Definition of Neuronal Circuitry Controlling the Activity of Phrenic and Abdominal Motoneurons in the Ferret Using Recombinant Strains of Pseudorabies Virus


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During a number of behaviors, including vomiting and some postural adjustments, activity of both the diaphragm and abdominal muscles increases. Previous transneuronal tracing studies using injection of pseudorabies virus (PRV) into either the diaphragm or rectus abdominis (RA) of the ferret demonstrated that motoneurons innervating these muscles receive inputs from neurons in circumscribed regions of the spinal cord and brainstem, some of which have an overlapping distribution in the magnocellular part of the medullary reticular formation (MRF). This observation raises two possibilities: that two populations of MRF neurons provide independent inputs to inspiratory and expiratory motoneurons or that single MRF neurons have collateralized projections to both groups of motoneurons. The present study sought to distinguish between these prospects. For this purpose, recombinant isogenic strains of PRV were injected into these respiratory muscles in nine ferrets; the strain injected into the diaphragm expressed β-galactosidase, whereas that injected into RA expressed green fluorescent protein. Immunofluorescence localization of the unique reporters of each virus revealed three populations of infected premotor neurons, two of which expressed only one virus and a third group that contained both viruses. Dual-infected neurons were predominantly located in the magnocellular part of the MRF; but were absent from both the dorsal and ventral respiratory cell groups. These data suggest that coactivation of inspiratory and expiratory muscles during behaviors such as emesis and some postural adjustments can be elicited through collateralized projections from a single group of brainstem neurons located in the MRF.

Key words: pseudorabies virus; diaphragm; abdominal muscle; emesis; dorsal and ventral respiratory groups; medullary reticular formation; raphe nuclei; dual-labeling immunofluorescence

The diaphragm and abdominal muscles typically contract out of phase as they induce inspiration and expiration, respectively. These contractions are regulated by the brainstem dorsal and ventral respiratory groups, which generate the respiratory rhythm and also impose that rhythm on respiratory motoneurons (Feldman, 1986). However, the diaphragm and abdominal muscles also contract in phase during a number of behaviors, including emesis (Gold and Hatcher, 1926; McCarthy and Borison, 1974), some postural adjustments (Grillner et al., 1978), and in response to vestibular stimulation (Yates et al., 1993; Rossiter et al., 1996). Brainstem respiratory group neurons are not responsible for eliciting simultaneous increases in inspiratory and expiratory muscle activity during at least some of these responses. For example, bulboinspiratory neurons in the dorsal and ventral respiratory groups are inhibited and mainly silent during emesis (Bianchi and Grélot, 1989; Miller et al., 1990). Furthermore, vestibular stimulation can activate respiratory muscles without modulating the firing of respiratory group neurons (Yates et al., 1994; Woodring and Yates, 1997), and lesions of the main respiratory groups do not abolish vestibulo-respiratory reflexes (Yates et al., 1995; Rossiter et al., 1996; Shiba et al., 1996; Woodring and Yates, 1997).

Previous neuroanatomical studies in the rat employing the transneuronal transport of pseudorabies virus (PRV) injected into the diaphragm have revealed the locations of inspiratory neurons in the brainstem and spinal cord of this species (Dobbins and Feldman, 1994). It was reported that rat inspiratory premotor neurons were mainly located in the dorsal and ventral respiratory groups, although a few labeled neurons were present in the raphe nuclei, medial reticular formation, and parabrachial nucleus. Nevertheless, differences in the organization of neurons presynaptic to phrenic motoneurons were recently demonstrated in an emetic species, the ferret. In contrast to the rat, the ventral portion of the ferret medial medullary reticular formation (MRF), particularly the magnocellular division, contained a substantial number of infected neurons after the injection of PRV into the diaphragm, although both species exhibited infection in the region of the ventrolateral reticular formation known to contain the ventral respiratory group (Yates et al., 1999). In another group of experiments, neurons in the MRF were also infected by transynaptic passage of PRV from the ferret rectus abdominis (RA) muscle, along with neurons in portions of the ventrolateral reticular formation known to contain the ventral respiratory group, the nucleus retroambiguus, and the raphe nuclei (Billig et al., 1999). The overlapping distribution of labeled MRF neurons after injection of PRV into either the diaphragm or RA raises two possibilities: that single neurons in this area could have collateralized projections to both inspiratory and expiratory motoneurons or that two populations of neurons with overlapping distributions provide parallel inputs to inspiratory and expiratory motoneurons, as is the case for cells in the ventral respiratory group (Feldman, 1986).

In the present study, two antigenically distinct recombinant strains of PRV were used in dual injection paradigms to distinguish between these two prospects. This experimental approach is based on the ability of the two recombinant viruses to coinfect neurons with common synaptology (Fig. 1A). Recent studies have demonstrated the utility of this approach in mapping autonomic and visual circuitry (Jansen et al., 1995; Levatte et al., 1998; Ueyama et al., 1999) while also identifying the factors that may contribute to the generation of false negatives (Kim et al., 1999; Mabon et al., 1999). Although these data reveal the need to be conservative in interpreting negative findings, the demonstrated ability of two recombinants to coinfect neurons in these dual infection paradigms.
provide a powerful means of addressing issues of collateralization that cannot be achieved with other anatomical methods. In the present study we have adapted this experimental approach to determine if a single population of brainstem neurons is presynaptic to two distinct populations of motoneurons involved in the control of respiration and emesis in the ferret.

MATERIALS AND METHODS

Animals. Experiments were conducted in 17 adult male ferrets (obtained from Marshall Farms, North Rose, NY). Animals were housed singly and allowed a minimum of 1 week acclimation to the animal facility before being injected with PRV. The experimental procedures used in this study conformed to regulations stipulated in the United States Department of Health and Human Services publication CDC 88–8395 and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Recombinant viruses. The experimental strategy is illustrated schematically in Figure 1A, and the organization of the genome of the recombinants used is shown in Figure 1B. Three recombinants of the Bartha strain of PRV (PRV-Bartha) that express either β-galactosidase (β-gal) or enhanced green fluorescent protein (EGFP) were used in this analysis. PRV-Bartha is an attenuated strain of PRV developed as a vaccine (Bartha, 1961). It has been used widely for transneuronal tracing (cf., Enquist et al., 1999; Card, 2000 for recent reviews), including in previous studies that provided the foundation for the present investigation (Billig et al., 1999; Yates et al., 1999). Preparation of PRV-Bartha, a recombinant Bartha strain that expresses β-gal, has been described previously (Standish et al., 1995; Kim et al., 1999). Briefly, this strain contains the lacZ gene at the gG locus such that EGFP is constitutively expressed using the cytomegalovirus immediate early promoter. The cell body, nucleus, and processes of cells infected with PRV-152 are filled with EGFP. PRV-154 expresses a novel antigen produced by PRV-154 (EGFP-US9). The genomic organization of the recombinant viruses used in this study is illustrated in Figure 1B. The genome of PRV contains unique long (UL) and unique short (US) regions. The parental strain used to produce the recombinants used in this study was an attenuated vaccine strain known as PRV-Bartha. PRV-Bartha has several well-characterized mutations and deletions that distinguish it from the wild-type virus (PRV-Becker). These alterations are mapped on the diagram. All recombinants involved insertion of the transgene at the gG locus of the viral genome. In PRV-Bartha, gG was replaced with the gene encoding β-galactosidase. In PRV-152 and PRV-154, the gene encoding green fluorescent protein was inserted into the gG gene, either alone (PRV-152) or as part of a fusion protein (PRV-154). The distribution of the reporters within two infected neurons that were both double-labeled. β-galactosidase expression in neurons infected with PRV-Bartha provided staining of neuronal perikarya and dendrites (red fluorescence, panels B and E). Two EGFP-expressing viruses (PRV-152 and PRV-154) labeled cells differently. PRV-152 produced staining of perikarya and dendrites comparable to that produced by PRV-Bartha (green fluorescence, C). In contrast, the fusion protein produced by PRV-154 (EGFP-US9) was differentially concentrated in the Golgi and rough endoplasmic reticulum of infected cells (green fluorescence, C). The majority of experiments reported here used PRV-152. Neurons containing both PRV-Bartha and PRV-152 or -154 appeared as having yellow fluorescence (A and D).

Figure 1. A, The experimental strategy used in this study. Two recombinant strains of pseudorabies virus were used to produce retrograde transynaptic infection of neurons presynaptically linked to motoneurons innervating the diaphragm and rectus abdominis muscles. Each viral strain expressed a unique reporter that could be detected with the use of monoclonal antibodies. B, The genomic organization of the recombinant viruses used in this study. The genome of PRV contains unique long (UL) and unique short (US) regions. The parental strain used to produce the recombinants used in this study was an attenuated vaccine strain known as PRV-Bartha. PRV-Bartha has several well-characterized mutations and deletions that distinguish it from the wild-type virus (PRV-Becker). These alterations are mapped on the diagram. All recombinants involved insertion of the transgene at the gG locus of the viral genome. In PRV-Bartha, gG was replaced with the gene encoding β-galactosidase. In PRV-152 and PRV-154, the gene encoding green fluorescent protein was inserted into the gG gene, either alone (PRV-152) or as part of a fusion protein (PRV-154). C, The distribution of the reporters within two infected neurons that were both double-labeled. β-galactosidase expression in neurons infected with PRV-Bartha provided staining of neuronal perikarya and dendrites (red fluorescence, panels B and E). Two EGFP-expressing viruses (PRV-152 and PRV-154) labeled cells differently. PRV-152 produced staining of perikarya and dendrites comparable to that produced by PRV-Bartha (green fluorescence, C). In contrast, the fusion protein produced by PRV-154 (EGFP-US9) was differentially concentrated in the Golgi and rough endoplasmic reticulum of infected cells (green fluorescence, C). The majority of experiments reported here used PRV-152. Neurons containing both PRV-Bartha and PRV-152 or -154 appeared as having yellow fluorescence (A and D).

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were thawed immediately before injection. Excess virus was inactivated with Clorox and discarded.

**Injection procedures.** Initial experiments were conducted using eight animals of each sex rendered muscle of the same animal. These animals were killed 4 (n = 1), 4.5 (n = 1), 5 (n = 5), or 5.5 (n = 2) d after inoculation. Further detail regarding the strain and volume of viruses injected in these experiments is provided in Table 1.

The injection procedures conformed to those detailed in our previous viral transneuronal analyses of premotor circuits that modulate the activity of diaphragm and RA motoneurons (Billig et al., 1999; Yates et al., 1999). Animals were initially anesthetized using a mixture of ketamine (25 mg/kg) and xylazine (2.5 mg/kg) injected intramuscularly; anesthesia was supplemented with 0.5–1% isoflurane, which was vaporized in O2 and administered through a face mask to maintain areflexia. In cases in which the diaphragm was injected with PRV, a midline incision was made through the linea alba, and the ventral surface of the left diaphragm was exposed by retracting the viscera. PRV-Bablu was injected beneath the peritoneal lining of the diaphragm using a 10 τ Hamilton syringe equipped with a 26 gauge needle. Injections of virus (1–2 μl) were administered at a rate of 0.5 μl/min. The injection site was confirmed by immersion in a 30% sucrose–PBS solution at 4°C for 2 d. Transverse 50 μm sections of the spinal cord and brainstem were cut with a 26 gauge needle. Injections of virus (1–2 μl/injection) were made at multiple sites in both the costal and crural regions of the diaphragm ipsilaterally. The total volume of virus injected was 60–100 μl. Similar procedures were used to inject 60 μl of PRV-152 or PRV-154 into the left RA; these injections were made beneath the connective tissue sheath surrounding the muscle and were restricted to the region within 3 cm of the diaphragm. After the injections, the abdominal musculature and skin were closed using sutures, and animals were maintained under Biosafety level II conditions for the balance of the survival period.

**Tissue preparation.** After their respective survival times, animals were deeply anesthetized using a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) injected intramuscularly, and then perfused transcardially with saline followed by paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974), as previously described (Billig et al., 1999; Yates et al., 1999). The brainstem and cervical, thoracic, and lumbar cord segments were removed, post-fixed 4–5 hr or overnight at 4°C in a combination of mouse anti-β-galactosidase (Sigma, St. Louis, MO; 1:150) and rabbit anti-green fluorescent protein (Clontech, Palo Alto, CA; 1:1000 or Molecular Probes, Eugene, OR; 1:250) to localize PRV-Bablu or either PRV-152 or PRV-154, respectively. Sections were then washed thoroughly in PBS before being incubated in affinity-purified secondary antibodies raised in donkey that were conjugated to either the CY3 (red) or CY2 (green) carboxyamine (Jackson ImmunoResearch). As a convention in this study, secondary antibodies conjugated to CY3 (concentration of 1:500) were used to visualize PRV-Bablu, and secondary antibodies conjugated to CY2 (concentration of 1:300) were used to visualize PRV-152 and -154. The incubations in these species-specific secondary antibodies were conducted simultaneously for 2 hr at room temperature before the sections were washed and mounted on gelatin-coated slides, and then dehydrated, cleared, and coverslipped with Cytoseal 60. An adjacent bin of brainstem tissue was stained for Nissl substance and fiber tracts using a modified Klüver-Barrera procedure (Kiernan, 1990), so that the precise boundaries of neuronal structures could be determined.

Several controls were included in the analysis to establish the efficiency of transgene expression and the subcellular localization of the reporter proteins. These included: (1) dual labeling immunofluorescence localization of viral antigens with either β-galactosidase or EGFP to demonstrate that the transgenes were efficiently expressed in all infected neurons, (2) immunoperoxidase localization of β-galactosidase or EGFP in sections adjacent to those processed for immunofluorescence, (3) immunoperoxidase localization of infected neurons in adjacent sections of brainstem with a rabbit polyclonal antiserum (Rb133) raised against acetoine-inactivated PRV, and (4) localization in PRV-154 infected neurons of EGFP to the Golgi apparatus and not to the processes or nucleus. Collectively, these localizations demonstrated that the distribution of infected neurons pro-
Figure 3. Photomicrographs of presumed motoneurons infected 4 and 4.5 d after injection of PRV-Bablu into the diaphragm and PRV-152 into rectus abdominis. A, B. Large presumed motoneurons immunostained with the red CY3 fluorophor (A) or immunoprocessed with peroxidase (B) after an injection of PRV-Bablu into the diaphragm. The presumed phrenic motoneurons were located in the ventromedial ventral horn of the C6 spinal cord segment ipsilateral to the side of the injection. These large neurons characteristically exhibited fasciculated bundles of dendrites that extended toward the central canal (CE) and sometimes crossed the midline to the contralateral side. C, D. Examples of a cluster of presumed motoneurons, immunostained with the green CY2 fluorophor (C) or immunoprocessed with peroxidase (D) after an injection of PRV-152 into the rectus abdominis. The cells were observed in ipsilateral lamina VIII of the T10 spinal cord segment. These large neurons exhibited large dendritic arbors, with some processes crossing the midline or reaching the central canal (CE). Scale bars, 200 μm.

Figure 4. Photomicrographs of presumed spinal cord interneurons infected 4.5 and 5 d after PRV-Bablu injection into the diaphragm and PRV-152 injection into rectus abdominis. These neurons were dually immunostained with the red CY3 and the green CY2 fluorochromes, indicating that they made synaptic connections with both phrenic and abdominal motoneurons. The cells were located in lamina VII of the T6 (A) and lamina VIII of the L1 (B) spinal cord segments, ipsilateral to the injections. A1–B2 show the cell under illumination that excites one of the two fluorophors, whereas A and B were photographed under illumination that excites both fluorophors. Scale bars, 200 μm.

Tissue analysis. The primary analysis was done on sections that were spaced 200 μm apart, both in animals injected with a single virus or two viruses. Previous studies in the ferret have shown that this frequency is sufficient for an accurate localization of all cell groups synaptically linked to motoneurons innervating the diaphragm (Yates et al., 1999) or the RA muscle (Billig et al., 1999). Two additional series of thoracic spinal cord tissue from all double virus-injected animals were processed using immunoperoxidase procedures to determine whether labeling was present in the lateral motor columns of cells harboring each reporter protein and in double exposures that revealed the cellular localization of both viruses (Fig. 1C). Verification that yellow fluorescence reflected the colocalization of both the CY2 and CY3 fluorophors and was not attributable to the presence of overlapping cells that each contained one of the fluorophors, was established using a 40× objective. Images were also digitized using a Dage MTI 3CCD camera (Mutech, Billerica, MA) and a Simple32 image analysis system (Compix, Lake Oswego, OR). Images were prepared for publication using Adobe Photoshop software. Individual images were adjusted for size and contrast, but color balance was not altered.

In the dual virus infection experiments, only a qualitative analysis of the locations of neurons expressing immunoreactivity to one or both viruses was attempted. As noted in the introductory remarks, infection of a neuron by one strain of PRV can lower its susceptibility to infection by a second strain (Kim et al., 1999). Thus, it is possible that some neurons with divergent projections to both diaphragm and RA motoneurons may not have been double-labeled in these experiments. The prospect of false negatives mandates a conservative interpretation of the data but does not detract from the significance of dual infected neurons. However, the presence of false negatives could produce misleading results regarding the relative proportion of a cell group that contains neurons that collateralize to innervate both populations of motoneurons. Therefore, we have refrained from making quantitative determinations of the number of dual-infected neurons. Nevertheless, it should be emphasized that the patterns of infection described and illustrated in this report are representative of all animals included in the analysis.

RESULTS

Involvement of autonomic pathways

A thorough analysis of both the thoracic spinal cord and brainstem revealed only minor infection within autonomic pathways after
Intracellular distribution of viral antigens in infected motoneurons was largely restricted to the nucleus, cytoplasm, and proximal dendrites of the infected neurons. This distribution pattern was observed in neuronal somata and proximal dendrites, including distal branches of the dendritic arbor. Because the intracellular distribution of viral antigens was a reflection of the timing of infection (i.e., early stages of infection associated with a restricted distribution of immunoreactivity) (Card, 1995; Card and Enquist, 1999), these data indicate that the replication and transsynaptic transport of virus through motor circuitry was more advanced than that in autonomic circuitry. Further evidence that brainstem labeling was not attributable to transneuronal passage of virus from symmetric preganglionic neurons lies in the distribution of infected neurons illustrated in the right panel; these sections were immunoprocessed using immunofluorescence. The right column illustrates infected motoneurons at the same location in sections immunoprocessed using immunoperoxidase. AI and A2 show neurons in nucleus retroambiguus that were infected after injecting PRV-152 into rectus abdominis on the contralateral side. The neurons were immunostained with the green CY2 fluorophor (A1) or with peroxidase (A2). B1 and B2 show premotor neurons in nucleus retroambiguus infected after injecting PRV-Bablu into the diaphragm on the contralateral side; these neurons were labeled with the red CY3 fluorophor (B1) or through immunoperoxidase processing (B2). C1 and C2 illustrate infected premotor neurons in the vicinity of nucleus ambiguus and the retrofacial nucleus that were immunostained with the red CY3 fluorophor (C1) or with peroxidase (C2) after injections of PRV-Bablu into the contralateral diaphragm. D1 and D2 show infected neurons in the ventrolateral portion of the nucleus of the solitary tract, which were immunostained with the red CY3 fluorophor (D1) or with peroxidase (D2) after injecting PRV-Bablu into the ipsilateral diaphragm. Scale bars, 400 μm. Figure 5. Examples of premotor neurons in regions of the brainstem known to contain the dorsal and ventral respiratory groups, which were infected 4.5 d after PRV injections into the diaphragm and rectus abdominis. A–D show photomicrographs of transverse brainstem sections located ~1 mm caudal to the obex (A, B) or at the level of the obex (C, D); these sections were stained with the use of a modified Klüver-Barrera method. Boxes on the photomicrographs indicate the locations of the infected neurons illustrated in the middle column; these sections were immunoprocessed using immunofluorescence. The right column illustrates infected motoneurons at the same location in sections immunoprocessed using immunoperoxidase. 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After injection of virus into the diaphragm, infected large presumed motoneurons were present in the ventral horn of segments C2 through C6 (but mainly C6) ipsilateral to the injection, whereas injection of virus into the proximal 3 cm of RA-infected neurons concentrated largely in the ventral horn of the T10 to T14 segments on the ipsilateral side. Examples of infected motoneurons are illustrated in Figure 3. The morphology and disposition of these neurons within the ventral horn also differed depending on the injected muscle. Infected diaphragm motoneurons were confined to a tight column in the ventromedial portion of the ventral gray and gave rise to fasciculated bundles of dendrites that exhibited a polarized trajectory toward the area immediately subjacent to the central canal. In contrast, infected RA motoneurons were more widely dispersed throughout the central and lateral portions of the ventral gray and gave rise to dendrites that extended radially from the somata. In both cases, the neuronal perikarya were 40–60 μm in widest diameter and exhibited morphological features classically defined for motoneurons using classical retrograde tracers. The number of infected cells exhibiting this morphology increased with advancing survival, but the segmental distribution and disposition
of the cells in the ventral horn remained projection-specific throughout the longest postinoculation intervals.

At postinoculation intervals extending to 5.5 d, transynaptic passage and replication of the recombinant viruses led to the appearance of infected neurons in the spinal cord and brainstem. The distribution of these neurons correlated with that demonstrated in our previous reports of this circuitry derived from injections of the parental virus into the diaphragm or RA (Billig et al., 1999; Yates et al., 1999). The infected neurons included local circuit neurons at multiple levels of the spinal cord (Fig. 4) as well as reproducible groups of neurons in the caudal brainstem (Figs. 5,6). The brainstem labeling was first apparent 4 d after injection of either muscle, and the number of infected neurons increased progressively through 5.5 d. Neurons were observed bilaterally with an ipsilateral propensity. In both paradigms, some infected neurons were observed in brainstem regions known to contain the dorsal and ventral respiratory groups in a variety of mammalian species (Feldman, 1986), and substantial numbers of neurons were observed in the MRF and along the midline in the vicinity of the raphe obscurus and raphe pallidus.

**Dual infection experiments**

Immunofluorescence analysis with filters selective for the CY2 and CY3 fluorophors and with a wide-band Omega filter that excites both fluorophors allowed a detailed analysis of neurons infected with one or both of the recombinant viruses. As noted in Materials and Methods, the CY2 fluorophor was used to identify neurons infected with either PRV-152 or PRV-154, whereas CY3 was used to localize the β-galactosidase reporter expressed by PRV-BaBlu. Additionally, PRV-BaBlu injection was restricted to the diaphragm, and the EGFP-expressing recombinants were only injected into RA. Thus, neurons that were a dedicated part of the diaphragm and the EGFP-expressing recombinants were only injected into RA. Thus, neurons that were a dedicated part of

![Figure 6](image-url)
polysynaptic circuits selectively innervating the diaphragm appeared red, those infected selectively by transynaptic passage of virus from RA appeared green, and neurons that collateralized to innervate motoneurons to both muscles appeared yellow. Each of the dual-injected animals that survived ≥4.5 d contained neurons that were replicating both recombinants. Furthermore, sections that contained dual-infected neurons always also contained neurons selectively labeled with β-galactosidase or EGFP. Consequently, we are confident that both recombinants had replicated and passed transsynaptically into comparable levels of the brainstem in all experimental animals. Nevertheless, we cannot preclude the possibility that previous infection of some neurons by one strain of virus made those cells refractory to replication of the second strain, as was recently demonstrated by Kim et al. (1999). However, it is noteworthy that the interference in viral replication demonstrated by Kim et al. (1999) was observed in animals in which one strain of virus was more virulent and transported at a faster rate than the other strain. In this study, both recombinants were produced from the same attenuated virus (PRV-Bartha) and exhibited the same relative rate of transport.

As noted above, some infected neurons were observed in brainstem regions known to contain the dorsal and ventral respiratory groups. Further immunofluorescence analysis revealed that these cells consisted of two projection-specific populations, and no cells contained reporters for both recombinants. Neurons selectively expressing either β-galactosidase or EGFP immunoreactivity were observed in the vicinity of the ventral respiratory group, whereas most neurons in the vicinity of the dorsal respiratory group ex-
pressed β-galactosidase immunoreactivity (Fig. 5). This observation
contrasted with the labeling observed in the more rostrally
situated sections through the MRF of the same animals. In each
animal, three populations of neurons were observed in the magnocellu-
lar tegmental field in the MRF: those replicating only one recombinant
and those that contained the reporter proteins of both recombinants
(i.e., dual-infected neurons were intermixed with cells that only expressed the EGFP or β-galactosidase reporters), as
illustrated in Figure 6. However, it was uncommon to observe
dual-labeled neurons along the midline in the vicinity of the raphe
cell groups, although neurons expressing immunoreactivity to one
of the two reporters were present in this area. Figure 7 shows the
distribution of infected neurons at selected levels of the brainstem
in two animals.

**DISCUSSION**

This study provides the first direct evidence that a population of
neurons in the brainstem, located mainly in the magnocellular part
of the MRF, provides inputs to both inspiratory and expiratory
motoneurons in the spinal cord. Furthermore, the patterns of dual
infection of brainstem neurons reported here support the conclu-
sion that cells synaptically linked to both the diaphragm and RA
exhibit a functional segregation from neurons that provide inputs
to only one of these muscles. This functional parcellation is char-
acterized by segregation of circuitry responsible for the generation
of the respiratory rhythm from that responsible for coordinating
cocoontractions of multiple respiratory muscles during behaviors
such as emesis.

Two caveats must be considered when interpreting the results of
this study. First, after the injection of the recombinant viruses into
the diaphragm and rectus abdominis the infection of neurons in the
dorsal and ventral respiratory groups was not as extensive as when
the parental strain, PRV-Bartha, was used (Billig et al., 1999; Yates
et al., 1999). It is possible that the recombinant viruses were not
transported as rapidly as the parental strain and that a longer
transneuronal travel time would have resulted in a more extensive infection of
the respiratory groups. Nonetheless, the distribution of labeling
produced by injection of PRV-Bartha and the recombinants PRV-
152, PRV-154, and PRV-Bablu into respiratory muscles was simi-
lar, and there is no indication that the general results would have
been different from those in the present experiments had longer
survival times been used. A second caveat is that a limited amount
of infection of sympathetic nervous system neurons did occur in
these studies, raising the prospect that some of the labeling of
brainstem neurons observed in this study was attributable to tran-
neuronal passage of virus from sympathetic preganglionic neurons
in the thoracic spinal cord. Although we cannot eliminate this
possibility, two lines of evidence raised in Results support our
conclusion that the MRF neurons were infected predominantly
by virtue of their collateralized projections to motoneurons control-
ing the diaphragm and rectus abdominis muscles.

Although previous experiments using PRV injections have
shown that overlapping populations of MRF neurons influence RA
and diaphragm activity (Billig et al., 1999; Yates et al., 1999), these
conventional transneuronal tracing studies using one virus could
not distinguish whether parallel projections from two groups of
neurons or collateralized projections from a single group provided
inputs to inspiratory and expiratory motoneurons. Furthermore,
the present findings have physiological importance, because they
shed light on lesion studies showing that inactivation of the MRF
abolishes simultaneous increases in diaphragm and abdominal
muscle activity during emesis (Miller et al., 1996). The current data
demonstrate that individual MRF neurons may simultaneously
activate inspiratory and expiratory motoneurons during vomiting,
opening the possibility that pharmacological agents acting on this
single neuronal population could abolish this behavior. Neverthe-
less, additional electrophysiological and lesion studies will be
required to determine the role of MRF neurons in coordinating
the contractions of the diaphragm and abdominal muscles during vom-
itng (Gold and Hatcher, 1926; McCarthy and Borison, 1974), as
well as during postural adjustments (Grillner et al., 1978), and
reaction to vestibular stimulation (Yates et al., 1993; Rossiter et al.,
1996). In addition, it remains to be determined whether abdominal
muscles other than RA receive substantial inputs from MRF pre-
motor neurons, including those that also influence diaphragm
activity.

It seems likely that the methodology used in this study can be
used to determine whether any two muscles or muscle groups
receive common influences from particular CNS regions. However,
several limitations are apparent in this approach. Because infection
of a neuron by one virus can lower its susceptibility to be infected by
a second virus (Kim et al., 1999), it is only possible to interpret
positive results (i.e., the presence of double-labeled neurons) when
using this methodology. Furthermore, because the immune system of the
ferret rapidly and effectively compartmentalizes and eliminates
neurons that are infected with PRV (Billig et al., 1999; Yates et al.,
1999), the temporal window during which infected neurons can be
detected is limited. As a result, experiments incorporating injection
of two virus tracers cannot be used in isolation to determine neural
pathways that are involved with coordinating movements. None-
theless, these experiments may provide clues regarding potential
neural pathways that can be investigated in subsequent lesion,
electrophysiological, and conventional neuroanatomical studies.

In conclusion, the present demonstration of dual infections of
MRF neurons by antigenically distinct recombinants injected into
the diaphragm and RA provides novel insights into the functional
organization of brainstem circuitry that controls respiratory mus-
cles. Furthermore, comparison of these data with those obtained
from previous studies in rat (Dobbins and Feldman, 1994) suggests
that the organization of this circuitry is species-specific. Finally,
the spatial separation of cell groups involved in the generation of
the respiratory rhythm from those in the MRF that collateralize to
innervate inspiratory and expiratory motoneurons suggests a func-
tional segregation in brainstem premotor respiratory neurons that
can be explored experimentally.

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