Nonimpulse-Mediated Synaptic Transmission During the Generation of a Cyclic Motor Program

Abstract. *A small neuronal network in the lobster stomatogastric ganglion, composed of impulse-producing motor neurons, gives rise to cyclic patterned outputs. This network continues to generate its cyclic motor program if impulse production within the ganglion is blocked. Continuously graded, nonimpulse-mediated, chemical synaptic transmission is sufficient to coordinate neuronal activity in a functioning pattern generator.*

Integrative neurophysiology has emphasized the role nerve impulses play in communication among neurons. Accordingly, information can be encoded by neurons and projected over long distances as the frequency, timing, or pattern of propagated nerve impulses. As individual impulses invade presynaptic terminals, the release of brief pulses of transmitter causes corresponding discrete postsynaptic potentials (PSP's) in the receiving neuron. Impulse coding is unnecessary if signals need travel only short distances (*1*). Many examples of chemical transmission from neurons in the absence of presynaptic impulse generation have been found (*2*). The principal features of this mode of synaptic action are (i) the passive spread of electrical signals from the input to the output regions of a neuron and (ii) the control of transmitter release by the continuously graded polarization of presynaptic sites (*3*).

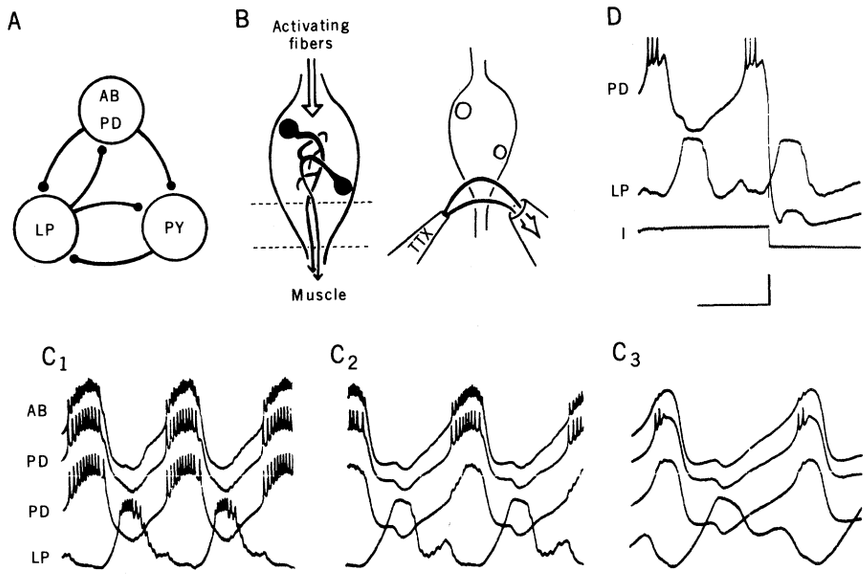

Fig. 1. (A) Major synaptic connections within the pyloric subsystem. Circles enclose classes of similar individually identified neurons. All chemical synaptic actions indicated are inhibitory. Abbreviations: *AB*, anterior burster; *PD*, pyloric dilator; *LP*, lateral pyloric; *PY*, pyloric. (B) Schematic of ganglion and experimental paradigm. Activating fibers enter via the anterior nerve, and pyloric motor axons exit via the posterior nerve. The impulse-initiating zones of the pyloric neurons are thought to lie between the dashed lines. Perfusion with TTX was confined to the posterior margin of the ganglion by ejecting the toxin solution from one pipette and retrieving it with another. (C_1) Simultaneous intracellular recordings under control conditions from the cell bodies of all the neurons in the AB/PD class and the LP. Descending inputs from the more central esophageal and commissural ganglia were responsible for the high level of spontaneous cyclic activity. Bursts of attenuated spikes ride on slower membrane potential oscillations driven by a mixture of synaptic events and endogenous membrane properties. (C_2) Spontaneous activity 7 minutes after the start of localized perfusion of $1.7 \times 10^{-5} M$ TTX across the posterior margin of the stomatogastric ganglion. Nerve impulses in the LP and one of the PD's have been suppressed. Note the marked graded inhibition of the AB/PD group by the LP. (C_3) All spontaneous activity ceased after 10 minutes of toxin perfusion, although brief cyclic activity could be induced by 1-second trains of 30-Hz shocks to the anterior input nerve. Nearly all impulse generation had been suppressed, but strong graded inhibition of the AB/PD by the LP is still evident, as is AB/PD inhibition of the LP. The PY group was responsible for the smooth inhibition of the LP between LP and PD times. (D) Graded transmission observed during a localized toxin experiment was reversed in sign by hyperpolarizing the postsynaptic neuron. In this example, spontaneous plateaus in the presynaptic LP mediated graded synaptic potentials in the postsynaptic PD. Current injection was accomplished through a second electrode in the PD. Calibration: 10 mV, 10 nA, 0.5 second.

I now report physiologically significant graded control of transmission from impulse-producing neurons operating under conditions approaching their normal functional environment. These graded synaptic interactions can coordinate the proper phasing of neuronal activity within a functioning pattern generator.

Both impulse-mediated and graded transmission are found in the stomatogastric ganglion of the spiny lobster, *Panulirus interruptus* (*4*). It is an accessible, well-characterized visceral ganglion composed of approximately 30 individually identified motor neurons and interneurons. It contains two nearly independent subsystems (pyloric and gastric), each of which generates a different cyclic motor output to the stomach musculature. Discrete, impulse-mediated PSP's are a prominent feature of neuronal communication within the ganglion, and corresponding monosynaptic pathways have been mapped (Fig. 1A) (*5*). Recent studies of the pyloric subsystem have demonstrated the graded control of transmission between neurons that were already known to interact through conventional impulse-mediated synapses (*6*). During normal behavior each pyloric neuron fires a high-frequency burst of nerve impulses at a set phase in the cyclic program. These bursts of impulses are driven by 15- to 25-mV oscillations in membrane potential arising (i) in response to synaptic inputs from other neurons in the pyloric network and (ii) from intrinsic membrane properties that give rise to prolonged regenerative depolarizations or "plateau potentials" (*7*). My primary goal was to determine whether the oscillations that drive impulses could by themselves, in the absence of impulses, cause a significant release of transmitter and whether this graded release would be sufficient to coordinate the proper phasing of neuronal activity within the cyclic program.

To assess the role graded release plays in the functioning pyloric pattern generator, a technique was devised that eliminates nerve impulses in otherwise normally active preparations. The technique relies on tetrodotoxin (TTX), which blocks voltage-sensitive sodium channels but does not interfere directly with transmitter release or the generation of synaptic potentials (*8*). Bath application of TTX rapidly blocks impulse conduction in all nerves, including the anterior (stomatogastric) nerve, which carries inputs from more central ganglia necessary for the natural activation of the pyloric pattern generator (*7*, *9*). However, if TTX is restricted to the

0036-8075/79/0720-0304$00.50/0

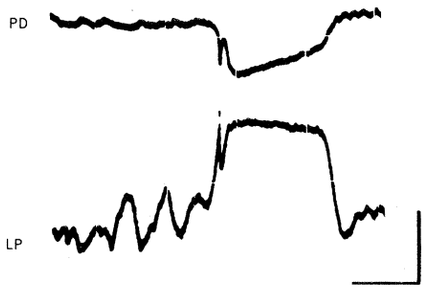

Fig. 2. Simultaneous intracellular recordings from the LP neuron and a PD neuron during the washing out of toxin 4 minutes after a localized application. Impulse generation failed during the course of a plateau potential in the LP. A single LP impulse caused a discrete PSP in the postsynaptic PD neuron and was followed by smooth inhibition of the PD mediated by the remainder of the LP plateau. Calibration: 10 mV, 1 second.

posterior margin of the stomatogastric ganglion, a region close to the presumed impulse-initiating zones of the pyloric motor neurons (*10*), pyloric impulse generation is blocked before descending activating units or cellular pacemaker potentials are grossly affected (Fig. 1B). Under these conditions, the membrane potential oscillates spontaneously without normal impulse production.

These experimental conditions allow graded synaptic transmission to be observed in naturally activated cycling preparations (*11*) (Fig. 1C). Three electrically coupled endogenous bursters, neurons with spontaneously oscillating membrane potentials, compose the AB/PD group of cells (*5*). This group acts as the pyloric pattern pacemaker, providing a source of strong cyclic inhibition to all other pyloric neurons. The LP cell is the only motor neuron in the ganglion that makes inhibitory synaptic connections onto the AB/PD group. Figure $1C_1$ shows normal spontaneous activity in these four neurons. Although impulse generation is vigorous, individual PSP's are difficult to distinguish in active preparations because the potentials overlap (*12*). Localized TTX perfusion blocked nerve impulse initiation in individual pyloric motor neurons one at a time, presumably as toxin diffused anteriorly and ventrally through the tissue. In Fig. $1C_2$ the LP neuron was no longer firing impulses, yet strong LP-mediated inhibition of the PD neurons is evident as a hyperpolarization of the PD's corresponding to depolarization of the LP. This inhibition was similar to that seen during LP impulse generation, but it lacked the slight ripple associated with impulse-mediated PSP's (Fig. $1C_1$). The magnitude of graded inhibition observed during localized TTX experiments was equal to or greater than impulse-mediated transmission seen in normal preparations. In Fig. $1C_3$, all spontaneous activity had ceased (*13*). Brief stimulation of the anterior nerve induced transient pyloric cycling resembling behavior observed in control conditions, except that impulse activity was suppressed. Even in the absence of impulses, pyloric neurons were depolarized in the same relative phases as during normal cyclic behavior (*14*). Continued localized toxin perfusion blocked any response to anterior nerve stimulation. At these times synaptic transmission could be evoked by depolarizing currents applied through an intracellular microelectrode.

Both impulse-mediated transmission and graded transmission observed when TTX is added to the bathing saline are chemically mediated in the pyloric subsystem (*15*). The chemical nature of graded release observed during localized TTX experiments is indicated by two observations: (i) postsynaptic waveforms are opposite in sign from the presynaptic potentials that drive them and (ii) postsynaptic waveforms can be reversed in sign with hyperpolarizing currents. Spontaneous plateau potentials in the LP neuron caused smooth potentials in a PD neuron, which were reversed in sign when the PD was tonically hyperpolarized with current (Fig. 1D).

During the normal operation of the pyloric pattern generator, does synaptic transmission mediated by nerve impulses or synaptic transmission mediated by slow membrane potential oscillations have a greater postsynaptic effect? The relative magnitudes of graded and impulse-mediated transmission can be compared during the early stages of TTX action, or as the toxin is washed out. The partial suppression of impulse generation at these times allowed the concurrent visualization of graded and impulse-mediated effects. In Fig. 2 the impulse generating mechanism of the LP neuron failed after a single impulse during a spontaneous plateau potential. The magnitude of the graded postsynaptic response evoked by the plateau potential alone was greater than the response evoked by the single impulse. Under control conditions, impulse amplitude of the LP neuron as measured in the soma of the same cell was the same as, and the PSP's evoked by LP impulses were smaller than, those in Fig. 2. In general, during the course of localized toxin experiments, graded synaptic effects and impulse-mediated effects appeared to be of comparable magnitude (*16*).

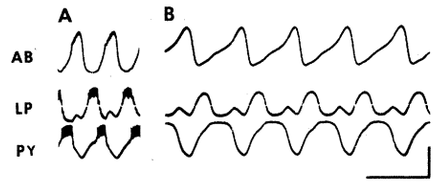

Fig. 3. (A) Simultaneous activity in three representative pyloric neurons in an isolated stomatogastric ganglion 0.5 second after a brief train of shocks to the anterior input nerve. (B) Simultaneous recordings from the same neurons after the ganglion was bathed in $5 \times 10^{-4}M$ dopamine and $2 \times 10^{-6}M$ TTX. Calibration: 25 mV, 1 second.

Even though pyloric neurons normally fire nerve impulses, graded release can, by itself, lead to the proper phasing of pyloric cell activity. This was most apparent when dopamine, a candidate substance for the natural induction of pyloric cycling (*17*), was used to induce rhythmic activity in preparations in which impulse generation was blocked by TTX. Bath application of TTX eliminates all cyclic activity. If both TTX and dopamine are present in the bathing solution, spontaneous pyloric cycling in the absence of impulse activity is observed (*18*). All of the major classes of neurons, AB/PD, LP, and PY, were depolarized in their appropriate phases within the pyloric cycle (Fig. 3). The oscillatory waveforms of any one of the important pyloric follower neurons (LP and PY's) could be reversed in sign for many cycles by the tonic injection of hyperpolarizing current. This result suggests that under these conditions their waveforms were primarily determined by extrinsic graded chemical transmission and not by intrinsic properties dependent on membrane potential. Thus, the correct phasing of pyloric neuron activity seen when the ganglion is bathed in dopamine and TTX can be attributed to strong graded synaptic interactions.

The presence of strong graded chemical transmission during normally activated or dopamine-induced pyloric pattern generation, and its ability to coordinate the proper phasing of neuronal activity, suggest that graded interactions normally play a role in the generation of the motor program. Pyloric neurons are part of a localized pattern-generating network confined to the ganglionic neuropil, and at the same time they act as motor neurons, driving muscles distant from the ganglion. Graded interactions can play an important role in intraganglionic function, whereas nerve impulses are necessary for the projection of information to the muscles. The mix-

ture of impulse-mediated and graded transmission observed within the ganglion may reflect these two functional features. Similar considerations suggest that mixtures of impulse-mediated and graded transmission might occur in other systems where projection neurons participate in local circuit interactions (*19*).

JONATHAN A. RAPER
Department of Biology, University of California at San Diego, La Jolla 92093

References and Notes

1. T. Bullock, *Science* **129**, 997 (1957).
2. For a review, see K. Pearson, in *Simpler Networks and Behavior*, J. C. Fentress, Ed. (Sinauer, Sunderland, Mass., 1976), p. 99.
3. For the purposes of this report, "graded release" is defined as the control of transmitter release by the continuously graded nonimpulse-mediated polarization of presynaptic sites.
4. D. Maynard and K. Walton, *J. Comp. Physiol.* **97**, 215 (1975); K. Graubard, *J. Neurophysiol.* **41**, 1014 (1978).
5. For a review of stomatogastric anatomy and physiology, see A. Selverston, D. Russell, J. Miller, D. King, *Prog. Neurobiol.* **7**, 215 (1976).
6. K. Graubard, J. A. Raper, D. K. Hartline, *Neurosci. Abstr.* **3**, 177 (1977). Nonimpulse-mediated transmission was found from the PD neurons to the LP and PY neurons, from the LP neuron to the PD and PY neurons, and from some PY neurons to the LP neuron. Nerve impulses were blocked by the bath application of TTX, and synaptic transmission was evoked by depolarizing presynaptic neurons with current.
7. D. F. Russell and D. K. Hartline, *Science* **200**, 453 (1978).
8. T. Narahashi, J. Moore, W. Scott, *J. Gen. Physiol.* **47**, 965 (1964); N. Ozeki, A. Freeman, H. Grundfest, *ibid.* **49**, 1319 (1966); B. Katz and R. Miledi, *Proc. R. Soc. London Ser. B* **167**, 8 (1967); J. Bloedel, P. Gage, R. Llinas, D. Quastel, *Nature (London)* **212**, 49 (1966).
9. D. Russell, thesis, University of California, San Diego (1977).
10. Stomatogastric neurons are monopolar; their major process dive into neuropil, branch, and then exit the ganglion. Synaptic sites are diffusely distributed on collateral branches in the neuropil [D. King, *J. Neurocytol.* **5**, 207 (1976); *ibid.*, p. 239]. Overshooting impulses are rarely observed with microelectrode penetrations of ganglionic neurites but can be observed where the pyloric motor axons exit the posterior of the ganglion (J. Miller, unpublished data).
11. The stomatogastric ganglion, the esophageal ganglion, and the commissural ganglia, their connectives, and some motor neurons were dissected free from the dorsal surface of the stomach and maintained in physiological saline. The stomatogastric ganglion and the base of the posterior output (dorsal ventricular) nerve were desheathed with fine forceps and conventional intra- and extracellular recording procedures were followed. Tetrodotoxin ($1.6 \times 10^{-5}M$) (Sankyo) was perfused across the base of the output nerve from one drawn pipette into another 100 to 400 μm in diameter. A stock toxin solution was diluted with saline, marked with fluorescein, and sometimes made isotonic with sucrose. Transverse lighting of the preparation facilitated visualization of the toxin solution as it traversed the path between pipettes.
12. One alternative explanation is that the graded control of transmitter release is the predominant mode of synaptic transmission in vigorously cycling preparations.
13. During the course of localized toxin experiments (5 to 15 minutes), toxin diffused deeper into the ganglionic tissue, first interfering with impulse initiation, then with the spontaneous generation of oscillating generator potentials, and later blocking the invasion of nerve impulses evoked by stimulation of the anterior input nerve.
14. D. Hartline and D. Maynard, *J. Exp. Biol.* **62**, 405 (1975). The pattern of activity is an alternation of impulse bursts between AB/PD neurons and the LP. Those PY neurons that most strongly inhibit the LP are active at the end of the LP burst and until they are inhibited in turn by the AB/PD group (D. Hartline and D. Gassie, in preparation).
15. K. Graubard, J. Raper, D. Hartline, in preparation. The criteria were (i) reversal of the postsynaptic waveform with the injection of hyperpolarizing current, (ii) abolition of the postsynaptic waveform in low-calcium–high-magnesium saline, and (iii) the selective blockade of some synaptic pathways with picrotoxin.
16. Plateau potentials could reach more depolarized levels in the absence of impulse generation than under normal conditions if the regions subserving impulse and plateau generation are electronically close to each other, allowing competition between their underlying ionic currents (*7*). Under these conditions, impulse generation could decrease net transmitter release by interfering with the full expression of the plateau characteristic.
17. P. Kushner and E. Maynard, *Brain Res.* **129**, 13 (1977); W. W. Anderson, *Neurosci. Abstr.* **3**, 171 (1977).
18. J. Raper, *Neurosci. Abstr.* **4**, 204 (1978); W. Anderson and D. Barker, in preparation.
19. Examples in vertebrates might include the mitral cells of the olfactory bulb or possibly the principal cells of the thalamus, since these projection neurons are thought to have output synapses on their dendritic arborizations [T. Reese and G. Shepard, in *Structure and Function of Synapses*, G. D. Pappas and D. P. Purpura, Eds. (Raven, New York, 1972), p. 121].
20. I thank D. K. Hartline for very helpful discussions and for criticizing the manuscript; C. Goodman, D. Berg, and R. Calabrese for criticizing the manuscript; and A. Selverston for his continuing encouragement. Supported by NIH grant NS13138 to D. K. Hartline, University of California grant BRSG, and predoctoral training grant PH 55-732-607-7153 to J.A.R.

6 October 1978; revised 23 February 1979

Transmitter-Specific Retrograde Labeling in the Striato-Nigral and Raphe-Nigral Pathways

Abstract. *Injecting radioactive transmitters into the rat substantia nigra led to retrograde neuronal labeling either in the dorsal raphe nucleus, after ^{3}H-labeled serotonin injection, or in the caudoputamen, after ^{3}H-labeled γ-aminobutyric acid injection. This differential labeling in projections whose transmitter has been established provides the basis for a histochemical tracing method indicating both connectivity and transmitter specificity of neural pathways.*

We have proposed that the connectivity and the chemospecificity of neural pathways could be established by transmitter-related retograde tracing (*1*). Such selective tracing could be based on specific terminal uptake after the administration of radioactively labeled transmitter, retrograde axonal migration, and retention of labeled material. The hypothesis of selective tracing originated from the observation of perikaryal labeling of neurons in the pigeon optic lobe after [^{3}H]glycine was applied to the terminal area (*1*). The release of exogenous and endogenous glycine when this pathway is electrically stimulated indicates its glycinergic nature (*2*). Furthermore, retrograde labeling has been found after administration of γ-[^{3}H]aminobutyric acid (GABA) in two neuronal systems, in which the transmitter has not been determined by well-established criteria (*1*, *3*). We have now tested our hypothesis in mammalian pathways characterized with respect to their transmitter.

The rat striato-nigral and raphe-nigral projections were chosen as models. Electrophysiological and biochemical investigations leave little doubt that GABA is a transmitter in the striato-nigral pathway (*4*, *5*). There is also good evidence for a serotoninergic projection from the raphe nucleus to the substantia nigra (*6*). According to the hypothesis tested, injection of [^{3}H]GABA into the substantia nigra should lead to perikaryal labeling in the caudoputamen, whereas application of [^{3}H]serotonin should label nerve cell bodies in the raphe nucleus.

We tested these predictions in female albino rats (160 to 190 g) anesthetized with Nembutal (40 mg per kilogram of body weight, injected intraperitoneally) for stereotaxic injections (coordinates were 2.6 mm anterior to the interaural line, 2.0 mm lateral to the midline, and 2.0 mm below the horizontal zero plane) (*7*). Two animals received [^{3}H]GABA (15 μCi in 0.05 μl, 5.6 mM, or 10 μCi in 0.1 μl, 1.85 mM, 4-amino-*n*-[2,3-^{3}H]butyric acid, 54 Ci/mmole; Radiochemical Centre, Amersham) and another two [^{3}H]serotonin (13 to 16 μCi in 0.05 μl, 24 to 30 mM, 5-hydroxy[G-^{3}H]tryptamine creatinine sulfate, 10.7 Ci/mmole; Radiochemical Centre). As a control, the unspecific retrograde marker substance horseradish peroxidase (HRP) [0.05 μl of 30 percent (weight to volume); Boehringer RZ3] was used to treat two rats. The survival time was 6 hours in the GABA and serotonin cases and 1 day in the HRP experiments. The animals were intracardially perfused, first with 5 percent Rheomacrodex solution (Pharmacia Uppsala) for 20 to 30 seconds and then with 400 ml of 5 percent phosphate-buffered (0.16M, pH 7.4) glutaraldehyde fixative. Transverse frozen sections (50 μm thick) were prepared for light microscopic autoradiography according to standard techniques and exposed in the dark for 6 and 12 weeks or stained for peroxidase activity (*8*).

The two injection sites after [^{3}H]-serotonin application differed in size from each other. Nevertheless, labeling

0036-8075/79/0720-0306$00.50/0