Published in final edited form as:

Brain Res. 2009 September 29; 1291: 40–52. doi:10.1016/j.brainres.2009.07.024.

Morphological and electrophysiological features of motor neurons and putative interneurons in the dorsal vagal complex of rats and mice

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Abstract

The dorsal motor nucleus of the vagus (DMV) contains preganglionic motor neurons that control viscera along the subdiaphragmatic digestive tract, but may also contain neurons that do not project to the viscera. Neurons that expressed EGFP 60–72 h subsequent to PRV-152 inoculation of vagal terminals in the stomach wall were targeted for whole-cell patch-clamp recording and biocytin filling in transverse brainstem slices from rats and their quantitative morphological and electrophysiological characteristics were compared with uninfected cells. Over 90% of PRV-152 labeled neurons were also labeled subsequent to intraperitoneal injection of FluoroGold, indicating most were preganglionic motor neurons. In reconstructed neurons with an identifiable axon trajectory, two cellular subtypes were distinguished. The axon projected ventrolaterally from the DMV in 44 of 49 cells and these were likely to be vagal motor neurons. Axons of other neurons ramified within the nucleus tractus solitarius (NTS) or DMV. These cells were smaller and otherwise morphologically distinct from putative motor neurons. Transgenic mice with GFP-expressing inhibitory neurons (i.e., GIN mice) were used to identify a GABAergic subset vagal neurons. These neurons had locally-ramifying axons and formed a morphologically distinct subset of DMV cells, which were similar in size and axon trajectory to GABAergic neurons in the NTS. Most neurons in the DMV therefore possess morphological features of motor neurons, but locally projecting cells and inhibitory neurons with distinct morphological features are also found within the DMV. These cells likely contribute to regulation of vagal function.

Keywords

GABA; morphology; patch-clamp; PRV; solitarius; vagus

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1. Introduction

Central parasympathetic control of the subdiaphragmatic viscera is accomplished mainly by activity of neurons in the dorsal vagal complex (DVC). Vagal afferents carrying viscerosensory information synapse in the nucleus tractus solitarius (NTS). Second order viscerosensory NTS neurons, in turn, synapse with neurons in a variety of areas, especially the dorsal motor nucleus of the vagus nerve (DMV) and within the NTS (Travagli and Rogers, 2001; Davis et al., 2003; 2004; Glatzer and Smith, 2005). Neurons in the NTS and DMV also receive inputs from a number of CNS regions, which can contribute to modulation of visceral function (Gillis et al., 1989; Grabauskas et al., 2004; Rogers et al., 1996). The parasympathetic preganglionic motor neurons in the DMV subsequently send vagal motor projections to postganglionic neurons controlling the subdiaphragmatic viscera from the lower esophagus to the colon (Travagli et al., 2006).

Motor neurons in the DMV are mainly large, multipolar, cholinergic cells (Browning et al., 1999). Putative interneurons with smaller somata have also been reported in the nucleus (McLean and Hopkins, 1981; 1982; Blessing et al., 1984; Blessing, 1990; Izzo et al., 1992; Fox and Powley, 1992; Huang et al., 1993; Streefland et al., 1998; Jarvinen and Powley, 1999), but axon trajectories of these cells have not been well-characterized. Several studies have indicated that DMV motor neurons have a small but detectable degree of heterogeneity with regard to functional, morphological, electrophysiological attributes (Huang et al., 1993; Fogel et al., 1996; Browning et al., 1999; Valenzuela, et al., 2004). The morphological details of putative interneurons have never been reported (Jarvinen and Powley, 1999). Likewise, primary control of DMV motor neurons is likely to arise from local GABA circuits (Travagli et al., 2006; Herman et al., 2009), but the morphology of intrinsic GABAergic neurons of the DVC is not well described.

Pseudorabies virus (PRV) is a useful tool for tracing the functional synaptic connections within the CNS and between the CNS and periphery. The attenuated Bartha strain of the virus infects axon terminal fields relatively specifically (versus axons of passage), and spreads retrogradely across synapses through central networks of functionally related neurons (Strack et al., 1989; Card et al., 1993; Enquist et al., 1998; Card, 1998; Smith et al., 2000; Ch'ng et al., 2007). Variants isogenic with PRV Bartha, which expresses a monomeric red fluorescent protein (mRFP1; Campbell et al., 2002) or enhanced green fluorescent protein (EGFP), have been developed to identify neurons in living tissue. The EGFP (i.e., PRV-152; Smith et al., 2000; Glatzer et al., 2003) or mRFP1 (i.e., PRV-614; Banfield et al., 2003; Glatzer et al., 2007) can be visualized in acute slices to target specific sets of neurons for electrophysiological recordings. In addition, inhibitory neurons can be identified in living brain using a transgenic mouse line with GFP-expressing Inhibitory Neurons (i.e., GIN mice; FVB-TgN (GADGFP) 45704Swn). Using these methods of neuron "pre-labeling", we tested the hypothesis that DMV neurons possess diverse morphologies, consistent with the existence of interneurons and motor neurons. These experiments were designed to clarify and obtain novel information about the morphological and electrophysiological properties of subgroups of principal neurons and of putative interneurons in the dorsal vagal complex (DVC).

2. Results

Stereologic features of Fluorogold- versus PRV-614-labeled DMV neurons

Combined intraperitoneal (i.p.) injection of FluoroGold (10 mg/kg) and inoculation of the gastric wall with PRV-614 was used to retrogradely label neurons with projections to the abdomen and also gastric-related neurons in the DMV, respectively. Pattern and timing characteristics of central PRV-614 labeling in the DVC was the same as for PRV-152 (Glatzer et al., 2007). The PRV-614-labeled (i.e., gastric-related) neurons made up $48.3 \pm 1.2\%$ of the

FluoroGold-labeled (i.e., motor neurons) motor neurons in the DMV ($n=6$; Fig. 1). Of the PRV-614 infected DMV neurons observed 72 hrs after stomach wall inoculation, $93.9 \pm 0.7\%$ were also labeled with FluoroGold, indicating that they were primarily motor neurons.

PRV-152 labeling of rat DMV after gastric wall inoculation

PRV-152 was injected into the rat stomach wall to infect the vagal terminals innervating the fundus and corpus, along the ventral margin of the greater curvature. This recombinant strain is a highly specific transneuronal retrograde tracer with little or no ability to infect axons of passage or non-neural elements, and with no anterograde or axo-axonal labeling capability (Smith et al., 2000; Pickard et al., 2002; Ch'ng et al., 2007). Central vagal neurons were infected in synaptic sequence after infection of the terminal fields of preganglionic vagal motor neurons (Card et al., 1993; Rinaman et al., 1993; Glatzer et al., 2003). The PRV-152-mediated expression of EGFP in DMV neurons was initially detected in the brainstem approximately 40 hrs after inoculation, with the number of EGFP labeled cells initially increasing with infection time. Whole-cell patch-clamp recordings were obtained from the EGFP labeled cells 60-72 hrs after inoculation (Fig. 1). At this post-inoculation time, sufficient numbers of DMV and NTS neurons were identifiable for recordings in slices while maintaining electrophysiological and morphological characteristics of uninfected cells (Rinaman et al., 1993; Davis et al., 2003; Glatzer et al., 2003; Derbenev et al., 2004). These neurons were thus considered gastric-related DMV neurons.

Neurons labeled with PRV-152, identified by their EGFP expression under epifluorescence illumination, could be readily observed and targeted for patch-clamp recordings in each transverse brainstem slice 60-72 hrs after gastric inoculation. Gastric-related neurons could also be identified post hoc by visualizing the recorded biocytin-filled cell using an avidin-rhodamine conjugate (i.e., red fluorescence) in concert with the EGFP (i.e., green fluorescence; Fig. 2) prior to converting the label to an opaque reaction product for morphological reconstruction. A total of 78 biocytin-labeled rat DMV neurons were reconstructed in three dimensions for morphological analysis (Figs. 2, 3). Of these, 25 were identified as gastric-related and 53 were unidentified neurons (i.e., were not labeled with PRV-152). The somata of all neurons were located within the boundaries of the medial DMV, -13.01 to -14.10 mm from Bregma. Recorded DMV neurons that were not prelabeled from the stomach had 1-6 primary dendrites. The dendrites were tapered and generally aspiny, although in some cases, a few dendritic spines of various shapes were observed. Average soma area for the pool of neurons that was not pre-labeled from the stomach was $290.2 \pm 14.8 \mu\text{m}^2$, and the average number of dendrites was 3.6 ± 0.2 ($n=53$). Average soma area for PRV-152 labeled neurons was $319.4 \pm 19.2 \mu\text{m}^2$ ($n=25$), and was not different than the unlabeled population ($p>0.05$). Each neuron had two to six primary dendrites, which were relatively aspiny, like unlabeled DMV neurons. There were no major differences in the pooled morphological features between the unlabeled and EGFP labeled groups (Table 1).

Morphology of DMV neurons with axons projecting into the NTS or DMV

Although axons of many neurons were cut near the soma during slice preparation, information about axon trajectory could be obtained in 49 of 78 neurons (37 of 53 unlabeled and 12 of 25 PRV-152 labeled). The axons of 32 unlabeled DMV neurons and 12 PRV-152 labeled neurons coursed ventrolaterally in the direction of the vagal efferent tract within the plane of the slice. The axons of these neurons were typically unbranched within the confines of the slice. They usually ended at the slice surface in the ventral medulla, indicating a relatively long planar trajectory within the plane of the slice preparation. Average axon length was $549.4 \pm 91.6 \mu\text{m}$ from the soma. These ventrolaterally-projecting neurons had a relatively large dendritic arbor with 2 to 6 primary dendrites; the soma area averaged $299.6 \pm 14.4 \mu\text{m}^2$.

Of the 49 DMV neurons with identifiable axon trajectory, five were observed to have axons that projected into NTS or ended within the confines of the DMV (Fig. 3), but did not exit the DMV ventrolaterally. In these cells, the axon ends were identifiable within the slice (i.e., not found at the cut edge of the slice). This group of neurons had significantly smaller cell bodies than those with axons projecting ventrolaterally ($163.3 \pm 46.1 \mu\text{m}^2$; $p < 0.05$). By comparison, the neurons with axons ramifying within the DVC and neurons with ventrolateral axon trajectory were significantly different groups with regard to several morphological features including soma area, dendritic length, dendrite branches, number of dendrites, and axon segment length (Table 2; Fig. 3). The remaining neurons ($n=29$) had axons with cut ends at the surface of the slice close to the soma within the DMV (i.e., they were likely cut when preparing the slice) and so were not characterized further with regard to axon trajectory.

Membrane properties and synaptic events of DMV

In order to describe electrophysiological features of the PRV-152 labeled group and unlabeled DMV group, resting membrane potential, input resistance, and spontaneous synaptic currents were assessed in 33 unlabeled DMV neurons and 16 PRV-152 labeled neurons. Synaptic currents were recorded at -60 to -70 mV for sEPSCs and 0 to -20 mV for sIPSCs. No significant differences were detected in membrane potential, input resistance, or spontaneous synaptic events between PRV-152 labeled and unlabeled neurons ($P > 0.05$; Table 3).

Morphology of DMV neurons from GIN mice

In order to better understand the subsets and morphological features of DMV neurons, we used transgenic GIN mice to visualize GABAergic DMV neurons for whole-cell patch-clamp recording and biocytin filling (Oliva et al., 2000; Williams et al., 2006; Glatzer et al., 2007). In each 300 μm thick slice, three to eight EGFP-expressing neurons were readily identified within each DMV bilaterally and were accessible for recording in the slices (Fig. 4). After reconstruction, the morphology of 12 unidentified (i.e. non-EGFP-labeled) and 11 identified GABAergic DMV neurons from GIN mice were analyzed. GABAergic neurons had one to four dendrites with a relatively simple dendritic arbor and a small soma area ($99.0 \pm 9.8 \mu\text{m}^2$; Table 4; Fig. 5). Of the 11 GABAergic DMV neurons, 5 axons were cut near the soma at the surface for the slice, and so their axon trajectories were not identified. In two neurons, the axon projected to the ipsilateral or contralateral NTS; the axon of one neuron projected to the midline. The remaining three neurons had a relatively extensive axonal arbor within the DVC, with multiple branches identified (i.e., >4 branch nodes). Axon branches extended to ipsilateral NTS, contralateral NTS, ipsilateral DMV, contralateral DMV, or toward midline or the central canal (Fig. 5); axons of identified GABAergic cells were not seen to project ventrolaterally from the DMV.

The unidentified mouse DMV neurons ($n=12$) had 2-5 dendrites and a larger soma area than GABAergic neurons (mean = $292.7 \pm 25 \mu\text{m}^2$; $p < 0.05$). The axon of unidentified neurons was unbranched and projected ventrolaterally from the DMV in seven neurons, with the axon of the remaining five cells being cut at the slice surface. The soma area and other morphological features of unlabeled neurons in GIN mice were statistically similar to those of unlabeled rat DMV neurons (Table 4).

Membrane properties and synaptic events of DMV neurons from GIN mice

To determine the basic membrane and synaptic input properties of GABAergic DMV neurons, membrane potential, input resistance, and the frequency and amplitude of sIPSCs and sEPSCs was examined. A comparison of unidentified DMV neurons with GABAergic cells indicated a significantly higher input resistance and lower frequency of sIPSCs and sEPSCs in GABAergic DMV cells ($P < 0.05$). Other features are shown in Table 3. There was no significant

difference in sIPSC or sEPSC amplitude or membrane potential between the two groups ($P>0.05$).

Morphology of NTS GABAergic neurons

The somatodendritic morphology of GABAergic neurons in the DMV was comparable to previous general descriptions of immunohistochemically identified GABAergic NTS neurons (Izzo et al., 1992; Kawai and Senba, 1999). To determine if GABAergic NTS neurons shared morphological characteristics with GABAergic neurons in DMV in this model, biocytin filled EGFP-labeled NTS neurons were examined ($n=10$; Table 4). In particular, soma area ($97.8 \pm 12 \mu\text{m}^2$) and number of dendrites (2-5) were similar for GABAergic cells in the NTS and DMV, although GABAergic neurons in the NTS had a less extensive dendritic tree than those in the DMV. The axons of GABAergic NTS neurons were observed to ramify across a wide area of the vagal complex, including to the DMV and into both ipsilateral and contralateral NTS (Fig. 5). Some electrophysiological features of GABAergic NTS neurons have been described (Glatzer et al., 2007; Bailey et al., 2006). As a comparison to DMV GABA neurons, membrane properties and synaptic inputs in NTS GABAergic neurons were also examined ($n=18$), and are summarized in Table 3. Input resistance and membrane potential, as well as the sEPSC and sIPSC frequency and amplitude in GABAergic NTS cells were all similar to those for GABAergic DMV neurons ($P>0.05$).

3. Discussion

Although the majority of DMV neurons are motor neurons, with many projecting to the subdiaphragmatic viscera, a distinct population of local neurons also exists in the DMV, whose axons do not appear to contribute to the vagal tract in the brainstem. In the mouse, these include a small number of GABAergic neurons. Overall, the properties of neurons retrogradely labeled subsequent to inoculation of the stomach with PRV-152 or PRV-614 were similar to those reported previously for gastric-projecting DMV cells (Browning et al., 1999; Valenzuela et al., 2004), validating this labeling technique in these cells. Whereas retrogradely transported labels such as FluoroGold are as likely to be taken up by injured axons or axons of passage as by terminals, PRV preferentially infects terminal fields over axons of passage (Card et al., 1993; Ch'ng et al., 2007), resulting in a larger pool of cells selectively labeled gastric-related neurons. When ejected onto the surface of the gastric wall or intraluminally, PRV-152 fails to consistently or specifically label significant numbers of DMV neurons (Glatzer et al., 2003; Davis et al., 2003). Similar injection of the PRV strain used here (i.e., Bartha) resulted in little or no labeling via uptake by fibers of passage (Pickard et al., 2001). Even though other strains of the virus are capable of axon sorting, neither orthograde transport across synapses nor transmission from axon to axon of the Bartha strain used here (i.e., PRV-152) can occur (Ch'ng et al., 2007). It is thus unlikely that non-specific labeling of axons unrelated to the stomach resulted in a measurable number of labeled neurons in the DMV. Once central neurons are infected, the retrograde spread of the virus to synaptically connected neurons is stereotyped, taking about 12 hours to label sequentially connected afferent neurons in the brain. Use of PRV-152 or -614, which express fluorescent proteins, were confirmed as relatively specific transneuronal retrograde tracers useful for obtaining viable recordings from labeled cells in circuits related to the control of the ventral gastric fundus (Rinaman et al., 1993; Card et al., 1993; Smith et al., 2000; Irnaten et al., 2001; Glatzer et al., 2003; 2007; Davis et al., 2003; Derbenev et al., 2004; Glatzer and Smith, 2005; Williams and Smith, 2006; Williams et al., 2007).

Previous estimates indicated that most neurons in the DMV were parasympathetic preganglionic motor neurons (Jarvinen and Powley, 1999), similar to our results (i.e., ~94% of PRV-614 infected neurons were also labeled with FluoroGold 72 hrs after stomach wall

inoculation). Logically, the percentage of the neurons labeled by either PRV-614 or FluoroGold is likely to be underestimation of the actual total projecting to the site of label placement due to variability in the labeling techniques (i.e., variability of label intensity, number of cells labeled by each method, transport variability, cellular degradation after FluoroGold or PRV label, etc.). Intracellular labeling indicated a ventrolateral axon trajectory in ~90% of neurons in which the axon trajectory could be identified, with the remainder of biocytin-filled neurons having only local projections, consistent with the hypothesis that the vast majority of neurons in the DMV are motor neurons. Jarvinen and Pawley (1999) reported that somata of putative interneurons were smaller than motor neurons with a more restricted dendritic tree and more dendritic spines. However, the Golgi-type labeling method used precluded identification of axons, so the local projections of these cells could not be confirmed. It seems reasonable to assume these cells with locally ramifying axons were interneurons, some of which probably participate indirectly in regulating gastric function. The present findings represent independent confirmation of the morphological features of gastric-projecting DMV cells using a labeling method not previously used for this purpose in the DMV and also describe the detailed morphology of more rarely encountered, putative interneurons.

The morphological features of ventrolaterally-projecting neurons in both rat and mouse were indistinguishable from each other and were similar to those reported previously for neurons projecting to the gastric compartment (Browning et al., 1999). However, ventrolaterally-projecting cells were morphologically distinct from those neurons whose axons projected toward NTS or DMV. The latter had significantly smaller soma size, fewer dendrites, shorter dendritic length, and a shorter axon within the slice. The relationship of the features of DMV motor neurons with their subdiaphragmatic visceral target, reflexive responses, neurochemistry, or responses to other input have been described (Fogel et al., 1996; Browning et al., 1999; Jarvinen and Powley, 1999; Zhang et al., 2002; Davis et al., 2003; Grabauskas and Moises, 2003; Valenzuela et al., 2004; Browning et al., 2005). The present data differentiate these cells from those whose axons do not obviously contribute to the vagus nerve.

In GIN mice, a subgroup of GABAergic neurons express EGFP under the control of the Gad1 gene promotor, encoding glutamic acid decarboxylase (GAD67), the synthetic enzyme for catalyzing the conversion of glutamate into GABA (Oliva et al., 2000). Identified GABAergic neurons in the DMV of this transgenic mouse strain were few in number, but were consistently present. In this model, PRV-614 inoculation of the stomach resulted in transsynaptic labeling of a subgroup of GABA neurons in the NTS (Glatzer et al., 2007). There are relatively few DMV GABA neurons, but a subset of these neurons (~25%) was similarly labeled after PRV-614 inoculation of the stomach (unpublished observations), suggesting that at least some of the GABA neurons in the DVC were gastric-related. We assume both groups of GABA neurons were labeled transneuronally and may thus function as ‘premotor’ inhibitory neurons.

In rat (Blessing et al., 1984; Tanaka et al., 2003), rabbit (Blessing, 1990), and cat (Izzo et al., 1992) small GAD-positive neurons were identified in the DMV, although an absence of GABA neurons in rat DMV was reported in another study (Fong et al., 2005). While it is possible that GABAergic DMV cells are not ubiquitously found across species, the presence of a few GABA neurons in the DMV of mice and other species is consistent with findings from nearly every other area of the mammalian brain, suggesting that local GABA neurons regulate neuronal function. Although GABAergic NTS neurons tended to have somewhat less extensive dendrites, morphological features of the GABAergic neurons in NTS and DMV were similar, suggesting that the GABA cells in these areas may be functionally or developmentally related. A previous study of immunohistochemically identified GABAergic NTS neurons in rats also found them to have relatively small somata (Kawai and Senba, 1999). Identified GABAergic neurons in both nuclei received both EPSCs and IPSCs, but they tended to receive less spontaneous synaptic input than the unidentified neuron population. They also had higher input

resistance, consistent with their smaller somatic area, and had an axon that ramified within the vagal complex, often bilaterally. These features would be expected to make them highly responsive to synaptic and modulatory inputs, underscoring the relevance of synaptic inhibition in controlling preganglionic motor neuron function (Travagli et al., 2006).

Although axon branching patterns do not necessarily equate with synaptic connectivity, the abundant local axon branches of GABA neurons in both NTS and DMV suggests a potentially divergent output to local neurons. Axons of GABAergic DMV neurons ramified in the DMV and widely into both ipsilateral and contralateral NTS. Identified GABAergic NTS neurons can be premotor to the DMV and they are also usually second order viscerosensory neurons (Glatzer et al., 2007; Bailey et al., 2008); their axonal branching patterns are consistent with this functional connectivity. The broad distribution of the axons, including into contralateral areas of the DVC, implicates them as potential integrators of viscerosensory input across several viscerotopic NTS and DMV regions. Indeed, activation of GABAergic cells in multiple areas of the NTS has been shown to increase inhibition to individual DMV neurons (Davis et al., 2004), and GABAergic input to individual NTS neurons often originates from within wide regions of the NTS (Glatzer and Smith, 2005). The morphological and electrophysiological attributes of GABAergic DMV neurons seem likely to make these local inhibitory cells important regulators of DMV motor neuron function, but may also provide feedback inhibition to neurons in the NTS. The physiological relevance of this widespread GABAergic connectivity in the DVC has recently been highlighted in studies showing removal of GABAergic tone in the NTS profoundly alters motor output from the DMV bilaterally (Herman et al., 2009). The present study provides a cellular and morphological correlate to that physiological analysis. The GABAergic neurons in the vagal complex appear positioned to coordinate functions of multiple parasympathetic output modalities and/or organ systems in addition to their proposed sensory integrative function.

Conclusions

An advantage of using PRV-152 is that the likelihood of labeling vagal terminals selectively (versus axons of passage) is high; it allows for labeling a relatively large proportion of the gastric-related neurons with minimal risk of incidental labeling of axons of passage that may innervate more distal parts of the gastrointestinal system. Putative interneurons, which had locally-ramifying axons, including subsets of GABAergic neurons in the DMV and NTS likely innervate the DVC bilaterally, providing an anatomical basis for a previously unappreciated feedback circuit from motor to sensory areas of the vagal complex as well as a functional commissural inhibitory connection between the components of the DVC.

4. Experimental Procedures

Animals and stomach inoculation

Male Sprague Dawley rats (Harlan, Indianapolis, IN), 4-8 weeks of age, and transgenic mice GIN (Oliva et al., 2000; strain name FVB-TgN (GADGFP)45704Swn; Harlan, Indianapolis, IN), 4-12 weeks of age, were housed under a standard 12-hour light/dark cycle, with food and water provided *ad libitum*. All animals were treated and cared for in accordance with the Tulane University and the University of Kentucky Animal Care and Use Committees and National Institutes of Health guidelines.

Labeling of gastric-related neurons

A fluorescently labeled viral vector (i.e., PRV-152 or PRV-614), which selectively labels neurons in a transsynaptic, retrograde manner, was used to identify gastric-related neurons for patch-clamp recordings and neuron labeling in slices (Jons and Mettenleiter, 1997; Smith et al., 2000; Pickard et al., 2002; Glatzer et al., 2003). Under sodium pentobarbital anesthesia

(Nembutal; 50 mg/kg, i.p., Abbott Laboratories; Chicago, IL), a laparotomy was performed and the gastric musculature was injected with the attenuated (Bartha) strain of PRV (PRV-152, generously supplied by Dr. L.W. Enquist), which was constructed to express EGFP (Smith et al., 2000). As initially described for gastric inoculation (Card et al., 1990; 1993), three to five 1- μ l injections of PRV-152 ($1-2 \times 10^8$ pfu/ml) were made into the gastric wall on the ventral surface of the stomach by using a 10- μ l Hamilton syringe fitted with a 26-gauge needle. Each injection was made over a 1-minute period, and the needle was left in place for an additional 30 seconds at each site before removal. The injection site was washed with saline and the abdominal wall closed with 6.0 silk sutures. Animals were maintained in a biosafety level 2 facility for up to 72 h postinoculation, where they were allowed to recover. Food and water were provided *ad libitum* and were monitored to ensure they were consumed at a normal rate.

Based on previous studies of neuronal health and infection stages following inoculation of the stomach with PRV (Card et al., 1993; Rinaman et al., 1993; Davis et al., 2003; Glatzer et al., 2003), labeling in the brainstem was examined at 60-72 h postinoculation. This time period resulted in labeling of neurons in the DMV sufficient to allow targeting of neurons for recording, but is more than 1 d before the time at which electron microscopic studies indicated signs of membrane damage from the virus (Rinaman et al., 1993). Previous studies showed no signs of altered membrane properties at this post-inoculation time point: membrane potential, input resistance, synaptic input patterns, responses to G protein-coupled receptor activation, Na^+ , K^+ , and Ca^{2+} channel function were all similar to uninfected cells (Smith et al., 2000; Irnaten et al., 2001; Wang et al., 2001; Glatzer et al., 2003; 2007; Davis et al., 2003; Derbenev et al., 2004; Glatzer and Smith, 2005; Williams and Smith, 2006; Williams et al., 2007).

In other cases, animals were injected i.p. with FluoroGold (Fluorochrome, Inc., Denver, CO, USA) to label subdiaphragmatic-projecting vagal motor neurons in the DMV. FluoroGold was dissolved in 20% lactose in 0.9% saline and was injected intraperitoneally (i.p.; two injections separated by 15 min; 10 mg kg^{-1} total), as described previously (Leong and Ling, 1990; Derbenev et al., 2004). After 7 d, a laparotomy was performed as above and the gastric musculature was inoculated with PRV-614 ($1-2 \times 10^8$ pfu/ml; generously supplied by Dr. B. Banfield), which reports mRFP (a generous gift of Dr. R. Tsien) with a timing similar to that for the green fluorescence of PRV-152 (Campbell et al., 2002; Banfield et al., 2003; Glatzer et al., 2007). Ten days after FluoroGold injection, and 72 h after inoculation of the stomach with PRV-614, rats were perfused transcardially with 4% paraformaldehyde in 0.15% sodium phosphate buffer, pH 7.4. The brains were removed and postfixed overnight at 4°C in paraformaldehyde, at which time they were rinsed in multiple washes of PBS, pH=7.2, equilibrated in 30% sucrose in PBS, and sectioned at 30 μ m on the freezing stage of a sliding microtome. Sections were mounted on slides, air dried, and coverslipped in Vectashield to retard photobleaching (Vector Laboratories, Burlingame, CA). Images were captured with a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) using filter sets for EGFP in GIN mice or in cells labeled with PRV-152, PRV-614, and for FluoroGold (Chroma Technology, Brattleboro, VT) on a Leica DMLB microscope (Leica, Nussloch, Germany).

Brainstem slice preparation

Whole-cell patch-clamp recordings were made by using brainstem slices prepared from infected and uninfected male Sprague-Dawley rats, 4-8 weeks of age, and GIN mice, 4-12 weeks of age. Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) or halothane (Sigma; St. Louis, MO) inhalation and then decapitated. The brain was removed and blocked on an ice-cold stand, and the medulla and cerebellum were glued to a sectioning stage. Transverse brainstem slices (300-400 μ m) containing the caudal vagal complex were made in 0-2°C, oxygenated (95% O_2 /5% CO_2) artificial cerebrospinal fluid (ACSF) by using a vibrating microtome (Vibratome Series 1000; Technical Products, Intl., St. Louis, MO). The

ACSF contained (in mM): 124 NaCl, 3 KCl, 2 CaCl₂, 1.3 MgCl₂, 1.4 NaH₂PO₄, 26 NaHCO₃, 11 glucose; pH 7.3-7.4, with an osmolality of 290-315 mOsm/kg. Slices were then incubated for at least 1 hour in warm (33-35°C), oxygenated ACSF. For recording, a single brain slice were transferred to a chamber mounted on a fixed stage under an upright microscope (Olympus BX51WI; Melville, NY), where they were continually superfused by room temperature ACSF.

Electrophysiological recording

Whole-cell voltage-clamp recordings were obtained in the DMV using patch pipettes with open tip resistances of 3-7 MΩ. Recording pipettes were pulled from borosilicate glass (0.45 mm wall thickness; Garner Glass Co., Claremont, CA, USA) and were filled with (mM): 140 K-gluconate (or Cs-gluconate), 10 HEPES, 1 NaCl, 1 CaCl₂, 3 KOH (or CsOH), 5 EGTA, 2-3 Mg-ATP, and 0.1% biocytin. Neurons in the DMV or NTS were targeted for recording under a 40x water-immersion objective (NA = 0.8) with fluorescence and infrared-differential interference contrast (IR-DIC) optics, as previously described (Davis et al., 2003; Glatzer et al., 2003; 2007; Derbenev, et al., 2004). For recordings from EGFP-labeled DMV neurons, initial visualization was made briefly under epifluorescence by using a FITC filter set. The epifluorescence illumination was then stopped and Nomarski (i.e., IR-DIC) illumination was used to guide the recording pipette onto the cell for whole-cell recording, exactly as for recordings in unlabeled neurons. Recorded neurons were visualized, and their EGFP content was documented on-line using a Spot RT Slider CCD camera (Diagnostic Instruments). Neurons were filled with biocytin, which diffused into the neuron from the recording pipette during whole-cell patch-clamp recordings.

Electrophysiological signals were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA), digitized at 88 kHz (Neurocorder, Cygnus Technology, Delaware Water Gap, PA) and low pass filtered at 2-5 kHz, recorded onto videotape and to a computer (Digidata 1320A, Axon Instruments, Foster City, CA) using pClamp 10 software (Axon Instruments). Recordings were analyzed with pClamp software (Axon Instruments) or MiniAnalysis (Synaptosoft, Decatur, GA). Seal resistances were typically 2-4 GΩ and series resistance, measured from brief voltage steps (5 mV, 5 msec) applied through the recording pipette, was typically <20 MΩ and monitored periodically during the recording. Recordings were discarded if series resistance changed by more than 20% over the course of the experiment. Input resistance was calculated using the slope of the current-voltage relationship obtained by measuring the voltage deflection from resting membrane potential at the end of brief (400-800 ms) current pulses of 5-25 pA. Resting membrane potential was determined by periodically monitoring the voltage at which no current was measured (i.e., removing voltage-clamp control of the neuron by switching to $I = 0$) during the recording. The criteria used for detecting and measuring excitatory and inhibitory spontaneous postsynaptic currents (i.e., sEPSCs and sIPSCs) in DVC neurons have been described (Smith et al., 1998; 2002). Membrane potential was clamped at -60 to -70 mV for sEPSC measurements and 0 to -20 mV for sIPSC measurements. At least 2 minutes of continuous spontaneous PSC activity was measured at each potential. All values are shown as mean ± SEM.

Histology

After recording, slices were fixed with 4% paraformaldehyde in 0.15 M sodium phosphate buffer overnight at 4°C (pH 7.4). After three rinses with 0.01 M phosphate buffered saline (PBS), slices (whole-mount) were immersed in avidin conjugated to AMCA or Texas Red (1:400; Vector Laboratories, Burlingame, CA) in PBS containing 0.5% Triton X-100 and incubated overnight at 4°C to identify biocytin-filled neurons. Slices were then rinsed 2-3× with PBS and mounted on slides to air dry for 10 minutes. The slices were then covered in Vectashield (Vector Laboratories) to reduce photo-oxidation during visualization with

fluorescent light and cover-slipped. Cells labeled with biocytin during a recording and/or with EGFP were identified with a Leica DMLB or Olympus BX40 microscope, and images were captured with a Spot RT camera (Diagnostic Instruments) using filters for the two fluorescent dyes and EGFP. Neurons were required to be positively identified and documented by one or both of these methods. The slices were then removed from the slides and rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the slices at 4°C for 1 hour in PBS containing 5% methanol, 5% ethanol, and 0.3% H₂O₂. They were then rinsed twice in PBS and incubated with avidin-biotin-horseradish peroxidase complex (ABC Elite; Vector Laboratories; 1:100) with 0.5-1% Triton X-100 for 24 hours. After 2-3 rinses in PBS, 0.06% diaminobenzidine tetrahydrochloride (DAB) and 0.003% H₂O₂ in PBS was applied for 15-30 minutes to visualize the cell. The reaction was stopped with PBS. The slices were then mounted and dried overnight on charged slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA), dehydrated in graded alcohol concentrations, and cover-slipped from xylenes in Permount.

Morphological analysis

Filled neurons were digitally reconstructed using images from a video camera (Cohu; San Diego, CA) mounted on an upright microscope with a 63× oil-immersion objective (Axioskop; Carl Zeiss, Thornwood, NY), motorized stage, and Neurolucida v4.05c software (Microbrightfield, Williston, VT) projected onto a monitor. The z-axis was calculated by measuring the focus changes in the camera and was calibrated by the software in reference to a coverslip of 0.22-mm thickness. The quantitative analysis was performed by Neuroexplorer 3.23b software (Microbrightfield) and based on information gathered about soma area, number of dendrites, total dendritic length, length of shortest primary dendrite, average length of primary dendrite, number of branch points, total axonal length, axon trajectory, and spine density (Glatzer et al., 2003). These features were chosen according to the previous studies on the morphology of DMV and NTS neurons (Fogel et al., 1996; Zhang, et al., 1998; Browning et al., 1999; Jarvinen and Powley, 1999; Kawai and Senba, 1999; Glatzer et al., 2003). The criteria for describing an axon were a process with all branches having relatively uniform thickness of approximately 2 μm. Soma area was used as a measure only if the soma was not obviously altered from its appearance before the cell was patched.

Stereology

Sections were compared with an atlas (Paxinos et al., 1999), and the distance from Bregma was noted. Images were captured with a Spot RT CCD camera (Diagnostic Instruments) using appropriate gold filter sets (Chroma Technology). Cell counts included sections from -13.01 to -14.06 mm from Bregma; sections were compared and analyzed at 270 μm increments, resulting in 5 rostro-caudal positions being analyzed per DMV. The cells were counted by using two-dimensional (profile) counting techniques with the StereoInvestigator 4.05a software (Microbrightfield) using the same setup and calibration as the Neurolucida (see above; Guillery, 2002). The counts were made of the entire DMV region within the section without sampling (Glatzer et al., 2003; Guillery, 2002). The contour area was drawn by hand around the apparent boundaries of the DMV with reference to the atlas.

Statistical analysis

Morphological and electrophysiological comparisons between the groups were made with an unpaired two-tailed Student's *t* test or one-way ANOVA. Significance for all measures was set at *P* < 0.05. All statistical measurements were performed with Microsoft Excel (Microsoft Corporation, Redmond, WA). Numbers are expressed as mean ± standard error of the mean (SEM), for statistical analysis.

Acknowledgements

This research was supported by funds from the NIH (DK056132) and NSF (IOB-0518209). Thanks to L. Enquist for providing the PRV-152, B. Banfield for providing the PRV-614, and R. Tsien for the mRFP1 construct used in making PRV-614.

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Figure 1.

Neuron labeling in the dorsal vagal complex after inoculation of the stomach wall with (PRV-152) and intraperitoneal FluoroGold. **A.** Green fluorescence (i.e., EGFP) indicates retrogradely labeled neurons in the dorsal vagal complex (DVC) 72 h after PRV-152 inoculation of the ventral gastric fundus. **B.** The region of the dorsal vagal motor nucleus (DMV) outlined by the box in **A** is shown in higher magnification. **C.** Stereological comparison of the total number of neurons in transverse sections from the DMV as a function of distance from bregma that were retrogradely labeled after intraperitoneal injection of FluoroGold or gastric inoculation with PRV-614. The number of neurons stained with both labels is also shown.

Figure 2.

Biocytin labeling of identified gastric-related rat DMV neurons. **A.** Fluorescence illumination after fixation reveals EGFP-labeled PRV-152 infected neurons 66hr after inoculation of the stomach wall. **B.** The same section showing biocytin-filled cell labeled with avidin-rhodamine. Arrows indicate the neuron that was recorded and filled with biocytin; scale bar in **B** applies to **A** and **B**. **C.** Identification of the same neuron after ABC-DAB reaction. **D.** Digital reconstruction of the same neuron. Arrow points to the axon.

Figure 3.

Examples of PRV-152 labeled and unlabeled rat DMV neurons. **A.** Digital micrograph of PRV-152 labeled rat DMV neurons filled with biocytin after whole-cell patch-clamp recording, shown in whole-mount. **B.** Digital reconstruction of the neuron in **A** superimposed on a diagram showing the relative position of the neuron in the DVC. **C.** Digital micrograph, and **D** reconstruction of an unlabeled rat DMV neuron. The neurons in **A-D** represent putative motor neurons, as the axon appears to exit the DMV ventrolaterally. **E.** Digital micrograph, and **F.** digital reconstruction of a neuron with axon projecting within the DMV. The scale bar in **A** applies to **A**, **C**, and **E**; scale bar in **B** applies to **B**, **D** and **F**. Arrows indicate axons. cc, central canal; DMV, dorsal motor nucleus of the vagus; Sol M, medial nucleus tractus solitarius; 4V, 4th ventricle.

Figure 4.

Identification of DMV neurons recorded from GIN mice. **A.** Fluorescence illumination of the neurons after slice fixation reveals EGFP-labeled GABAergic neurons. **B.** The same section showing biocytin-filled cell labeled with avidin-rhodamine. The scale bar in **A** applies to **A** and **B**. **C.** Identification of the same biocytin-filled neuron after ABC-DAB reaction. **D.** Distribution of GABAergic DMV neurons recorded from GIN mice filled with biocytin at three rostrocaudal levels of the DMV. AP, area postrema; cc, central canal; DMV, dorsal motor nucleus of the vagus; Gr, nucleus gracilis; SolC, commissural nucleus tractus solitarius; SolM, medial nucleus tractus solitarius; TS, tractus solitarius; XII, hypoglossal nucleus.

Figure 5.

Morphology of biocytin-filled GABA neurons in the DVC. **A.** Diagram showing the digital reconstruction of the GABAergic DMV neuron shown in **Fig 5 A-C** (left side) and another GABAergic neuron recorded in a different slice (right side). Reconstructions are superimposed on a map of the DVC to illustrate the neurons' relative positions in the DMV and also the variability in the amount of local axon arbor. Red indicates dendrites, and black color indicates axons. **B.** Diagram showing the digital reconstruction of an unidentified DMV neuron from GIN mice and the position in the dorsal vagal complex. Red color indicates dendrites, and black color indicates the axon; *, axon truncated for clarity. **C.** Diagram showing the digital reconstruction of a GABAergic NTS neuron and the position in the dorsal vagal complex. Red color indicates dendrites, and black color indicates the axon. Scale bar in **C** applies to **B** and

C. D. Confocal image of EGFP-labeled GABAergic NTS neurons. **E.** Confocal image of an axon from a biocytin-filled GABAergic NTS neuron. The neuron was recorded in the NTS contralateral to the imaged axon. **F.** Merged image of confocal sections (1 μ m optical sections) showing the axon and a GABAergic NTS neuron in close apposition.

Table 1**Morphological features of DMV neurons**

Features	Unlabeled (n=53)	EGFP-labeled (n=25)
Soma area (μm^2)	290.2 $\pm$ 14.8	319.4 $\pm$ 19.2
Total dendritic length (μm)	1151.0 $\pm$ 63.2	1360.1 $\pm$ 134.6
No. of dendrites	3.6 $\pm$ 0.2	4.0 $\pm$ 0.2
No. of branch points	4.7 $\pm$ 0.4	4.6 $\pm$ 0.4
Total axonal length (μm)	381.1 $\pm$ 58.8	463.1 $\pm$ 150.4
Axon out of vagal complex	32 of 53	12 of 25
Length of shortest primary dendrite (μm)	27.6 $\pm$ 5.7	15.5 $\pm$ 2.3
Average distance to first dendritic branch (μm)	82.0 $\pm$ 11.0	65.7 $\pm$ 7.2
Spine density (spines/ μm)	0.0030 $\pm$ 0.0005	0.0025 $\pm$ 0.0009

DMV, dorsal motor nucleus of the vagus. EGFP, enhanced green fluorescent protein.

Table 2

Morphological features of rat DMV neurons with different axon trajectories

Features	VL (n=44)	DVC (n=5)
Soma area (μm^2)	299.6 $\pm$ 14.4	163.3 $\pm$ 46.1 [*]
Total dendritic length (μm)	1111.5 $\pm$ 66.7	610.4 $\pm$ 129.3 [*]
No. of dendrites	4.0 $\pm$ 0.2	2.6 $\pm$ 0.4 [*]
No. of branch points	4.2 $\pm$ 0.4	1.6 $\pm$ 0.7 [*]
Total axonal length (μm)	549.4 $\pm$ 91.6	347.2 $\pm$ 78.8
Average axonal segment length (μm)	539.0 $\pm$ 91.2	282.7 $\pm$ 27.5 [*]
Length of shortest primary dendrite (μm)	24.9 $\pm$ 6.3	52.1 $\pm$ 23.2
Average length of primary dendrite (μm)	84.4 $\pm$ 12.6	147.9 $\pm$ 44.7
Spine density (spines/ μm)	0.0021 $\pm$ 0.0005	0.0053 $\pm$ 0.0018

DVC, dorsal vagal complex; VL, ventrolateral projection; NTS, nucleus tractus solitarius

^{*} Significant difference from neurons with VL trajectory ($p<0.05$).

Table 3

Electrophysiological features of DVC neurons

Features	Rat DMV		GIN mice NTS		GIN mice NTS	
	Unlabeled (n=17-33)	EGFP-labeled (n=8-16)	Unidentified (n=7-13)	GABAergic (n=8-11)	GABAergic (n=6-18)	
sEPSC frequency (Hz)	4.6±1.4	3.4±1.4	6.1±1.6	2.0±0.5*	3.9±1.4	
sEPSC amplitude (pA)	22.9±6.0	23.5±8.5	14.9±1.1	14.3±1.8	14.1±2.6	
sIPSC frequency (Hz)	2.8±0.6	1.7±0.5	3.5±0.7	1.2±0.3*	2.0±0.5	
sIPSC amplitude (pA)	25.8±2.1	25.7±3.8	21.9±2.6	18.6±2.8	26.0±3.8	
RMP (mV)	-45±2	-43±3	-49±3	-51±4	-53±2	
Input resistance (MΩ)	357±26	359±25	519±107	1227±185*	1180±144*	

sEPSC or sIPSC, spontaneous excitatory or inhibitory postsynaptic current; RMP, resting membrane potential.

* Significant difference versus unidentified murine DMV neurons (p<0.05).

Table 4

Morphological features of unidentified and GABAergic mouse DMV neurons and GABAergic mouse NTS neurons

Features	DMV		NTS
	Unidentified (n=10-12)	GABAergic (n=10-11)	GABAergic (n=7-10)
Soma area (μm^2)	292.7 $\pm$ 25.4 [*]	99.0 $\pm$ 9.8	97.8 $\pm$ 12.0
Total dendritic length (μm)	1045.6 $\pm$ 87.1 [*]	634.2 $\pm$ 70.3	423.1 $\pm$ 61.2 ^{**}
No. of dendrites	3.5 $\pm$ 0.3	2.7 $\pm$ 0.4	2.9 $\pm$ 0.4
No. of branch points	4 $\pm$ 0.5	3.1 $\pm$ 0.5	2.5 $\pm$ 0.7
Average segment length (μm)	144.2 $\pm$ 7.2 [*]	113.8 $\pm$ 10.5	82.2 $\pm$ 9.0 ^{**}
Total axonal length (μm)	747.8 $\pm$ 190.8	967.5 $\pm$ 506.8	726.7 $\pm$ 363.3
No. of axon branch points	0	4.7 $\pm$ 3.0	3.5 $\pm$ 2.6
Avg axon segment length (μm)	747.8 $\pm$ 190.8 [*]	163.7 $\pm$ 36.9	134.1 $\pm$ 33.0

* Significant difference between unidentified DMV neurons and both other groups ($p<0.05$).

** Significant difference between GABAergic neurons in DMV versus NTS ($p<0.05$).