads from neuron to neuron at points of synaptic contact in functionally connected circuits (reviewed by Enquist, 1994). The only other study (Ter Horst *et al*, 1993) in which a virus was used to trace the cardiac circuitry employed a similar strain to one we used in our previous study which does not well define the dendritic organization of neurons. The majority of other studies have primarily used conventional tracers such as horseradish peroxidase and its conjugates which can not cross synapses thus are incapable of defining functionally connected neural circuits. With limited to no information available regarding the dendritic morphology of cardiac-related neurons, we now aim to extend our previous finding with improved methods to more

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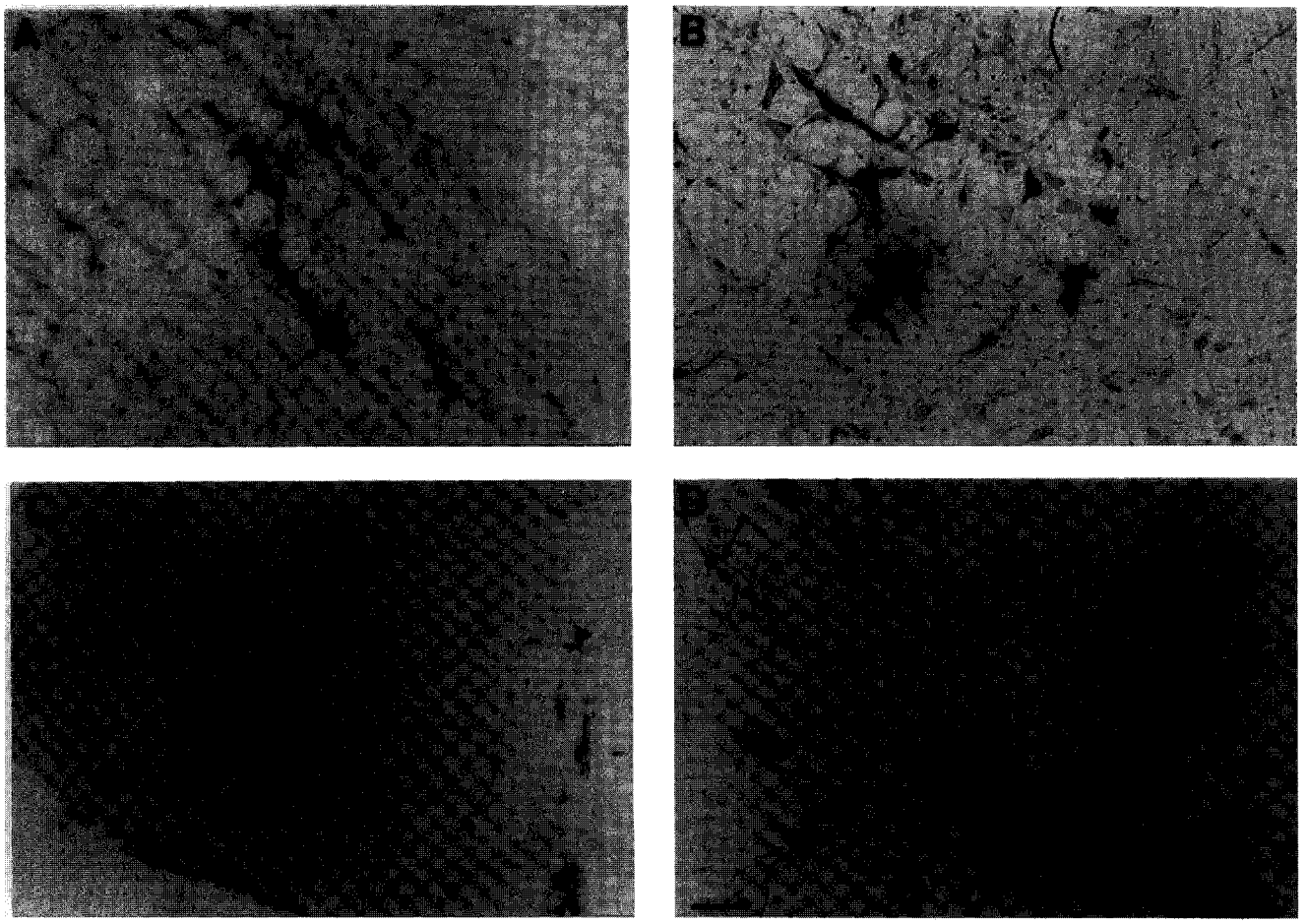

Figure 1 Differences in neuronal dendritic labeling after cardiac injection of PRV-BaBlu (~90 hours post-injection). Tissue sections were processed A: for PRV antigen (Rb134) B: for β-galactosidase enzyme activity C and D: for β-galactosidase antigen. Sections were taken from similar regions in the NA. Tissue shown in B was counterstained with Neutral Red. Scale bar 40 μm (A); 50 μm (B, D); 250 μm C.

closely examine details of the cardiac neural circuitry.

Mettenleiter and Rauh (1990) constructed a derivative of PRV-Bartha called B80 that expressed the E. coli lacZ gene encoding β-galactosidase. They showed its utility in detecting transneuronally labeled CNS neurons using histochemistry that detected enzyme activity (Loewy *et al*, 1991). For our studies, we constructed a similar viral strain following the method of Mettenleiter and Rauh (1990). Our PRV Bartha lacZ recombinant is called PRV-BaBlu and expressed β-galactosidase after infection. The present study was conducted to examine the utility of this new virus and, in particular, to develop a better method to reveal the dendritic morphology of infected neurons.

Results

When PRV-BaBlu was injected into the heart, the virus was taken up and transported to functionally connected neurons that innervate the heart. We observed consistent and reliable patterns of neuronal labeling after cardiac injection using both histochemical and immunohistochemical procedures to identify infected neurons. The patterns and distributions of labeled neurons were very consistent with results achieved using PRV-Bartha. We found that anti-β-galactosidase immunohistochemistry was superior to colorimetric detection of β-galactosidase enzyme activity and to PRV-antigen localization in defining the extent and branching patterns of dendrites of cardiac related motoneurons, interneurons, and NTS neurons. Figure 1 shows differences in dendritic labeling after cardiac injection of PRV-BaBlu resulting from tissue processing for PRV antigen (Rb134) (1A) for β-galactosidase enzyme activity (1B), for β-galactosidase antigen (1C and 1D). Tissue sections clearly show that β-galactosidase antigen (1C and 1D) revealed the dendrites better than the other two methods of processing. Dendrites could be traced for distances over 750 μm

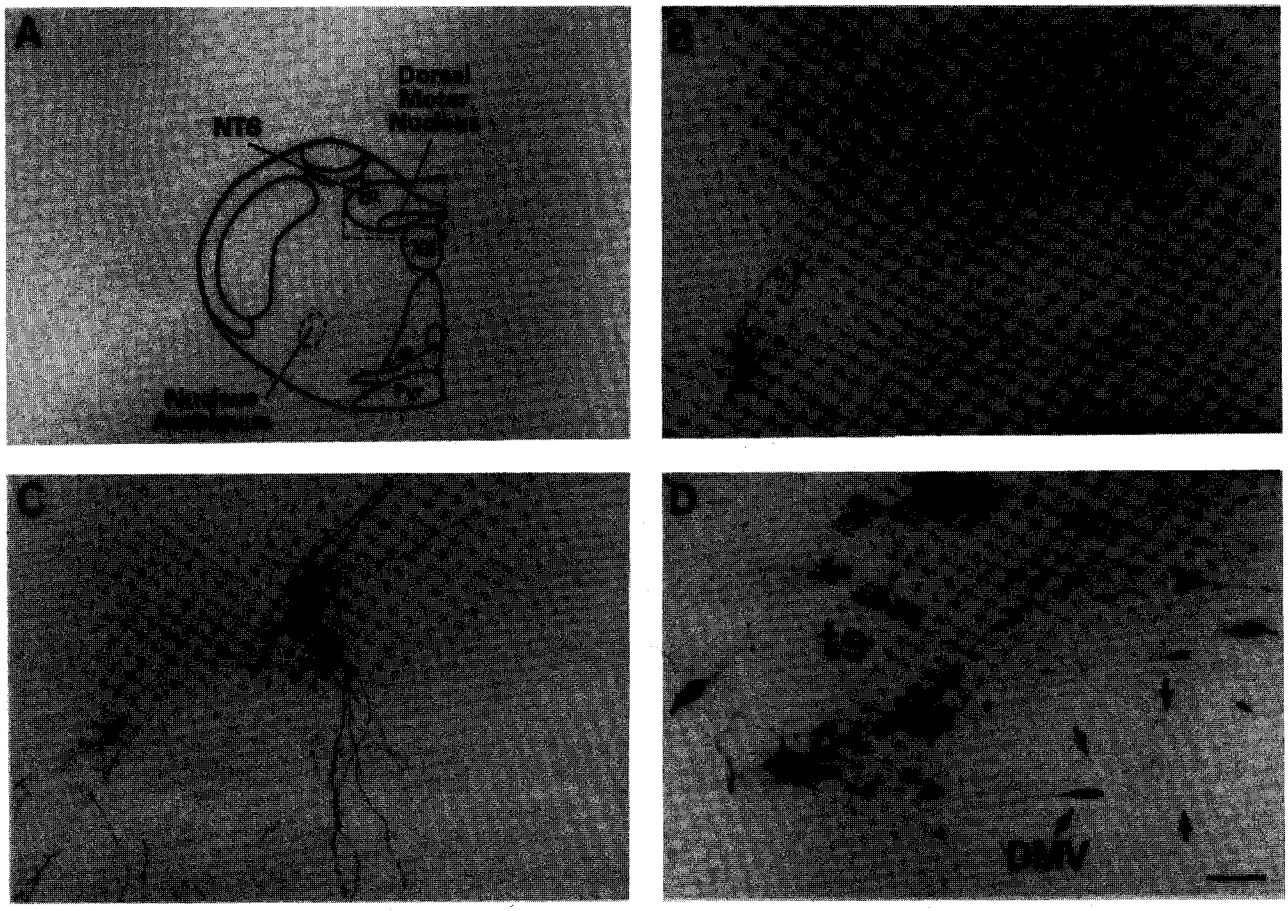

Figure 2 Labeled cardiomotoneurons after cardiac injection of PRV-BaBlu detected using β-galactosidase antisera. A: drawing of a transverse section through the caudal medulla. B: labeled cardiac vagal motoneurons in the caudal NA at 56 h post cardiac injection, C: dendrites of some labeled NA neurons were intertwined with adjacent dendrites from other labeled neurons. D: a DMV neuron labeled by PRV-BaBlu at 88 h post cardiac injection. The arrows point to the boundaries of the DMV; ts, tractus solitarius. Scale bar 250 μm (B); 60 μm (C); 50 μm (D).

following tissue processing for β-galactosidase antigen. Furthermore, beautiful results were achieved by combining the colorimetric detection of β-galactosidase enzyme activity with anti-β-galactosidase immunohistochemistry to yield a double labeled neuron (data not shown). The somas were a striking blue-brownish color and the dendrites were primarily brown especially as they travelled a distance from the soma. Thus, in most cases, the β-galactosidase antigen appeared well down the dendritic processes where enzyme was not. To obtain these results the tissue was first processed with x-gal to yield the blue reaction product. The tissue was then placed in antiserum to β-galactosidase and the reaction carried out using the standard immunohistochemical protocol.

PRV-BaBlu labeled motoneurons in the brain approximately 49 h after cardiac injection. The trans-synaptic spread (uptake, transport and replication) of PRV from infected motoneurons was relatively slow thus first, second and third order neurons can be segregated based on time post infection (Card *et al*, 1990; Enquist, 1994, Standish *et al*, 1995; Dobbins and Feldman, 1994). The pattern resulting from cardiac injection fell into three time categories. At 'early' survival times, approximately 47–60 h post-injection, vagal cardiomotoneurons were labeled; at 'intermediate' times, approximately 65–75 h post-injection, interneurons were labeled; and 'late' times approximately, 80–95 h post-injection, presumed barosensitive neurons in the NTS were well labeled.

Cardiomotoneurons

Vagal preganglionic motoneurons were the first neurons labeled by PRV-BaBlu and were found primarily in the external division of the NA both caudal and rostral to the obex. Figure 2 shows the dendrites of infected neurons were clearly defined after tissue processing for β-galactosidase antigen. Figure

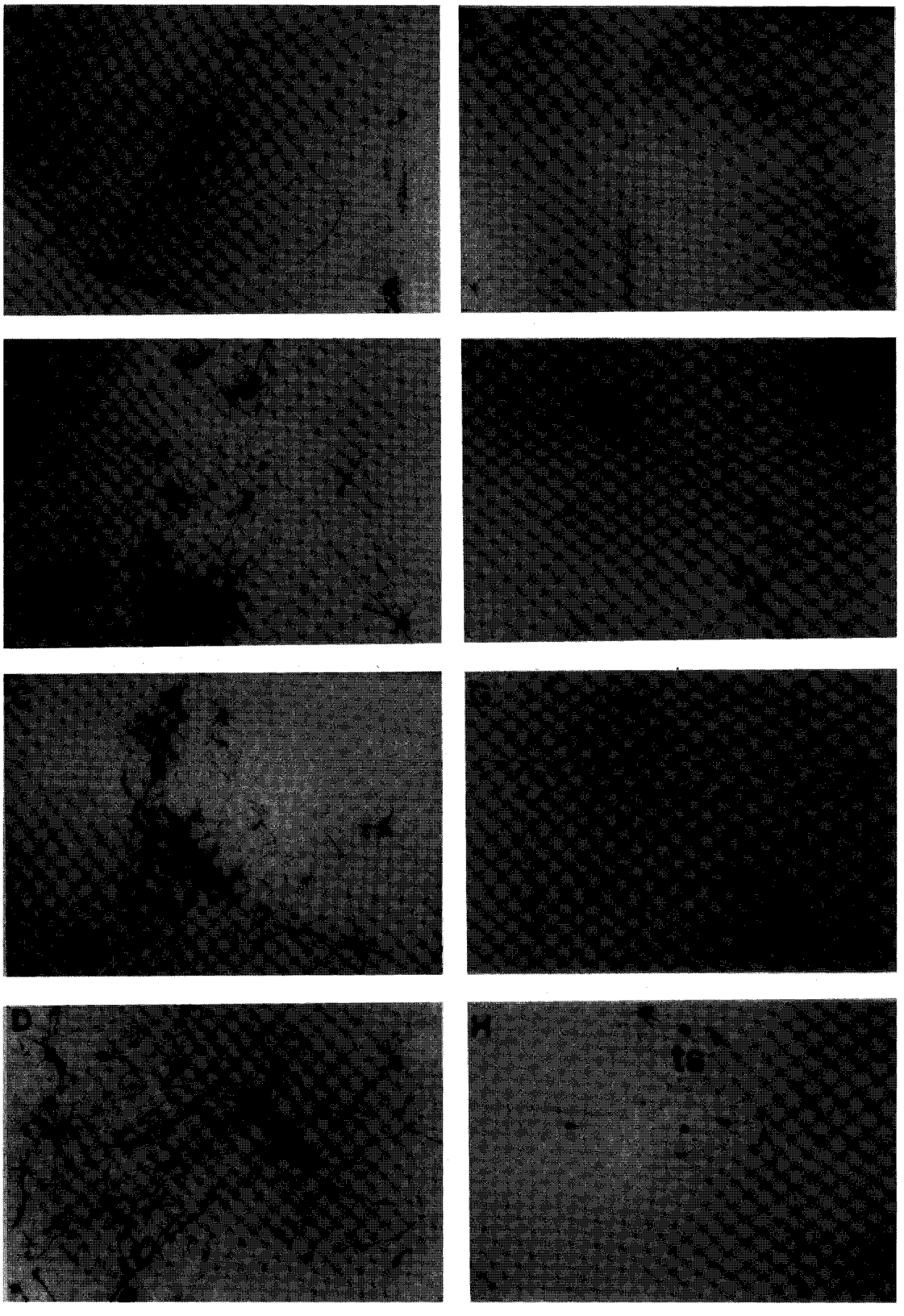

Figure 3 Dendritic morphology of labeled interneurons located in the NA regions and in the lateral reticular arc more than 72 h after cardiac injection of PRV-BaBlu. A–D: show labeled interneurons intermixed with labeled cardiomotor neurons in the NA region. E-H: show interneurons located along the reticular arc. The dendrites of the labeled interneurons shown in F extended for 760 μm. The dendrite of the interneuron shown in H extends into the ventral regions of the NTS. Scale bar 250 μm (A,E,G): for 100 μm (B,C,F,H); 50 μm (D).

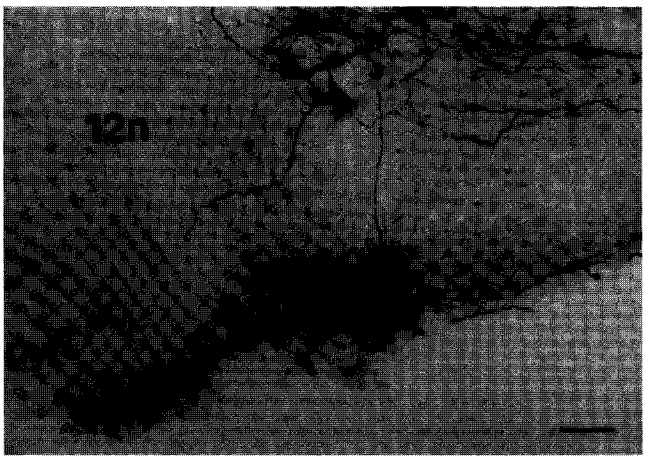

Figure 4 Labeled neurons on the ventral surface of the medulla located lateral to the exist of the hypoglossal nerve (12n), 91 h post cardiac injection. The dendrites of one neuron on the brainstem surface extends into the ventral medulla. pyr, pyramidal tract. Scale bar 50 μm.

2 shows labeled NA neurons and their labeled dendrites at the level of the area postrema. Dendrites were usually traced 200–500 μm from the soma. Typically we found between 4-5 large primary dendrites extending in all directions from the soma. Figure 2B shows several labeled neurons in the caudal NA. Figure 2C shows that the dendrites of some labeled neurons are intertwined with adjacent dendrites from other labeled neurons. Sometimes there was the appearance of swellings on some dendrites of labeled cardiomotor neurons in the NA and lateral reticular arc at higher magnification.

Only a few labeled neurons were present in the dorsal motor nucleus of the vagus (DMV). These neurons were spindle shaped and typically their dendrites extended 75–150 μm in the medio-lateral plane. Figure 2D shows a DMV neuron labeled by PRV-BaBlu.

Interneurons

PRV-BaBlu infected interneurons in the NA regions

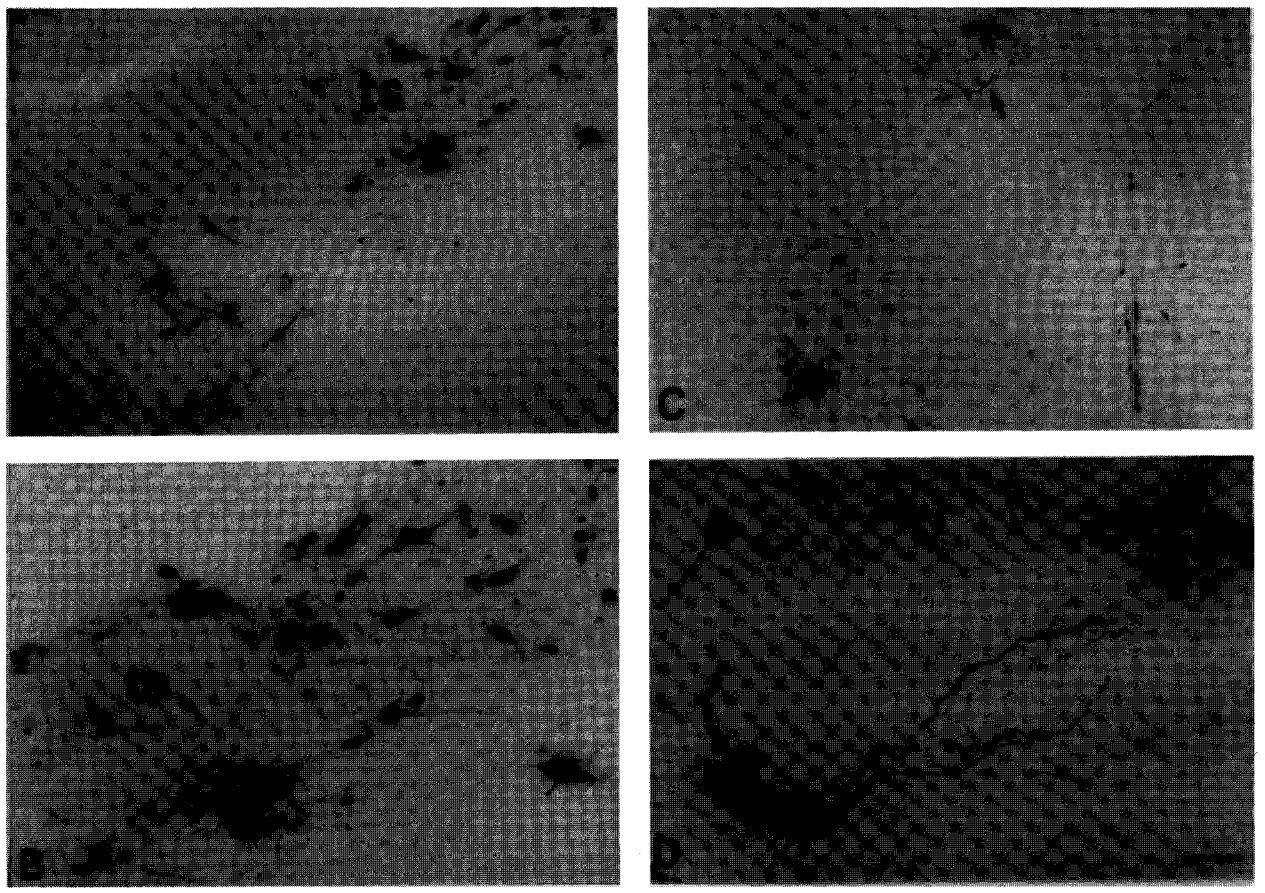

Figure 5 Dendrites of labeled NTS neurons after 90 h post cardiac injection of PRV-BaBlu. A and B: at the level of the AP heavily infected neurons around and dorsal to the tractus solitarius, ts. Dendrites of these neurons appear relatively compact. C: labeled neurons in the ventral and dorsal regions of the NTS and labeled NA neurons rostral to the obex. Arrow points to neuron shown in D. D: some dendrites of NTS neurons were tortuous especially near their termination point. Scale bar for 100 μm (A); 50 μm (B); 250 μm (C); 25 μm (D).

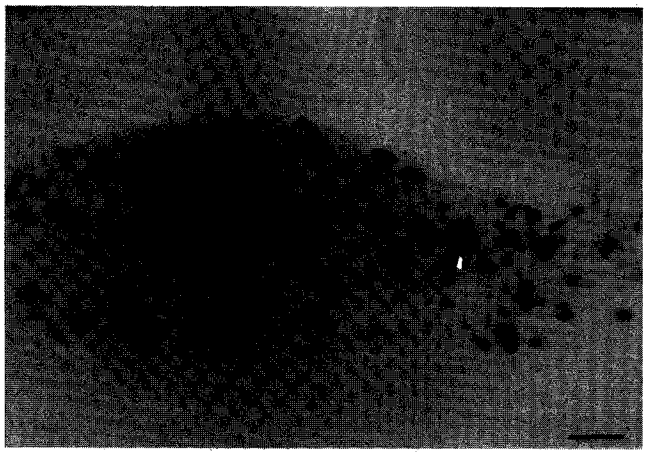

Figure 6 Labeled cells in the nodose ganglion 91 h post cardiac injection. Scale bar 100 μm.

and also distributed in the lateral reticular arc, the region between the NA and the DMV, after 72 h post-cardiac injection. The cell body size, shape and dendritic patterns of motoneurons were clearly distinguishable from interneurons. We also compared the somatic and dendritic morphology of virally labeled neurons to neurons labeled by cardiac ganglionic injection of CT-HRP which only labels motoneurons (Escardo *et al*, 1991; unpublished observations). Figure 3 shows infected interneurons and their dendritic trees in the NA regions and in the lateral reticular arc. Interneurons in these regions tended to be small (15–25 μm) elongated cells but also include some stellate shaped cells. The dendrites of interneurons in the NA region and lateral reticular arc could often be traced for up to 500 μm. Typically 2–3 primary dendrites were present on these interneurons. Dendrites which extended dorsomedially typically were located along the axis of the lateral reticular arc. Figures 3A and 3E show labeled cardiomotor neurons and interneurons in the caudal and rostral medulla respectively. Motoneurons were distinguished from interneurons based on cell size and dendritic morphology. Figures 3B, 3C, and 3D show labeled interneurons located in the NA regions, many times intermixed with labeled motoneurons. Labeled interneurons were also located along the lateral reticular arc. Figure 3F shows an elongate interneuron in the lateral reticular arc with its dendrites projecting both dorsomedially and ventromedially along the arc. The dorsomedially projecting dendrite extends over 800 μm in length ending in the ventral NTS region. In some cases, the dendrites of other interneurons could be traced to the ventral regions of the NTS (Figures 3G and 3H). Labeled interneurons were consistently found in the ventral regions of the NTS.

At intermediate survival times, PRV-BaBlu also infected other medullary neurons such as raphe pallidus and raphe obscurus neurons and gigantocellular neurons by presumed retrograde spread from infected cardiomotor cells. The dendrites of labeled neurons in the raphe pallidus extended in the dorso-ventral plane along the midline and ranged in length from 50–220 μm. The dendritic domain of raphe obscurus neurons was quite small as seen in the transverse plane. Raphe obscurus neurons had dendrites that typically extended along the midline in the dorso-ventral direction with a few dendrites extending laterally for a short distance. Figure 3B shows the extensive dendrites of labeled neurons in the gigantocellular reticular nucleus, and lateral paragigantocellularis nucleus (termed parapyramidal and lateral parapyramidal nucleus) that extended approximately 500–800 μm in the transverse plane of section. Figure 4 shows labeled neurons located along the superficial ventral medullary surface lateral to the exit of the hypoglossal nerve. Dendrites of these labeled neurons were often observed extending dorsally into the ventral medulla.

NTS neurons

Approximately 86–94 h post cardiac injection neurons in the interstitial, dorsal, dorsomedial and commissural 'cardiorespiratory' subdivisions of the NTS were heavily labeled with β-galactosidase antigen. The dendritic architecture of labeled neurons in the dorsomedial regions of the NTS was somewhat variable, and both compact dendritic trees and more extensive arborizations were observed in the transverse plane of section. Neurons were triangular, or fusiform in shape ranging in size from approximately 15–25 μm. Figure 5 shows the dendrites of infected neurons in NTS regions in the transverse plane of section. Figure 5A shows labeled neurons in the NTS in both ventral, dorsal and dorsomedial to the tractus solitarius at the level of the area postrema. Figure 5B shows a higher magnification of Figure 5A showing the abundant number of labeled neurons dorsomedial to the tractus solitarius. Rostral to the obex NTS neurons were also labeled (Figure 5C). Some dendrites of labeled dorsal NTS neurons were tortuous especially near their termination point (Figure 5D).

Neurons in the area postrema were also labeled at longer survival times. The dendrites of these neurons typically extended 15–30 μm from soma in all directions in the transverse plane of section (see Figure 1).

Nodose ganglia

Cells within the nodose ganglia were also infected following cardiac injection of PRV-BaBlu. Blue cells were readily detected following the histological reaction to detect enzyme activity of β-galactosidase in a whole mount of the nodose ganglia. This method which shows a dramatic contrast of blue infected cells on a white background was superior

in identifying infected cells in a whole mount preparation than using the Rb134 antibody combined with the standard immunohistochemical procedure which shows dark brown infected cells on light brown background. Figure 6 shows that histochemical procedures easily identified cells in the nodose ganglia that were positive for the β-galactosidase enzyme. Labeled nodose cells were found at 56–90 h post cardiac injection. Labeled cells were located throughout the medial aspect of the nodose ganglia. We could not detect any labeled axons.

Discussion

The combination of PRV-BaBlu as a transneuronal tracer with antibody detection of β-galactosidase provides a superior method to define the dendrites and dendritic arborizations of cardiac related motoneurons and interneurons. This virus had similar neurotropic properties as the Bartha strain and both viruses are very useful for the study of functionally connected circuits. However, the intense blue enzymatic reaction product and antibody detection of β-galactosidase used singly or combined provides an excellent method to label neurons and reveals dendritic organization significantly better than PRV-Bartha. Since a goal of our group is to create a computational model of neural cardiac control, defining the dendritic organization of cardiac related neurons plays a key role. PRV-BaBlu has proven to be of great value.

Cardiomotor neurons

Labeled cardiomotoneurons were found primarily in the external formation of the NA and secondarily in the DMV. These results are consistent with our previous results injecting PRV-Bartha (the parent of PRV-BaBlu; Standish *et al*, 1994, 1995) and CT-HRP (Escardo *et al*, 1991) into cardiac ganglia. Our present results demonstrate extensive dendritic arborizations of motoneurons in the NA. In some cases adjacent labeled motoneurons appear closely associated and dendrites were intertwined. Lewis (1994) demonstrated that there is dye-coupling between adjacent vagal motoneurons within the compact region of the NA. While the direct coupling of cardiomotor neurons remains unclear, communication between these NA neurons could provide a mechanism for sychronous activity in the control of heart rate. These results suggest that the extensive dendritic arborization of cardiac motoneurons provides the potential for a wide range of afferent contact that can modulate their activity.

In the DMV neurons that were labeled following cardiac injections the dendritic arborization of these neurons was quite limited compared to the more extensive dendritic field of NA neurons. Typically two primary dendrites extended in the medio-lateral plane and the dendrites tended to stay within the boundaries of the nucleus. Similarly the dendrites of the more widely studied gastric DMV neurons have dendritic arborizations that tended to stay within the boundaries of the DMV however a portion of gastric DMV dendrites extend into NTS regions (Shapiro and Miselis, 1985; Fox and Powley, 1992).

Interneurons

Neurons labeled at intermediate survival times but not at early survival times were defined as interneurons which presumably project onto cardiomotoneurons. One particular population of interneurons located in the caudal ventrolateral medulla and along the lateral reticular arc have extensive dendritic extensions. Usually for this subset of interneurons the dorsally extending dendrite could be traced for a long distance usually extending into the NTS region. The ventrally extending dendrite projects to area where the cardiac vagal mononeurons are located. These interneurons, given their location between the NA and the NTS, may be interneurons which form a connectional link between the NTS neurons and the motor output in the baroreceptor reflex. We infer that there must be at least one interposed synapse between the dorsal crNTS neurons and the cardiomotor neurons based on time to infection after cardiac PRV injection. Physiological studies have also demonstrated that there is an additional synapse since the interval between the onset of pulse synchronous activity of baroreceptor nerves and the discharges of antidromically identified cardiomotoneurons in the NA could be as long as 100 msec (McAllen and Spyer, 1978).

Cardiac-related interneurons in other areas of the brainstem include the raphe nuclei and catecholaminergic regions such as A5, locus coeruleus and A7, paragigantocellular neurons. The dendritic fields of these neurons were all quite different. Dendrites of labeled neurons in the raphe nuclei were quite restricted while the dendritic field of neurons in the paragigantocellular region and A5 were extensive. These above mentioned interneurons are likely infected from synapses on the cardiomotoneurons.

Nucleus tractus solitarius

We observed that labeled cardiac-related NTS neurons have variable dendritic arborizations. Similarly Champagnat and coworkers (1986) described diversity in size and branching patterns of neurons in the NTS by intracellular filling of NTS cells that responded to solitary tract stimulation. While these experiments examined different types of NTS neurons, our present results likely show labeled neurons from several neuronal classes in the dorsal crNTS, for example projection neurons, local circuit neurons, inhibitory interneurons and second order neurons. The varied range of dendritic branching of

NTS neurons may provide an anatomical substrate for the existence of different types of cardiac-related NTS neurons.

Direct connections from intermediate and ventral NTS to the ventrolateral medulla and regions where cardiac vagal motoneurons are located has been demonstrated (Ross *et al*, 1985, rev. Dampney, 1994). We observed labeled neurons in the more ventral NTS regions at intermediate survival times and labeled neurons in the dorsal/dorsomedial, cardiorespiratory NTS (crNTS) region at later survival times. The ventral NTS regions may be a relay for crNTS neurons. Some of these more ventrally located neurons as well as neurons located on the ventral surface of the medulla have been implicated in respiratory control (rev. by Feldman, 1986). Inputs from these regions to the cardiomotor NA neurons suggest a highly organized control for the co-ordination of cardio/respiratory systems.

Nodose ganglia

It is clear that the PRV-Bartha strain can be transported by either retrograde or anterograde mechanisms but retrograde transport is faster (Card *et al*, 1990, Card *et al*, 1991). Rotto-Percelay *et al* (1992) demonstrated that PRV-Bartha can potentially be transported via anterograde transport since viral DNA was detected in dorsal root ganglion cells after injection of the medial gastrocnemius muscle. The survival times used in our studies did not allow sufficient time for anterograde transport of PRV-BaBlu to the CNS. However, we did find infected cells in the nodose ganglia, a process which involved retrograde transport of virus from the cardiac injection site. We do not have any direct evidence for anterograde transport of virus from these ganglia cells to afferent terminals or to cardiac related NTS neurons.

Technical limitations

There are three important technical limitations in this study. First, our methods have inherent problems for assessing and reconstructing the exact dendritic arborizations of neurons with the methods described in this study. For instance, some of the tissue sections analyzed were 40 μm in thickness thus some of the dendrites that extended out of the tissue section could appear to terminate resulting in an underestimation of their length. We attempted to make 3-D reconstructions of some of the neurons taken from tissue that was serially sectioned. The results from these analyses still may not have completely overcome this limitation since tracing a labeled dendrite into new sections in abundantly labeled tissue was quite difficult. Secondly, PRV-Bartha does not infect all neurons in the cardiac circuitry for reasons that are not understood. It is well known that PRV-Bartha contains a a number of known and unknown mutations that affect its neurotropism and virulence (Wittmann and Rziha 1989, Enquist, 1994). Previous reports have demonstrated that PRV-Bartha may not infect certain classes of neurons that can be infected by PRV-Becker, the field strain of PRV (Card *et al*, 1991; Standish *et al*, 1994, 1995). As far as we can tell, PRV-BaBlu is indistinguishable from PRV-Bartha in its pattern of infections of rodent neural circuits in that strains selectively infect a subset of cardiac related neurons whereas PRV-Becker infects both cardiomotor populations in the DMV and NA (Standish *et al*, 1994a, 1994b). Specifically, PRV-Bartha and PRV-BaBlu only infect a small number of DMV neurons, about 8–10% of the total cardiomotor population. Lastly, while antibody detection of β-galactosidase expressed by PRV-BaBlu is superior in defining the dendritic morphology it is not as sensitive in detecting the total number of infected neurons as Rb134, the polyvalent antiserum generated against the entire virion. Double label experiments showed that Rb134 antisera detected more labeled neurons than histochemical procedures or immunohistochemical to detect β-galactosidase (data not shown). Although the patterns and distribution of labeled neurons were similar the procedures to detect β-galactosidase in BaBlu infected neurons may underestimate the total number of infected neurons.

Further extensions for use of PRV

In addition to using viruses as neuroanatomical tools, the neurotropic features of PRV provides additional approaches to study the mammalian nervous system. For example, since neurons die when infected with most strains of PRV, circuit-specific virus spread may be used to eliminate connected neuronal populations. Since PRV can be introduced from the periphery, it should be possible to assess the brain's response to neuronal damage to defined groups of connected neurons without confounding experimental intrusions. In addition, the neurotropic herpesvirus are being used to deliver foreign genes into specific chains of neurons in mammalian central nervous systems. Work by Loewy *et al* (1991) and by our own laboratory shows that functionally connected mammalian neurons can express the E. coli lacZ gene encoding β-galactosidase after infection with PRV recombinants. In principle, any gene of interest can be introduced and expressed via PRV in this manner. Careful choice of foreign genes and engineering of attenuated and non-cytocidal PRV derivatives should facilitate considerable progress in developing novel tracers of neural connections and probes of neuronal function.

Materials and methods

Animals

Twenty-one male Wistar rats (120–250 grams) were anesthetized with an intraperitoneal injection of chloral hydrate (200 mg kg^{-1}) and pentobarbital (50 mg kg^{-1}). Experimental procedures involving PRV

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

Virus

PRV-BaBlu was constructed essentially as described by Mettenleiter and Rauh (1990) and Loewy *et al* (1991) except that the plasmid carrying a portion of the upstream protein kinase gene, the PRV gG promotor, the first seven codons of gG and the downstream gD gene were derived from PRV-Becker and not from strain Ka. The lacZ expression cassette of the gG promotor and the first seven codons of PRV-Becker gG fused to the E. coli lacZ gene was crossed onto the genome of PRV-Bartha of the gG locus by homologous recombination. Recombinants that expressed active β-galactosidase were easily identified. A single recombinant was selected, plaque purified three times and named PRV-BaBlu. The genome structure was verified by restriction enzyme analysis and Southern blotting. Thus, in PRV-BaBlu the gG gene is disrupted by the insertion of the lacZ gene in addition to other deletions and mutations in the parental Bartha. For our studies, PRV-BaBlu stocks were prepared in PK15 cells and diluted to an average titer of 1×10^8 plaque forming units (PFU) per ml as determined on PK15 cells. Viral stocks were kept frozen at –70°C until use.

Cardiac injections and perfusion

Surgical preparation and injection locations are described in greater detail elsewhere (Standish *et al*, 1994b). Briefly, following a midline bisection of the sternum, the animal was placed on a ventilator and PRV-BaBlu was injected using a Hamilton syringe with an attached glass micropipette tip. Injections were made into either the ganglia around the heart or the left ventricular muscle. For the ganglionic injections, approximately 2–4 μl of PRV was injected into each site (about $1–5 \times 10^5$ total plaque forming units). The 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. These areas 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 effective injection sites. For the cardiac ventricular muscle wall injections 2–8 μl (approximately $1–7 \times 10^5$ total plaque forming units) was injected at various locations in the musculature of the heart. After injection, the sites were swabbed with sterile cotton applicators to reduce nonspecific viral spread. Following a survival period of 3–5 days, the animals were deeply anesthetized and transcardially perfused with 100 ml of 0.1 M phosphate buffer (pH 7.4) followed by 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 h and placed in 20% sucrose solution for at least 10 h. Brain tissue was cut in the transverse plane using a freezing microtome. Alternate sections (40 μm) in the brainstem approximately 1.5 mm caudal and 1.5 mm rostral to the area prostrema were analyzed.

Histochemistry and immunohistochemistry

Enzyme activity of β-galactosidase was determined in PRV-BaBlu infected neurons. Tissue sections were incubated in a solution containing: 40 mg/ml of 5-bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Life Technologies, Gaithersburg, MD) in dimethyl sulfoxide, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 0.01% deoxycholic acid, 0.02% nonidet P-40, 2M $MgCl_2$ in 0.1M PO_4 buffer at pH 7.3–7.4. Tissue sections were incubated for 3 hours at 32°C in the dark. After several rinses in 0.1M PO_4 buffer the tissue was mounted on slides or placed into antibody for double label experiments.

The antisera used in these studies included a rabbit polyvalent antiserum to β-galactosidase (5 Prime – 3 Prime, Boulder, CO) and a rabbit polyvalent antiserum generated against the entire virion (Rb134) (Card *et al*, 1990). Antibody was diluted to a final concentration of 1:2000 with 0.1M PO_4 buffer containing 1% normal goat serum and 0.3% Triton X-100. After approximately a 48 h antibody incubation at 4°C, the tissue was processed with the avidinbiotin 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, Fairlawn, NJ).

Double label experiments were performed in 10 of the cases. In the majority of these experiments, double labeling of neurons was achieved by detection of β-galactosidase enzyme activity which yielded a blue reaction product and anti-β-galactosidase immunohistochemistry which yielded a brown reaction product. Other double label experiments were performed using histochemical procedure to detect enzyme activity of β-galactosidase combined with immunohistochemical procedures to detect PRV antigen in neurons using Rb134, the polyvalent antiserum generated against the entire virion.

Controls

Many investigators have shown that PRV infects functionally connected neurons with little to no evidence of non-specific infection or spread to inappropriate areas of the nervous system (see review by Enquist, 1994). We have also performed numerous control experiments to insure that the neuronal label we observed was from transport along the

vagus nerve and to be certain that we did not observe CNS labeling from nonspecific diffusion of virus to adjacent nervous tissue, spread to adjacent nerves, or transfer into the blood. Such controls are discussed at length in our previous paper (Standish *et al*, 1994b).

Data analysis

Cases were analyzed with the aid of computer-based mapping and graphics to map cell location and dendritic organization (Neurolucida, MicroBrightField). 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 ganglionic injections (Escardo *et al*, 1991; unpublished observations).

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