# Viral Tracing of Innervation

A. Standish *et al.* (*1*) use the viral tracing technique to identify the neurons in the medulla oblongata involved in the control of the heart in rats. Three different strains of a herpes virus called pseudorabies virus (PRV) were injected into the ventricular myocardium and, after several days, transneuronal labeling was identified in two different regions of the medulla: the nucleus ambiguus and dorsal vagal nucleus. Unexpectedly, extensive cell body labeling was found throughout the dorsal vagal nucleus. Prior to this report, it was generally concluded that this central nervous system site provided the parasympathetic outflow to the subdiaphragmatic organs and was not involved in the autonomic control of the heart (*2*).

The statement (*1*) that the wild-type form of PRV (Becker PRV, or PRV-Be) is a specific transneuronal marker is unfounded. We know of no study demonstrating that this virus can be used as a specific retrograde transneuronal marker. In contrast, two studies (*3*) suggest that the attenuated virus called Bartha PRV (PRV-Ba) can be used in some systems in this manner, but even this virus is not a perfect transneuronal marker because it can produce false-positive results (*4*).

In the report by Standish *et al.* (*1*), two crucial control experiments are missing. First, because neurons in the full mediolateral extent of the dorsal vagal nucleus innervate the stomach and pancreas (*5*), experiments are needed to demonstrate whether or not the labeling of cardiac dorsal vagal neurons is specific. When PRV-Be is retrogradely transported in the cardiac vagal nerve fibers, does it cause infections in functionally unrelated fibers that lie within the vagus nerve? If so, this would produce false-positive labeling in the dorsal vagal nucleus. A double-labeling experiment in which the stomach or pancreas or both were injected with one retrograde marker and the heart with PRV-Be would directly test this issue.

Second, the report could be read as implying that PRV can be used in a straightforward manner. This is misleading because PRV-Be is transported in the anterograde and retrograde directions (*6*). Thus, the data obtained by Standish *et al.* could have been the result of anterograde transneuronal labeling. Again, experiments in which either the vagal afferent fibers or nodose ganglion were destroyed would have ruled out this potential concern.

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*Response:* Jansen and Loewy say that PRV-Be has not been shown to be a specific transneuronal tracer, but Card *et al.* (*1*) demonstrated that after injection of PRV-Be into the tongue, stomach, and esophagus, the pattern of viral labeling recapitulated precisely the known circuits specific for those organs as defined by injection of conventional tracers. A later, detailed electron microscopy analysis by Card *et al.* (*2*) of serial sections of rat brain stem labeled by injection of PRV-Be and PRV-Ba into the stomach demonstrated that both viruses gave highly specific infections and that release of virus from infected neurons occurred preferentially at sites of afferent contact. No evidence of focal spread in the dorsal motor nucleus of the vagus (DMV) or nucleus tractus solitarius (NTS) was observed. There was no nonspecific diffusion of virus from even the most severely infected cells. They proposed that release of virus was restricted by astrocytes and other nonneuronal cells responding to the local infection. Rinaman *et al.* (*3*) showed that the PRV-Be and PRV-Ba strains were highly specific transneuronal tracers. Card *et al.* (*4*) demonstrated that the PRV-Be was a specific transneuronal tracer of well characterized, retino-recipient regions of the brain. They showed that PRV-Ba was restricted in its ability to label these regions and that it showed remarkable restricted neurotropism, which suggests PRV-Be is more efficient at labeling known pathways, whereas PRV-Ba might not label all neurons participating in the circuitry.

Jansen and Loewy also argue that PRV-Ba is not a perfect transneuronal tracer because it has produced false-positive results (*5*). We are not sure what a perfect tracer would be, but we do know that viruses are not simple transneuronal tracers—they are complex infectious agents and as such demand careful analysis of the kind suggested by Jansen and Loewy. Rotto-Percley *et al.* (*5*) showed that PRV-Ba labeled some neurons that were unexpected by classical studies, but it is not at all certain that these results are false positive.

Controls such as those suggested by Jansen and Loewy were completed before we submitted our report. The test for organ-specific labeling was completed as described in the first paragraph above. Additional controls included the use of vagotomized animals to eliminate vagal transport, spinal cord transections to eliminate brainstem label of sympathetic origin, topical application of virus onto surgically exposed nerves, injections into other organs innervated by the vagus and other cranial nerves, and comparison of viral data with those obtained after injection of cholera toxin–horseradish peroxidase conjugate. We made the following observations: (i) no evidence of infection in nearby, functionally unrelated vagal motoneurons, (ii) no evidence of any axon-axon spread within the nerve, (iii) no evidence of central neuronal labeling after topical application of PRV-Be directly onto the vagus nerve, and (iv) no evidence of labeled motoneurons on the vagotomized side of the brain, which indicates that infections depend on intact vagus nerves.

There is other evidence that supports the conclusion that the PRV-Be labeling is cardiac-specific. (i) The non-DMV labeling by PRV-Be is similar to that seen for other strains, for example, PRV91 and PRV-Ba. (ii) PRV91 is isogenic with PRV-Be except that a single gene encoding the gE glycoprotein is deleted. After deleting this single gene, the distribution of infected neurons is similar to infection by PRV-Ba and dissimilar from that seen for the parental Becker strain. A single gene can effect specific labeling of neurons. We argue that the labeling of the DMV by PRV-Be is not a case of nonspecific, random labeling, but rather a case of specific host-virus interactions. (iii) The DMV labeling in the PRV-Be cases represents less than 2% of the DMV population, but increases the proportion of DMV cardiac vagal motor neurons from approximately 10% to 50%.

The final concern expressed by Jansen and Lowey is that the PRV-Be strain is transported in both the retrograde and anterograde direction, which can produce misleading results. This is an important consideration for both PRV-Be and PRV-Ba viral strains, but the evidence shows that retrograde transport is faster (*1*, *4*, *5*). Thus, we performed detailed time series experiments to collect data reflecting the earliest (presumably retrograde transport) labeling of neurons. We observed simultaneous labeling of both nucleus ambiguus and DMV neurons and found few NTS

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