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SPONTANEOUS BIOELECTRIC ACTIVITY OF NEURONS IN CEREBELLAR CULTURES: EVIDENCE FOR SYNAPTIC INTERACTIONS.

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INTRODUCTION

Neurons of mammalian and avian central nervous tissue maintained as thick organ cultures, e.g., by the Maximow chamber technique (26), may differentiate and mature (25), they exhibit bioelectric activity (10), and the cultures retain at least some of their biochemical properties (1,40). Cellular associations such as myelin sheaths (4,29,32) and synapses (3,11) develop in vitro. The thickness of these organ cultures (100μ to 300μ) may be a disadvantage for some investigations especially with regard to the accurate placement of electrodes near or into specific parts (such as dendrites) of a neuron.

By using the roller tube technique (8,19) most areas of the culture rapidly thin out into a nearly two-dimensional layer of nerve cell bodies embedded in a matrix of glial cells and neuronal and glial processes, therefore allowing good visibility of individual neurons and their processes under the phase contrast microscope. In spite of the transformation of the tissue from a thick slice to a membrane-like flat arrangement, which is achieved by this latter culture method, a variety of distinct types of neurons survive and mature and myelin forms in vitro (17). While Hild and Tasaki (19) emphasized the absence of electrophysiological evidence for synaptic interactions in this type of culture, synaptic endings were later observed with the electron microscope in similar cerebellar cultures (5,22). Neurons in such cultures have excitable

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membranes, and many of the nerve cells exhibit spontaneous bioelectric spike activity (19,6,22).

Mammalian cerebellum is a desirable starting material for tissue cultures since the structure and function of the mammalian cerebellum has been studied in considerable detail (12). Therefore, comparisons between the cultured tissue and its in vivo counterpart may allow an evaluation of the degree of differentiation of the culture.

This paper presents an analysis of the spontaneous bioelectric activity of neurons and their interactions in cultures of rat cerebellum.

MATERIALS AND METHODS

1. Tissue Culture Methods. - Roller tube cultures (8) were prepared from the cerebellum of three to four day old rats (Sprague-Dawley, Simonsen, Gilroy, California) by a method similar to the one described by Hild and Tasaki (19). Sterile conditions were observed throughout the explanting and culturing procedure. The rats were decapitated, the roof of the cranium was removed, and the entire cerebellum was cut out with iridectomy scissors and placed in a drop of Gey's balanced salt solution (Microbiological Associates) enriched with glucose to a final concentration of 5.5 mg/ml. The cerebellum was cut sagitally into 6-8 pieces with triangular knives made from stainless steel razor blades. Two slices were then placed side by side in a drop of heparinized chicken plasma (Baltimore Biological Laboratories) on a 12 x 50 mm #1 Gold Seal coverslip and clotted with a drop of chicken embryo extract (Baltimore Biological Laboratories). After the clot had solidified, the coverslips were inserted into Falcon Plastic culture tubes and fed with 2 ml of culturing medium consisting of 75% Gey's balanced salt solution (Microbiological Associates) and 25% heat inactivated calf serum (North American Biological), with glucose concentration increased to approximately 5.5 mg/ml. No antibiotics were used.

The cultures were incubated at 36°C ± 0.5°C in roller drums tilted about 4-5° with respect to the horizontal axis, and rotating at 1/5 rev/min. Since Falcon Plastic culture tubes are permeable to CO_2, the atmosphere in the incubator was kept at approximately 4-5% CO_2 in humid air to insure control of the bicarbonate buffered medium at a pH near 7.0. The medium was exchanged weekly. After 12-16 days of incubation most cultures had thinned enough for clear phase contrast visualization of nerve cell bodies, myelinated axons, and some dendrites. At various times after that, representataive cultures were removed from the roller tubes, viewed under a phase contrast microscope and stained as whole mounts with Nissl stains (cresyl violet, toluidine blue, Einarson's gallocyanin chromalum, thionine and methylene blue-azure blue), with silver stains such as Bodian's protargol (26) and Holmes' silver impregnation (36), and with Sudan black B for myelin sheaths (31).

2. Electrophysiological Methods.- For electrophysiological experiments, the cultures were examined with phase optics and suitable areas for exploration with microelectrodes were selected. The coverslip bearing the culture was then mounted, culture down, on a bridge attached to the stage of a modified Bausch and Lomb phase contrast microscope with a 40X Zeiss objective. The open sides of this bridge allowed positioning of the microelectrodes under visual control (Fig. 1). The bathing solution (approximately 0.65 ml Gey's balanced salt solution enriched with glucose) was held in the chamber by surface tension and was exhanged frequently in order to avoid pH changes and accumulation of waste products, and to provide freshly aerated balanced salt solution. Ports for perfusion and exchange of the bathing solution were provided through the bridge mounts. A manual syringe pump consisting of two pairs of back-to-back 10 ml plastic syringes allowed the exchange of the bathing solution. All experiments were carried out at room temperature (24° to 27°C).

Glass micropipettes with tips of 4-6 μ filled with 0.9% NaCl and having a resistance of approximately 10 megohms were used to record extracellular action potentials. Two recording microelectrodes were placed under visual control simultaneously near two neuronal cell bodies. A platinum wire inserted into the shaft of each microelectrode was connected to a low noise, high input impedence, a.c.-coupled FET preamplifier (Applied Cybernetics Model 4UAH) mounted very close to the microelectrodes. A coil of platinum wire in the bathing solution served as reference electrode. The outputs of the preamplifiers were connected to separate channels of a storage oscilloscope (Tektronix model 564) and each, through Tektronix 122 amplifiers, to one channel of an 8-channel tape recorder (Precision Instruments model PI-6208) and to a discriminator pulse generator to make square pulses which were simultaneously recorded on the tape recorder for computer analysis.

3. Statistical Methods.- Statistical analysis of the spike trains was performed with PDP 8/I general purpose computer, using a specially written program. In addition to interspike interval histogram for each spike train the computer constructed first order "cross-interval histograms" for simultaneous spike trains from two nerve cells. For each such cross-interval histogram a null hypothesis cross-interval histogram was then calculated (30) and a Kolmogorov-Smirnov test (33) was performed to test whether the null hypothesis (H_0, independent firings in the two spike trains) should be rejected.

RESULTS

A. Morphological Characteristsics of Cultured Nervous Tissue. Within 24 hours after explantation, mesenchymal cells derived from the meninges and blood vessels, and glial cells began migration from the explant. The tissue flattened as macrophages engulfed debris and as cells migrated peripherally

with large nerve cells generally staying behind. Flattening was sufficient for transillumination for phase contrast microscopy starting about the tenth day in vitro, and by the third week, most parts of the culture were usually thin enough to permit the observation of considerable cellular detail. In most areas, the nerve cell bodies became arranged in a two-dimensional layer dispersed between glial cells and an extensive network of glial and neuronal processes. After 6-10 days in vitro many axons became myelinated.

Neurons were usually found in groups or nests and often in rows, within the confines of the original explant. Dendrites were often visible in areas of lower cellular density and could sometimes be followed for some distance from the perikaryon. The identification of cells as neurons in phase contrast can be confirmed by staining the culture as whole mounts with Nissl stains (Fig. 2) or by silver impregnation (Fig. 3 and 4). Neurons resembling Purkinje cells could be seen in almost every culture. Their characteristics in culture include (a) location in rows or groups, usually toward the periphery of the explant appropriate to their cortical origin; (b) often a pear-shaped cell body with a large oval nucleus and distinct nucleolus; and (c) a dendritic tree to one side of the cell body in a much reduced form compared to the in vivo morphology (Fig. 4). In central areas of the cultures, large multipolar cells, often surrounded by myelinated axons, and presumably derived from the sub-cortical nuclei of the cerebellum, were regularly found. No attempt was made to rigorously classify the other neuronal cell types of the cerebellar cortex.

B. Spontaneous Bioelectric Activity

1. Extracellular recordings from individual neurons. - By positioning the tip of the microelectrode under visual control within a few micra of the soma

of a neuron (Fig.5), spontaneous extracellular action potentials were recorded from a majority of the nerve cells tested. Spontaneous activity was found in cultures of 12 to 34 days in vitro; younger cultures were usually still too thick to allow accurate visual placement of electrodes; and much older ones often became spread too thinly and may have started to degenerate. With respect to the reference electrode, the spikes were sometimes negative, most often biphasic negative-positive, or triphasic with a small positive deflection proceeding the major negative-positive potential, frequently with an inflection in the negative swing. At times, large positive or positive-negative potentials were seen (Fig. 6). Spikes of two different amplitudes could sometimes be simultaneously recorded with the same electrode, presumably the smaller one originating from another nearby neuron. With the aid of a pulse height discriminator, the smaller potentials could easily be eliminated from the analysis. Spontaneous bioelectric activity was recorded from more than 250 neurons during the course of this study.

When the extracellular microelectrode was withdrawn from the immediate vicinity of the neuron soma, the amplitude of the spike decreased rapidly with increasing distance. Beyond a certain distance (more than 10 to 15μ, with electrode just sitting in balanced salt solution), no signals have ever been detected, nor have any signals ever been found by placing the electrode extracellularly onto presumed glial or mesenchymal cells.

In order to assess the origin of the extracellular spikes, the cell body near the recording electrode was damaged by puncturing it with a second (ultrafine) electrode from the opposite side. In every case the large action potentials (as shown in Fig. 7) recorded extracellularly disappeared following the entry of the damage-inflicting electrode tip, at times immediately, at other times after a few high frequency "injury" discharges. The injured cell body could then be seen to disintegrate (it turned rapidly granular under phase illumination) and seemed

subsequently to disappear. Thus it can be assumed with reasonable certainty that the recorded spikes are actually from the cell body near the electrode tip and not from any other small undetectable neurite.

2. Patterns of spontaneous bioelectric activity. - Spike activity could be recorded undiminished and presumably from the same unit, for five hours or longer at room temperature in balanced salt solution from large neurons (presumably mostly Purkinje cells and sometimes possibly Golgi cells), as shown in Figures 2, 3, and 4 and from neurons in denser areas of cerebellar cultures profusely surrounded by myelinated axons.

The average spike frequency recorded at room temperature in Gey's balanced salt solution from various neurons in different cultures ranged from a spike every few seconds to about 20 to 30 spikes per second. The frequency and pattern of firing usually remained fairly constant during the course of a recording session. Frequencies of 1 to 5 per sec were most often found, most often with highly non-uniform spike intervals (Fig.7a). In some cultures, neuronal spikes occurred in trains, with silent periods of sometimes several seconds between bursts and spike frequencies of up to 100/sec within the bursts. Sometimes doublet and triplet spikes, with the second and third spikes usually somewhat smaller in amplitude than the first one (Fig.7b), were found, and at other times, a very regular spike pattern with little variation in average frequency was recorded (Fig.7c). Neurons tested within the same area of a culture generally had a similar pattern, e.g., if one nerve cell showed a bursting activity, another cell in the vicinity also tended to discharge with a bursting pattern, although the average spike frequency could sometimes be quite dissimilar (see Figs.10 and 11). In Figs. 8 and 9 typical interspike interval histograms are shown demonstrating the irregular distribution. Short interval peaks are quite common (due to double spikes) usually accompanied by broad secondary peaks and tails resulting from long intervals.

3. Simultaneous extracellular recording from two neurons. - When unit activity was simultaneously recorded extracellularly with two microelectrodes from two randomly selected neurons, the somas of which were 20μ to 200μ apart (Fig.5), their spike activities were in most cases statistically correlated, i.e., the two simultaneous spike trains were not independent.

Fig. 10 shows the simultaneous recording of the bursting activity of two nearby neurons. In this culture, the bursts started and ended almost in unison with common silent periods between bursts. Within the bursts there was no complete synchrony between the spikes of the two trains; at times spikes could be seen in one neuron and not in the other. Representative examples of cross-interval histograms of pairs of neurons are shown in Figs. 11 and 12. It can be seen that the cross-interval histogram distributions vary considerably and that the deviation from the calculated null hypothesis distribution is different in every case, although the deviation is usually most significant near zero. A high probability of near-coincident spikes in the two neurons was a common feature in these cultures. During the course of this investigation, simultaneous extracellular recordings with two electrodes were made from 64 pairs of neurons (from over 50 cultures). The records of 41 pairs of cells were analyzed with the computer of which 29 cases showed a clear-cut, cross-correlation, i.e., the null hypothesis (H_0 = the two spike trains are independent) could be rejected with an $\alpha < 0.01$ (Kolmogorov-Smirnov two-tailed test). In 5 cases a moderate degree of correlation between the spike trains was found ($0.01 < \alpha < 0.20$) and in 7 cases no cross-correlation existed ($\alpha > 0.20$). Of the 23 pairs of neurons not analysed with the computer, 20 pairs had obvious cross-correlation, while no conclusion could be reached about the other three pairs. Therefore 77% of the neuron pairs tested showed statistically correlated spike activity.

In a few cases, a neuronal cell body was irreversibly damaged with the recording electrode while recording simultaneously from a second nearby perikarya. The activity in the damaged cell stopped, sometimes after a few typical injury discharges. The second cell always continued its spontaneous spike activity, although the discharge pattern sometimes seemed to change. Not enough experiments were done to assess the effect of distruction of one cell on the pattern of activity of another nearby neuron.

DISCUSSION

Hild (18), Cechner (6), Lumsden (22) and Mamoon (23) have described the morphological development of cerebellar roller tube cultures that are very similar to the ones used in this study and the present morphological description confirms and extends their results.

A majority of the neurons in a healthy culture exhibit a spontaneous spike pattern which can be recorded with carefully placed extracellular electrodes. The shape and duration of the biphasic negative-positive potential is very similar to the in vivo recordings from cerebellar Purkinje cells (13). At times, usually larger positive-negative spikes (Fig.6), were seen, resembling the extracellular potentials recorded by Woodward, Hoffer and Lapham (39) in the Purkinje cell layer of rat cerebellum. Crain (9) and Hild and Tasaki (19) have reported both negative-positive and positive-negative extracellular unit spikes recorded from cultured neurons. Both the shape and the average frequency of the action potentials recorded extracellularly from these cultures are temperature sensitive (15).

A variety of different discharge patterns are found in neurons of cerebellar cultures varying from patterns with uniform spike intervals to the more common highly non-uniform patterns (Fig.7). It seems that more uniform spike intervals are usually found in neurons situated in extremely thin areas of the cultures, whereas neurons embedded in a dense fiber matrix tend to have more irregular patterns (16). The irregular interspike interval histogram obtained from most neurons tested may be due to an endogenous mechanism, but it seems more likely that the observed patterns are the result of interacting units, in particular to synaptic inputs from a network containing excitatory and

inhibitory pathways; with at least some cells acting as pacemakers. Increased levels of Mg^{++} in the bathing solution or the addition of pentobarbitol to these cultures leads to a reversible regularization of the firing pattern (16). A similar phenomenon has been observed in vivo (24).

The correlation (more or less simultaneous firing) of the individual spikes from two nearby neurons (separated by 20 μ to 200 μ) even more strongly suggests the presence of nets of interacting units. The degree of correlation, i.e. the deviation of the cross-interval histogram from the calculated distribution can vary qualitatively and quantitatively from cell pair to cell pair although coincident firings are found most often (Figs. 11 and 12). The units sampled with microelectrodes represent two nearby points in the postulated net, possibly receiving inputs from common and from independent sources, in addition to their possible endogenous spontaneous activity. The exact connections probably vary greatly from culture to culture but one may assume that both excitatory and inhibitory synapses are involved.

It is probable that the spike correlations found in the cultured presumed Purkinje cells are due to common excitatory granule cell input as is observed in vivo (14). The observed spike discharge correlation could also be mediated by Purkinje axon collaterals impinging on Golgi cells to cause recurrent facilitation by disinhibition of granule cells, in turn causing discharges in other Purkinje cells as postulated by Llinas and Precht (21). In our cultures the density and organization of the granule cell input is certainly much decreased and its exact timing may have been lost. Cerebellar granule cells cultured under similiar conditions have been shown to have spontaneous bioelectric activity (22). In cultured mouse cerebellum, granule cells mature, migrate organotypically, and form axospinous synapses with Purkinje cells (37,38).

It is likely, although not directly demonstrated in this study, that the synapses mediating the observed interactions have developed in culture. In the mouse cerebellum, most of the synapses, especially the intracortical ones, develop between 7 and 14 days postnatally (20), that is after the tissue has been put in culture.

Although non-synaptically mediated interactions between units in these cultures cannot be completely ruled out, the vastly increased extracellular spaces in these cultures make interactions mediated by electric fields (34) or by ionic changes in the extracellular spaces (28) unlikely. Even if present such interactions would show up only with a small probability among two randomly selected neurons.

Electrotonic junctions between neurons have been found in the mammalian brain(2), and such junctions could result in a correlation of the firing pattern. Glial cells and a special class of inexcitable neurons in cultures similar to ours seem to be coupled by electrotonic junctions (35). The electrical response of these cells was similar to that of cultured glial cells (19). Cultured neurons with electrophysiological properties similar to _in vivo_ neurons (excitable membranes, etc.) as described in this paper did _not_ seem to be coupled electrotonically to each other or to other cells.

This investigation suggests that in spite of the transformation of the explant from a three-dimensional block of tissue into a thin, more or less two-dimensional structure, synaptic interactions between the neurons in culture persist or may even develop _de novo_. This result is in apparent conflict with the observations of Hild and Taski (19) who found no electrophysiological evidence for synaptic interaction in similar cerebellar cultures. Since they were primarily interested in the electrophysiological parameters of individual cultured neurons and their dendrites, it seems likely that they chose cells in areas of low cellular density where dendritic processes could be

followed over long distances (see, e.g., their text figures 6 and 10), while our data is from areas of the culture which are of higher cellular density (figures 2-5) Gahwiler (16) has found that neurons in sparsely populated areas of such cerebellar cultures tend to have regular firing patterns, uninfluenced by elevation of the Mg++ concentration in the bathing medium from 0.9 to 8 mM/l, whereas neurons in denser area of the same culture usually exhibit irregular firing patterns (as in figures 8 and 9) which become reversibly regular with increasing Mg++ concentration. They interpreted this differential response to Mg++ as possibly being due to a paucity of synaptic inputs to spontaneously active Putkinje cells situated in peripheral areas of their cultures.

Nerve tissue cultures, especially these thin roller tube cultures are convenient material for the study of environmental effects on the cells and their interactions and they lend themselves quite naturally to the investigation of the development of intercellular interactions, such as formation of synapses, under controlled environmental conditions. The thinness of these cultures greatly facilitates the accurate electrode placement under visual control near or into specific parts of a neuron, while at the same time, organotypic tissue organization and function seem to maintained to a large degree.

SUMMARY

Explants from the cerebellum of newborn rats were cultured in plasma clots on flying coverslips in roller tubes.

Spontaneous bioelectric spike activity was recorded extracellularly from individual cultured neurons. The patterns of unit spike activity included bursts with irregular silent periods of sometimes several seconds, doublet and triplet spike patterns with intervals as short as 4-5msec, and at times regular firings. At room temperature, the average frequency ranged from a few spikes per minute to up to 100 spikes per second in some of the bursts. 50-300 spikes per minute was the most common. In general, the inter-spike interval histogram showed a non-uniform distribution of intervals. Spikes simultaneously recorded with two microelectrodes from two neurons separated by as far as 200μ showed, in 77% of the neuron pairs tested, varying degrees of correlation with each other, i.e., spikes from one neuron were accompanied within a small time interval by spikes from the other neuron more often than expected on the basis of chance.

From the close correlation of simultaneous spike trains from two neurons, it is concluded that the neurons in these thin cultures of rat cerebellum are synaptically connected to form complex nets of interacting units. They represent a simplified, isolated neural net accessible to visual observation and electrode exploration under controllable environmental conditions.

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BIBLIOGRAPHY

1. Appel, S.H.; and Silberberg, D.H.: Pyrimidine synthesis in tissue culture. J. Neurochem. 15: 1437-1443 (1968).
2. Baker, K. and R. Llinás: Electrotonic Coupling between Neurones in the Rat Mesencaphalic Nucleus. J. Physiol. (London) 212: 45-63, 1971.
3. Bunge, M.B., Bunge, R.P., and Peterson, E.R.: The onset of synapse formation in spinal cord cultures as studied by electron microscopy. Brain Res.6: 728-749 (1967).
4. Bunge, R.P., Bunge, M.B., and Peterson, E.R.: An electron microscope study of cultured rat spinal cord. J. Cell Biol. 24: 163-191 (1965).
5. Callas, G., and Hild, W.: Electron microscopic observations of synaptic endings in cultures of mammalian central nervous tissue. Z. Zellforschung. 63: 686-691 (1964).
6. Cechner, R.L. Neurons in vitro: A Tool for Basic and Applied Research in Neural Electrodynamics. Ph.D. Dissertation, Case Inst. of Technology, Cleveland, Ohio, 1967.
7. Cechner, R.L., and Fleming, D.G.: Long term spontaneous unit activity in cultures of neonatal rat brain. Biophys. Soc. Meeting, Feb. 1967.
8. Costero, I., and Pomerat, C.M.: Cultivation of neurons from the adult human cerebral and cerebellar cortex. Amer. J. Anat. 89: 405-467 (1951).
9. Crain, S.M.: Resting and action potentials of cultured chick embryo spinal ganglion cells. J. Comp. Neurol. 104: 285-329 (1956).
10. Crain, S.M.: Development of "organotypic" bioelectric activities in central nervous tissues during maturation in culture. Internat. Rev. of Neurobiol. 9: 1-43 (1966).
11. Crain, S.M. and Peterson, E.R.: Onset and development of functional interneuronal connections in explants of rat spinal cord - ganglia during maturation in culture. Brain Res. 6: 750-762 (1967).

12. Eccles, J.C., M. Ito and J. Szentagothai, The Cerebellum as a Neuronal Machine: Springer, New York, 1967.

13. Eccles, J.C., R. Llinás and K. Sasaki: Parallel Fibre Stimulation and the Responses Induced thereby in the Purkinje Cells of the Cerebellum. Exp. Brain Res. 1: 17-39, 1966.

14. Freeman, J.A.: The Cerebellum as a timing device: An Experimental Study in the Frog. In (R. Llinás, ed) Neurobiology of Cerebellar Evolution and Development, Chicago: Am. Med. Assn. Educ. & Res. Fdn. 1969.

15. Gähwiler, B.H., A. Mamoon, W.T. Schlapfer and C.A. Tobias: Effects of Temperature on Spontaneous Bioelectric Activity of Cultured Nerve Cells Brain Res. in press. 1972.

16. Gähwiler, B.H., A.M. Mamoon, and C.A. Tobias: Discharge patterns of spontaneously active Purkinje cells in cultures of rat cerebellum. Biophys. Soc. Meeting, Feb. 1972.

17. Hild, W.: Myelinogenesis in cultures of mammalian central nervous tissue. Z. Zellforsch. 46: 71-95 (1957)

18. Hild, W.: Cell types and neuronal connections in cultures of mammalian central nervous tissue. Z. Zellforsch. 69: 155-188 (1966).

19. Hild, W. and Tasaki, I.: Morphological and physiological properties of neurons and glial cells in tissue culture. J. Neurophysiol. 25:277-304 (1962)

20. Larramendi, L.M.H.: Analysis of synaptogenesis in the cerebellum of the mouse. In (R. Llinás, ed.) Neurobiology of Cerebellar Evolution and Development, Chicago: Am. Med. Assn. Educ. & Res. Fdn. 1969.

21. Llinás, R. and W. Precht: Recurrent facilitation by disinhibition in Purkinje cells of the cat cerebellum. In (R. Llinás, ed.) Neurobiology of Cerebellar Evolution and Development, Chicago: Am.Med.Assn.Educ.& Res.Fdn.1969.

22. Lumsden, C.E.: Nervous tissue in culture. In The Structure and Function of Nervous Tissue, Vol.1, ed.G.H.Bourne. Academic Press, N.Y., 1968, pp. 67-140.

23. Mamoon, A.: Effects of ionizing radiations on myelin formation in rat brain cultures, Ph.D. Dissertation. University of California, Berkeley, Calif. 1969.

24. Murphy, J.T., and N.H. Sabah: Spontaneous firing of Cerebellar Purkinje cells in decerebrate and barbiturate anesthetized cats. Brain Res. 17: 515-519 (1970).

25. Murray, M.R.: Nervous tissues in vitro. In Cells and Tissues in Culture Vol. 2, edited by E.N. Willmer. Academic Press, New York, 1965, pp.373-455.

26. Murray, M.R. and Stout, A.P.: Characteristics of human Schwann cells in vitro. Anat. Rec. 84: 275-293 (1942).

27. Murray, M.R. and Stout, A.P.: Adult human sympathetic ganglion cells cultivated in vitro. Amer. J. Anat. 80: 225-273 (1947).

28. Orkand, R.K., Nicholls, J.G., and Kuffler, S.W.: Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. J. Neurophysiol. 29: 788-806 (1966).

29. Périer, O. and deHarven, E.: Electron microscope observations on myelinated tissue cultures of mammalian cerebellum. In Cytology of Nervous Tissue Proc. Anat. Soc. GB and Ireland, Annual Meeting, 1961, pp. 78-81.

30. Perkel, D.H., Gerstein, G.L., and Moore, G.P.: Neuronal spike trains and stochastic point processes. II. Simultaneous spike trains. Biophys. J. 7: 419-440 (1967).

31. Peterson, E.R. and Murray, M.R.: Myelin sheath formation in cultures of avian spinal ganglia. Amer. J. Anat. 96: 319-355 (1955).

32. Ross, L.L., Bornstein, M.B. and Lehrer, G.M.: Electron microscopic observations of rat and mouse cerebellum in tissue culture. J. Cell Biol. 14: 19-30 (1962).

33. Siegel, S.: Nonparametric Statistics for the Behavioral Sciences. McGraw Hill, New York, 1956

34. Terzuolo, C.A. and Bullock, T.H.: Measurement of imposed voltage gradient adequate to modulate neuronal firing. PNAS 42: 687-694 (1956).

35. Walker, F.D. and Hild, W.: Neuroglia electrically coupled to neurons. Science 165: 602-603 (1969).

36. Wolf, M.K.: Differentiation of neuronal types and synapses in myelinating cultures of mouse cerebellum. J. Cell Bio. 22: 259-279 (1964).

37. Wolf, M.K.: Anatomy of cultured mouse cerebellum. II. Organotypic migration of granule cells demonstrated by silver impregnation of normal and mutant cultures. J. Comp. Neur. 140: 281-298 (1970).

38. Wolf, M.K. and M. Dubois-Dalcq: Anatomy of cultured mouse cerebellum. I. Golgi and electron microscopic demonstrations of granule cells, their afferent and efferent synapses. J. Comp. Neurol. 140: 261-280 (1970).

39. Woodward, D.J., Hoffer, B.J., and Lapham, L.W.: Postnatal development of electrical and enzyme histochemical activity in Purkinje cells. Exp. Neurol. 23: 120-139 (1969).

40. Yonezawa, T., Bornstein, M.B., Peterson, E.R., and Murray, M.R.: A histochemical study of oxidative enzymes in myelinating cultures of central and peripheral nervous tissue. J. Neuropath. Exp. Neurol. 21: 479-487 (1962).

FIGURE CAPTIONS

Fig. 1: Microscope stage for electrophysiological recordings from covership cultures. The chamber consists of a glass plate as floor and the coverslip bearing the culture as roof separated by two teflon blocks. The chamber is filled with balanced salt solution held by surface tension and the recording electrodes are introduced through the open sides.

Fig. 2: Group of nerve cells embedded between glial cells in a dense fibrillar matrix in a 19-day old cerebellar culture. The neuronal cell bodies are laid down in a two-dimensional arrangment on the covership. A: Living culture photographed under phase contrast; B: same area after staining as whole mount with cresyl violet.

Fig. 3: Group of cerebellar neurons from a 20-day old culture. A: Living, phase contrast; B: same area after fixation and staining with Holmes' silver impregnation. Note the rather dense axonal network with many terminals surrounding the neuron perikarya.

Fig. 4: A: Living neurons (Purkinje cells) from 26-day old culture photographed in phase contrast. Note characteristic dendritic tree under (d), pear-shaped cell bodies and large rounded nuclei. B: Bodian stained culture, 11 days in vitro showing dendritic tree (d) and axon (a) emerging from soma.

Fig. 5: Typical arrangement of extracellular recording electrodes near two neuronal cell bodies in a group of nerve cells.

Fig. 6: Examples of action potentials recorded extracellularly from cultured cerebellar neurons. In this and all following records positive deflections are upward. A:is a multiple trace photograph, B and C are single traces.

Fig. 7: Examples of spike patterns recorded extracellularly from cultured cerebellar neurons. A: Irregular, bursty distribution most often found; B: doublet pattern; C: rarer, very regular spike intervals.

Fig. 8: Interspike interval histogram with excerpt of the recording of the spontaneous activity of a nerve cell in a 22-day old cerebellar culture. Bin size: 5 msec., N = 3980 spikes, sampling interval: 11 min. 19 sec.

Fig. 9: Spontaneous spike activity recorded from a neuron in a 14-day old culture. Interspike interval histogram with 5 msec. bin size, N = 1630 counts and 4 min. 18 sec. sampling interval.

Fig. 10: Correlated bursting activity simultaneously recorded with two extracellular microelectrodes from two neurons (traces 1 and 2, respectively) approximately 50 μ apart. a: bursts recorded at slow sweep speed; b: beginning of a burst, and c: within a burst.

Fig. 11: Cerebellar culture, 22 days *in vitro*. A: Sample traces of spontaneous activity recorded simultaneously from two neurons 150 μ apart. B: Cross-interval histogram (histogram of first-order forward and backward waiting times as defined in reference 30). The intervals between any spike in trace 1 and the next subsequent spike in trace 2 are plotted to the right of zero, and the time between a spike in trace 1 to the most recent spike in trace 2 is platted to the left of zero *versus* the probability of the interval falling within the specified time bin. The solid line is the calculated null hypothesis (H_o = independence of firing of neurons 1 and 2) assuming random occurence of a spike in trace 1 with respect to the distribution of spike train 2. The cross-interval histogram is significantly different from the calculated distribution ($\alpha < 0.001$, Kolmogorov-Smirnov two-tailed test). Bin size: 5 msec. Number of counts in trace 1: 3980, in trace 2: 2677, sampling interval 689 sec.

Fig. 12: Cross-interval histograms with calculated null hypothesis distributions (solid lines) obtained as described in fig. 12. A: Recorded from two neurons approximately 150 μ apart in a 19-day old culture. Bin size: 5 msec., 700 and 1824 spikes recorded in neurons 1 and 2, respectively. Sampling interval: 284 sec. B: Two neurons 60 μ apart in culture 16 days *in vitro*. Bin size: 2 msec. Counts from neuron 1: 1939, from neuron 2: 4280. Sampling interval 343 sec. In both histograms the cross-interval distribution is significantly different from the calculated null hypothesis ($\alpha < 0.001$, two-tailed Kolmogorov-Smirnov test).