Bioelectric Activity in Long-Term Cultures of Spinal Cord Tissues

Abstract. *Fragments of embryonic spinal cord (human, rat, and chick) can regenerate and differentiate in tissue culture. Complex bioelectric activity evoked by electric stimuli indicates that nerve cells in cultures may maintain, for months in vitro, not only the capacity to propagate impulses along their neurites but also a remarkable degree of functional organization resembling the activity of synaptic networks of the central nervous system.*

Electrophysiologic studies of cultured chick spinal ganglia demonstrated that neurons can retain their characteristic property of conducting nerve impulses even after months of isolation from the living organism (*1*). Similar results have been recently obtained in tissue cultures of mammalian cerebellum (*2*). Although the cultured cerebellar neurons could propagate impulses (some showing spontaneous, repetitive spike discharges), no sign of neuronal interactions characteristic of the central nervous system (CNS) *in situ* were detected. Hild and Tasaki concluded, "A neuron in vivo is always part of a neural network, whereas a neuron in tissue culture no longer has synaptic connections with other neurons" (*2*). This statement, however, appears to be based on studies of tissues which have spread, in culture, into an extremely thin layer. Our experiments with thicker explants of spinal cord demonstrate that neurons in at least some types of CNS cultures can maintain, during months of isolation in vitro, a high degree of functional organization resembling complex synaptic activity *in situ* (*3*). Spontaneous, rhythmic bioelectric activity has been recorded from fragments of embryonic and adult brain maintained in vitro up to 2 weeks (*4*). Although strychnine enhanced and anesthetics blocked these spontaneous waves, the data are difficult to interpret since no attempt was made to utilize microelectrode techniques and electric stimulation to demonstrate characteristic neuronal responses.

Our cultures consist of cross sections (0.5 to 1 mm) of spinal cord from human (6th week) and chick (9th day) embryos and rat (17th to 19th day) fetuses. The fragments were explanted onto cover glasses coated with collagen gel (*5*) and maintained in Maximow slide chambers. The culture medium, containing human placental serum and chick embryo extract (*6*), was changed twice weekly. The cord segment was frequently explanted with its attached dorsal-root ganglia (Fig. 1*A*). A large number of cord neurons survive and mature in vitro (*7*). Many of the axons become myelinated in both the explant and the outgrowth zones. Although the neuronal somas generally remain thickly invested in a framework of glia, the explants become thin enough to permit at least some of the perikarya to be visible in the living state, at high magnification (Fig. 1*B*). Well-organized ultrastructure has been seen in preliminary electron microscopy of neurons in these long-term cord cultures, including evidence of synaptic junctions between neurites and somas, as well as between one neurite and another (*8*). The bioelectric experiments were carried out (at 36°C) in a moist chamber attached to the mechanical stage of a compound microscope. The culture cover glass was mounted as a "hanging-drop" preparation. Microelectrodes were inserted through holes in the side walls of the chamber and brought up to the tissue by micromanipulators (*1*). Platinum or chloridized silver electrodes with 10- to 25-μ tips were generally used but many of the phenomena have also been recorded with 1- to 3-μ pipettes filled with electrolyte. One electrode was positioned (under direct visual control) against (or inside) the tissue, while an indifferent electrode was placed in the fluid nearby (a chloridized silver wire in the periphery served as a ground electrode). Electric stimuli (0.1 to 0.3 msec) were applied locally through 10- to 15-μ pipettes filled with saline, and bioelectric signals were recorded with differential-input preamplifiers and an oscilloscope.

Figure 2 illustrates some of the response patterns evoked in our cultures by various modes of stimulation. The action potential (100 to 200 μv in amplitude) which occurs within 5 msec after the stimulus at *a* in Fig. 2*A* is typical of the simple spike responses previously recorded in cultures of spinal ganglia (*1*) and cerebellum (*2*). The response evoked in this cord explant, however, is far more complex and lasts much longer (*b* and *c* in Fig. 2*A*). The spikes in *a* are actually superimposed on a long-duration (about 40 msec) negativity (*b* in Fig. 2*A*), and the latter is followed by a second "slow wave," more than 100 msec in duration and 200 μv in amplitude (*c* in Fig. 2*A*). The response patterns vary throughout

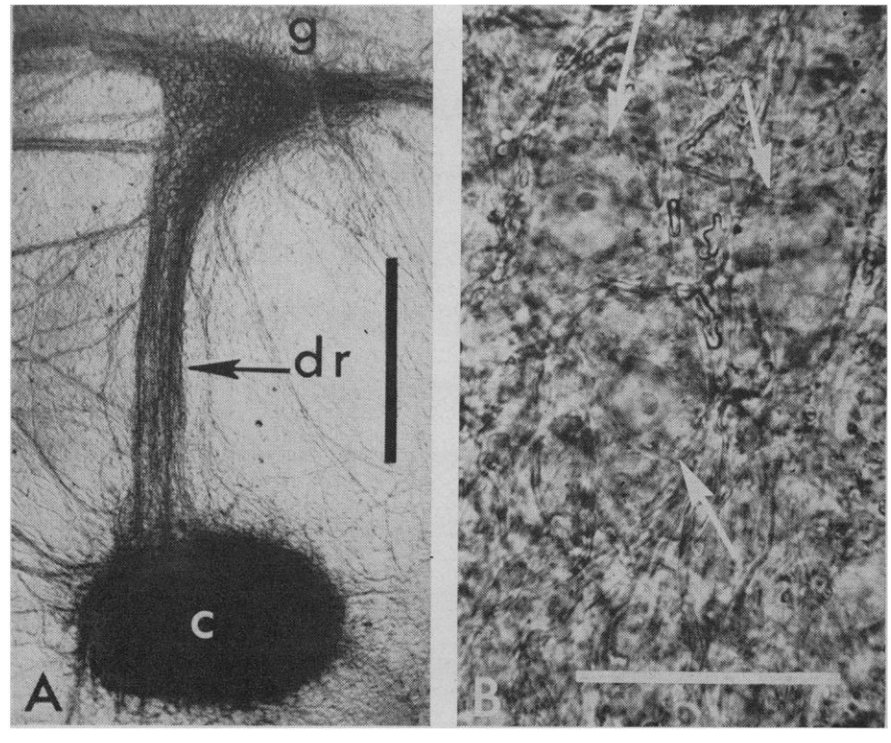

Fig. 1. Photomicrographs of living, unstained cultures of rat spinal cord, 3 months in vitro. *A*, Low-power view of semi-cross-section of cord (*c*) plus attached dorsal-root ganglion (*g*). Note long dorsal root (*dr*) and thinner bundles of neurites emerging from explant. Scale, 500 μ. *B*, High-power view of typical region within cord explant. Note group of neuron somas (at arrows) with prominent nuclei and nucleoli; also, myelinated axons nearby. Scale, 50 μ.

each cord explant and from one explant to another, but discharges continuing for 100 to 500 msec after termination of the stimulus have been seen in over 20 long-term cultures. Whereas the simple spike potentials could be regularly evoked at stimulus rates of well over 10 per second, the complex barrage responses were quite labile, often requiring rest periods of 1 to 10 seconds between stimuli. In some cases, the barrages were triggered at a critical threshold, with little increase in discharge amplitude or duration at higher

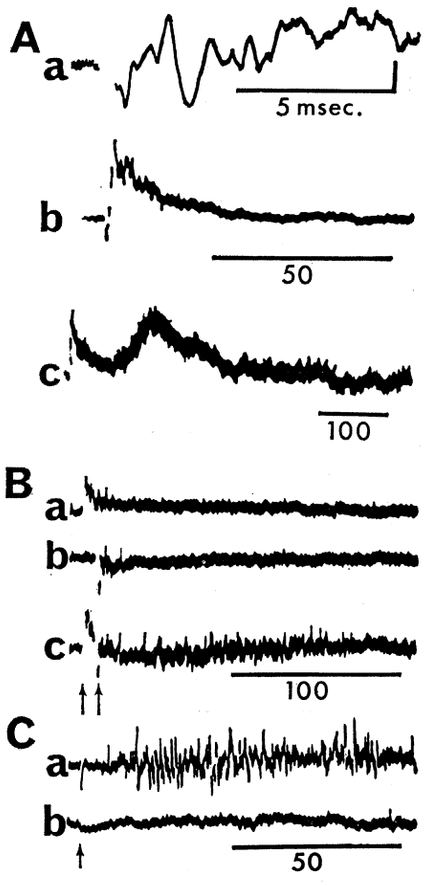

Fig. 2. Complex, bioelectric responses to electric stimuli recorded with extracellular microelectrodes in cultured rat (*A,C*) and chick (*B*) spinal cord tissue (1 to 3 months in vitro). *A*, Barrage of spikes following single stimulus (0.1 msec duration) applied to nearby region of explant (about 150 μ from recording site) at fast (*a*), medium (*b*), and slow (*c*) sweeps. Shock artifact appears as discontinuity in base line. *B*, Long barrage response (*c*) to dual stimuli (at arrows) compared with brief responses to each stimulus alone (*a* and *b*). *C*, Barrage response (*a*) evoked by brief stimulus applied (at arrow) in dorsal-root ganglion, 2 mm away from cord explant. Note 7 msec latency. Reversal of stimulus polarity eliminates response (*b*). Time base, in milliseconds; amplitude calibration (*A,a*), 100 μv. (Upward deflection indicates negativity at active recording electrode.)

stimulus intensity. These in vitro response patterns are similar in many respects to spinal cord after-discharges recorded *in situ* after decerebration or spinal transection (*9, 10*).

Figure 2*B* illustrates another prominent CNS property which is observed in these isolated cord cultures. A barrage of spikes lasting about 150 msec is evoked by two brief shocks applied, through the same stimulating electrodes, 9 msec apart (*c*). Application of either stimulus alone, on the other hand, elicits only a brief response terminating in less than 20 msec (*a, b*). Facilitation could be demonstrated, at times, even when the second stimulus was delayed for more than 100 msec. Addition of strychnine to the culture medium (1 to 10 μg/ml)resulted in increased excitability within a few minutes. The threshold for evoking barrage responses was generally decreased, the duration of the discharge was greatly prolonged, and facilitation could be produced with the stimuli at longer intervals. In some cases, strychnine resulted in the sporadic appearance of repetitive discharges. Moreover, in cultures where only brief spike responses could be detected after electric stimulation, long barrages were readily evoked after addition of strychnine.

When dorsal-root ganglia were explanted together with spinal cord (Fig. 1*A*), it was possible to stimulate locally in the ganglion and to record characteristic barrage responses in the cord explant (Fig. 2*C,a*). The latency of 7 msec between the end of the stimulus and the beginning of the response is only partly attributable to conduction time along the 2 mm of dorsal root connecting the ganglion and cord. Measurements of the conduction velocity along dorsal-root fibers in these cultures give values greater than 1 m/sec. Thus, the major delay probably occurs as the nerve impulses invade the cord and activate the neurons in the vicinity of the recording electrode.

Although only simple, short-latency spike potentials could be evoked in a human spinal cord culture tested 1 month after explantation, cultures maintained for 3 to 4 months in vitro showed activity comparable to that illustrated in Fig. 2. In some cases, the response to a single, brief stimulus was even longer-lasting with a 100-msec triphasic potential (Fig. 3*A,a*) followed by a series of repetitive waves, at 5 per second, with gradually increasing amplitude (Fig. 3*A,b*). The latter sequence shows interesting similarities to "hyperexcitable" responses recorded, *in situ,* in neuronally isolated slabs of cerebral neocortex in immature animals (*11*). In one culture (4 months in vitro) these rhythmical groups of discharges continued for more than 1 minute and occurred spontaneously as well as in response to an electric stimulus (Fig. 3*B*). Similar sporadic bursts have been seen in some of the long-term cultures of rat spinal cord. Facilitation was effective in human cord cultures over intervals as long as 400 msec, as in Fig. 3*C,b* where a barrage begins several hundred milliseconds after the second stimulus. When the test interval was increased to 600 msec, no response occurred (Fig. 3*C,a*). A still longer response latency is shown in Fig. 3*D*, which was obtained under conditions similar to those of Fig. 2*C*. Although the distance between the stimulating leads in the dorsal-root ganglion and the recording electrodes in the cord explant was only about 2 mm, the response at two different recording sites, 1 and 2 (about 200 μ apart), did not begin to develop un-

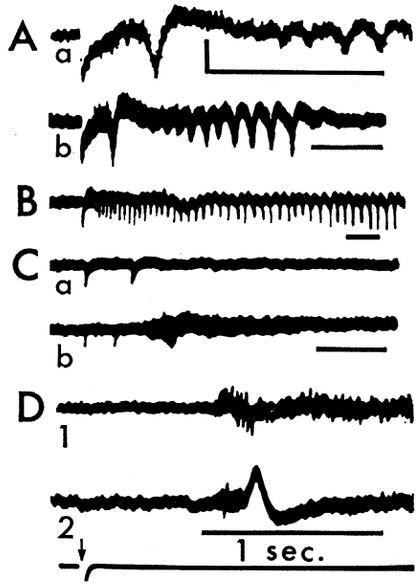

Fig. 3. Long-duration responses to electric stimuli in human spinal cord cultures, 3 to 4 months in vitro. *A*, Large triphasic potential (*a*) followed by series of repetitive waves of increasing amplitude (*b*) elicited by single 0.1-msec shock. *B*, Part of a series of repetitive potentials lasting nearly 1 minute. *C*, Barrage response evoked by a pair of brief stimuli 400 msec apart (*b*). Note absence of response when test interval is increased to 600 msec (*a*). *D*, Simultaneous records from two sites in cord explant (about 200 μ apart) in response to electric stimulus applied to dorsal-root ganglion (2 mm away). Note extremely long latency of both responses after shock (at arrow). Time bases, 1 second; amplitude calibration (*A,a*), 50 μv.

til well over 500 msec after the shock (at arrow). Since higher stimulus intensities (as well as multiple stimuli at low intensity) decreased the response latency to less than 50 msec, most of the delay is probably due to slow spread of the activity within the cord explant.

These experiments demonstrate the remarkable degree to which the bioelectric activities of cultured human, rat, and chick spinal cord tissues resemble complex CNS patterns *in situ*, even after months of isolation in vitro. A powerful new experimental approach is therefore available to supplement *in situ* studies of CNS function, especially those aspects concerned with deafferented or other types of neuronally isolated CNS regions (*9–11*). The complexity of the long-lasting excitatory phenomena evoked in cultured cord tissue suggests sequential activation through multiple chains of synaptically linked neurons (*12*). Other factors which may be responsible for repetitive neural activity *in situ* must also be considered here, such as sustained oscillation of local membrane potential, differential repolarization, and persistence of humoral transmitters (*9*). Further analysis of the bioelectric activities of cultured CNS tissue will be of great interest since a relatively small number of neural elements can be studied as an isolated, model nervous system, in a controlled chemical environment, during direct observation of cytologic details of the constituent neurons and glia. Furthermore, since the tissues can be explanted at early embryonic stages, correlative studies of neural structure and function can be made during critical stages of differentiation and maturation in vitro. This approach is being extended in current electrophysiologic experiments with long-term cultures of neonatal mouse cerebral neocortex where a high degree of structural and functional organization develops after explantation in vitro (*13*). It should be emphasized that the techniques used in the preparation and long-term maintenance of healthy CNS cultures require meticulous control over many laboratory procedures, but we believe that the research potentialities of this method warrant the required effort and expense (*14*).

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14. Aided by grants NB-03814 and NB-00858 and research career program award (NB-K3-2904) from the National Institute of Neurological Diseases and Blindness.

6 May 1963

Signal Analysis of Evoked Potentials Recorded from Cats during Conditioning

Abstract. *Correlation coefficients were computed between average response waveforms recorded from different brain regions of trained cats, before and after a specific stimulus acquired cue value. Application of signal-analysis techniques to the correlation matrix shows marked increase in the similarity between waveshapes evoked by that stimulus in sensory-specific and nonsensory-specific regions.*

Numerous reports (*1*) have described extensive changes in electrophysiological responses to an intermittent stimulus as it acquires informational significance by being used as the conditioned stimulus during behavioral training. Electrical activity in sensory-specific structures appears to reflect closely certain attributes of the actual physical stimulus, such as the frequency of a flickering light. During conditioning, the electrical activity in nonsensory-specific structures increases markedly in response to the conditioned stimulus. In cats trained to differentiate between two frequencies, the response of nonsensory-specific regions may or may not correspond to the actual stimulus. When the frequency correspondence is good, behavioral response tends to be appropriate to the intermittent conditioned stimulus actually present. When the correspondence is poor, behavioral response tends to be inappropriate. On such occasions, activity in nonspecific regions sometimes displays the frequency of the stimulus appropriate to the behavior displayed by the cat. On the basis of evidence from such studies, John and Killam (*2*) proposed that such discrimination behavior involved a comparator system, possibly localized in the cortex, which estimated the degree of congruence between the temporal patterns of electrical activity in sensory-specific and in nonsensory-specific structures of the brain.

During further work, the impression was gained that a number of diverse anatomical regions, which initially displayed appreciable differences in evoked responses to a particular stimulus, came to display markedly similar electrical responses as meaning was attached to that stimulus during conditioning. Similar observations have been described recently by Galambos and Sheatz (see *3*).

We devised a technique to obtain a quantitative estimate of similarity between electrical waveforms recorded from different brain areas and to describe the set of organized relationships inherent in the configuration of similarities.

A Mnemotron four-channel average response computer was used to obtain average response waveforms from a large number of electrodes ($N = 14$ to 34) chronically implanted into cats. Each average response computation was based on 200 iterations of the stimulus. In four cats, averages were obtained at various stages during the elaboration of differential avoidance response. At each behaviorial stage, N average response waveforms were obtained from each animal, one average from each electrode derivation. The waveforms were then read out, in digital form, from the average response computer.

The data processing was carried out on the I.B.M. 7070 digital computer at the University of Rochester Computing Center. The waveforms were normalized to equate the differences in signal strength. Cross-correlation coefficients (the Pearson product-moment correlation coefficient) were computed for all pairs of waveforms within a given stage of behavioral response. Correction for differences in latency of the evoked potentials had a negligible effect, because of the relatively low frequency of the waveforms. Distributions of these correlation coefficients, before