NEURONAL TUMOR CELLS WITH EXCITABLE MEMBRANES GROWN IN VITRO

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Abstract.—Mouse neuroblastoma cells grown *in vitro* are capable of generating action potentials in response to electrical stimulation. A wide spectrum of responses was observed with different cells ranging from passive responses to action potentials. A hypothesis is proposed concerning the acquisition of electrical excitability as neuroblasts mature into neurons.

Tissue culture methodology affords many opportunities to study molecular aspects of information processing by cells from the nervous system.[1, 2] During the last few years we have investigated the properties of tumor cells and normal cells derived from the nervous system and grown *in vitro*. During the course of these studies, Augusti-Tocco and Sato told us that they had established clonal lines of cells from a transplantable mouse neuroblastoma[3] that contain choline acetylase, acetyl cholinesterase, and tyrosine hydroxylase.[4] Human neuroblastomas also have been cultured *in vitro*[5] and have been shown to contain enzymes related to norepinephrine synthesis and catabolism.[6–8]

We have investigated electrophysiologic properties of the mouse neuroblastoma of Augusti-Tocco and Sato and wish to report that neuroblastoma cells, grown *in vitro*, are capable of generating action potentials that are characteristic of neurons.

Materials and Methods.—Neuroblastoma C-1300 is a spontaneous tumor maintained since 1940 by serial transplantation in strain A/J mice. Mice bearing this tumor were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine.

Neuroblastoma cells were dissociated and grown *in vitro* as described by Augusti-Tocco and Sato[4] except that Dulbecco's modification of Eagle's medium[9] was used in place of F-10, and cells were grown in an atmosphere of 10% CO_2 and 90% air. Cells have not been cloned. The average doubling time of cells is 18 hr; hence, cells have been repeatedly trypsinized and subcultured.

The medium contains inorganic ions in the following concentrations (mM): Na^+, 154; K^+, 5.4; Ca^{++}, 1.8; Mg^{++}, 0.8; Cl^-, 118; and HCO_3^-, 44.

Plastic petri dishes, 35 or 60 mm in diameter and containing neuroblastoma cells in the medium described above, were placed in a plastic chamber on the stage of a Zeiss inverted microscope. The temperature of the medium, measured with a thermistor probe, was maintained at 35°C with a heating element below the microscope stage. A humidified mixture of 10% CO_2 and 90% air flowed over the surface of the culture.

Glass micropipette electrodes with tip diameter $<0.5\ \mu$ and filled with 3 M KCl were used to record transmembrane potentials from the cultured cells. The resistance of the electrodes ranged from 10 to 200 megohms; most were from 20 to 80 megohms when measured in the culture medium. The microelectrode was arranged in a bridge circuit and connected to a Bak unity gain amplifier. This circuit allows transmembrane potentials to be recorded while currents are passed across the cell membranes through the impaling microelectrode.[10]

Results.—The morphologic characteristics of mouse neuroblastoma cells cultured *in vitro* resemble those described by Augusti-Tocco and Sato.[4] How-

ever, the cells used in the present study are not clonal cell lines, so a heterogeneous cell population is expected.

The following procedure was used to study the electrophysiologic properties of neuroblastoma cells *in vitro*. The cell was photographed and the tip of a microelectrode was inserted in the interior of the cell; another electrode was immersed in the extracellular medium; within several minutes, when stable records could be obtained, the difference in voltage across the cell membrane recorded by the intracellular electrode was determined. Pulses of current 50–100 msec in duration were then passed through the electrodes and the changes in voltage across the cell membrane were determined as a function of time. Steady current was then passed through the electrodes to adjust the voltage across the cell membrane to about −60 mv. Pulses of current again were passed through the electrodes and perturbations in voltage across the cell membrane were determined. This procedure allowed us to test the excitable properties of the cell membrane at the voltage level obtained after penetration of the cell by the electrode and at a standard level of −60 mv.

Our objective was to determine whether neuroblastoma cells are electrically excitable *in vitro* and whether they generate neuron-like action potentials in response to stimuli. Of 259 cells that were examined, 149 were studied in detail. Most of the cells were found to have "active" membranes; however, the degree of activity varied widely. Excitable cells were found that were capable of generating action potentials, as well as cells with less active membranes. An example of an excitable cell generating action potential is shown in Figure 1*A* and *B*. A resting potential of −40 mv was obtained upon penetration of the cell by the electrode. A steady current of about 2 namp was passed through the electrode to adjust the voltage across the cells membrane to −65 mv. Then a series of five pulses of stimulating current approximately 70 msec in duration, but differing in intensity, were passed across the membrane (the upper traces of Fig. 1*B*), evoking the changes in membrane voltage corresponding to the lower traces of Figure 1*B*. The weakest stimulus, labeled *a* in the Figure, evoked a small, smoothly increasing perturbation in voltage. The most intense stimulating pulses elicited action potentials. Partial responses were elicited by stimuli of intermediate intensity.

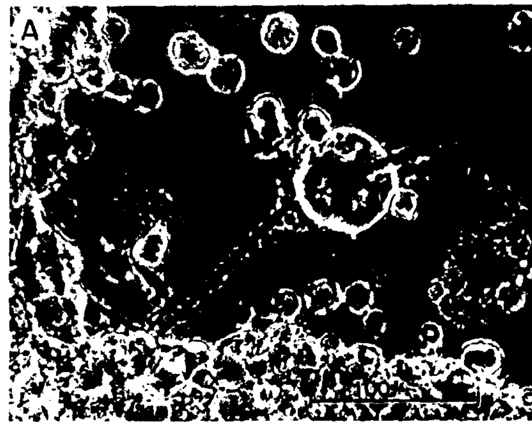
Nine per cent of the cells examined were capable of generating action potentials. Stimulation of such cells for five msec, a pulse of current briefer than the duration of the action potential, elicited an action potential. Action potentials were elicited two days after cells were dissociated with trypsin and then subcultured.

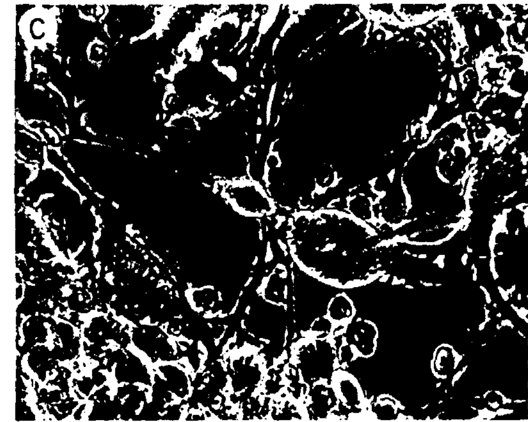
A cell exhibiting only a partial response is shown in Figure 1*C* and *D*. A resting potential of −20 mv was found on penetration of this cell, but even when the voltage across the membrane was adjusted to −60 mv with steady current, a pulse of stimulating current elicited only a partial response. A fully developed action potential could not be evoked in this cell.

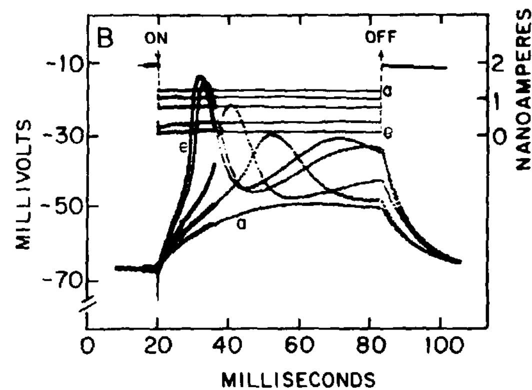
The observed response to a pulse of current is dependent upon the level of transmembrane voltage at the time of stimulation (Fig. 2). When the voltage across the membrane of the cell shown in Figure 2*A* was adjusted to −60 mv, a current pulse of one namp evoked a partial response (Fig. 2*B*). A current pulse

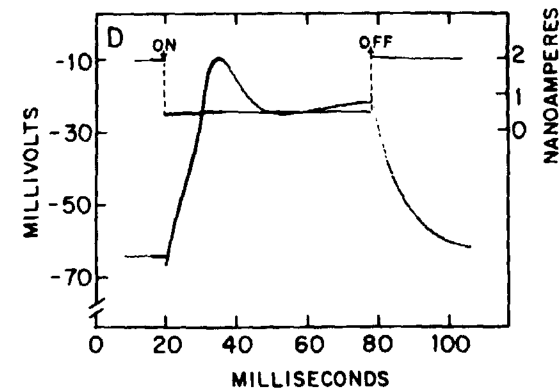
FIG. 1.—(A, B). The large cell near the center of the photograph was studied electrophysiologically. Cells had been subcultured 7 days earlier and were past the logarithmic phase of growth. Total time *in vitro* was 103 days. The 100 μ bar applies to both (A) and (C). Intracellular recordings from the cell are shown in (B). Five oscilloscope traces are superimposed. The lines shown in the upper part of this and subsequent figures correspond to the currents that were passed through the electrodes across the cell membrane (*right ordinate*); the lower curves indicate the changes in voltage across the cell membrane (*left ordinate*) evoked by these currents. After the resting potential was determined, 2 namp of steady current were passed through the electrodes to adjust the voltage across the cell membrane to −65 mv. Then the cell membrane was stimulated with pulses of current as indicated by ON and OFF. The weakest stimulating pulse, labeled *a*, elicited a small passive voltage response, also labeled *a*. The most intense stimulating pulses (*e*) elicited action potentials.

(C, D). The large cell near the center was examined electrophysiologically. The cell is from the same culture described in (A). The pulse of stimulating current is indicated by the upper lines, the resultant change in membrane voltage by the lower curve. The inflection on the rising phase of the voltage curve corresponds to a partial response of the cell membrane.









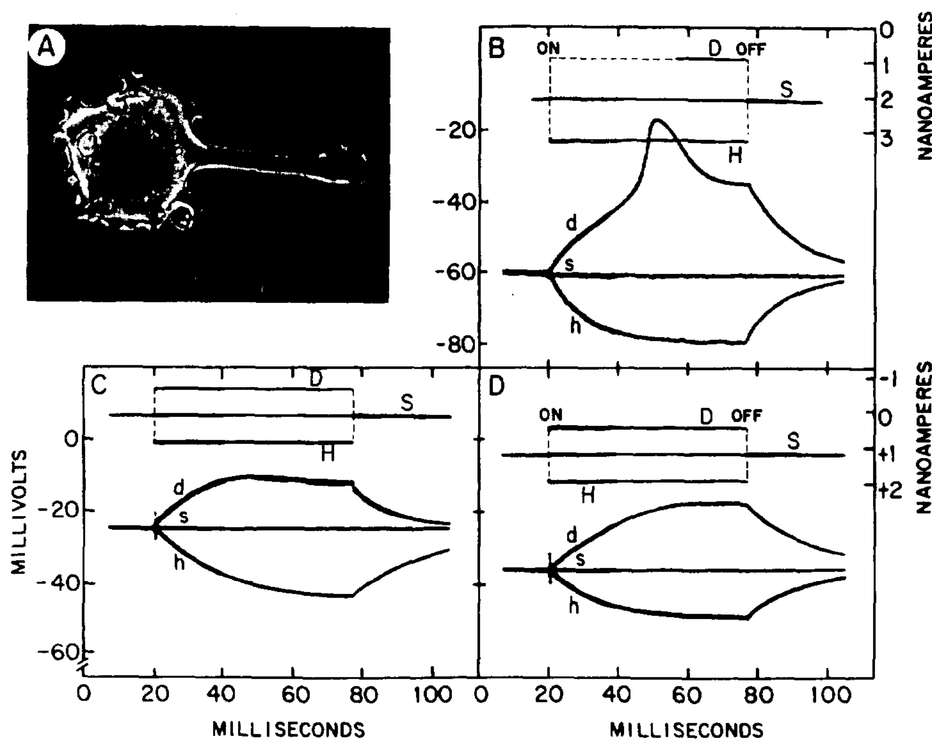


FIG. 2.—(*A*). The cell is from a culture that had been treated with trypsin to dissociate the cells 5 days earlier. The cell was examined electrophysiologically soon after the culture had passed the logarithmic phase of growth. Total time *in vitro* was 108 days.

(*B*, *C*, *D*). An active response is shown in (*B*); examples of rectification in (*C*, *D*). The lines labeled *D* and *H* indicate pulses of current that evoke changes in voltage across the cell membrane labeled *d* and *h*, respectively. *S* designates the steady current used to adjust the voltage across the cell membrane, *s*.

of the same intensity but of opposite direction elicited a different wave form, a smoothly changing, uninflected, voltage transient. In Figure 2*C*, the steady voltage across the cell membrane was adjusted to −25 mv. Then, current pulses that increased or decreased transmembrane voltage did not evoke partial responses; however, the decrease in transmembrane voltage was smaller than the increase. When the steady voltage was adjusted to −35 mv (Fig. 2*D*), asymmetric responses (rectification) were obtained, but the direction of asymmetry was reversed. However, at a steady membrane voltage of −15 mv, symmetric responses to pulses of current were obtained (not shown).

The cell illustrated in Figure 3*A* generated repetitive action potentials in the absence of a stimulating current (Fig. 3*B*) and also in response to a pulse of stimulating current (Fig. 3*C*). The action potential shown in Figure 3*D* was elicited by turning *off* a pulse of current that increased the voltage across the cell membrane. The response appears to be identical to the "off-excitation" phenomenon exhibited by many neurons.

Action potentials, partial responses, and rectification are characteristic of

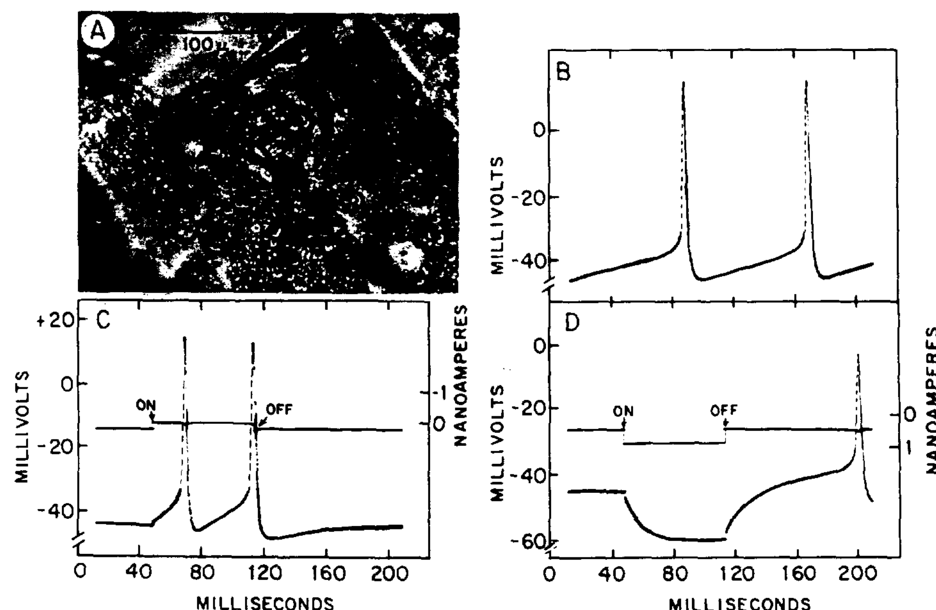


FIG. 3.—(*A*). The large round cell shown in the upper left part of the photograph and indicated by the microelectrode was studied. The culture was in the stationary phase of growth; total time *in vitro* was 66 days.

(*B*). Repetitive action potentials recorded soon after the cell was penetrated by the microelectrode. The action potentials occurred in the absence of stimulating current. The dashes in this and the following figure represent portions of the records that were filled in during reproduction of the records.

(*C*). Two action potentials elicited by a pulse of current.

(*D*). An example of "off-excitation" is shown. An action potential was evoked by turning *off* the pulse of current. The top of the action potential is not shown.

"active" cell membranes and are due to voltage-dependent changes in the permeability of cell membranes.[11] "Active" membranes could not be demonstrated in 36 per cent of the cells studied. Such membranes are termed "passive" because even intense pulses of current produce changes in membrane voltage that have the simple form of voltage trace *a* in Figure 1*C* or trace *h* in Figure 2*C* and are symmetrical.

The relative frequencies of membrane responses are summarized in Table 1. "Active" responses were observed with 65 per cent of the cells examined. Neuron-like action potentials were observed with 9 per cent of the cells, partial

TABLE 1. *Summary of cell membrane responses.*

Membrane properties	Number of cells	Total (%)	Resting potential (mvolts)
Active membranes			
Action potential	13	9	40
Partial response	25	17	24
Rectification	58	39	20
Passive membranes	53	35	17
Total	149	100	22

responses with 17 per cent, and rectification with 39 per cent of the cells. Thirty-five per cent of the cells examined had "passive" membranes.

Discussion.—The results show that mouse neuroblastoma cells grown *in vitro* are electrically excitable and are capable of generating action potentials. Neurons with action potentials have been found in explant[12, 13] and dissociated cell[14] cultures. However, normal mature neurons usually do not divide, whereas at least some neuroblastoma tumor cells retain the ability to divide *in vitro* and also exhibit properties expected of neurons. Augusti-Tocco and Sato[4] have cloned these cells and have shown that they contain choline acetylase, acetyl cholinesterase, and tyrosine hydroxylase. We have confirmed these observations and have also shown that the cells synthesize catechols and catechol derivatives *in vitro.*[15]

Sixty-five per cent of the cells examined electrophysiologically had "active" membranes. However, a wide spectrum of responses was observed with different cells, even when membrane properties were studied at the same transmembrane voltage and under relatively stable recording conditions. Although cell injury may have contributed to the variability of results, it is unlikely that such a wide spectrum of responses would be observed with mature neurons.

We propose as a working hypothesis that the range of membrane properties observed is expressed in the following sequence as neuroblasts mature into neurons: passive responses, delayed rectification, partial responses, and action potentials. The molecular events responsible for these phenomenon are not fully understood. It is possible that some neuroblastoma cells differentiate *in vitro* and one or more genetic programs corresponding to functional action potentials are expressed, or that events related to cell division affect the capacity of cells to generate action potentials. Alternatively, cultures may contain many neuroblastoma cell lines that differ genetically and are fixed at different stages of differentiation. Experiments are in progress to resolve these questions and also to determine whether neuroblastoma cells are capable of forming synapses *in vitro.* The mouse neuroblastoma cell system of Augusti-Tocco and Sato and other cell lines derived from the nervous system provide many opportunities to explore various aspects of neurobiology.

Note added in proof: Similar results have now been obtained with clonal neuroblastoma lines.

We would like to express our appreciation to Gabriella Augusti-Tocco and Gordon Sato for telling us about their experiments with neuroblastoma cells prior to publication.

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