Neuron Function Inferred from Behavioral and Electrophysiological Estimates of Refractory Period

Abstract. *The refractory period of neurons mediating an electrically elicited behavior (self-stimulation) was estimated by varying the intrapair pulse separation in a stimulating train made up of pulse pairs and measuring the intensity of the elicited behavior. Two neuronal systems with different refractory periods were indicated. Single-unit recording in acute preparations stimulated through self-stimulation electrodes revealed primarily two classes of units. Each class gave refractory period estimates characteristic of one of the behaviorally indicated systems. The experiments illustrate a technique for establishing functional relations between single units in the brain and gross behavior.*

Before the responses of single neuronal units can be related to an animal's behavior, the behavioral perspective appropriate to a unit must be determined. We have developed a technique for ascertaining which behavioral perspective is most likely to be appropriate to a neuron excited by electrical stimulation of the central nervous system.

The neuronal refractory period is the period following a suprathreshold-stimulating pulse to a neuron during which a second pulse will not excite another action potential. When the interval within a pair of pulses is less than the refractory period of a neuron, the pair of pulses will excite one action potential instead of two. If 100 pairs of pulses are delivered each second to a neuron with a refractory period of 1.0 msec, the firing rate of the neuron should fall from 200 to 100 action potentials per second when the intrapair interval (IPI) is reduced from slightly more than 1.0 msec to slightly less. Reducing the firing frequency of neurons can decrease the intensity of the behavioral and neurophysiological effects mediated by the neurons. Thus, measurement of the behavioral and neurophysiological effectiveness of stimulation at different IPI's will characterize populations of neurons by their refractory periods (*1*). The neurons for which electrophysiologically estimated refractory periods agree with behaviorally estimated refractory periods can then be analyzed.

We used self-stimulation to evaluate this technique because self-stimulation behavior appears to have two components. For example, the running speed of a rat traversing a 1.8-m alley for brain stimulation reward (BSR) depends on (i) the amount of BSR it receives as a reward for running and (ii) the amount and recency of BSR given to the rat just before each run (called "priming" stimulation) (*2*). To measure the refractory period of neurons mediating the first effect, we held priming stimulation constant and varied the IPI of the reward stimulation. To measure the refractory period of neurons mediating the second effect, we held reward constant and varied the IPI of the priming stimulation. In both cases, we adjusted the duration of the train of stimulating pulses so as to maximize the decrease in running speed produced by halving the pulse frequency from 200 to 100 pulses per second. Then we delivered *pairs* of pulses at 100 pairs per second and varied the IPI (Fig. 1A).

Our results agree with those of Deutsch (*3*), who first applied this technique to self-stimulation. The refractory period was 0.53 to 0.64 msec for the reward effect and 0.9 to 1.1 msec for the priming effect. Deutsch used different electrode placements, different stimulating parameters (voltage and train duration), and a different behavior (bar pressing) to assess the reward and priming refractory periods. We conclude that these refractory-period values are unlikely to be artifacts of any special choice of parameters (*4*).

We looked for direct evidence that the stimulation was activating individual neurons with the indicated refractory periods. Albino male rats (475 to 560 g) were placed in a stereotaxic instrument, where one side of the skull top was removed. Monopolar electrodes were lowered into self-stimulation sites along the medial forebrain bundle on the exposed side of brain and were cemented to the skull on the unexposed side. The skin was sutured over the exposed dura. After a postoperative recovery period of 4 to 10 days, we determined the voltage and current that yielded self-stimulation (*5*). We then anesthetized the rat with urethane (1.2 g/kg), replaced it in the stereotaxic instrument, and exposed the brain again.

We recorded with a pair of tungsten microelectrodes placed 0.5 to 1 mm apart. They were connected to a differential amplifier to reduce the size of the stimulus artifact. The signal was displayed on the free-running upper beam of a double-beam oscilloscope. The action potentials triggered the lower beam. The trigger output from the oscilloscope drove a gated digital counter to record the number of action potentials over previously selected intervals.

We recorded the activity of single units while stimulating through the macroelectrode at the self-stimulation intensity. Many neurons showed no re-

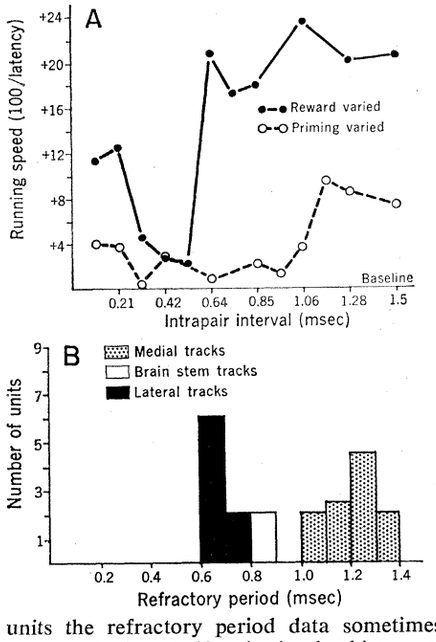

Fig. 1. (A) Total running speed of a male albino rat as a function of IPI in the reward stimulation (solid line) and as a function of IPI in the priming stimulation (dotted line). Each point is the mean over four blocks of ten trials each, minus the mean of blocks of ten "baseline" trials, in which the second stimulus pulse in each pair was omitted. The speed scores are 100/(total latency). The reward curve rises more than the priming effect curve because the total latency (start box plus running time) was more sensitive to a twofold variation in the reward stimulation than to a twofold variation in the priming stimulation. (When reward was low the rat hesitated for long periods before leaving the start box.) The curves based on running latency alone (not shown) have the same form as these curves, but show equal increments in running speed. The electrode tip was in the medial forebrain bundle above the mammillary bodies. (B) Frequency of occurrence (*9*). In refractory period determinations from transsynaptically driven units the refractory period data sometimes indicated a spread over 0.2 msec. These were plotted as half units in the histogram (columns 1.1 to 1.2 and 1.2 to 1.3). Of the 19 units, 4 were directly driven, and 15 were transsynaptically driven.

sponse to the stimulation. Those that responded formed two classes: (i) units assumed to be directly driven and (ii) transsynaptically driven units. Directly driven units showed a short-latency (0.2 to 0.5 msec) action potential after each stimulus pulse in a train of pulses. Transsynaptically driven units responded after a longer latency (1 to 20 msec) with either a temporary increase or decrease in their ongoing rate. These excitatory or inhibitory bursts outlasted a 0.1 to 0.9-second train of stimulus pulses.

We measured the refractory periods of the directly driven neurons by stimulating with a train of pulse pairs and reducing the IPI until the second stimulus pulse in each pair ceased to fire the neuron (Fig. 2A). Increasing the stimulating voltage did not reduce the refractory period, which thus was the absolute refractory period.

We studied the transsynaptically driven neurons with a method analogous to the behavioral technique for estimating refractory periods. The refractory period which this method yields need not be the refractory period of the target neuron under examination; rather, we assume that it is the refractory period of the directly stimulated neurons in a chain of cells leading to the target neuron (*6*). For this method, we first adjusted the frequency and duration of the stimulating train so as to maximize the change in burst size produced by cutting in half the frequency of the stimulus. We then set the gate of the counter to open at the end of stimulation and close after the estimated average duration of the burst. Settings ranged from 0.5 to 10 seconds. We next plotted the number of spikes in the count interval as a function of the IPI of the stimulation. In the example shown in Fig. 2B the average burst size increased when the IPI exceeded 1.0 msec.

We validated the burst-size method by applying it to a transsynaptically driven unit near the directly driven unit of Fig. 2A. The average burst size in the transsynaptically driven unit (Fig. 2C) increased when the IPI exceeded 0.6 msec (*7*). In other words, the refractory period inferred from the burst-size method agreed with that obtained from a directly driven unit nearby. Thus, the burst-size method provides refractory period labels for following a neural system beyond its directly stimulated parts.

In many transsynaptically driven units, variablility in spontaneous firing rate prevented refractory period determinations. Figure 1B summarizes the refractory periods obtained from the 19 units which were analyzed. Two directly driven units in the reticular formation of the mid-brain had refractory periods of 0.8 to 0.9 msec. This value does not correlate with the refractory periods of the two behavioral components of self-stimulation. Thus, these units are apparently not involved in the priming and reward effects in self-stimulation. All neurons yielding refractory period estimates corresponding to the behavioral reward effect ($\sim$ 0.6 msec) were found in penetrations around the lateral thalamic nucleus (3.2 to 3.7 mm lateral to the sinus). The priming effect neurons were found in more medial penetrations into the dorsal thalamus around the nucleus parafascicularis (*8*).

The 0.6-msec units were found laterally in three out of seven rats, and 0.9- to 1.1-msec units were found medially in five of these rats. The stimulating electrodes in these preparations were 3.0 mm to 6.0 mm behind bregma. The A-P coordinates of the recording electrodes ranged from 0.5 to 4.1 mm behind bregma. Thus, the results probably are not an artifact of the macroelectrode-microelectrode geometry.

These experiments demonstrate a new approach to the problem of establishing the functional identity of single units mediating behavior elicited by electrical stimulation of the brain. An appropriate refractory period label does not guarantee that a unit is functionally related to the behavior, but it increases the likelihood. The functional distinctiveness of units in different refractory period classes is suggested by their anatomical segregation and by preliminary data indicating that transsynaptically driven neurons in different refractory period

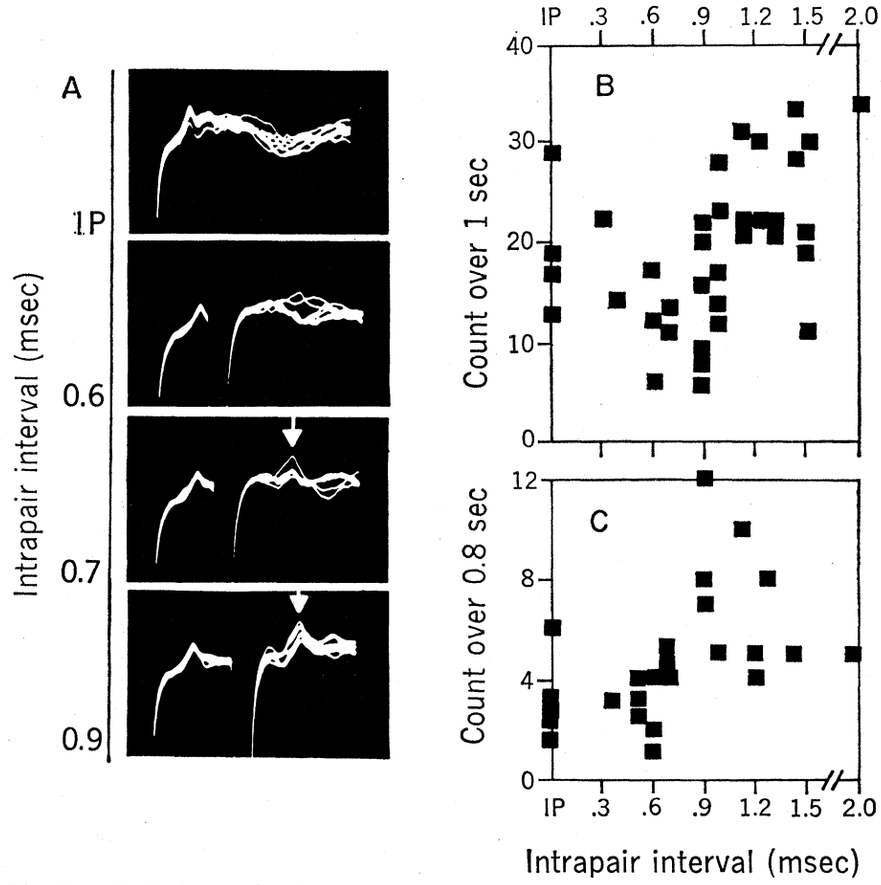

Fig. 2. (A) Refractory period determination from a directly driven unit. Arrows indicate action potential following second-stimulus pulse. At an IPI of 0.6 msec, an action potential does not follow the second stimulus pulse in four of the five sweeps. At 0.7 msec, the second action potential has a greater latency due to relative refractoriness. At 0.9 second, the second pulse has a heightened amplitude, perhaps attributable to a hyperpolarization following the relative refractory period. Sweep time, 2 msec. (B) Burst size in a transsynaptically driven neuron (medial track) as a function of IPI. The *IP* at the origin of the abscissa indicates the condition in which the second stimulus pulse of each pair was omitted. (C) Burst size in a transsynaptically driven neuron (lateral track) as a function of IPI. This unit was within 0.2 mm of the unit in (A).

classes respond differently to tests involving repeated stimulation and the manipulation of the rat's level of arousal.

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References and Notes

1. This technique was used extensively in neurophysiological studies from Helmholz to the early 1950's. K. Lucas [*J. Physiol.* **51**, 1 (1917)] used it to show that there were two populations of neurons innervating the adductor muscle of the claw of *Astacus*. For references verifying Lucas' conclusions, by single unit recording, see E. J. Furshpan, in *Handbook of Physiology: Neurophysiology*, J. Field and H. W. Magoun, Eds. (American Physiological Society, Washington, D.C., 1959), vol. 1, pp. 240–242.
2. C. R. Gallistel, *J. Comp. Physiol. Psychol.*, in press.
3. J. A. Deutsch, *ibid.* **58**, 1 (1964).
4. There is both internal and external evidence consistent with our interpretation of these data. The refractory period value for the reward effect is characteristic of mammalian A fibers with diameters of 2 to 4 μm [H. Grundfest, *Annu. Rev. Physiol.* **2**, 213 (1940)]. The period of latent addition in such fibers is 0.2 msec. The hump near the abscissa, which we ascribe to latent addition in the fibers receiving subthreshold excitation from the first pulse, has a width of 0.2 msec. The refractory period value for the priming effect is characteristic of mammalian A fibers with diameters of 1 to 2 μm. The period of latent addition in these fibers is also 0.2 msec—again consistent with the width of the hump near the abscissa. Finally, the refractory period estimates are consistent with the following histological and pharmacological data. The self-stimulation system probably utilizes norepinephrine as a transmitter substance [L. Stein and C. D. Wise, *J. Comp. Physiol. Psychol.* **67**, 189 (1969); C. D. Wise and L. Stein, *Science* **163**, 299 (1969)]. The noradrenergic fibers in the medial forebrain bundle area have diameters ranging from 1 to 4 μm [K. Fuxe, *Acta Physiol. Scand.* **64**, suppl. No. 247, 47 (1965).
5. The fixed parameters of stimulation during the self-stimulation test were: train duration, 0.3 second; pulse width, 0.1 msec; 100 pulse/sec. The pulses throughout this study were negative-going, but fed through a large capacitance, to prevent polarization of the electrode.
6. Since the first synapse probably acts as a nearly perfect temporal integrator over the relevant range (0.1 to 0.2 msec), refractory period effects probably arise before the first synapse and remain independent of the characteristics of subsequent units in the chain. The Helmholz nerve-muscle preparation yields the neural refractory period, even when the muscle refractory period is twice as long [H. C. Bazett, *J. Physiol.* **36**, 414 (1908)].
7. The refractory period was estimated by eye from the plotted data on burst size. In doubtful cases (including Fig. 2C) we applied a statistical decision rule based on the χ^2 test.
8. The neurons shown in Fig. 1B with refractory period values of 0.7 msec and 1.2 to 1.4 msec do not agree with the behaviorally determined refractory period values of the neurons mediating the rewarding and priming effects, respectively. But both sets of neurons were in the same brain sites and responded to further tests in the same manner as the neurons with refractory periods agreeing with the behavioral determinations. Since urethane prolongs the refractory period of nerve bundles [I. Tasaki, *Nervous Transmission* (Thomas, Springfield, Ill., 1953), p. 104], one might include these units in the appropriate populations. However, their inclusion must remain tentative.
9. This histogram was compiled from units recorded in rats tested for self-stimulation and in rats with stimulating electrodes lowered into highly reliable self-stimulation sites at the start of the acute experiments. Results from tested and untested preparations were alike.
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Laughing Gull Chicks: Recognition of Their Parents' Voices

Abstract. *Laughing gull chicks between 6 and 13 days old responded to the calls of their own parents with orientation toward the sound, approach, increased locomotion, and vocalization. In response to the same kinds of calls from other adults they tended to orient away from the sound, withdraw, and sit or crouch. Chicks as young as 6 days can identify their parents from individual characteristics in the calls of adult gulls.*

Laughing gulls *Larus atricilla* breed colonially (*1*). Consequently, when the young become mobile, they frequently encounter adults other than their parents. Because the young are usually cared for only by their parents and are treated with hostility by other adults, it must be assumed that there is some means by which parents and young are directed to one another in a gullery. Laughing gull chicks behave differently toward their parents than the way they do toward other adults, even at the same place of encounter (*2*), which implies that the chick can recognize its parents. I now report an experiment to test the possibility that laughing gull chicks can distinguish their parents on the basis of individual characteristics of voice. This possibility was suggested by the fact that individual characteristics are discernible in some of the types of calls given by adults (*2*), and by the fact that the vegetation height and density in the gullery after hatching are such that parents and young are often cut off visually from one another. Recognition by chicks of the voices of their parents has been conclusively demonstrated by experiment with the guillemot *Uria aalge aalge*, a colonially breeding bird in the same order (*3*). Less-direct evidence has been obtained for it in black-billed gulls *Larus bulleri* (*4*), sandwich terns *Sterna sandwicensis* (*5*), and king penguins *Aptenodytes patagonica* (*6*).

Laughing gull chicks were taken from their nest areas (*7*) and tested indoors, one at a time, with recordings of calls of their parents and other adults. The testing situation was a wooden box (120 by 30 by 30 cm) open at the top and with the two end walls consisting of cheesecloth screens. The interior of the box was flat gray. The floor was marked off transversely into 24 strips (5 cm wide) and longitudinally into 2 strips (15 cm wide) to give a reference grid for noting the position of a chick in the box. A portable speaker-amplifier (Nagra DH) connected to a portable tape-recorder (Nagra III) was used to broadcast calls through the cheesecloth screen at one end or the other of the box during a test. The volume of the broadcast sound approximated that of natural calls and was the same in each test, but no measurements of the sound intensity in the box were made. An overhead light source gave even illumination over the floor of the box. The observer sat behind a screen and was not directly visible to a chick in the box. A mirror enabled him to observe the position and behavior of the chick being tested.

The chicks tested came from nests at which recordings of the calls of the parents had been obtained (on the Nagra, with a Sennheiser MKH404 microphone). These chicks were captured in the field and tested at ages which ranged from 6 to 13 days. This range was selected because within it chicks show the first clear signs of being able to recognize their parents. Twelve chicks were tested. For each test session two chicks were selected which were from parts of the gullery remote from one another, and whose ages were as close as possible. Each chick was tested with two tapes—one was a recording of calls of its parents (its "parental" tape); the other was a recording of calls of the other chick's parents (the "foreign" tape). Thus each test tape was used both as a "parental" tape and as a "foreign" tape. This strategy was used to balance out the possible effects of differences between tapes incidental to the identities of the adults recorded.

The tapes were played for 5 minutes, and each contained instances of all the types of call that, as field observation had suggested, might influence filial responses of a chick. Before each test with a tape (sound test) a chick was