Impulse Blockade in Frog Cardiac Ganglion Does Not Resemble Partial Denervation in Changing Synaptic Organization

Abstract. *Partial denervation of parasympathetic neurons in the frog heart by surgical section of one vagus nerve results in a marked reorganization of functional synaptic connections made by the remaining vagus nerve. These changes are not simply due to a lack of impulse activity per se in the sectioned nerve because blockage of impulses in one vagus with tetrodotoxin-impregnated cuffs did not cause similar changes in the innervation pattern of the ganglion. Furthermore, tetrodotoxin-blocked vagal fibers retain their ability to sprout and can form new synapses on denervated neurons.*

When denervation occurs as a result of damage to surrounding nervous tissue, remaining intact axons sprout and grow new terminals to contact the denervated cells (*1*). Investigators have examined synaptic transmission at sprouting nerve terminals and have shown that functional connections are established by the proliferating endings (*2*). An important consequence of sprouting is that novel axonal pathways, perhaps quite dissimilar to the original connections, are established within the nervous system. We have investigated this phenomenon in the cardiac ganglion of the frog, a parasympathetic ganglion that mediates vagal inhibition of the heart, and have found that crushing one vagus nerve causes a marked change in the functional synaptic connections from the other vagus nerve; the remaining intact vagal axons, which normally innervate roughly half the ganglion cells, form functional synapses with all the neurons after unilateral vagotomy (*3*).

The stimulus that initiates this change in innervation pattern after partial denervation is not known. One important consequence of cutting one vagus nerve is that the pattern of preganglionic nerve impulses reaching the cardiac ganglion is altered, and this may be an important factor in the remodeling of ganglionic innervation. We have devised a method to block propagated activity in intact vagus nerves of the frog for several days, leaving the nerves otherwise intact, to test whether cessation of impulses per se in one vagus nerve mimics partial denervation and leads to a functional reorganization of synapses from the other, actively conducting, vagus nerve.

The cardiac ganglion of the frog (*Rana pipiens*) is well suited for these studies. When the ganglion is dissected from the animal and stretched in a small chamber containing Ringer solution, individual cells can be seen and impaled with microelectrodes under direct visual observation (*4*). Vagal preganglionic fibers are the only synaptic inputs. Left and right vagus nerves can be drawn into separate suction electrodes and stimulated independently in the isolated preparation. Furthermore, the ganglion is divided into two distinct branches, and each branch is innervated predominantly by the ipsilateral vagus nerve (*3*). For example, 88 percent of the nerve cells on the left branch of the ganglion are innervated by the left vagus nerve, and 52 percent receive inputs from the right vagus nerve; about one-third of the cells receive innervation from both vagus nerves. Ganglion cells usually receive a single suprathreshold input and often one or more subthreshold ones. Frequently we can evoke each input separately by carefully adjusting the stimulus intensity to the vagus nerve. The methods for stimulating and recording intracellularly from cardiac ganglion cells are described elsewhere (*3*, *4*).

We made a detailed census of all the vagal inputs to ganglion cells in 15 frogs that had not been operated on, using intracellular records of evoked responses. Neurons were grouped into five categories depending on whether they were innervated (i) only by the right vagus nerve, (ii) predominantly by the right vagus, (iii) equally by right and left vagus nerves, (iv) predominantly by the left vagus, and (v) only by the left vagus nerve. Figure 1A shows that the ipsilateral vagus nerve contributes the predominant input but that many cells also receive subthreshold or suprathreshold innervation from the contralateral vagus. Only neurons on the left branch of ganglia have been included in this report to simplify the analysis. Figure 1B shows the vagal dominance distribution of ganglia 6 to 26 days after the ipsilateral (left) vagus nerve was crushed; nearly all the cells are innervated by the remaining intact contralateral axons (*3*).

We next tested whether this change in ganglionic innervation occurred when impulse propagation per se in the ipsilateral vagus nerve was blocked. We blocked propagated action potentials over a period of up to 10 days by implanting small Silastic cuffs impregnated with tetrodotoxin (TTX) (*5*) around the left vagus near its exit from the skull (about 1 cm from the cardiac ganglion). The frogs were anesthetized with 10 percent ethanol during the procedure. It has been shown by others that TTX has a specific effect on impulse propagation and does not, for example, impede axoplasmic transport (*6*). Control experiments consisted of implanting Silastic cuffs impregnated with NaCl or with citrate buffer, *p*H 4.8, around the left vagus nerve in another group of animals (*7*). Cuffs impregnated with NaCl or citrate buffer did not block nerve conduction.

Cuffs that contained TTX effectively blocked propagated impulses in the vagus nerves of some frogs for at least 10 days, a period of sufficient duration to test for possible reorganization of ganglionic synapses comparable to that which occurs after partial denervation. Data from animals in which we could not confirm that impulses were blocked or that the block was only partial at the time of the experiment are not included in this report. The effect of the cuff was tested by reanesthetizing the frog, exposing the TTX-treated nerve, and electrically stimulating the vagus above and below the cuff while recording the electrocardio-

Table 1. Vagal innervation of cardiac ganglia (left branch) in control frogs that were not operated on and in frogs with Silastic cuffs implanted on the left vagus nerve for 5 to 10 days. The 1.5 percent TTX-impregnated cuffs blocked vagal impulse propagation for the duration of the implantation. Control cuffs contained either 9 percent dry citrate buffer or 20 percent NaCl and did not block impulse conduction. Entries are the average percentages (± standard error of the mean) for 13 to 19 ganglia in each line; the total number of neurons recorded in these experiments is listed in the second column.

Group	Number of ganglion cells	Percentage innervated by			Percentage with no inputs
		Left vagus	Right vagus	Both vagi	
Control	373	87.6 ± 3.3	51.9 ± 6.2	39.9 ± 5.4	0.5 ± 0.4
TTX cuff on left vagus	412	77.3 ± 5.1	66.2 ± 7.8	45.2 ± 5.7	1.8 ± 0.8
NaCl or buffer cuff on left vagus	573	80.5 ± 4.0	67.7 ± 4.8	50.0 ± 5.0	1.7 ± 0.6

0036-8075/78/1006-0066$00.50/0

gram (ECG) with a concentric needle electrode. The central connections of the vagus nerve were severed before recording the ECG. Vagal stimulation proximal to an effective TTX cuff had no effect on the heart rate or had an effect only when intense stimulation strong enough to spread electronically below the cuff was applied (that is, 60 to 90 V, 10 Hz, 1-msec pulses). On the other hand, stimulation applied distal to an effective TTX cuff, or stimulation of the untreated right vagus promptly stopped the heartbeat at low-intensity stimulation (4 to 5 V). In all cases, the effectiveness of each TTX implant was tested in situ in this manner before the cardiac ganglion was removed and vagal innervation mapped (*8*).

Figure 2A shows the vagal dominance diagram for ganglia from TTX-treated frogs. Although the dominance diagram for TTX-treated animals differs significantly ($P < 1$ percent, χ^2 test) from that for animals that were not operated on (Fig. 1A), the data are indistinguishable from those obtained when we implanted control (NaCl- or citrate buffer–impregnated) cuffs as shown in Fig. 2B. Neither NaCl- nor buffer-containing cuffs had a measurable effect on impulse conduction.

These data are summarized in Table 1. The slight increase in right vagal innervation observed after implanting either TTX- or salt-impregnated cuffs is probably due to some degeneration caused by the cuff technique, as supported by electron microscopic inspection of the treated nerves. Thus TTX cuffs applied to one vagus nerve and blocking impulse conduction do not cause changes in preganglionic connections comparable to those seen after partial denervation.

We also tested whether the absence of impulses would affect the ability of vagal axons to form new functional connections. This differs from the above experiments in that the left nerve was severed and the TTX cuff was implanted on the remaining intact right nerve, which normally forms new connections with the ganglion cells (see Fig. 1B). In four animals in which the left vagus was crushed and in which we confirmed that the TTX cuff on the remaining vagus was effective, we observed the expected increase in right vagal innervation after 6 to 8 days. Thus, intact preganglionic axons were still able to form new functional synapses in response to one vagus nerve being cut even though propagated activity had been blocked in those fibers.

These experiments show that blocking propagated impulses in vagus nerve axons does not mimic partial denervation and does not stimulate the formation of new functional synapses from the other, actively conducting, preganglionic fibers. With physiological techniques we cannot exclude the possibility that TTX treatment leads to collateral sprouting without functional synapses being formed (*9*). The signals that initiate collateral sprouting in axons and cause a functional reinnervation of neurons in a region of damaged tissue are still obscure. Cajal thought that degenerating nervous tissue and surrounding Schwann cells released chemotropic substances which stimulated both collateral and regenerative sprouting (*10*). More recently, other investigators have proposed that nerve terminals may be in a constant state of growth and retraction (*11*). In this case, one might expect to find factors released from intact terminals which normally prevent or impede sprouting in adjacent nerve endings and thus tend to

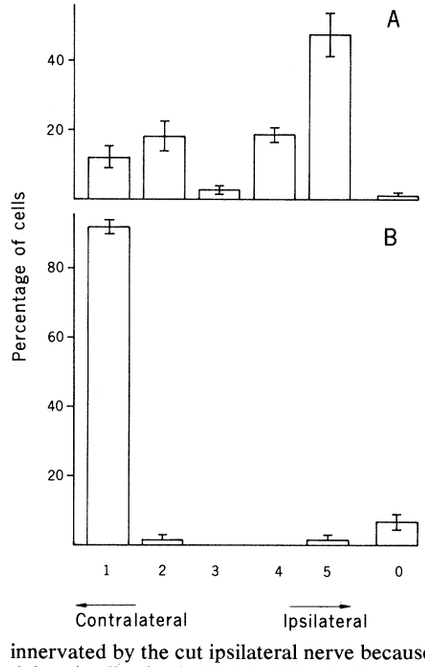

Fig. 1. Distribution of preganglionic vagal terminals in the left branch of cardiac ganglia from (A) control frogs that had not been operated on and (B) frogs in which the ipsilateral (left) vagus nerve had been crushed 6 to 26 days previously. Ganglion cell responses evoked by stimulating the vagus nerves were recorded with intracellular microelectrodes. Cells of group 1 were driven only by the contralateral (right) vagus nerve. For cells of group 2, the contralateral vagal responses were suprathreshold and the ipsilateral vagal responses were subthreshold. In group 3 there was about equal input from the two vagus nerves. For cells of group 4 there were subthreshold inputs from the contralateral nerve and suprathreshold ones from the ipsilateral nerve. In group 5 the cells were driven only by the ipsilateral vagus nerve. In some experiments, a small proportion of cells received no inputs, presumably because of slight damage they received when the cardiac branches of the vagus nerves were dissected from the animal. These cells are included in group 0. When the ipsilateral vagus nerve was cut, the contralateral preganglionic terminals rapidly sprouted and innervated the entire ganglion, as is shown in (B). A few cells in (B) are still innervated by the cut ipsilateral nerve because preganglionic degeneration was not complete at 6 days in all animals. In (A), 373 cells from 15 control animals are included, and in (B), 140 cells from six animals with their left vagus nerves cut are included. Bars indicate standard error of the mean.

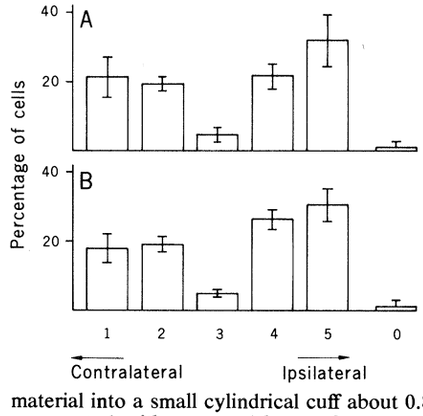

Fig. 2. Distribution of preganglionic vagal terminals in the left branch of cardiac ganglia from (A) frogs with a TTX-impregnated Silastic cuff implanted on the left vagus nerve and (B) animals with NaCl- or citrate buffer–impregnated cuffs implanted on the left vagus. The cuffs were implanted for 5 to 10 days before the vagal innervation was mapped. This time interval was chosen because our earlier experiments (*3*) showed that if impulse blockade were to mimic cutting the left vagus nerve, preganglionic sprouting would be nearly complete by this time. Cells are organized in groups as described in Fig. 1. The TTX cuffs were constructed by mixing 1.5 percent TTX by weight with Dow Corning Silastic A RTV plus catalyst No. 4 and forming the material into a small cylindrical cuff about 0.8 mm in diameter with a lumen of 0.35 mm. This was coated with a second layer of untreated Silastic. Individual cuffs contained about 3 to 5 μg of TTX. Control cuffs contained either 9 percent dry citrate buffer or 20 percent NaCl and were constructed similarly to TTX cuffs. For TTX-treated frogs, the blockade of propagated impulses in the left vagus nerve was effective within a few hours and lasted for the entire period of implantation. Impulse conduction in the contralateral nerve was not blocked. Sodium chloride and citrate buffer cuffs had no measurable effect on impulse propagation. The proportion of cells innervated by the right vagus nerve (groups 1 through 4 inclusive) increased somewhat in both the animals with TTX cuffs and those with control cuffs when compared with control frogs (Fig. 1A). In (A), 412 cells from 13 frogs are included, and in (B), 573 neurons from 19 frogs are included. Bars indicate standard error of the mean.

stabilize the population of synapses. When a nerve terminal is destroyed, the regulator would no longer be present and adjacent terminals could then proliferate and occupy the vacated synaptic sites. Diamond and his colleagues have presented some experimental evidence for such an interpretation (*12*). The results of our studies suggest that propagated action potentials do not play the only role in controlling the release of putative chemotropic or regulator substances and in maintaining normal synaptic connections since blocking conducted activity in one vagus nerve with TTX neither caused a remodeling of functional ganglionic innervation nor did it prevent sprouting in the TTX-treated nerve after the opposite vagus was destroyed.

S. ROPER
C.-P. KO

Department of Anatomy and
Physiology, University of Colorado Medical Center, Denver 80262

References and Notes

1. G. Raisman and P. M. Field, *Brain Res.* **50**, 241 (1973); G. S. Lynch, D. Matthews, S. Mosko, T. Parks, C. W. Cotman, *ibid.* **42**, 311 (1972); J. G. Murray and J. W. Thompson, *J. Physiol. (London)* **135**, 133 (1957); Y. Nakamura, N. Mizuno, M. Sato, *Brain Res.* **82**, 298 (1974).
2. G. Lynch, S. Deadwyler, C. Cotman, *Science* **180**, 1364 (1973); F. Murakami, Y. Fujito, N. Tsukahara, *Brain Res.* **103**, 147 (1976).
3. K. Courtney and S. Roper, *Nature (London)* **259**, 317 (1976); S. Roper, *ibid.* **261**, 148 (1976).
4. U. J. McMahan and S. W. Kuffler, *Proc. R. Soc. London Ser. B* **177**, 485 (19 M. J. Dennis, A. J. Harris, S. W. Kuffler, *ibid.*, p. 509.
5. E. D. Robert and Y. T. Oester, *J. Pharmacol. Exp. Ther.* **174**, 133 (1970); T. Lømo and J. Rosenthal, *J. Physiol. (London)* **221**, 493 (1972).
6. P. A. Lavoie, B. Collier, A. Tenenhouse, *Nature (London)* **260**, 349 (1976); A. Pestronk, D. B. Drachman, J. W. Griffin, *ibid.*, p. 352.
7. Tetrodotoxin-impregnated cuffs contained 1.5 percent TTX by weight. Commercially prepared TTX samples, in addition to 1 mg of the drug contained 5 mg of dry citrate buffer, *p*H 4.8. Hence, a 1.5 percent TTX cuff will also contain 7.5 percent dry buffer. Our citrate buffer control cuffs contained 9 percent buffer by weight, and our NaCl control cuffs contained 20 percent NaCl by weight.
8. In some experiments, vagal stimulation proximal to an implanted TTX cuff blocked the heart rate at low (4 to 5 V) or intermediate (for example, 10 to 40 V) intensities. Data from these experiments were not included in the final analysis. Our criterion for accepting the results of any one experiment was that the stimulus intensity applied central to the TTX cuff had to be at least one order of magnitude greater than that which was just effective when applied distal to the cuff.
9. M. C. Brown and R. Ironton, *Nature (London)* **265**, 459 (1977). These investigators showed that TTX cuffs applied to motor nerves in mice cause some degree of morphological sprouting at the treated end plates, but without accompanying functional changes. We have examined zinc iodide–stained cardiac ganglia from frogs with TTX cuffs implanted around one vagus nerve. To date, our material has not revealed any morphological changes comparable to those seen by Brown and Ironton. However, if a small number of fine nerve terminal sprouts occurred in TTX-treated cardiac ganglia, they might not be revealed by the somewhat capricious nature of zinc iodide staining.
10. S. Ramon y Cajal, *Degeneration and Regeneration of the Nervous System* (Oxford Univ. Press, London, 1928).
11. D. Barker and M. C. Ip, *Proc. R. Soc. London Ser. B* **163**, 538 (1966); A. R. Tuffery, *J. Anat.* **110**, 221 (1971); C. Sotelo and S. L. Palay, *Lab. Invest.* **25**, 653 (1971).
12. J. Diamond, E. Cooper, C. Turner, L. Macintyre, *Science* **193**, 371 (1976).
13. We thank A. R. Martin, S. Frenk, and W. Proctor for assistance in preparing this manuscript. This work was supported by the Edward Mallinckrodt, Jr., Foundation, National Institutes of Health (grant NS 11505), and the Colorado Heart Association.

6 March 1978; revised 16 May 1978

Induction of Ovalbumin Synthesis in Immature Chicks by Actinomycin D and Thioacetamide

Abstract. *Actinomycin D and thioacetamide induced ovalbumin synthesis and increased serum progesterone concentrations in immature chicks. The increase in progesterone induced by the carcinogens actinomycin D and thioacetamide may account for the induction of ovalbumin synthesis.*

We recently reported a novel effect of the administration of the carcinogen ethionine in immature chicks (*1, 2*). The effects of ethionine on the oviducts of immature chicks that have previously been stimulated with estrogen and subsequently withdrawn from estrogen administration for 3 to 4 weeks are similar to those caused by the administration of progesterone. Ethionine induces synthesis of the egg white proteins ovalbumin and conalbumin and also produces cellular changes in the oviduct characteristic of hormone administration. The effect of ethionine on the oviduct appears to be an indirect consequence of its influence on steroid hormone metabolism: it produces tenfold increase in serum progesterone concentrations (*2*). We show here that two other carcinogens, thioacetamide and actinomycin D, induce ovalbumin synthesis in estrogen-treated chicks and increase serum progesterone concentrations.

Estrogen administration to immature chicks results in cytodifferentiation of tubular gland cells which synthesize the egg white proteins ovalbumin, conalbumin, ovomucoid, and lysozyme. The continuous presence of estrogen is required for sustained synthesis of these proteins in immature chicks. Discontinuation of estrogen administration results in gradual decline in cell-specific protein synthesis. Ovalbumin synthesis is not detected after 3 to 4 weeks of estrogen withdrawal. Either estrogen or progesterone can induce the synthesis of major egg white proteins when given as a secondary stimulation to chicks withdrawn from estrogen stimulation (*3–5*).

Four-day-old Calhorn chicks (from Meyer Brothers' Hatchery) were injected intramuscularly below the knee daily with 1 mg of estradiol benzoate in sesame oil (*3*) for 10 days (primary estrogen stimulation). After 3 to 4 weeks of estrogen withdrawal the chicks were used in the experiments; at this stage, ovalbumin synthesis in the oviducts is not detected. The chicks were decapitated and their oviducts removed. The magnum portion of the oviduct was freed of adjoining tissue and was cut into small pieces. The magnum explants were incubated for 3 hours in vitro (*3*), 100 to 200 mg of tissue being placed in 2 ml of medium in 25-ml rubber-stoppered Erlenmeyer flasks. The temperature was maintained at 37°C, and the flasks were subjected to constant shaking and were gassed with 95 percent O_2, 5 percent CO_2, at hourly intervals. At the end of the incubation period the pieces of tissue were blotted on filter paper, homogenized in a Potter-Elvehjem homogenizer with 2 ml of 15 m*M* sodium chloride and 10 m*M* sodium phosphate, *p*H 7.5, and centrifuged at 100,000*g* for 1 hour. The supernatant obtained at high speed was used for subsequent analyses.

Administration of thioacetamide or actinomycin D to immature chicks 4 weeks after primary estrogen stimulation induced ovalbumin synthesis and increased progesterone concentrations (Table 1). Labeled ovalbumin was determined by specific immunoprecipitation with monospecific antiserum against purified ovalbumin (*1, 3*). The identity of the ovalbumin precipitated by antiserum was confirmed by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel with authentic markers (Fig. 1). An increase in serum progesterone brought about by actinomycin D in immature chicks has also been observed by Elo *et al.* (*6*). Progesterone is known to induce ovalbumin synthesis in chicks when it is given as a secondary stimulation to chicks withdrawn from estrogen stimulation (*4, 5*). The increase in serum progesterone brought about by actinomycin D and thioacetamide may account for the induction of ovalbumin synthesis in chicks. The lag period for the induction of ovalbumin synthesis is much longer with actinomycin D (1 day), thioacetamide (2 days, data not shown), or ethio-

0036-8075/78/1006-0068$00.50/0