In all five replicated populations, experimental and control, the fitness index was lower for the experimental line than its paired control. Within each of experiments 1, 2, and 3, this difference is statistically significant (Fig. 2). Furthermore, across the five paired experiments there is a strong consensus that 35 generations without recombination leads to lowered fitness [$P < 0.0006$, two-tailed consensus combined probability test (*13*)]. The estimated mean and standard deviation of the magnitude of fitness index reduction, 47 and 25%, respectively, are large and may substantially overestimate net fitness reduction because sample sizes in the fitness assays were small (Fig. 2) and a fitness index rather than net fitness was measured. It remains clear, however, that fitness declined in experimental relative to control populations.

Because reduced fitness was manifest in heterozygous individuals, these experiments extend prior work (*14*) by demonstrating genetic decay by means of the accumulation of nonrecessive mutations in response to the elimination of recombination. Studies of asexual genomic haplotypes in natural populations (*15*) also support the conclusion that once a haplotype stops recombining it becomes susceptible to deterioration through mutation accumulation.

The mechanism by which mutations accumulated in the experimental, nonrecombining populations cannot be unambiguously determined, but Muller's ratchet (*3–6*) and changes in the equilibrium mutational load (*8, 16, 17*) are likely candidates. Although the opportunity of sampling drift to fix deleterious mutations was the same in the control and experimental populations, the latter could not express mutations in the homozygous state, and this may have contributed to faster accumulation of partially recessive mutations. Part of the evolutionary advantage of sexual species may be due to the lack of recombination in asexual species, which therefore accumulate more deleterious mutations than do their sexual counterparts.

REFERENCES AND NOTES

1. J. J. Bull, *Evolution of Sex Determining Mechanisms* (Benjamin Cummings, London, 1983).
2. W. R. Rice, *Science* **256**, 1436 (1992).
3. H. J. Muller, *Mutat. Res.* 1, 2 (1964).
4. J. Felsenstein, *Genetics* **78**, 737 (1974).
5. J. Haigh, *Theor. Popul. Biol.* **14**, 251 (1978).
6. B. Charlesworth, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5618 (1978).
7. W. R. Rice, *Genetics* **116**, 161 (1987).
8. J. F. Crow, *Mathematical Topics in Population Genetics*, K.-I. Kojima, Ed. (Springer, Berlin, 1970), pp. 128–177.
9. M. Lynch and W. Gabriel, *Evolution* **44**, 1725 (1990).
10. T. Mukai, *Genetics* **50**, 1 (1964).
11. O. Ohnishi, *ibid.* **87**, 529 (1977).
12. L. Chao, *Nature* **348**, 454 (1990).
13. W. R. Rice, *Biometrics* **46**, 303 (1990).
14. T. Dobzhansky and B. Spassky, *Evolution* 1, 191 (1947).
15. R. C. Vrijenhoek, in *Population Biology and Evolution*, K. Wohrmann and S. Jain, Eds. (Springer-Verlag, Berlin, 1990), pp. 175–197.
16. A. S. Kondrashov, *Nature* **336**, 435 (1988).
17. B. Charlesworth, *Genet. Res.* **55**, 199 (1990).
18. The wild-type stock had been maintained for the first year at large size (in 15 half-pint culture jars that were mass transferred, with mixing among jars every 14 days) and for the year immediately prior to use at small size (in a single 8-dram culture vial by means of mass transfer every 14 days).
19. D. L. Lindsley and E. H. Grell, *Genetic Variations of* Drosophila melanogaster (Carnegie Institution of Washington, Washington, DC, 1968).
20. W. R. Rice, *Biometrics* **44**, 1 (1988).

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Innervation of the Heart and Its Central Medullary Origin Defined by Viral Tracing

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The vagus nerve exerts a profound influence on the heart, regulating the heart rate and rhythm. An extensive vagal innervation of the cardiac ventricles and the central origin and extent of this innervation was demonstrated by transynaptic transport of pseudorabies virus with a virulent and two attenuated pseudorabies viral strains. The neurons that innervate the ventricles are numerous, and their distribution within the nucleus ambiguus and dorsal motor nucleus of the vagus is similar to that of neurons innervating other cardiac targets, such as the sino-atrial node. These data provide a neuroanatomical correlate to the physiological influence of the vagus nerve on ventricular function.

The innervation of the heart by the vagus nerve has been investigated since profound vagal cardiac influences were demonstrated by the Weber brothers in 1845 (*1*). The vagus nerves exert important control over cardiac rhythm and rate, and the baroreceptor vagal reflex is crucial for normal blood pressure regulation (*2*). In addition, vagal stimulation elicits an appreciable frequency-dependent negative inotropic effect on the left ventricle (*3*). However, the vagal cardiac innervation has been a continuing source of controversy, and uncertainties remain as to whether the cardiac ventricles receive a significant vagal innervation. Vagal innervation of the ventricles has been thought to be absent or at least greatly less than that of the atrium (*4*). Further, questions remain as to the location and distribution of central medullary vagal cardiac preganglionic motoneurons innervating specific cardiac targets. Neurons of the dorsal motor nucleus of the vagus (DMV) and of the nucleus ambiguus (NA) innervate the heart as shown by various physiological, traditional tract tracing, and degeneration techniques. Cardiac vagal preganglionic neurons located in both the DMV and NA (*5, 6*), mostly in the NA (*7*), or primarily in the DMV (*8*) have been reported. Species variations may account for some of these differing results. However, conventional tracing studies with cholera toxin–conjugated horseradish peroxidase (CT-HRP) as a tracer have suggested a rather minor involvement of the DMV in the rat (*9, 10*), and recent reviews have reached a consensus that cardiac neurons arise mainly in the NA, with little contribution from the DMV (*11, 12*). This conclusion is consistent with the evidence that most, if not all, cells of the DMV appear to project to subdiaphragmatic, not cardiac, structures (*11*).

It has been difficult to resolve questions concerning cardiac, particularly ventricular, innervation by the usual retrograde tracing techniques because the postganglionic neurons at the heart break the link between the medullary vagal motoneurons and their cardiac targets. The postganglionic neurons are widely scattered at the heart (*13*). We have been able to circumvent these problems by using pseudorabies virus (PRV), a neurotropic herpesvirus, as a transynaptic tracer. Herpesviruses have been used to map synaptically linked neuronal circuits both centrally and peripherally (*14*). PRV can be injected directly into a peripheral organ, in this case the myocardium, and the virus transported from the specific cardiac site to postganglionic neurons that innervate that target. PRV replicates in these postganglionic neurons, then retrogradely crosses synapses and is transported to the specific vagal preganglionic neurons innervating the postganglionic neurons. Transneuronal spread of virus in

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the brain results from specific passage of virions at the synapse and not from passage through the extracellular space (*15*).

Forty-three Wistar rats (200 to 225 g) were anesthesized with a mixture of chloral hydrate (375 mg per kilogram of body weight) and pentobarbital (75 mg/kg) administered intraperitoneally. A tracheostomy was performed, and the rat was mechanically ventilated. The heart was exposed after a midline bisection of the sternum, and PRV was injected with a Hamilton syringe with an attached glass micropipette tip. Three strains of PRV were used, the virulent Becker (PRV-Be) and two attenuated strains, Bartha (PRV-Ba) and PRV-91. PRV-Ba has multiple mutations affecting virulence, whereas PRV-91 is isogenic with the virulent strain PRV-Be but is deleted for one gene encoding the viral envelope protein gE (previously called gI), a protein not required for viral replication in cell culture (*16*). The cardiac ventricular muscle wall was injected [2 to 8 μl; ~1 × 10^5 to 7 × 10^5 total plaque-forming units (PFU)] with PRV at various locations, including the apex of the heart. Some of the rats received one injection with 2 μl of PRV at various ventricular locations or received multiple injections (up to three) with 2 to 3 μl of PRV. In other rats, the fat pads around the heart where the parasympathetic ganglia are located (*13*) were injected (four sites around the great vessels, 2 to 4 μl per site) with PRV. After all cardiac injections, the sites were swabbed with sterile cotton applicators. The average titer of the PRV stocks was 1 × 10^8 PFU per milliliter. At different survival times, the animals were reanesthetized and killed by transcardial perfusion (*17*). Because the virus strains differ in virulence, the range of survival times were chosen to produce comparable amounts of neuronal infection and were 29 to 55 hours for PRV-Be, 51 to 78.5 hours for PRV-91, and 47 to 94.5 hours for PRV-Ba. Infected neurons were localized in the medulla with light microscopic immunohistochemistry (*18*). There was no obvious sign of lesions or infection of cardiac tissue at the cardiac injection sites. A diagram of the vagal cardiac circuit is shown in Fig. 1. Motor cells were distinguished from transynaptically labeled interneurons by comparison of somatic and dendritic morphology and cell location with cells labeled by CT-HRP cardiac injections (*10*).

Injection of each of the three strains of virus into the ventricular muscle wall, the sino-atrial node, or the area of cardiac vagal postganglionic neurons all produced abundant viral labeling of neuron vagal motoneurons in the medulla at the level ±2 mm of the obex, and of interneurons in the medulla and more generally within the visceral neuraxis. All 43 animals were used for data collection, and the results were consistent among animals. At short survival times, principally cardiomotor neurons and no (or very few) interneurons were labeled, but the shortest survival time at which labeling was observed depended on the injection location and the virus. For example, ventricular injections of PRV-Be, PRV-91, and PRV-Ba yielded substantial vagal cardiomotor labeling at 44, 51, and 72 hours, respectively. After injection of the ganglia around the heart, labeled neurons were first observed at shorter survival times, 8 to 20 hours before that seen after ventricular injection. Vagal cardiomotor neurons were located (typically bilaterally) in the DMV, NA, and in the tegmental field between these two nuclei (the intermediate zone) (Fig. 2). Neurons in the DMV (ranging from 15 to 25 μm) were spindle shaped with dendrites typically extending mediolaterally as observed in transverse sections, whereas those in the NA and intermediate zone were larger (ranging from 20 to 30 μm) with prominent multipolar dendrites. Distributions of labeled neurons were similar whether the ganglia around the heart (Fig. 2, A and D) or the cardiac ventricles (Fig. 2, B and E) were injected. Likewise, bilateral distributions of infected neurons were similar regardless of the location of injection on the left ventricle, including just the apex of the heart (Fig. 2F).

However, different distributions of vagal cardiomotor neurons were labeled by the different viral strains. In particular, cardiac injection of the virulent strain PRV-Be resulted in neuronal labeling in both cardiomotor populations, within the regions of the NA and the DMV (Fig. 2, A through C). In contrast, both attenuated strains of PRV readily infected neurons in the NA but rarely infected neurons in the DMV (Fig. 2, D through F). Both the virulent

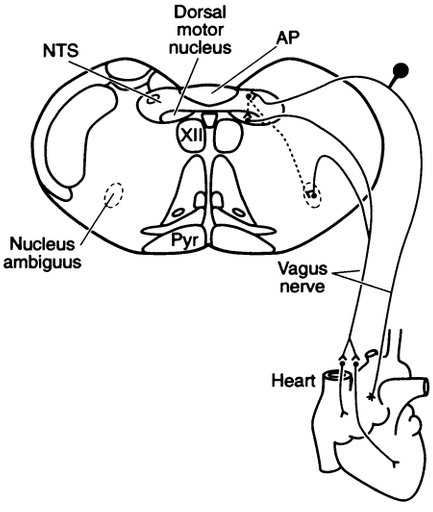

Fig. 1. Schematic diagram of the circuit involved in the baroreceptor vagal reflex. Cardiac preganglionic neurons are located in the dorsal motor nucleus (DMV), the nucleus ambiguus (NA), and the intermediate zone between these two populations. Second order neurons in the nucleus tractus solitarius (NTS) synapse on the cardiomotor neurons, most likely through at least one interneuron.

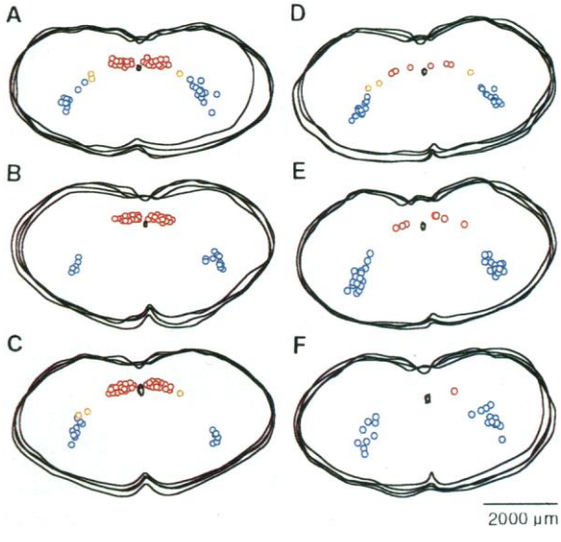

Fig. 2. Computer-based mapping of the distribution of labeled neurons after cardiac injection of the pseudorabies virus PRV. Maps are representative of the number of neurons observed in four sections located ~1 mm caudal to the obex. (**A**) PRV-Be injected into fat pads. Labeled neurons were located in both the DMV (red circles) and NA (blue circles). Injections were made into the fat pads surrounding the major vessels of the heart: 4 μl between the superior vena cava and the aorta, 1.5 μl at the junction of the aorta and the heart muscle, 1.5 μl between the aorta and pulmonary artery, and 3 μl at the crossing of the precaval vein, the pulmonary artery, and the left ventricle for a total of 6 × 10^5 PFU. A few labeled neurons were also located in the intermediate reticular zone (orange circles). (**B**) PRV-Be injected into the left ventricle labeled neurons in both the DMV and NA. A total of 8 μl (4.8 × 10^5 total PFU) was injected at three sites (2 to 3 μl per site) along the left ventricle. (**C**) PRV-Be injection into the SA-node resulted in virally infected neurons in both cardiomotor populations. (**D**) Injection of PRV-91 into the ganglia and (**E**) injection of PRV-91 into the left ventricle readily infected neurons in the NA but few in the DMV. Injection sites and amounts of virus used were similar to those for PRV-Becker. (**F**) Injection of 2 μl of PRV-Ba into the apex of the ventricle produced neuronal labeling primarily in the NA.

and attenuated strains infected neurons in the NA and intermediate zone. Approximately 65%, 15%, and 5% of the total population of cardiomotor neurons were labeled in the DMV for PRV-Be, PRV-91, and PRV-Ba, respectively, after cardiac ventricular injection (Fig. 3). The DMV labeling was largely at intermediate levels of the nucleus within ±1 mm of the obex. Rostrocaudal columns of labeled neurons were not clearly seen in the DMV, but many labeled neurons were located in the periphery of the nucleus along dorsal or lateral boundaries.

At longer survival times, all three types of PRV produced labeling of cells in the dorsal and dorsomedial nucleus tractus solitarii (NTS), a region that receives cardiovascular sensory inputs. Most of these neurons were labeled only at longer survival times, suggesting the interposition of another interneuron between these apparent second order afferent neurons and the cardiomotor output neurons involved in cardiovascular reflexes.

The neuronal labeling seen after injection of PRV results from retrograde transport. Both NA and DMV neurons were labeled simultaneously at short survival times by PRV-Be. The specificity for PRV-Be as a retrograde tracer has been proven by various controls. Control experiments were done on five rats to ensure that the observed neuronal labeling was due to viral transport up the vagus nerve. Immediately after PRV heart injections, a unilateral cervical vagotomy was performed. In these animals there was an absence of label in the NA, intermediate zone, and the DMV on the vagotomized side of the brain, whereas there was significant labeling on the intact side. Further, injections into the chest cavity and into the lung tissue, topically onto the vagus nerve and directly into the blood, did not label central neurons. PRV is neurotropic, does not diffuse far from the injection site, and labels neurons in distinct central regions dependent on the injected target organ (*16*).

Our findings clarify the current debate about vagal innervation of the heart by showing that cardiac ventricular sites, including the apex, receive an extensive vagal innervation distributed within the NA and DMV in a manner similar to that of neurons innervating other cardiac targets, such as the sino-atrial node. The vagus has profound effects on cardiac function, including beat-to-beat regulation of cardiac rate (*3*), but until now the vagal ventricular innervation has not been possible to establish anatomically. Because cardiac postganglionic neurons have not been found in the ventricles (*4, 13*), ventricular injections of conventional tracers would not be expected to label centrally located vagal preganglionics. This prediction was confirmed by injection of CT-HRP into the ventricles; no labeled cells were seen. This result contrasts with the abundant label after viral injection. Our results provide an anatomical substrate for the controversial but potentially important inotropic function of vagal cardiac innervation and thus support the physiological results of Levy and others (*2, 3*). This may indicate that the neurons projecting to the ventricles and to the atria and nodal sites participate in similar central control circuits.

Our different results in DMV neurons with different viral strains point to selective uptake, transport, and labeling by the DMV and NA: PRV-Be labels the DMV relatively extensively as compared with CT-HRP or the other attenuated viral strains. This difference in DMV labeling among viral strains is a potentially powerful tool for exploring the differences in the DMV and NA cardiac populations. For example, selective labeling may correlate with functional differences: DMV neurons have primarily unmyelinated axons that mediate slow vagal effects on the heart, whereas NA neurons are myelinated and mediate fast effects. The PRV-Be and PRV-91 strains differ only in the deletion of the gE gene, and this gene is required for efficient infection of DMV cardiomotor neurons. Our virally selective findings are consistent with results from Card and co-workers which demonstrate that selective infection of a functionally distinct subset of neurons innervating the eye is due to the gE gene (*16, 19*). Indeed, the PRV glycoprotein gE is clearly critical in distinguishing NA neurons from DMV neurons that innervate the heart.

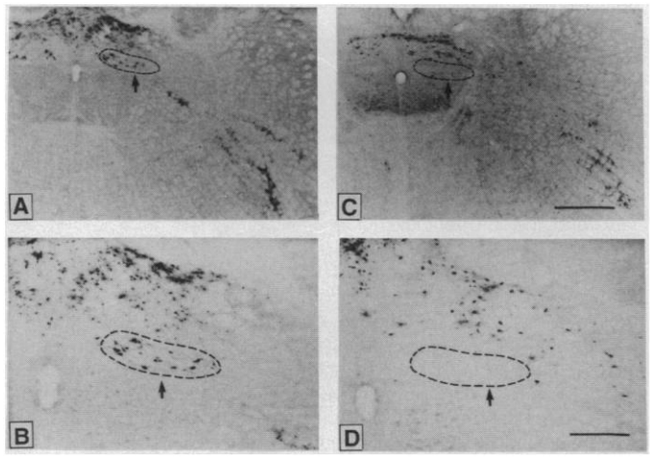

Fig. 3. Photomicrographs of the virally infected cardiomotor populations after injection of PRV at sections caudal to the obex. (**A** and **B**) Both the DMV and NA were labeled 52 hours after ventricular injection of PRV-Be. (**C** and **D**) Only the NA was labeled 78 hours after ventricular injection of PRV-91. Scale bar, 500 μm for (A) and (C) and 250 μm for (B) and (D). Dashed black circles outline the region of the DMV.

REFERENCES AND NOTES

1. H. E. Hoff, *Ann. Med. Hist.* **8**, 138 (1936).
2. M. Levy and P. Martin, in *Nervous Control of Cardiovascular Function*, W. C. Randall, Ed. (Oxford Univ. Press, New York, 1984), pp. 68–94.
3. H. DeGeest, M. N. Levy, H. Zieske, R. I. Lipman, *Circ. Res.* **17**, 222 (1965).
4. K. Loffelhoz and A. J. Pappano, *Pharmacol. Rev.* **37**, 1 (1985).
5. G. S. Geis and R. D. Wurster, *Circ. Res.* **46**, 606 (1980); J. Ciriello and F. R. Calaresu, *Am. J. Physiol.* **238**, R57 (1980).
6. S. Nosaka, K. Yasunaga, S. Tamai, *Am. J. Physiol.* **243**, R92 (1982).
7. M. R. Thomas and F. R. Calaresu, *ibid.* **226**, 1344 (1974); R. M. McAllen and K. M. Spyer, *J. Physiol.* **258**, 187 (1976).
8. F. R. Calaresu and M. K. Cottle, *J. Physiol. (London)* **176**, 252 (1965); K. Todo *et al.*, *Brain Res.* **130**, 545 (1977).
9. P. N. Izzo, J. Deuchars, K. M. Spyer, *J. Comp. Neurol.* **327**, 572 (1993).
10. J. A. Escardo *et al.*, *Soc. Neurosci. Abstr.* **17**, 993 (1991).
11. D. A. Hopkins, in *Cardiogenic Reflexes*, R. Hainsworth, P. N. McWilliams, D. A. S. G. Mary, Eds. (Oxford Univ. Press, New York, 1987), pp. 185–203.
12. A. D. Loewy and K. M. Spyer, in *Central Regulation of Autonomic Function*, A. D. Loewy and K. M. Spyer, Eds. (Oxford Univ. Press, Oxford, 1990), pp. 68–87.
13. T. Burkholder *et al.*, *Anat. Rec.* **232**, 444 (1992).
14. G. Ugolini, H. G. J. M. Kuypers, A. Simmons, *Brain Res.* **422**, 242 (1987); A. M. Strack *et al.*, *ibid.* **491**, 156 (1989); J. P. Card *et al.*, *J. Neurosci.* **10**, 1974 (1990).
15. J. P. Card *et al.*, *J. Neurosci.* **13**, 2515 (1993).
16. J. P. Card, M. E. Whealy, A. K. Robbins, L. W. Enquist, *J. Virol.* **66**, 3032 (1992).
17. Each animal was deeply anesthetized and transcardially perfused with 100 ml of 0.1 M phosphate buffer (pH 7.4) and then with 200 to 300 ml of paraformaldehyde-lysine-periodate (PLP) fixative [I. W. McLean and P. K. Nakane, *J. Histochem. Cytochem.* **22**, 1077 (1974)].
18. Serial sections (40 μm thickness) were cut with a freezing microtome. Free-floating tissue sections were incubated with polyclonal antisera and processed according to the avidin-biotin peroxidase method (ABC; Vectastain, Vector Laboratory, Inc.).
19. J. P. Card, M. E. Whealy, A. K. Robbins, R. Y. Moore, L. W. Enquist, *Neuron* **6**, 957 (1991).

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