

# MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF NEURONS AND GLIAL CELLS IN TISSUE CULTURE<sup>1</sup>

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THE METHOD of tissue culture offers a unique opportunity for investigating electrophysiological properties of single cells by means of microelectrodes under direct visual control. Crain (13) has published microelectrode recordings of the action potentials from spinal ganglion cells of chicken embryos. Hild *et al.* (26) have reported electrophysiological findings on glial elements from the mammalian brain maintained *in vitro*. Recently, Cunningham and Rylander (14) investigated electric properties of brain tissue from chicken embryos (10 days incubation) which was kept *in vitro* for up to 120 hours; these investigators were, however, interested more in the behavior of the tissue as a whole and did not investigate at the cellular level.

It seemed of great interest to extend our earlier observations since it is possible at present to maintain neurons *in vitro* in good condition for extended periods of time. In many instances protoplasmic processes of living neurons can easily be observed under a phase-contrast microscope. It is generally easy to distinguish dendrites from axons in such preparations. It is possible, therefore, to investigate the electrophysiological properties of various parts of a nerve cell under direct visual control. Obviously, the present method differs greatly from that in previous studies in which properties of the neuron soma and dendrites were inferred from the electric responses recorded from the gray matter with macroelectrodes or from the data of single cell recordings without any knowledge as to the position of the microelectrode relative to the cells under study.

The first half of the present paper deals with the results of our observations on the neuron soma and the dendrite by this method. In the second half, we are concerned with a comparison of electrophysiological properties of neurons with those of neuroglial cells and of macrophages.

## METHOD

1. *Material used.* Explants were prepared from the cerebella of 7-14 day old kittens and new born rats. The brains were quickly removed from the animals and immersed in Gey's balanced salt solution (BSS). The meninges were removed as much as possible under the dissecting microscope in order to minimize the outgrowth of mesenchymatous cells.

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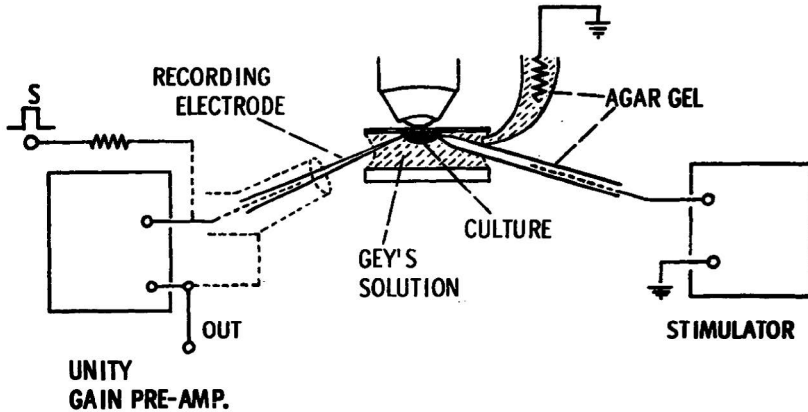


FIG. 1. Simplified diagram illustrating experimental arrangements. Chamber carrying culture was mounted on stage of phase-contrast microscope. Illumination was from beneath.

From one rat cerebellum six explants were cut, whereas from the cerebella of kittens only the lateral (floccular, parafloccular, semilunar and paramedian) parts were taken, yielding about 25–30 explants. The white matter was avoided as much as possible, although in several instances parts of the dentate nucleus were included in the explants.

The cultivation was done in two ways. About half of the explants used in this study were grown in a plasma clot affixed to a  $12 \times 50$  mm. cover slip in roller tubes (flying cover-slip method); the other half was grown in the same setup but instead of the plasma clot a reconstituted collagen gel as described by Ehrmann and Gey (16) and Bornstein (5) was used. The latter method was employed because we thought that it would present much less difficulty in penetration of microelectrodes since the cells grow on top of the collagen gel instead of within a plasma clot. It soon became apparent, however, that certain difficulties in penetration which we had encountered in our earlier study (26) existed in both kinds of explants to the same degree. They are due to the presence of collagenous fiber sheets which, in some areas of the cultures, are produced by fibroblastic elements which cannot be excluded from the explants. In general, however, this interference was not serious and if it occurred another area of the culture was chosen where penetration was easily possible.

The cultures were fed with a fluid nutrient medium consisting of 50% BSS, 45% inactivated calf serum, and 5% embryonic extract (from eight-day chicken embryos). The glucose content of the nutrient medium was raised to about 300 mg.%. According to previous experience, this relatively high amount of glucose enhances neuronal survival, glial viability and myelin formation around axons (24, 25). The cultures were incubated at  $37^\circ\text{C}$ ., the roller drums turned 10 times per hour, the fluid medium was changed once weekly. The cultures to be used for recordings were selected from the incubator on the basis of their gross morphological appearance, preference given to the most flattened cultures. They ranged in age between 10 and 30 days.

2. *Optical and mechanical set-up.* The cover slips bearing the cultures were taken from the roller tubes and mounted on a microscope slide for close inspection under a high power phase-contrast microscope. Favorable areas were marked and the cover slip was then mounted in a chamber as described previously (26). This chamber was then placed on the stage of a phase-contrast microscope which was modified in such a way as to permit the introduction of microelectrodes into the open sides at an angle of about 20 degrees. The culture was suspended from the roof of the chamber formed by the cover slip on which the culture was grown. The chamber was filled with BSS held by capillary forces (Fig. 1). The placement of the electrodes in or on the cells was done under visual control at a magnification of  $600\times$  with Zeiss phase-contrast optics. This permitted the recognition of details of form and structure of the cells under investigation and made possible the accurate place-

ment of the electrodes on various parts of the cell soma and its extensions (Figs. 2, 3, 6, 8, 11, 12). The microscope and micromanipulators were mounted on a vibration-free table standing in sand buckets. All instruments were bolted to an aluminum plate of 0.5 inch thickness. To reduce vibration further, this plate was loaded with a total of about 125 lbs. of lead brick.

3. *Electrical set-up.* Microelectrodes used for recording intracellular potentials had a tip diameter of less than  $1\mu$  (30); they were filled with 3 M. KCl solution (32) by the alcohol method (36). The resistance of the electrodes which gave reasonably large resting potentials were usually between 4 and 10 M $\Omega$ . The chlorided silver wire in the microelectrode was led to the input of a unity-gain preamplifier described by Bak (3). The metallic shield of the input was driven by the output of the preamplifier (see Fig. 1).

A large Ag-AgCl electrode, enclosed in a glass tubing of 3-4 mm. in tip diameter, was used to ground the fluid around the culture. There was a small battery and a potential divider (not shown in the diagram) between this electrode and ground; this was used to compensate the small potential difference between the recording microelectrode and the ground electrode.

Intracellular stimulation of neurons through the recording microelectrode was accomplished by connecting a 1000 M $\Omega$  resistance between the output of a Grass stimulator and the input of the preamplifier. A Wheatstone bridge was used to reduce the shock artifact caused by the flow of currents through the microelectrode. The duration of the stimulating pulses was approximately 0.1 msec.

For delivering stimulating current pulses extracellularly, glass pipette electrodes of about  $10\mu$  in tip diameter were used. When filled with BSS Gey-agar gel, the resistance of the stimulating electrode was in the range 1.2 to 1.5 M $\Omega$ . In order to avoid mechanical injury to the cells on contact, the edge around the orifice of the stimulating electrode was fire-polished.

A Tektronix dual-beam oscilloscope (Model 502) and a Grass camera were used to record simultaneously the time course of the stimulating current and the responses of the cell. The temperature of the culture was kept at about 36°C. in several experiments. However, since a slight fall in the temperature did not affect the amplitude of the electric responses (see Fig. 7), most of the later experiments were carried out at 26°C.

In one series of experiments, the electric impedance of a brain slice was measured by the use of a straight-forward A.C. Wheatstone bridge (see Fig. 15). The A.C. source was a beat-frequency oscillator (General Radio Co., Type 1304-A). The bridge unbalance was amplified by the use of a Tektronix low-level preamplifier (Type 122), filtered with a variable electronic filter (Spencer-Kennedy Laboratories, Model 302) and recorded with a Tektronix oscillograph.

## RESULTS

1. *General description of cultures.* There was no basic difference in the mode of outgrowth between the cultures grown on collagen and those set up in a plasma clot. The two media did not influence the shape or structure of the cells. The only minor difference was that, due to partial liquefaction of the plasma clot during the first few days, the cultures which were grown in this medium developed a ring-shaped outer zone which was heavily populated by mesenchymatous cells, whereas in cultures on collagen this ring formation did not take place. The outer zone showed a more radial arrangement of the cellular elements.

Outgrowth from the original explants could be observed as early as 24 hours after explantation. The first cells to emigrate were mesenchymatous elements derived from blood vessel walls and unremoved parts of the meninges. Glial elements soon thereafter started migrating and macrophages began phagocytizing debris from degenerating cells which were injured or destroyed during the preparation of the explants. Beginning with the 5th or

6th day of cultivation, some of the explants had flattened and spread out sufficiently to permit the recognition of distinct cell types under the phase-contrast microscope. By the 10th to the 12th day, most areas of the explants were so thinned out that transillumination and microscopic observation was possible without difficulties. The large cell somata of the neurons (Purkinje cells, basket cells, Golgi cells, cells of the dentate nucleus) with their large vesicular nuclei and distinct nucleoli were easily distinguishable (Figs. 2, 3, 6, 8, 11, 12). In many instances the dendritic main stems were recognizable, whereas the finer arborizations of the dendritic tree were interwoven in the general glial network in which the neurons were suspended. The origin of the axon from the cell soma could be made out in many cases and could clearly be distinguished from the origin of the main dendrites. Axons are much thinner than dendrites and originate more abruptly from the cell body, whereas the dendrites are attached to the perikaryon by much broader bases. Moreover, axons, if they could be followed for some distance, had a fairly uninterrupted course in contrast to the dendrites which soon gave off a larger or smaller number of branches. In these areas in the cultures where the original orientation of a group of Purkinje cells was maintained to a considerable degree, the axonal end of the cells pointed in one direction whereas all the dendritic ramifications were directed toward the other side. Since the cell processes traversed the glial network in a three-dimensional space, it was difficult to photograph every part of a neuron which was visible with the play of the micro-adjustment of the microscope.

Beginning on the 7th or 8th day, an increasing formation of myelin around the axons could be observed. This myelination, however, proceeded most vigorously in thicker areas of the cultures which were only of secondary importance during this study.

Glial cells formed three-dimensional, yet to some extent flat, networks in which individual elements could be recognized with ease. We do not attempt here to correlate the appearance of living glial cells with conventionally recognized types, like astrocytes or oligodendrocytes, but use only the term "neuroglia" since there are many transitional forms recognized in living preparations. The electrical responses from all such glial cell types were similar and followed a uniform pattern.

Macrophages were found in all areas of the cultures, but were fairly numerous only in liquefaction pools in cultures grown in a plasma clot. They were easily distinguished by their characteristic polymorphism due to extension or withdrawal of pseudopodia. Their cytoplasm mostly was filled with phagocytized material which is highly refractile under phase-contrast optics.

2. *Intracellular recording of neuronal action potentials.* When the recording microelectrode penetrated the surface of a neuron soma successfully, there was a sudden potential shift on the oscillograph screen. The resting potentials of the neurons in culture observed in this manner were about 50 mV. or less. After penetration, the normal morphological characteristics of the neuron were found to change gradually. In all cases the morphological

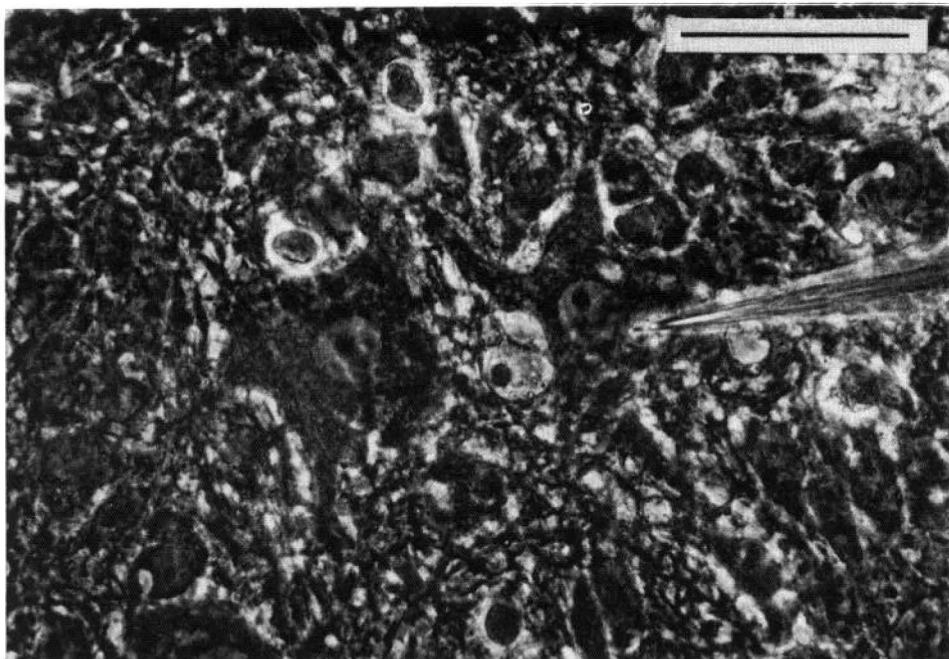


FIG. 2. Microscopic field showing two neurons in a 25-day-old culture of cat cerebellum grown on collagen. One neuron is impaled by hyperfine recording electrode. This picture illustrates electrode arrangement for records such as shown in Fig. 4. Bar in all photomicrographs represents distance of 50  $\mu$ .

changes were accompanied by a simultaneous fall in the resting potential. After withdrawal of the electrodes from the cell there was a marked morphological change in the cells indicating cell death (Figs. 2, 3). This change consisted of increased granularity probably due to demixing of cytoplasmic constituents as well as to an increase in thickness and irregularity of the nuclear membrane. In such cases where the cell membrane was torn instead of just pierced, the cellular outlines were rapidly lost and the original appearance was completely disorganized. All such cells showed neither resting nor action potentials.

In the impaled neurons showing relatively large resting potentials, action potentials were observed on stimulation through the recording microelectrode. Three examples of such intracellular recordings of action potentials are presented in Fig. 4. Immediately following successful penetration, the action potentials evoked were roughly all-or-none. After deterioration of the impaled neuron or when there was difficulty in the process of penetration of a neuron, variable responses with no clear-cut threshold were observed. In some of the action potentials (*e.g.*, Fig. 4C), there was a slight break in the rate of the potential rise; this break is similar to that observed in the spinal motoneurons and was used to subdivide the response into A and B components (*see e.g.*, 17).

The amplitude of the action potential recorded from neurons was be-

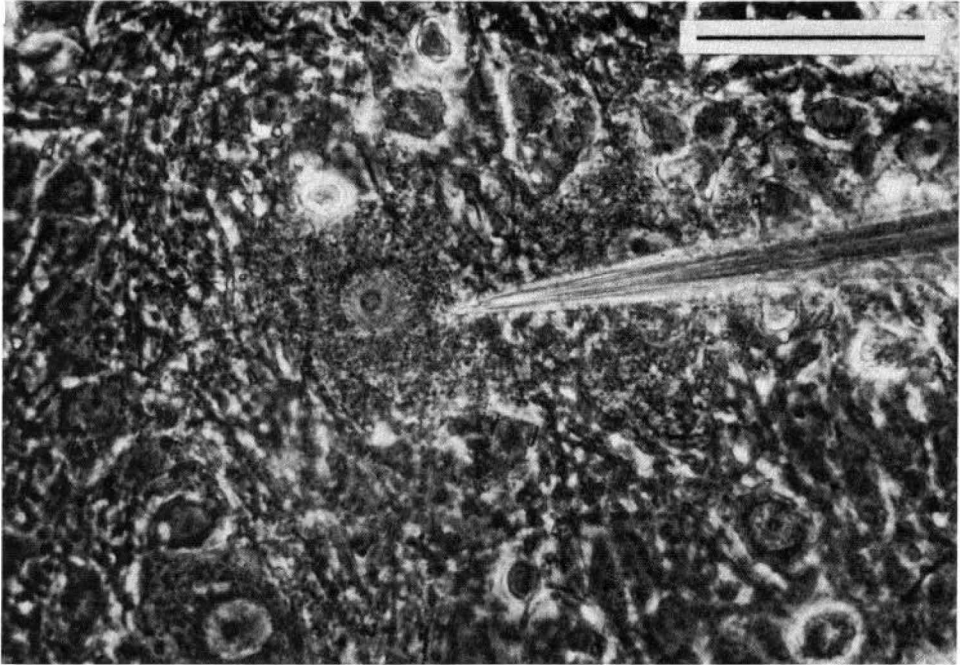


FIG. 3. Same field as in Fig. 2. Marked morphological changes in the two neurons indicate cell death after cells had been impaled and electrode was withdrawn. The same changes could be observed when a dendritic stem or an axon were torn. Sometimes this occurred inadvertently in cells lying in vicinity of neurons which were under investigation.

tween 50 and 70 mV. in most of our successful penetrations; it fell with time because of deterioration of the cell. The duration of these action potentials was between 1.5 and 3 msec. at about 36°C.; it was our impression that the duration depended upon the degree of injury caused by penetration. At 26°C., the duration was in general slightly longer; but because of the large variability among different neurons, it was difficult to make a direct comparison of the results obtained at two different temperatures (see Fig. 4).

In the early stage of the present investigation the method of external stimulation was also employed. The recording microelectrode was pushed into a neuron while cathodal shocks were being delivered repetitively to the surface of the neuron. The amplitude and the duration of the action potential were similar to the values obtained by intracellular stimulation.

We attribute the difficulty of maintaining the resting and action potentials of the neuron soma partly to the presence of some fibrous material on the neuronal surface preventing a clean penetration of a fine microelectrode (see Methods). A similar difficulty was reported also by Crain (13) who investigated chicken dorsal root ganglion cells in tissue culture. Working on Purkinje cells *in vivo*, Granit and Phillips (23) encountered "giant extra-cellular responses" which were sometimes as large as 50 mV. in amplitude.

Under the conditions of our experiments, such giant extracellular responses were never observed (see later).

On several occasions, attempts were made to introduce the microelectrode into large dendrites. It was found that the dendrites were far more sensitive to injury caused by penetration. On two occasions we could record distinct action potentials of 30–40 mV. in amplitude from the portions of dendrites approximately 50  $\mu$  away from the soma.

3. *Spontaneous discharge of impulses from neurons.* During the course of experiments with relatively high recording sensitivity, it was found that a fine glass pipette microelectrode staying on the surface of a neuron without piercing registered small action potentials appearing at irregular intervals in the absence of artificial stimulation. The amplitude of such spontaneously fired action potentials, recorded extracellularly, was usually less than 0.5 mV. (peak to peak). The frequency of impulses observed was usually not more than 15/sec. In some neurons, there was only occasional firing at intervals of several sec. There were also neurons which did not show any spontaneous activity but which could be excited by way of their dendrites (see later).

Three examples of spontaneously fired action potentials of neuron somata are presented in Fig. 5. The most common configuration of these action potentials was, as in the example in Record B in the figure, of the positive-negative type, the negative phase being larger than the first positive phase. In some cases, the negative phase was followed by a distinct positive phase.

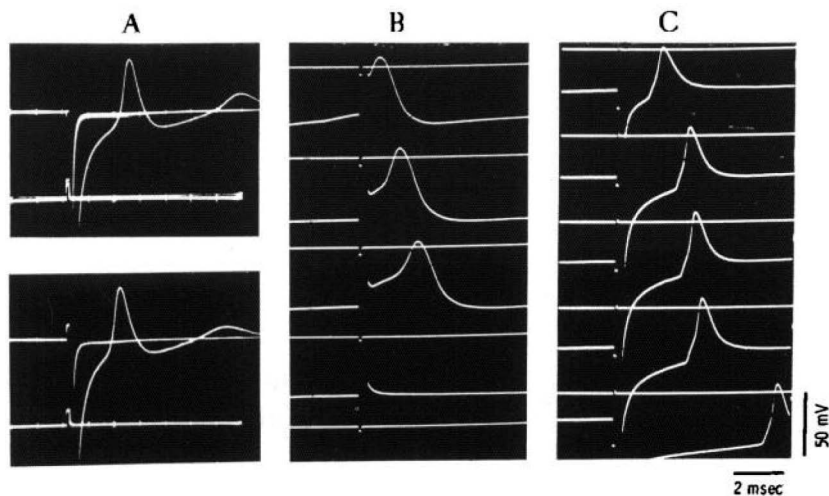


FIG. 4. Action potentials of neuron somata recorded with intracellular microelectrodes as shown in Fig. 2. Stimuli were 0.05–0.1 msec. in duration, repeated 7–8 shocks/sec. and delivered through recording microelectrode. Records A were taken from 20-day-old culture of cat cerebellum, record B from 12-day-old culture of cat cerebellum, and record C from 14-day-old culture of cat cerebellum. Threshold intensity for outward current pulses was approximately  $7 \times 10^{-8}$  A in this case. Records A and B were obtained at 26°C. and records C at 36°C.

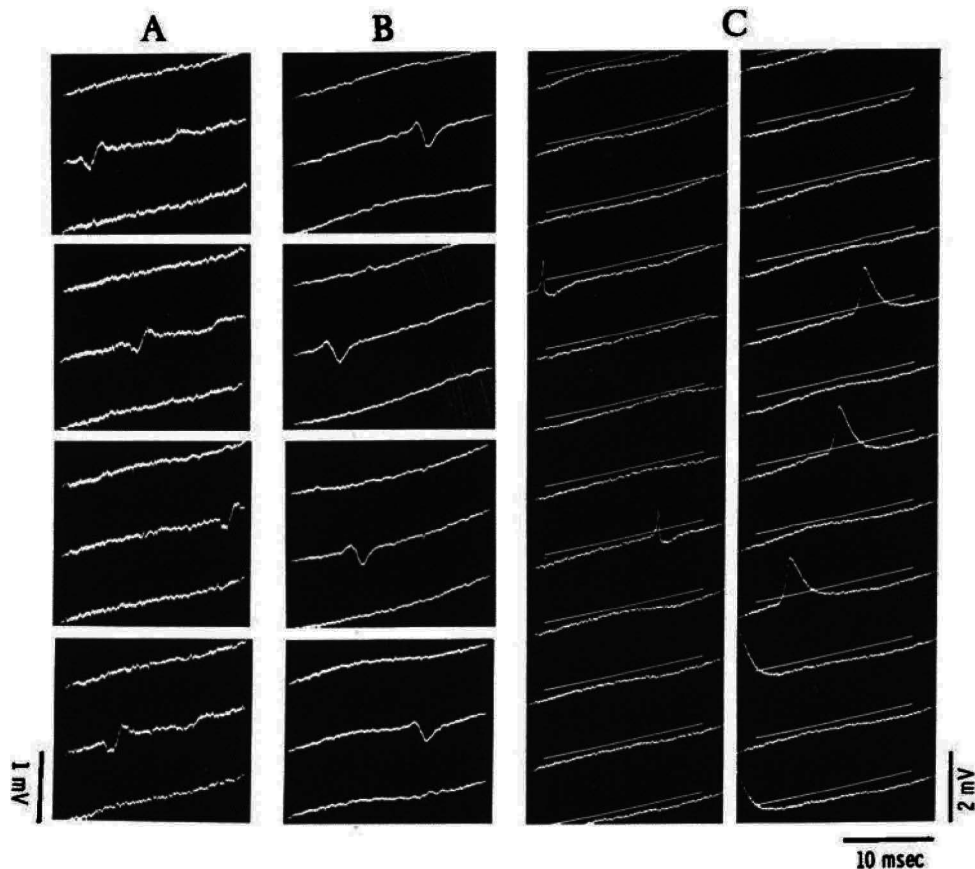


FIG. 5. Several types of spontaneously fired action potentials recorded with glass microelectrode making contact with surface of neuron somata. Upward deflections represent positivity of recording electrode. Records A were taken from neuron in 13-day-old culture of rat cerebellum, records B from 10-day-old culture of rat cerebellum, and record C from 15-day-old culture of rat cerebellum. Sudden change in configuration of recorded action potentials in record C was induced by pushing recording electrode harder against neuron surface. Oscilloscope sweep speed was same for all records; deflection sensitivity for B was same as that for C.

These configurations were observed previously in neurons in the cerebral cortex, in the geniculate body, in the cerebellum etc. *in vivo* (36, 19, 23).

The presence of a large negative phase in the extracellularly recorded action potentials indicates that there is an inward-directed membrane current at the point of contact with the recording electrode (see later). These action potentials were never observed unless the recording electrode was actually dimpling the neuronal membrane; this dimpling is expected to increase the electric resistance of the path of the membrane current [thus increasing the amplitude of the recorded response as in the case of giant extracellular response observed by Granit and Phillips (23)]. The first posi-



tive phase in the extracellularly recorded action potential is the indication of an outward-directed membrane current, caused by the response which has not yet reached the site of recording.

The last column in Fig. 5 shows an example of injury discharge caused by excessive pressure applied to the cell membrane by the recording electrode. Under ordinary recording conditions, spontaneous discharges of impulses could last almost indefinitely without any appreciable change in the configuration of the potentials. When the recording electrode was pressed harder against the cell surface, the amplitude of the potential variations could increase slightly without a change in the frequency of the discharge. It sometimes happened that such a procedure led to a discharge of large, positive, monophasic action potentials, repeating at a relatively regular, rapidly declining frequency, followed by an irreversible damage to the cell. The absence of a negative phase in such an injury discharge is an indication of the loss of a physiological response at the site of recording.

4. *Action potentials of neuron somata elicited by stimulation of dendrites.* When the existence of spontaneous discharge of impulses in neurons in tissue culture was established, the following question was asked immediately: is it possible to influence these spontaneously discharged impulses by stimulation of the dendrite? With the experimental arrangement of Fig. 1, attempts were made to stimulate one of the dendrites of the neuron when the recording microelectrode was picking up spontaneous action potentials from the surface of the soma. In these experiments both the stimulating electrode (approximately 10  $\mu$  in tip diameter) and the recording electrode (hyperfine glass pipette) were extracellular (Fig. 6).

It was found by this technique that, whenever there were spontaneously fired action potentials in a neuron soma, stimulation of the dendrite could induce similar responses in the same soma. It was also possible to record evoked action potentials from neuron somata which were not firing spontaneously. Figure 7 is an example of the records obtained from neurons which were firing impulses spontaneously at a low frequency. Stimulating current pulses applied to the dendrite were cathodal and were repeated usually at a frequency of 5–20/sec. At threshold of stimulation, the interval between the shock (0.05–0.1 msec. in duration) and the response showed a large fluctuation. Such a fluctuation in the latency in threshold stimulation is well known to occur in other excitable cells since Blair and Erlanger (4) first described this phenomenon in the frog phalangeal nerve preparation. At higher stimulus intensities the latency became shorter and more or less insensitive to changes in the intensity.

We could not obtain any response with anodal current pulses—namely, with the stimulating electrode connected to the anode of the stimulating circuit (up to five times the threshold strength for cathodal stimuli). When the neuron under study had a long, clearly discernible dendrite (see Fig. 6), it was possible to move the stimulating electrode along the dendrite and show that the latency increased with the length of the intervening portion of

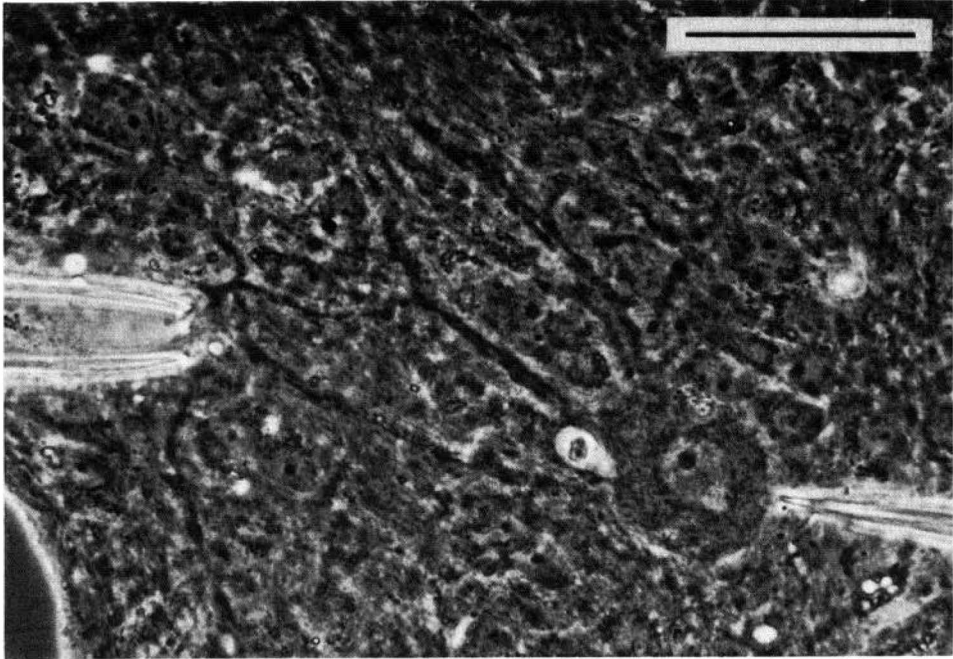


FIG. 6. Neuron with three branching dendrites in 22-day-old culture of rat cerebellum grown in plasma clot. Origin of axon is not clearly visible in this microphotograph because it was out of plane of focus at lower left circumference of cell body. Hyperfine recording electrode is close to cell membrane. Larger stimulating electrode is located close to dendritic branch. This picture illustrates electrode arrangement for records such as shown in Fig. 7. Bar represents 50  $\mu$ .

the dendrite. The following example will show the procedures of our measurements on the neurons in tissue culture.

A shock-response interval of approximately 3.3 msec. was obtained when electric shocks (0.1 msec. in duration and about 50% above threshold intensity) were delivered to a spot on a dendrite approximately 100  $\mu$  away from the soma; when the distance between the site of stimulation and the soma was reduced to approximately 60  $\mu$ , the corresponding latency decreased to about 2 msec. This neuron had two more dendrites and an axon arising from the soma; stimulation of those dendrites at a distance of about 50  $\mu$  away from the soma gave rise to all-or-none responses in the soma with a stable latency of 1.5–2 msec. The configurations of the action potentials elicited by way of different dendrites were—within the limit of our resolution about the same, indicating that the configuration was determined by the excitability pattern of the soma itself.

Based on 12 measurements of this type, the ratio of the length of the dendrite and the shock-response interval was found to be of the order of 0.03 m./sec. at 26°C. The diameter of the neuron soma (30  $\mu$  in average) was not included in this calculation. It is important to note also that our meas-

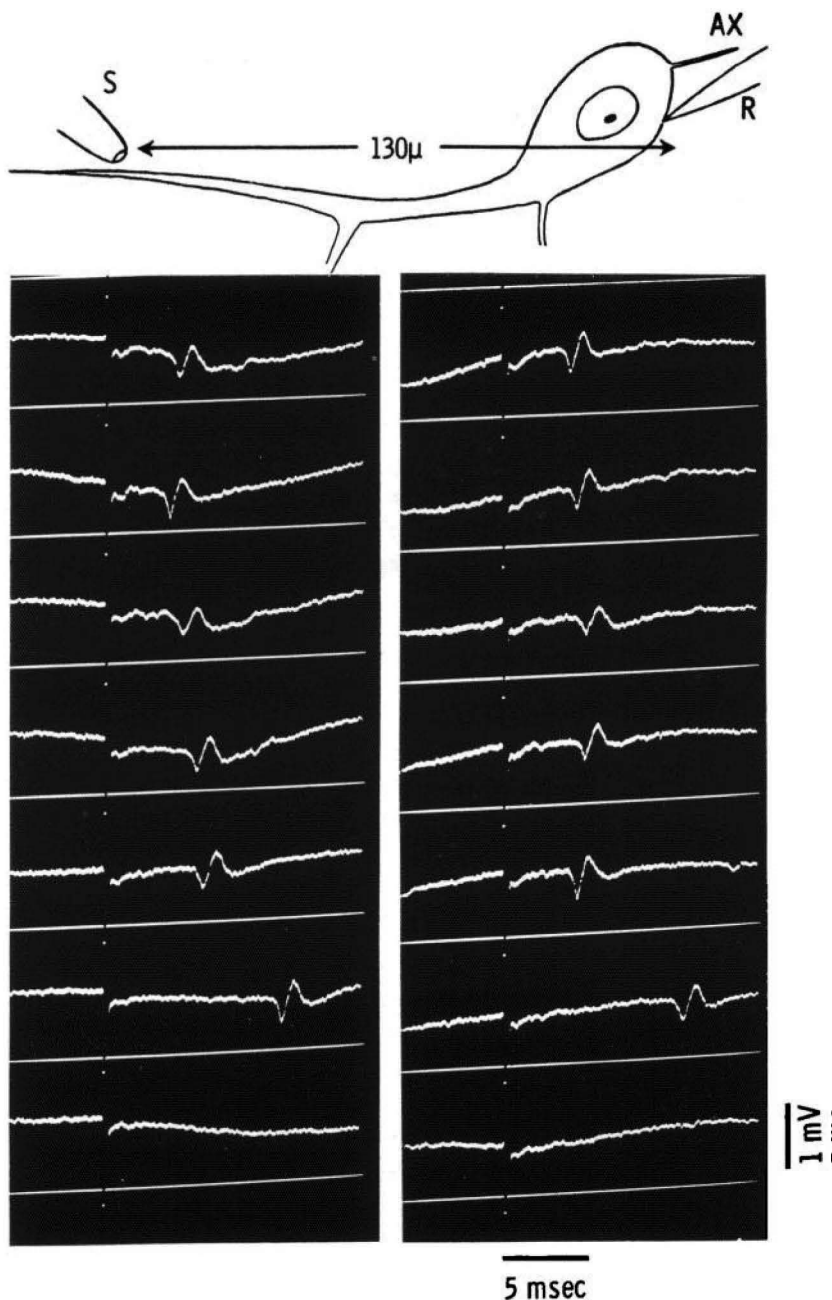


FIG. 7. Extracellularly recorded action potentials of neuron soma evoked by stimulation of dendrite. Arrangement of stimulating and recording electrodes (both extracellular) is shown in diagram above; only clearly discernible dendrites are shown. S represents stimulating electrode, R recording electrode and Ax axon. Fourteen-day-old culture of rat cerebellum. Stimulating pulses were 0.1 msec. in duration and intensity at threshold was in order of  $25 \mu\text{A}$ .

urements are limited to the basal (up to  $110 \mu$ ) portion of the dendrite which is  $5-8 \mu$  in diameter. Because of technical difficulties, we were not able to examine the properties of fine terminal portions of the dendrites.

Taking the all-or-none response of the neuron soma as an index of excitation, it was possible to determine the relationship between stimulus duration and threshold strength. For durations shorter than about 1.5 msec., the relationship could be approximated by Weiss' hyperbolic formula:

$$i = \left( 1 + \frac{\tau}{t} \right) b,$$

where  $i$  is the threshold intensity for a current pulse of the duration  $t$ ,  $\tau$  is the chronaxie and  $b$  is the rheobase. The value of  $\tau$  was found to be 0.15–0.3 msec. (Note that the chronaxie is known to depend on the size of the stimulating electrode.) The rheobase  $b$  was found to vary markedly with the distance between the orifice of the stimulating electrode and the surface of the dendrite. With a stimulating electrode of about  $10 \mu$  at the tip gently touching the surface of the dendrite, the rheobase was usually in the range between 5 and  $15 \mu\text{A}$ .

5. *Propagation of impulses along dendrites.* It was found possible in some neurons to record spontaneously fired action potentials with a glass pipette microelectrode pushing gently against the surface of a dendrite. Extracellular recording from the dendrite was more difficult than recording from the soma surface because of the greater susceptibility of the dendrite to mechanical injury. In order to pick up small (less than 0.5 mV.) extracellular responses in the culture, it was necessary to dimple the surface of a dendrite. In other words, the dendritic response recorded in the present experiment is not an indication of the variation of the diffuse potential field around a neuron; instead, it is an indication of a flow of electric current through the portion of the dendritic membrane at the site of recording.

The configuration of extracellularly recorded dendritic responses was either nearly monophasic (negative) or slightly diphasic (small positive phase preceding the large negative phase). This type of response was recorded previously by Tasaki *et al.* (36) in the cat geniculate body and was tentatively (and correctly) attributed to the activity of the dendrite. The existence of a large negative phase in the extracellular dendritic response is an indication of a strong inward-directed membrane current at the site of recording. Since such an inward current cannot be generated by an electric response localized in the neuron soma, there seems no doubt that the dendritic membrane is excitable and can produce an inward-directed membrane current when an impulse travels along this portion (see Discussion).

In dendrites showing a spontaneous discharge of impulses, it was simple to demonstrate that stimulation of the neuron soma could elicit dendritic responses of the same configuration (Fig. 8). An example of the dendritic responses to stimuli applied to the neuron soma is presented in Fig. 9. Except

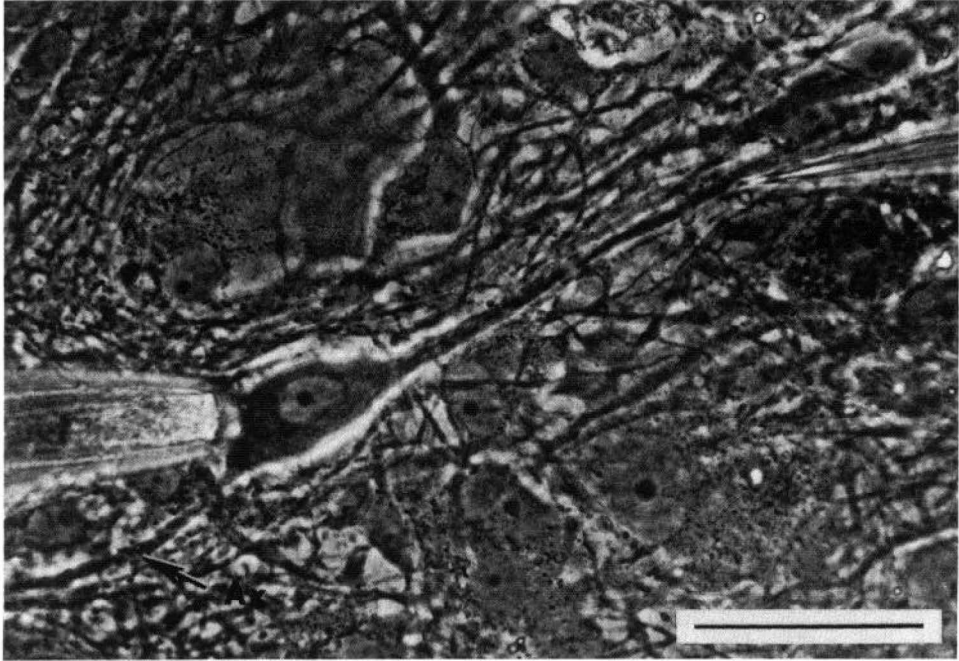


FIG. 8. Neuron showing axon (Ax) and dendritic main stem in 12-day-old culture of cat cerebellum grown on collagen. Hyperfine recording electrode is pushed against dendrite. Stimulating electrode is located close to cell soma. This picture illustrates electrode arrangement for records such as shown in Fig. 9. By moving stimulating electrode to dendrite, one would have arrangement as in diagram in Fig. 10, left side. Bar represents 50  $\mu$ .

on a few occasions in which responses from short dendrites (which were apparently injured) were rapidly deteriorating, the responses to stimuli applied to the soma were always all-or-none. When a long portion of a dendrite was discernible it was possible to record dendritic responses evoked by stimulation of the same dendrite. An example of this type of experiment is furnished in Fig. 10, left. In neurons with long bifurcating dendrites (Fig. 11), we were able to stimulate one branch of a dendrite and to record from the other branch of the same dendritic stem. Figure 10, right, shows an example of observations made with this type of electrode arrangement. In neurons with two or more dendrites arising from the soma (Fig. 12), we had no difficulty in demonstrating propagation of impulses initiated by stimulation of a dendrite to a different dendrite via the neuron soma.

The diameter of the dendrite usually decreases with the distance from the neuron soma. In a rapidly tapering dendrite, it is expected that propagation of a nerve impulse toward the neuron soma is slower than in the reverse direction. With this possibility in mind, we examined the results of nine measurements in which the length of the dendrites between the stimulating and recording electrodes was about 100  $\mu$ . The observed shock-response

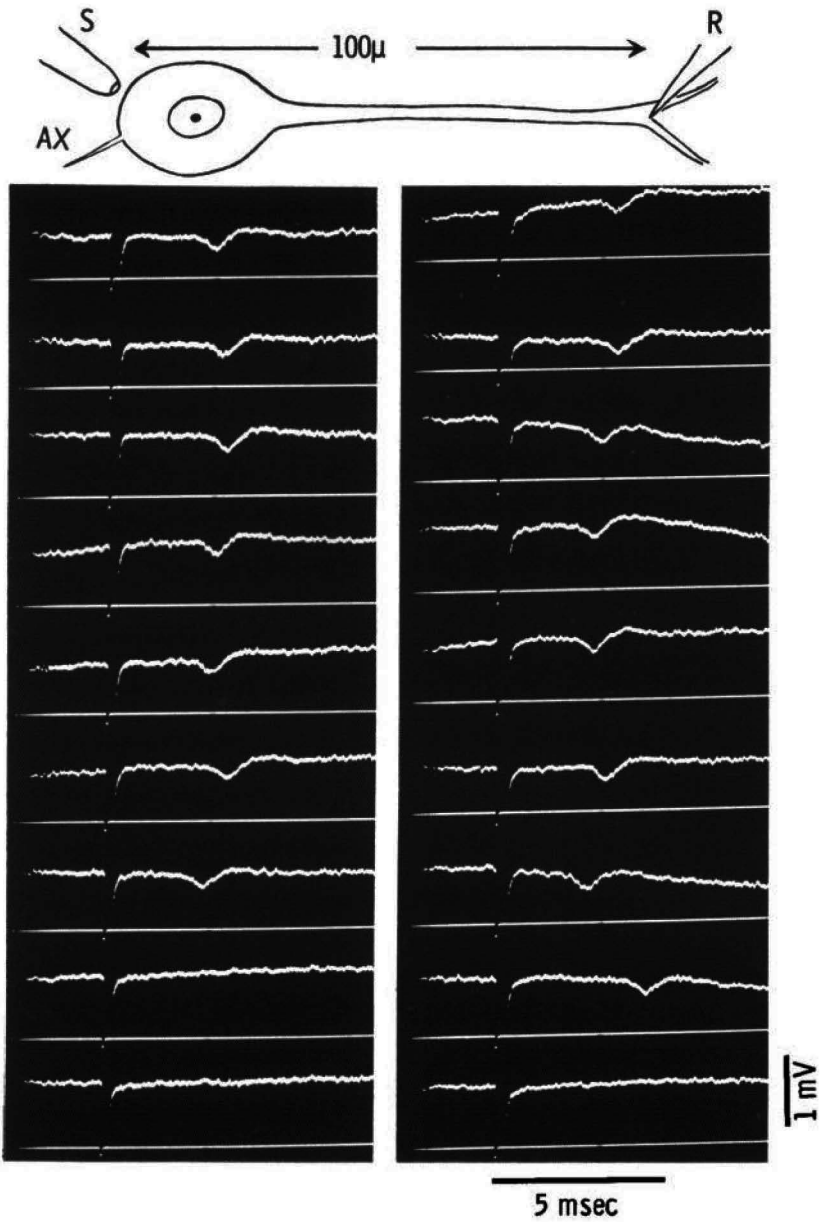


FIG. 9. Action potentials recorded at surface of dendrite on stimulation of neuron soma. Stimulating shocks were 0.05 msec. in duration, approximately  $20 \mu\text{A}$  at threshold and repeated at rate of 7/sec. Arrangement of electrodes (both extracellular) and major, clearly discernible, portion of dendrite are shown. Ten-day-old culture of cat cerebellum.

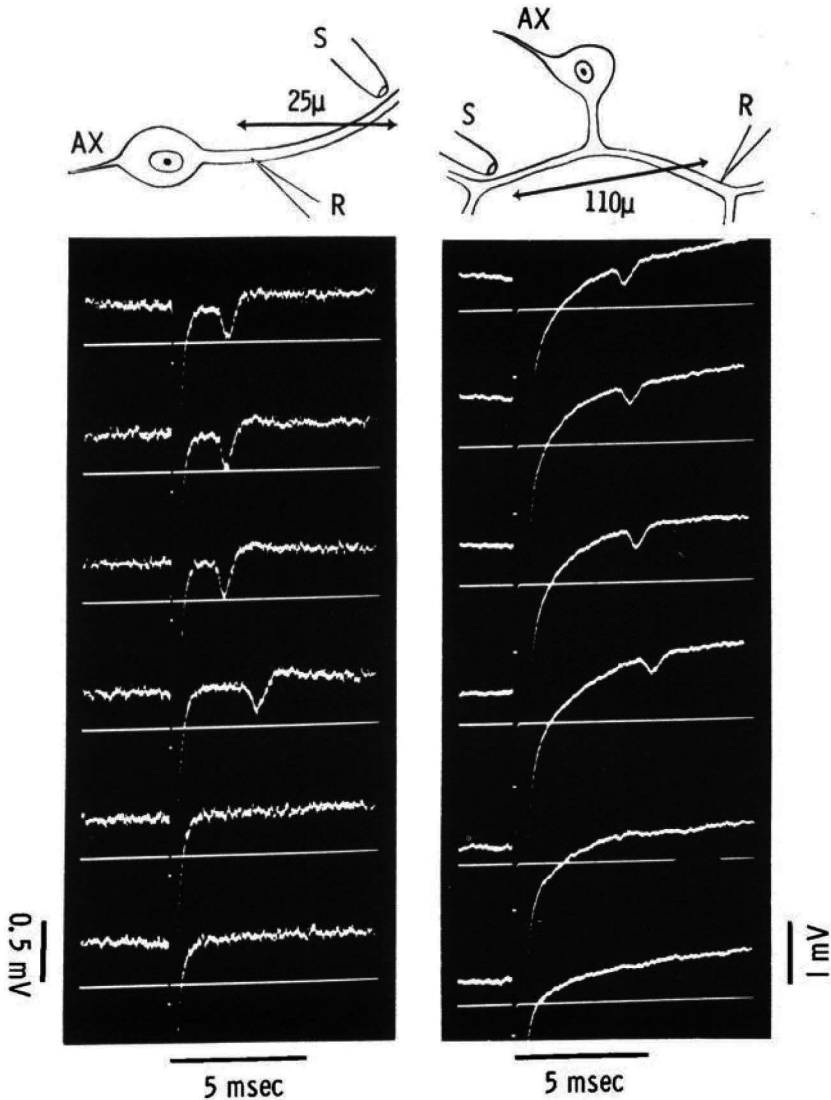


FIG. 10. Action potentials evoked by stimulation of dendrites and recorded from surface of dendrites. Arrangement of stimulating (S) and recording (R) electrodes is shown in diagram. Short, downward deflections of current trace show intensities of stimulating current pulses (0.1 msec. in duration).

intervals varied between 3 and 5.5 msec. and the correlation with the direction of propagation was not clear.

6. *Resting potential and membrane resistance of glial cells.* Neuroglial cells were present abundantly in our cultures. Although glial cells are generally smaller than neurons, it was easier to obtain reproducible values of resting potentials from them than from neurons. When there was no fibrous layer

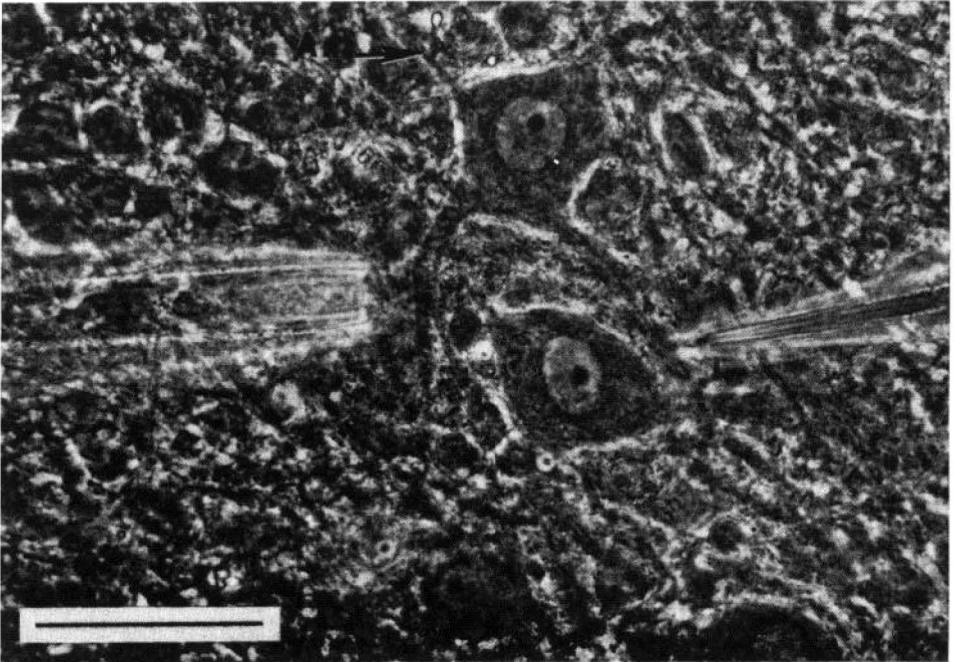
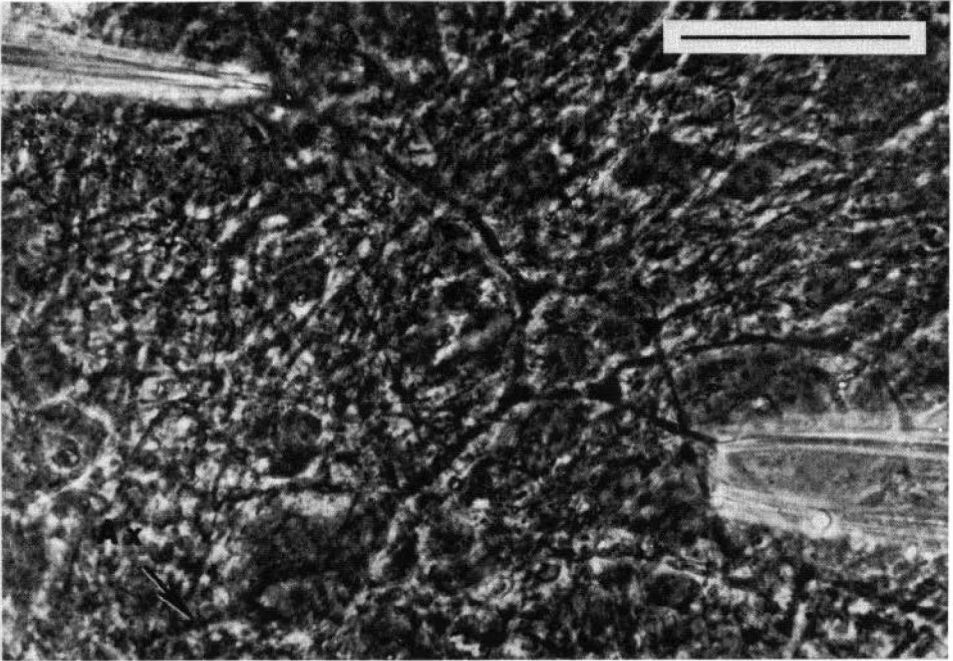




FIG. 11. Neuron showing branching dendrite and origin of axon in 19-day-old culture of cat cerebellum grown in plasma clot. Cell body seems somewhat distorted because it lies outside focal plane of dendrites on which camera was focussed. Hyperfine recording electrode is applied to Y-shaped branching of dendrite. Larger stimulating electrode is located close to other branches of dendrite. This picture illustrates electrode arrangement for records such as shown in Fig. 10, right side. Bar represents 50  $\mu$ .

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preventing smooth penetration of the microelectrode, D.C. potential shifts of 50–70 mV. were obtained from a number of glial cells in one explant. The resting potential of the glia was reduced when the potassium concentration in the media was increased (26).

Encouraged by the relative ease of penetrating glial cells with microelectrodes, attempts were made to measure their membrane resistance by introducing two microelectrodes into a single cell. Passage of a pulse of constant current through one of the intracellular microelectrodes is expected to bring about across the plasma membrane a potential variation which is a measure of the membrane resistance. Such potential variations can be measured directly by the use of the second (recording) intracellular microelectrode.

The arrangement for recording the intracellular potential was the same as that used in the preceding experiments. The microelectrode for passing current pulses was connected to a source of constant voltage pulses through a 35 M $\Omega$  resistor. Tests were made to show that the current-electrode could carry a current of the order of 0.1  $\mu$ A without diverging from linearity. The current delivered to the cell was calculated from the voltage employed divided by the total resistance (35 M $\Omega$  plus electrode resistance) or measured directly by recording the potential drop across a 400  $\Omega$  resistor inserted between the large electrode and ground.

An example of the records obtained is furnished in Fig. 13. When two microelectrodes were placed on the surface of a glia cell (10–15  $\mu$  apart), passage of a constant current of the order of 0.1  $\mu$ A did not bring about any distinct deflection of the oscillograph beam (Record 1). (In this record, the long rectangular deflection of the current trace shows the time course of the

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FIG. 12. Two neurons in 25-day-old culture of rat cerebellum grown on collagen. One cell displays three clearly visible dendrites and an axon (Ax). Hyperfine recording electrode and a larger stimulating electrode are placed close to two different dendrites.

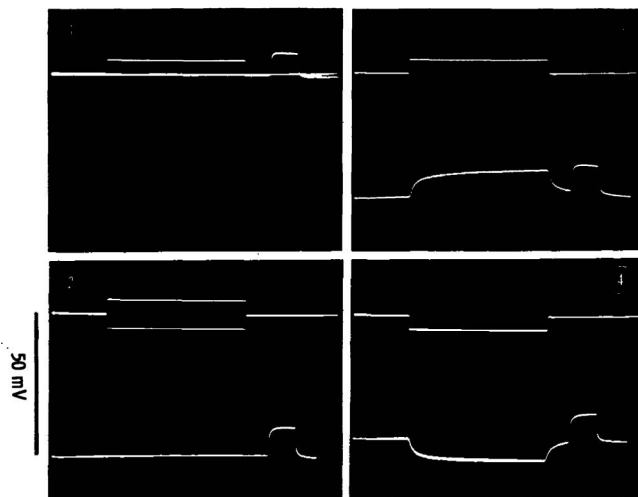


FIG. 13. Variations in membrane potential of glial cell produced by passage of constant current pulses. Both current and potential electrodes were intracellular. Record 1 was taken when both microelectrodes were immediately outside cell membrane. In record 2 potential electrode was introduced into cell; there was a shift of 52 mV. in recorded potential; two records with different current polarities were superposed. In record 3 current electrode was also introduced into cell. In record 4 polarity of current was reversed. Applied current shown by upper oscillograph beam was  $7 \times 10^{-9}$  A in intensity and 5 msec. in duration. Rectangular deflection of 1 msec. duration of potential trace is measure of resistance of potential electrode (approximately  $7 \text{ M}\Omega$ ).

applied current; the short, roughly rectangular deflection of the potential trace is the measure of the resistance of the microelectrode and of the degree of neutralization of the input-capacity of the unity-gain cathode-follower.) Because of the volume conductor around the cell there should be, under these conditions, a potential variation of the order of 0.5 mV. in the potential trace; but this is too small for the present recording sensitivity which was 10 mV./cm. When the recording microelectrode was pushed into the cell, there was a D.C. shift representing the appearance of the resting potential (Record 2); passage of a current pulse through the other (extracellular) microelectrode had no effect upon the membrane potential under these conditions. At the moment when the current-electrode was also pushed into the cell, there was a slight fall in the resting potential (Record 3). Application of a current pulse produced under these circumstances a change in the membrane potential. As long as the potential variation did not exceed 20 mV. or so, the potential variation was roughly proportional to the intensity of the applied current and a reversal of the polarity of the current reversed the deflection without changing its size (Record 4).

The membrane resistance of glial cells was calculated by dividing the potential change at the end of 5 msec. current pulses by the current intensity. Since there was no clear steady level in the membrane potential during the period of current flow, there was some ambiguity in the resistance deter-

mined in this manner. In six measurements in which the fall in the resting potential on the second penetration did not exceed 10 mV., the membrane resistance of a glial cell of approximately 15  $\mu$  diameter was found to be from 0.5 to 1.4 M $\Omega$ . There was no significant difference in the membrane resistance between cells from cat brain and those from rat brain.

As was pointed out earlier, there was usually a slight fall in the observed resting potential when the second microelectrode was introduced into the cell. This indicates that there was some leakage of current through the space between the microelectrode and the cell membrane. Assuming that the leakage resistances caused by the two successive penetrations are equal, it is possible to estimate the magnitude of such leakage. When the fall in the observed resting potential is from 50 mV. to 45 mV., the leakage resistance is estimated to be eight times or more of the membrane resistance of the cell. With the two leakage resistances connected in parallel, the membrane resistance excluding the leakage is 25% (or less) higher than the observed value. The resistance of the glial cell with this correction introduced is then 0.6–1.7 M $\Omega$ .

Since the shape of the glial cell body *in vitro* can be regarded as being intermediate between a flat disc and a sphere (disregarding the varying number of processes), the surface area  $A$  was estimated by the formula

$$A = (3/4)\pi D^2$$

where  $D$  is the diameter of the cell measured under the microscope. Introducing

$$D = 15 \times 10^{-4} \text{ cm.},$$

the area is found to be roughly  $5 \times 10^{-6} \text{ cm}^2$ . Therefore the resistance of the glial membrane of a unit area is found to be 3–10 ohm·cm<sup>2</sup>. This figure is only 1/100 to 1/500 of the generally accepted value of the membrane resistance of various neurons (see Discussion).

7. *Electric stimulation of glial cells.* In our previous report (26), it was shown that the resting potential of the glial cell could be reversibly lowered by strong electric stimulation. The extent of lowering depended, within a certain limit, upon the intensity of the stimulus. The time course of the process of restoration of the resting potential was very slow; it took 3–5 sec. Delivery of two shocks at a short interval gave rise to a summated potential change. With an extracellular stimulating electrode, both cathodal and anodal shocks were effective; but in many cases cathodal stimuli were more effective. Figure 14 shows three examples of such observations using more recent cultures and techniques. The microelectrodes and the preamplifier employed were the same as those used in the preceding experiments. The stimulating electrode was a glass capillary with an orifice of about 10  $\mu$  filled with BSS-agar gel. The resistance of the recording microelectrode was monitored repeatedly during observation. In order to induce a reversible potential fall of about 10 mV., it was necessary to apply a current pulse of about

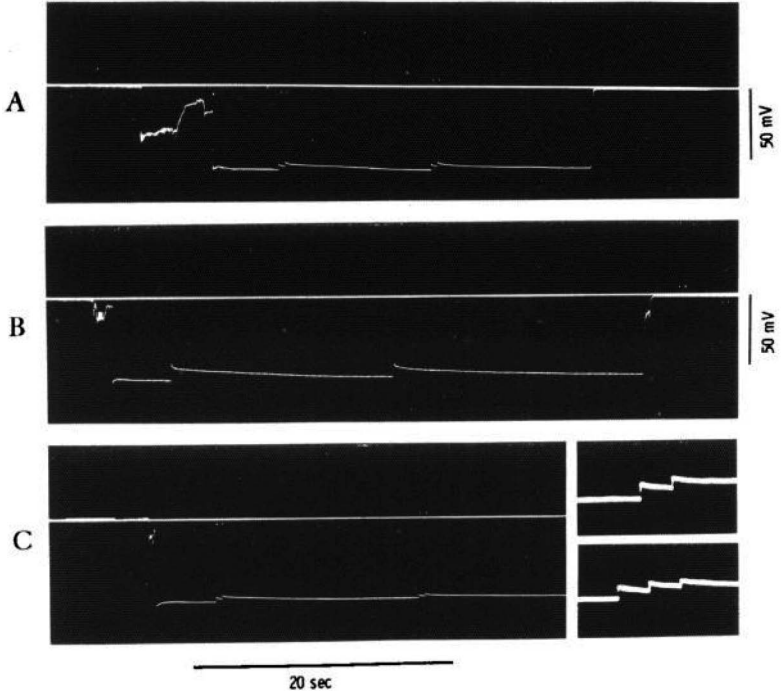


FIG. 14. "Electric responses" of glial cells to electric stimulation. Sudden falls in recorded potential indicate penetration of cells with glass microelectrodes; sudden rises indicate withdrawal of recording microelectrodes from cells. Intensity and polarity of electric shocks are shown by brief deflections (seen as dots) of second, thicker, oscillograph beam. Stimulating pulses were 1.5 msec. in duration, 15–25  $\mu$ A in intensity and were repeated (except in B) at 1-sec. intervals. Note in record B that shocks of same intensity delivered after withdrawal of recording electrode from cell did not produce any recognizable potential variation. In record C, "responses" to subsequent stimulation were enlarged photographically by a factor of 5. The three records were taken from three different glial cells in 9-day-old culture of rat cerebellum.

10  $\mu$ A or more (approximately 1 msec. in duration) through the stimulating electrode placed in the vicinity of a glial cell.

The main difficulty in this observation was the problem of maintaining the resting potential. As is well known, the surface membrane of the glial cell is not stationary; the cell migrates and sometimes shows pulsating movements. Because of such continuous movements, the recording microelectrode did not stay in the cytoplasm indefinitely and the observed resting potential of the cell fell to a low value or even to zero within about 0.5 min. after penetration. When the recording microelectrode was no longer picking up the full-size resting potential, as the result of such spontaneous movements, the stimulating current pulse of the same intensity did not bring about any detectable D.C. potential shift; this shows that we are actually dealing with a fall in the resting potential and not with an artifact in our recording system caused by a flow of a strong current. At the right-hand end of Record B in

Fig. 14, it is seen that stimuli of the same intensity delivered after withdrawal of the recording microelectrode from the cell interior did not bring about any displacement of the base-line of the potential trace.

In view of the fact that the potential variation in the glial cell caused by electric stimulation is very different from the ordinary all-or-none action potential in neurons, questions may be raised. Does this potential variation have anything in common with the ordinary process of excitation? Or do glial cells *in vivo* produce similar potential variations in response to electric stimulation? Does this kind of potential variation exist in non-neural cells in the culture, such as fibroblasts and macrophages?

The last question was easily answered. When a recording microelectrode was pushed into large macrophages (20–30  $\mu$  in diameter) under direct visual observation, it was found that the observable resting potential was between 5 and 15 mV. Strong stimuli (up to 50  $\mu$ A through a 10  $\mu$  stimulating electrode) did not affect the recorded D.C. potential. The electrophysiological properties of the macrophage are evidently very different from those of the glial cells.

The question whether or not the glial cells *in vivo* have a similar property was given a partial answer in a short note published elsewhere (37). It was shown that a strong electric shock applied to the cat's cortex affects the resting potential of some cells near the stimulating electrode in the manner similar to that shown in Fig. 14. Since glial cells make up the bulk of cellular elements in the grey matter and since neurons are known not to behave thus, it was inferred that the glial cells *in vivo* behave like those in tissue culture. An experiment to be described later provides further support to this view.

The finding that some glial cells respond to electric stimuli with a slow contraction (10) suggests that there exists a certain behavioral similarity between muscle cells and glial cells. The contraction phase of the glial cell was found to be 1–2 min. and its relaxation phase was 7–13 min. This indicates that the reaction in the glial cell proceeds far more slowly than it does in cardiac or smooth muscles.

The absence of a discrete threshold and of all-or-none responses in the glial cells does not necessarily indicate that neuroglia are entirely different from other excitable tissues and cells. Whenever a muscle or nerve fiber is heavily narcotized or injured, the discrete threshold is lost and the all-or-none responses change into graded responses. The low membrane resistance of the glial cell can be regarded as a factor which favors graded responses.

8. *Impedance measurements with brain slices.* In ordinary excitable tissues, electric responses are elicited by outward-directed (depolarizing) membrane currents but not by inward-directed currents. Electric responses are accompanied invariably with a decrease in the membrane resistance (or impedance). The next question is, do glial cells behave in accordance with these general rules established in other cells?

An experiment designed to answer this question was carried out by J. J.

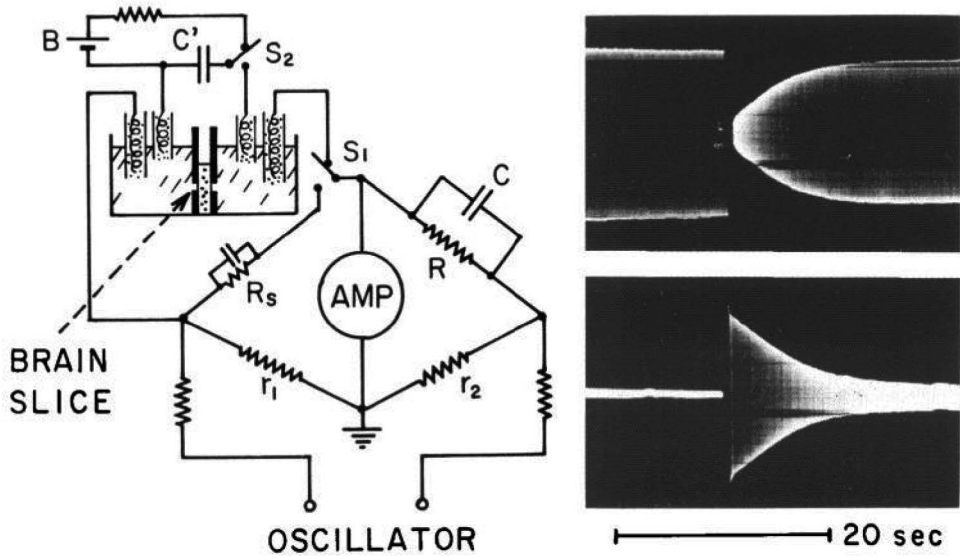


FIG. 15. Left: Diagram of A.C. Wheatstone bridge used to measure impedance change of brain slice caused by electric stimulation. Ratio arms  $r_1$  and  $r_2$  were resistors of 40 and 200  $\Omega$  respectively. Switches  $S_1$  and  $S_2$  were used to avoid passage of stimulating currents through impedance electrodes. Right, bottom: Bridge unbalance caused by stimulation of brain slice. Resistor  $R$  in bridge was 6411  $\Omega$ ,  $C$  0.0054  $\mu\text{F}$ , A.C. frequency 300 c./sec., stimulating battery  $B$  14 V., capacitor  $C'$  1  $\mu\text{F}$ , stimulating electrodes 450  $\Omega$  each, impedance electrodes 250  $\Omega$  each. Temperature 26°C. Right, top: Similar to lower record except that bridge was brought off balance by changing  $R$  from 6352  $\Omega$  (best balance at rest) to 6322  $\Omega$  and  $C$  from 0.0053  $\mu\text{F}$  to 0.0051  $\mu\text{F}$ .

Chang in collaboration with one of us (I.T.) in 1958. Since the result was not published at that time and since the experiment was repeated more recently, a somewhat detailed description will be made here of the design and the results of the experiment.

The material used was brain slices of young cats or guinea pigs. Slices of 1–1.4 mm. thickness were obtained from the grey matter of the cerebral cortex by using a sharp razor blade moved tangentially along the surface of the cortex. In some cases, the layer immediately beneath the pia was chosen; in other cases the pia was left intact on one side. The slice was sandwiched between two thin glass plates, each of which had a hole of 2.5–3.5 mm. diameter in the middle. The pack of two glass plates and a brain slice was introduced into a Lucite box as is shown by the diagram in Fig. 15. The fluid (Gey's solution) in the box was divided by the pack into two compartments. An electric current flowing between two compartments passed through the holes in the glass plates and the brain slice. Two large electrodes of the Ag-AgCl agar-gel type were introduced into each compartment; one pair of the electrodes was used to stimulate the brain slice by electric currents and the other pair was used to measure the electric impedance or the potential difference across the slice. The stimulating electrodes were connected to a

battery of 6–45 V. (B in the figure) through a switch ( $S_2$ ) and a resistor. When the switch was in the position shown in the figure, capacitor C (1–2  $\mu\text{F}$ ) was charged by the battery. When  $S_2$  was thrown into the other position, the electricity stored on the capacitor was discharged through the slice. The other pair of the electrodes were normally connected to the A. C. Wheatstone bridge as illustrated in the diagram. By adjusting resistance R and capacitor C, the bridge could be balanced for the impedance of the brain slice. The bridge A.C. frequency was 100–1000 c./sec.; in most of the later experiments, it was 300 c./sec. The intensity of the bridge A.C. was not allowed to exceed about 100 mV. across the brain slice. There was a continuous slow change in the bridge balance during the experiment (due to swelling of the slice).

The procedure of the impedance measurement was as follows. When the bridge was in balance, switch  $S_1$  was thrown into the alternative position, thereby disconnecting one of the impedance electrodes from the circuit. Next, the stimulating current was delivered by using switch  $S_2$ . Finally, both switches were brought to the original positions. It took approximately 1 sec. or less to operate these switches manually. The bridge unbalance was recorded continuously on a slowly running film. The detail of the electric equipment is described in Methods.

The records in the right-hand column of Fig. 15 show the time course of the impedance loss of the brain slice following electric stimulation. The lower record was taken with the bridge balanced to the resting impedance of the slice. A short break in the record indicates the time at which a stimulating shock (discharge of a 1  $\mu\text{F}$  capacitor charged to 14 V.) was delivered. There was a distinct unbalance of the bridge following the shock. The course of the A.C. envelope was slow and resembled the course of the return of the intracellular potential shown in Fig. 14. In the upper record, the best bridge balance was obtained immediately after the end of stimulation; this was done by reducing resistance R below the value for the best balance before stimulation. Evidently the observed change in the impedance represents a decrease in the electric resistance of the slice. A slice which had been killed by heating produced no impedance changes on stimulation.

The magnitude of the impedance change, as well as the intensity of stimuli necessary to bring about impedance changes, varied markedly from preparation to preparation. In some preparations, a shock of about 3 V. (1 msec. in duration) across the slice was sufficient to produce a detectable impedance change (*i.e.*, approximately 0.1% of the resting value). In other preparations, much stronger shocks (20 V. or more) were needed. In a given slice, the impedance loss was found to vary with shock intensity. A strong shock was found to produce first an impedance decrease and then long-lasting impedance increase which could be interpreted as being associated with a contraction of the glial cells. These impedance changes were of the order of 5% of the resting value and did not vary appreciably with the reversal of the polarity of the applied current.

With a D.C. recording set-up instead of a Wheatstone bridge, it was found that an electric stimulus strong enough to produce impedance changes evoked a slow variation in the potential difference between the two sides of the slice. The procedure of electric stimulation was the same as in the preceding experiment. The time course of the slowly declining potential variation (up to a few mV.) across the slice was, in many instances, very close to that of the impedance changes. The polarity of this potential variation was reversed when the stimulating current was reversed, the compartment in which the stimulus cathode was immersed being always negative to the other compartment.

Since glial cells are the most numerous cellular elements in the brain (outnumbering neurons by a factor of 10) and since most of the neurons are undoubtedly damaged during the process of slicing the cortex, it seems reasonable to attribute the impedance and potential variations in the slice to the activity of glial elements. This in turn suggests that the neuroglia have several properties which are common to excitable cells. The similarity between the glial response and the "response" in a slime mold, *Physarum polycephalum*, has been suggested previously (26).

Demonstration of an electric response following direct stimulation of the cerebral cortex is well known (1, 9, 6). Freygang and Landau (21) demonstrated in the cat's cortex that a response to direct stimulation was accompanied by a simultaneous fall in the resistance of the cortical layer. Owing to the structural complexity of the intact cerebral cortex, it is probable, however, that the responses observed *in vivo* are more complex than those in the slice. It is also known that electric stimulation of a brain slice accelerates the tissue metabolism (31).

#### DISCUSSION

Neurons in tissue culture differ from those *in vivo* in one important aspect. 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. Although this is undoubtedly a shortcoming of our experimental material, this situation makes it extremely simple to interpret the experimental results obtained from tissue culture material. It is only under these experimental conditions that we can investigate the properties of central neurons without being confused by the effects of nerve impulses arriving at the synapses.

We have shown that neurons of the central nervous system in tissue culture, in response to intracellular stimulation, give rise to action potentials which resemble the responses obtained from neurons *in vivo* by previous investigators (see *e.g.*, 18). The finding that some neurons in tissue culture are firing impulses spontaneously (Fig. 5) is not surprising for investigators in the field of tissue culture. It is well known that isolated skeletal muscle fibers in tissue culture twitch spontaneously (28, 29). The present investigation indicates that spontaneous firing in neurons does not necessarily arise from the synaptic membrane.



In recent years there have been many confusing reports concerning the excitability of the neuronal membrane. Among the investigators who employed gross metal electrodes for recording cortical responses, Claire and Bishop (11), Purpura and Grundfest (33) and others believe that dendrites are not capable of developing action potentials. Chang (9), on the contrary, argued that the surface-negative responses to direct cortical stimulation derived from propagated impulses in the dendrites. More recently Fatt (17) working on the cat's motoneuron and Andersen (2) analyzing the electric responses from the hippocampus came to the conclusion that nerve impulses can invade the dendrites. Eccles (15) also expressed the view that dendrites are excitable.

Neurophysiologists who investigated the excitability of various neurons with hyperfine microelectrodes are also divided into two groups. Recording from the cat's geniculate body and cerebral cortex, Tasaki *et al.* (36) suggested that slow negative (or slightly diphasic) extracellular responses may arise from dendrites. Quite recently, Terzuolo and Araki (38), using two intracellular microelectrodes, recorded full-size action potentials from two points of a single neuron simultaneously and expressed the view that the dendritic responses were longer in duration than the soma responses. However, Freygang and Frank (19, 20), working on the cat's geniculate body and spinal motoneurons, asserted that not only the dendrites but also the soma membrane were inexcitable. In generalizing the results of recent experiments on invertebrate neurons, Bullock (7) came to the conclusion that the dendrite and soma membrane was incapable of developing all-or-none responses and that this was the basis of integrative action of the higher nervous system.

Under the conditions of the present experiments, both the neuron soma and the dendrites were found to be excitable and capable of carrying impulses (see Figs. 4, 5, 7, 9, 10). Since the response recorded from the dimpled surface of the neuronal membrane disappeared completely when the direct contact of the recording microelectrode with the surface was broken, there seems no doubt that the observed negativity represents the existence of an inward-directed membrane current at the site of recording. Such a current cannot have been derived from an action potential localized in a remote area. At least the basal (about 100  $\mu$  long) portions of the dendrites are capable of carrying nerve impulses in both directions. We could not explore the properties of the portions of the dendrites far from the neuron soma.

One might argue that the dendrites *in vivo* were inexcitable but they became excitable *in vitro*; but, in view of the experiments suggesting the presence of excitability in the dendrites *in vivo*, such an argument appears very weak.

The velocity of the nerve impulse travelling along the dendrite was found to be 0.02–0.05 m./sec. at 26°C. At body temperature, it is probably of the order of 0.1 m./sec. It is possible that the dendrites *in vivo* are capable of carrying impulses at a faster rate. The velocity obtained by Chang (9) and by Andersen (2) is between 0.05 and 0.2 m./sec.

The surface of membranes of neurons *in vivo* are in contact with glial

cell processes and presynaptic fibers. It was found in the present investigation that the resistance of the glial membrane (3–10 ohm·cm.<sup>2</sup>) was far smaller than the accepted values for the neuron. Coombs *et al.* (12) and Frank and Fuortes (18) reported a resistance of 400–2000 ohm·cm.<sup>2</sup> for the cat spinal motoneurons. The values for other neurons are also in this range (cf. 35). According to Rall's (34) rigorous analysis, the neuronal membrane resistance is higher than the values estimated by previous investigators. This means that the resistance of the glial membrane is less than a few per cent of the neuronal membrane resistance. Because of this low resistance, electric currents produced by neurons are expected to flow through the glial cytoplasm and not through the gap between the neuronal and glial membranes.

We have presented experimental results showing that a short, strong pulse of electric current evokes a kind of response in the glia cells—namely, a reversible reduction in the resting potential associated with a reduction in the membrane resistance. This "glial response" was very slow, lasting for several seconds after stimulation. The intensity of the extracellular electric field necessary to evoke such responses was 5–15 V./mm. in the brain slice; it was of the same order of magnitude in glial cells in culture (based on the assumption of a spherical symmetry of the field produced by a current of 10  $\mu$ A at a distance of about 25  $\mu$ ). This strength of stimulus is within the range employed by many neurophysiologists for direct cortical stimulation. For instance, Burns (8, p. 52) applied 0–25 V. across a distance of 0.5–1 mm. Clare and Bishop (11, p. 86) used up to 15 V. across two points only 50  $\mu$  apart.

A stimulus strength of 5–15 V./mm. corresponds to a field of 75–225 mV. across a glia cell of 15  $\mu$  in diameter. Since the resistance of the glial membrane is found to be very low, the current intensity necessary to elicit a "glial response" is probably far stronger than the value a neuronal membrane can normally produce. For this reason, it seems unlikely that such large potential variations in glial cells as those shown in Fig. 14 appear in intact animals. It is possible, however, that a long continuous activity of neurons can alter the state of the surrounding glial cells and, conversely, a continuous activity of glial cells may alter the state of neurons. Recently, Hydén and Pigon (27) and Galambos (22) emphasized the importance of the neuroglia in brain physiology in general.

#### SUMMARY

Neurons and neuroglia from young cat and rat cerebella were cultivated *in vitro* either in a plasma clot or on the surface of reconstituted collagen gel. Morphological properties of the neurons in tissue culture were discussed. By means of hyperfine glass-pipette microelectrodes, action potentials from neuron somata and dendrites were recorded intracellularly under direct visual observation. By the use of extracellular microelectrodes, the existence of spontaneous firing of impulses in the neurons in tissue culture was demon-

strated. It was shown that both neuron somata and dendrites were excitable and capable of producing all-or-none propagated impulses under these experimental conditions. The velocity of impulse propagation in the basal part of the dendrite was of the order of 0.1 m./sec. The membrane resistance of neuroglial elements was measured by using two intracellular microelectrodes and was found to be 3–10 ohm · cm<sup>2</sup>. Strong electric stimuli applied to neuroglial cells caused a transient reduction of the resting potential which recovered to the normal level in about 5 sec. Evidence was presented indicating that such a "glial response" was accompanied by a reduction in the membrane impedance.

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