Transneuronal tracing of neural pathways influencing both diaphragm and genioglossal muscle activity in the ferret

T. Shintani, A. R. Anker, I. Billig, J. P. Card, and B. J. Yates. Transneuronal tracing of neural pathways influencing both diaphragm and genioglossal muscle activity in the ferret. J Appl Physiol 95: 1453–1459, 2003. First published June 27, 2003; 10.1152/japplphysiol.00558.2003.—In prior experiments that employed the transneuronal transport of isogenic recombinants of pseudorabies virus (PRV), we demonstrated that neurons located ventrally in the medial medullary reticular formation (MRF) of the ferret provide collateralized projections to both diaphragm and abdominal muscle motoneurons as well as to multiple abdominal muscle motoneuron pools. The goal of the present study was to determine whether single MRF neurons also furnish inputs to diaphragm motoneurons and those innervating an airway muscle with inspiratory-related activity: the tongue protruder genioglossus. For this purpose, PRV recombinants expressing unique reporters (β-galactosidase or enhanced green fluorescent protein) were injected into either the diaphragm and PRV-152 injected into the rectus abdominis (3–6). Thus some MRF neurons have the connectivity to simultaneously alter the activity of multiple inspiratory and expiratory muscles that participate in breathing.

METHODS

All experimental procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee. Eleven adult male ferrets (Marshall Farms, North Rose, NY) weighing 1.4–2.8 kg (median of 1.8 kg) were included in the analysis. PRV recombinants. The characteristics of the two recombinants of the Bartha strain of PRV used in this study,
PRV-BaBlu and PRV-152, have been published elsewhere (5, 12, 22). Both viruses were the generous gift of Dr. Lynn Enquist (Princeton University, Princeton, NJ). PRV-BaBlu expresses β-galactosidase, and PRV-152 expresses enhanced green fluorescent protein, under the gG and cytomegalovirus immediate early gene promoters, respectively. Both recombinants of PRV were grown in pig kidney (PK15) cells and were adjusted to a final concentration of 1 x 10⁶ plaque-forming units/ml.

**Experimental design.** Two series of experiments were performed. In an initial experiment carried out with four animals, we determined the minimal survival time required after injection of PRV-152 into the left genioglossal muscle to produce transnaptic infection of premotoneurons in the brain stem. These animals were euthanized 2, 3, 4, and 5 days after inoculation; brain stem and spinal cord sections from these cases were processed for immunohistochemical localization of infected neurons by using procedures described below. A similar temporal analysis previously performed after injections of PRV into the diaphragm revealed that a 5-day postinoculation survival period was necessary to infect a substantial number of MRF neurons (5, 26). It was essential to determine the minimal latency needed to infect genioglossal premotoneurons in the MRF so that survival times could be balanced such that PRV injected into genioglossus and the diaphragm would reach the nucleus of these neurons at approximately the same time. This was important because a prior study (12) demonstrated that infection of a neuron by one virus can lower its susceptibility to infection by a second virus. Thus timing PRV injections such that the two viral recombinants reached MRF neurons at approximately the same time optimized the possibility of observing dual-infected cells. The results of these initial experiments determined the temporal sequencing of the dual viral inoculations used in the second set of seven animals, in which PRV-BaBlu was injected into the left diaphragm and PRV-152 was injected into the left genioglossus during separate recovery surgeries. In the latter cases, animals typically survived 5 days after diaphragm injections and 4 days after genioglossus injections. However, in one animal, the inoculations were separated by 48 h, such that animals survived 5 days after diaphragm injections and 3 days after genioglossus injections.

**Surgical procedures.** Animals were acclimatized in the animal housing facility for at least 1 wk before surgery. All surgeries were performed using aseptic procedures in a dedicated operating suite. For each surgery, animals were anesthetized by an intramuscular injection of ketamine (25 mg/kg) and xylazine (2.5 mg/kg) and intubated; subsequently, deep anesthesia was maintained with 0.5% – 1.5% isoflurane vaporized in oxygen to produce areflexia and stable heart rate. For PRV-152 injection into the left genioglossus, a midline incision was made over the ventral surface of the jaw, and superficial musculature was retracted; 10 μl of virus were injected into the muscle belly with the use of a 10-μl Hamilton syringe equipped with a 26-gauge needle. PRV-BaBlu (100 μl) was injected into the diaphragm as described in previous manuscripts (3, 5, 26). Briefly, a midline incision was performed through linea alba, and the viscera were retracted to expose the left diaphragm; PRV was then injected at multiple sites into both the costal and crural portions of the muscle. After completion of the incisions were sutured closed, and 3 mg/kg ketoprofen was injected to provide analgesia. Animals were maintained under biosafety level II conditions until they were killed. After the appropriate survival times, animals were deeply anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (2 mg/kg), and were perfused transcardially with 1 liter of 0.15 M NaCl followed by 2 liters of 4% paraformaldehyde-lysine-periodate fixative (13). The brain and spinal cord segments C1–L4 were subsequently removed, postfixed for 4–5 h in 4% paraformaldehyde-lysine-periodate fixative, and then cryoprotected by incubation for 2 days in 30% aqueous sucrose at 4°C.

**Tissue processing.** The brain stem and spinal cord segments removed from all animals were sectioned coronally at 35-μm thickness by using a freezing microtome; tissue sections were collected in six sets so that spacing between adjacent sections in a set was 210 μm. Tissue sections were stored at –20°C in cryoprotectant (25) until they were immunoprocessed.

One tissue set from each animal employed for dual injection experiments was processed by using a carbocyanine double-fluorescence method described in detail elsewhere (5) to localize the distribution of neurons infected by injection of PRV-BaBlu into the diaphragm and PRV-152 into genioglossus. Briefly, sections were incubated in a primary antibody solution for 2 days at 4°C. A mouse monoclonal antibody (1:1,500; Sigma Chemical, St. Louis, MO) identified the unique reporter of PRV-BaBlu, whereas a rabbit polyclonal antibody that recognized enhanced green fluorescent protein (1:1,000; Molecular Probes, Eugene, OR) identified the unique reporter of PRV-152. Tissue was then washed in 0.1 M sodium phosphate buffer and incubated for 2 h at room temperature in a solution containing donkey anti-mouse secondary antibody conjugated to the CY3 carbocyanine (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-rabbit secondary antibody conjugated to Bodipy (1:300; Molecular Probes). Thus neurons infected with PRV-BaBlu exhibited red fluorescence, and neurons infected with PRV-152 exhibited green fluorescence. Neurons containing both fluorophors appeared yellow. To provide a more complete mapping of the distribution of genioglossal and diaphragm premotor neurons in the brain stem, two other wells of tissue were processed by using immunoperoxidase procedures; this method was also utilized in initial single-injection experiments where genioglossus was inoculated with PRV-152. This processing made use of the same primary antibodies employed for the double fluorescence method described above along with the avidin–biotin modification of the peroxidase-antiperoxidase procedure (11). Affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories) and Vectastain reagents (Vector Laboratories, Burlingame, CA) were employed for these immunoperoxidase localizations. On completion of the immunohistochemical processing, the tissue was mounted on gelatin-coated slides, dehydrated, cleared, and coverslipped with Cytoseal 60 (VWR Scientific, West Chester, PA).

**Tissue analysis.** Immunoreacted tissue sections were examined and photographed by using a Zeiss Axiosplan microscope equipped with epifluorescence and filters that selectively excited Bodipy or CY3 and with a filter that allowed for the excitation of both fluorophors. As noted above, the red fluorescence of CY3 was used to identify cells infected after injection of PRV-BaBlu into the diaphragm, whereas the green fluorescence of Bodipy was used to identify neurons infected by PRV-152 injected into the genioglossal muscle. Putative dual-infected neurons were examined by using a x40 objective to ensure that yellow fluorescence signal reflected the intracellular localization of both CY3 and Bodipy and was not due to the overlap of neurons differentially labeled with each fluorophor. Digital photographs of neurons were obtained with a Hamamatsu camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Simple-32 PCI image anal-
double-labeled neurons in the MRF are indicated in Fig. 3. All animals, thoracic spinal cord sections were included in our sample. Because these presumed sympathetic preganglionic neurons were few in number and in an early stage of infection, it seems unlikely that they served as a conduit for transmission of PRV to cells in the brain stem. Furthermore, the distribution of infected brain stem neurons was very similar in all animals. Thus it appears as though all brain stem labeling noted in these experiments was the result of transport of PRV through motor circuitry. We also examined cervical and thoracic spinal cord sections to establish the locations of PRV-BaBlu infected diaphragm motoneurons. These neurons were located in the C5–C6 spinal segments, as described in our laboratory’s previous studies (5, 26). In two cases, however, a very limited number of PRV-BaBlu infected motoneurons was also present at the midthoracic level, indicating that our PRV injections involved the intercostal muscles adjacent to the costal diaphragm. Nonetheless, because the number of infected cervical motoneurons greatly exceeded that of thoracic motoneurons in these cases, and the distribution of labeled brain stem neurons was similar in all animals as well as to that reported in our laboratory’s previous studies that involved PRV injections into the diaphragm (5, 26), these data were included in our sample.

**Labeling of hypoglossal motoneurons produced by PRV-152 injections into genioglossus.** Injection of PRV-152 into the genioglossal muscle produced infection of large presumed motoneurons in the ipsilateral hypoglossal nucleus. These infected neurons were present at all survival times employed in this study. Figure 1A illustrates labeling of motoneuron cell bodies and processes in one case. Figure 1B shows reconstructions of the locations of infected hypoglossal motoneurons in two animals. Infected motoneurons were present from −0.5 mm caudal to the obex to 1.25 mm rostral to the obex but were absent in the caudal portion of the hypoglossal nucleus extending from −0.5 to 1.25 mm caudal to the obex. As illustrated in Fig. 1B, the infected motoneurons were concentrated in the ventrolateral portion of the hypoglossal nucleus.
Labeling of brain stem premotor neurons produced by PRV injections into the genioglossal muscle and the diaphragm. In initial experiments, the relative number of neurons infected outside of the hypoglossal nucleus was compared for survival times ranging from 2 to 5 days after injection of PRV-152 into the genioglossal muscle. After a 2-day survival time, all infected neurons were confined to the hypoglossal nucleus. Infected premotoneurons were first evident in the MRF 3 days after injection of genioglossus. The number and intensity of labeling of these neurons increased with survival times of 4–5 days, although by 5 days some MRF neurons showed cytopathic damage, indicating the presence of an advanced infection. On the basis of these findings, we decided that a 4-day survival period after genioglossal muscle injections was optimal for defining premotor neurons infected by transneuronal passage of PRV from hypoglossal motoneurons. We confirmed that this survival time was ideal in an additional control experiment in which an animal was euthanized 3 days after injection of PRV-152 into genioglossus and 5 days after injection of PRV-BaBlu into the diaphragm. The intensity of labeling resulting from infection of MRF neurons by PRV-BaBlu was considerably stronger than that produced by PRV-152 in this case. In contrast, the infection of MRF neurons by the two recombinants was balanced when animals were killed 4 days after the genioglossus injections and 5 days after the diaphragm injections.

Figure 2 illustrates examples of single and dual labeling of MRF neurons infected by injection of PRV-152 into genioglossus and PRV-BaBlu into the diaphragm, and Fig. 3 shows a reconstruction of the locations of infected MRF neurons from the same animal. The distribution of MRF cells infected by PRV-152 and PRV-BaBlu was largely overlapping; most of these neurons were located from 1 to 4 mm rostral to the obex, as indicated in Fig. 4. The labeled MRF neurons were observed bilaterally, with a slight ipsilateral propensity.

Table 1 summarizes the number of MRF cells infected with one or both viral recombinants in all animals (n = 6) in which the survival time after PRV-152 injections into the genioglossal muscle was 4 days and the postinoculation time after PRV-BaBlu injections into the diaphragm was 5 days. Because the distribution of single- and double-labeled cells was similar in the ipsilateral and contralateral MRF, neuronal counts from both sides were pooled. The relative number of MRF neurons infected with PRV-152 and PRV-BaBlu varied between animals, as did the fraction of the neurons that was infected by both recombinants. However, dual-infected cells that presumably were premotor to both genioglossal and diaphragm motoneurons

![Figure 3](image-url)
were present in every animal. Overall, ~10% of the MRF neurons that were infected by either PRV-152 or PRV-BaBlu also expressed the other recombinant. In addition to the MRF, the medullary lateral tegmental field also contained infected cells, mainly in the vicinity of nucleus retroambigualis, nucleus ambiguus, and the retrofacial nucleus. However, across all experiments, only a total of 79 PRV-152 and 38 PRV-BaBlu infected neurons were observed in this region. A comparison of these cell counts to those for the MRF presented in Table 1 shows that labeled neurons were far more prevalent in the medial MRF. Furthermore, only two double-infected neurons were observed in the lateral tegmental field across all animals.

**DISCUSSION**

The major finding of this study is that a small population of neurons in the MRF of the ferret provides divergent inputs to both genioglossal and diaphragm motoneurons. It should be noted, however, that infection of a neuron by one virus can make it less permissive to being infected by a second virus (12). Thus, although recombinants with similar virulence were employed in the study (5, 12, 22), and survival times were optimized so that the two viruses infected MRF neurons at approximately the same time, it is possible that the number of MRF neurons that provide inputs to both diaphragm and genioglossal motoneurons was underestimated. Nonetheless, the present data in combination with those from our laboratory's previous transneuronal tracing studies (5, 6) suggest that some MRF neurons make synaptic connections with multiple populations of motoneurons innervating two or more respiratory muscles, whereas others influence only a single muscle.

In addition to MRF neurons, some cells located in the lateral tegmental field in the vicinity of nucleus retroambigualis, nucleus ambiguus, and the retrofacial nucleus were infected by PRV injected into either the genioglossal muscle or the diaphragm. The majority of neurons in the ferret with activity related to the respiratory cycle are located in this region (21), and thus it seems likely that many of the infected cells we observed in the lateral tegmental field were respiratory group neurons. The preponderance of these cells was infected by only one viral recombinant, suggesting that individual respiratory group neurons typically are not responsible for imparting inspiratory-related firing on both genioglossal and diaphragm motoneurons. This observation is in keeping with physiological evidence that suggests that respiratory control of hypoglossal motoneurons and the diaphragm originates from different neurons in the lateral tegmental field (16).

The motoneurons infected by PRV-152 injected into the genioglossal muscle were located in the ventrolateral MRF, medullary reticular formation; PRV, pseudorabies virus.

Table 1. Number of infected neurons in the MRF after injection of PRV-BaBlu into the diaphragm and PRV-152 into the genioglossal muscle

<table>
<thead>
<tr>
<th>Case No.</th>
<th>PRV-152</th>
<th>PRV-BaBlu</th>
<th>Double Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>136</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
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<td>2</td>
</tr>
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<td>4</td>
<td>48</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>108</td>
<td>66</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>317</td>
<td>319</td>
<td>36</td>
</tr>
</tbody>
</table>

Neuronal counts were obtained from 1 of 6 bins of brain stem tissue collected. Survival times were 5 days after diaphragm injections and 4 days after genioglossal muscle injections. Neuronal counts from both the ipsilateral and contralateral sides were pooled.
eral portion of the hypoglossal nucleus. This observation is in accordance with previous anatomical studies in the rat and cat (1, 9, 23, 24), suggesting that our PRV-152 injections selectively targeted genioglossus. However, the distribution of transneuronally infected genioglossal premotor neurons was more restricted in our experiments than was previously reported in the rat (9). In particular, we noted little labeling in nucleus tractus solitarius, the spinal trigeminal nucleus, or pontine or mesencephalic regions, although infection of neurons in these regions was reported 3.5–4 days after injection of PRV into the rat genioglossal muscle. These differences in results are likely due to the fact that brain size is much smaller in the rat than in the ferret, which allows PRV to be transported across more synaptic connections in the former species within a fixed postinjection survival period. Unfortunately, an analysis of brain stem labeling produced after a variety of times subsequent to inoculation of the rat genioglossal muscle with PRV is not available, and thus it cannot be ascertained whether similar populations of premotor neurons provide direct inputs to genioglossal motoneurons in the rat and ferret.

The present data showing that individual MRF neurons can provide inputs to both genioglossal and diaphragm motoneurons provide clues regarding the role of this region in respiratory control. Prior studies have demonstrated that individual MRF neurons provide collateralized projections to both diaphragm and abdominal motoneurons (5) or to multiple abdominal muscle motoneuron pools (6). The present findings indicate that some of these cells also send axonal branches to upper airway motoneurons. MRF neurons with highly divergent projections must play a fairly generalized role in respiratory control, such as globally adjusting the excitability of respiratory motoneurons such that they are more or less responsive to other inputs. Some postural changes in quadrupeds, such as nose-up tilts of the body, can produce increases in activity of both the diaphragm and abdominal muscles to maintain the diaphragm at a constant resting length (19), as well as increases in activity of genioglossus to maintain airway patency (20). Collateralized projections of MRF neurons are ideally suited for producing such posturally related changes in respiratory muscle activity, particularly since a majority of neurons in this region receive inputs from the vestibular system (7, 17, 18). However, some cells in the MRF only appear to provide inputs to particular respiratory motoneurons, and could serve to elicit responses that involve the contractions of specific muscles. Because neurons in the ferret MRF do not have firing that is correlated with the respiratory cycle (21), the functions of these cells do not appear to be related to the generation of breathing-related discharges of respiratory muscles. One possibility is that these neurons engage in the regulation of the contractions of respiratory muscles that are unrelated to respiration, such as those that occur during emesis. Support for the latter postulate lies in the observation that some MRF neurons fire during the bursts of activity of respiratory muscles that generate vomiting, and lesions placed in this area impair the ability to vomit (15). Nonetheless, physiological studies that involve recording the behavior-related activity of MRF neurons whose axonal projections have been mapped through antidromic stimulation will be required to fully elucidate the functions subserved by this area.

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DISCLOSURES

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REFERENCES


