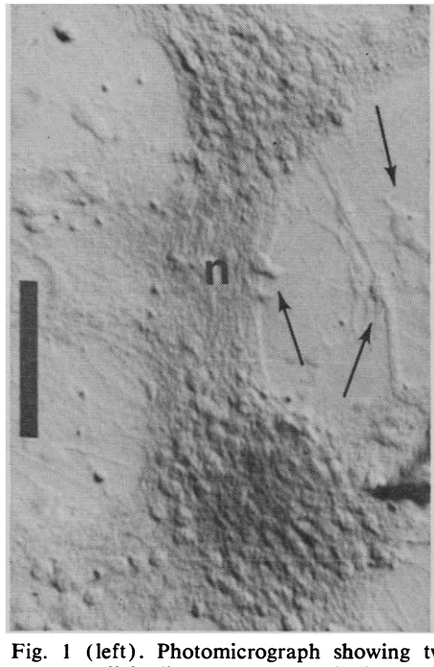
Organotypic Bioelectric Activity in Cultured Reaggregates of Dissociated Rodent Brain Cells

Abstract. *Complex repetitive-spike or slow-wave discharges can be evoked, and can also occur spontaneously, in small clusters of neurons which reaggregate in vitro after dissociation of cerebral cortex, brainstem, or spinal cord from the fetal mouse. Even after random dispersion in culture, these cells still form functional synaptic networks with bioelectric discharge patterns and pharmacologic sensitivities characteristic of the organ (that is, organotypic).*

Explants (~ 1 mm^3) of tissues from the central nervous system (CNS) of mammalian fetuses develop the capacity to generate synaptically mediated repetitive-spike or slow-wave discharges, characteristic of the organized CNS (organotypic), during maturation in culture (*1*). This occurs even when the tissue is isolated at stages prior to the formation of synapses in situ. Microelectrode recordings have also now been made on small clusters of neurons after reaggregation in vitro of cells, dissociated by trypsin, that were obtained from the cerebral neocortex of 18-day fetal mice or from spinal cord or brainstem of 13- to 14-day fetal mice ("presynaptic" stages). In studies of dissociated cells from the spinal cord and muscle of chick embryo in culture (*2*) intracellular postsynaptic potentials were obtained "which, in some cells, occurred in definite patterns and at regular intervals." Our data extend these experiments to cells of the fetal mammalian spinal cord and brain, and they demonstrate that complex organotypic patterns of bioelectric discharges can be generated by small reaggregated clusters developing from randomly dispersed neurons in culture.

The CNS tissues were dissociated with trypsin and cultured as described for chick neural tissues (*2, 3*), except that the cover glasses were coated with collagen and sealed into Maximow depression-slide chambers (*4*). The tissues were exposed to trypsin (0.25 percent) and repeated pipetting, and then suspensions of cells (~ 10^6 cells per milliliter of culture medium) were explanted onto collagen films, using 0.05 to 0.1 ml per cover glass (22 mm in diameter). Microscopic observation immediately after explantation indicated that the cells had been completely dissociated prior to culture. Cytologic studies indicated development of characteristic neurons and glial cells (*5*), both within the clusters and in the connecting neuritic bridges (Fig. 1). Development of abundant axon-dendrite and axon-soma synapses has been demonstrated by electron microscopy of these reaggregated neurons, as in intact explants of fetal cerebrum (*5, 6*), and in cultured reaggregates of dissociated cells from the spinal cord, retina, and spinal ganglion of the chick (*3*). Well-organized histologic patterns have also been observed in larger reaggregates developing in rotatory suspension cultures of dispersed cells from the brain of fetal mice (*7*).

Electrophysiologic studies on dissociated neurons from the CNS were carried out with similar techniques as with intact explants of the CNS (*1, 8*). Extracellular recordings were made with silver–silver chloride electrodes using pipettes (1- to 5-μm tips) filled with saline; the amplifier passband ranged from 0.2 hz to 10 khz. Electric stimuli (0.1 to 0.5 msec) were applied through



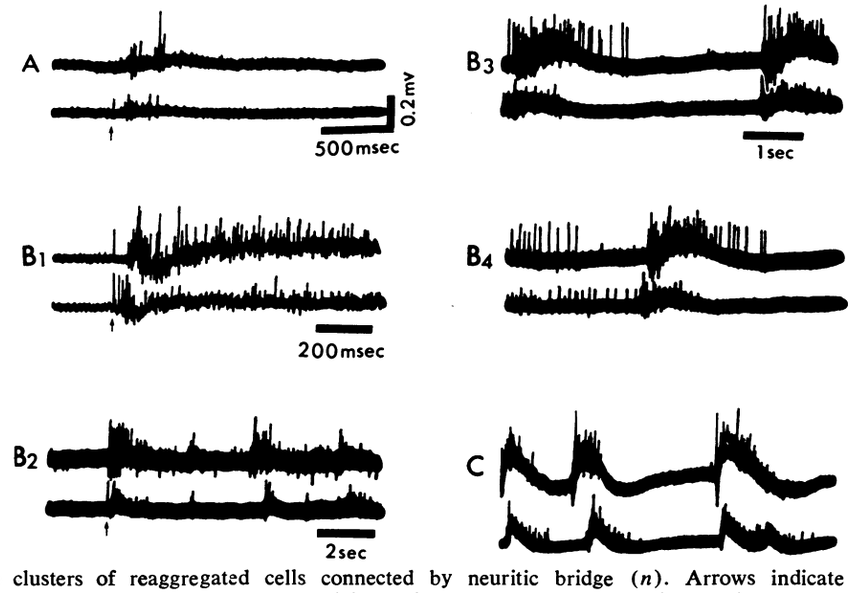


Fig. 1 (left). Photomicrograph showing two clusters of reaggregated cells connected by neuritic bridge (*n*). Arrows indicate neuron cell bodies near clusters in looser array. Scale, 100 μm. Fig. 2 (right). Complex bioelectric discharges in cultured reaggregates of brainstem and spinal cord tissues from fetal mouse, 2 weeks after dissociation. (A) Simultaneous microelectrode recordings of repetitive-spike barrages and long-lasting, negative, slow-wave responses in two similar clusters of reaggregated neurons (3 mm apart), elicited by a single stimulus applied to the intervening tissue. (B) After addition of strychnine to medium (10 μg/ml), evoked discharges were enhanced in amplitude, duration, and complexity (B_1 and B_2), and similar potentials also occur spontaneously, and synchronously, between these distant regions of the neuronal network (B_3 and B_4). (C) Records of similar complex, spontaneous discharges from two reaggregated clusters (~ 3 mm apart) in another culture of dissociated brainstem and cord tissues, under influence of strychnine. (Time and amplitude calibrations apply to all succeeding records, until otherwise noted.) Upward deflection indicates negativity at focal recording electrode; onset of stimulus is indicated by pulse (or arrow) below second sweep.

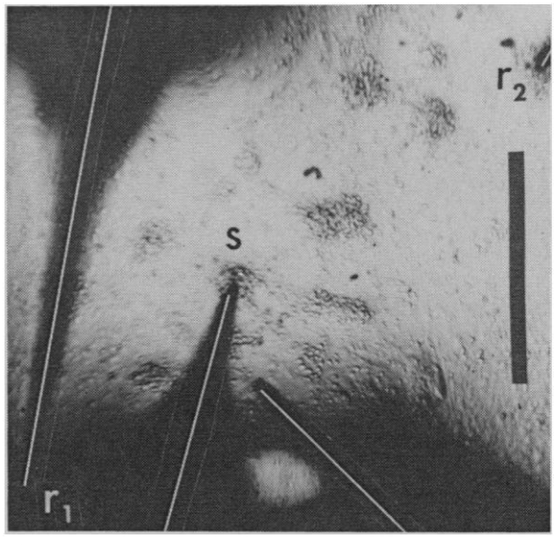
pairs of similar pipettes with 10-μm tips. The cover glass was transferred from the Maximow slide to a larger, closed, chamber that was mounted on an inverted microscope. Electrodes, attached to small micromanipulators incorporated into this sealed chamber, were brought up to individual cells (under microscopic observation) (Fig. 3) by the use of external magnetic controls (*9*, *10*). Recordings were made while the cells were in a bath containing about 0.5 ml of physiological salt solution (Simms balanced salt solution equilibrated with 3 percent CO_2 to maintain *p*H around 7.2), at 34° to 35°C.

After the cells were in culture 2 to 4 weeks, we recorded complex, evoked and spontaneous, repetitive-spike discharges from dozens of discrete neuronal clusters that were attached to the cover glass over an area of about 1 cm^2, and that were connected to one another by neuritic bridges (Figs. 2A and 4A). In the larger clusters with dozens of neurons, characteristic long-lasting potentials were often observed in association with the spike barrages. The complex bioelectric potentials recorded from each cluster were clearly generated within the cell cluster and were not merely due to impulses propagating along neurites passing through the cluster.

After introduction of strychnine into the medium (1 to 10 μg/ml) the amplitude of these slow waves, and the duration and complexity of the discharge sequences, were greatly enhanced (Figs. 2B and 4B). The complex bioelectric activities were rapidly blocked, on the other hand, by raising the Mg^{2+} concentration of the medium from 1 to 5 m*M* (Fig. 4, C and D), although spike potentials with short latent periods could still be evoked. The sensitivity of these reaggregated CNS neurons to pharmacologic agents is similar to that observed in larger intact CNS explants (*1*, *11*); the data suggest that the complex discharges generated in both types of CNS cultures are based upon synaptic network activity as occurs in situ (*12*). In some of the reaggregates, oscillatory (~ 10 to 15 per second) afterdischarge patterns occurred spontaneously, as well as in response to stimuli (Fig. 4B). These stereotyped, yet complex, rhythmic discharges have, until now, been observed only in well-organized, undissociated explants from the CNS (*1*, *13*), and resemble repetitive discharge patterns of neuronally isolated slab preparations of cerebral cortex of the neonatal cat (*14*). Analyses of similar rhythmic bioelectric activities in various regions of the CNS, in situ, suggest that they may be produced by complex circuits involving sequential generation of inhibitory, as well as excitatory, postsynaptic potentials (*15*).

Spontaneous and evoked activities in the neuronal reaggregates were often synchronized, even between clusters that were 2 to 3 mm apart (Figs. 2 and 4). The marked variation in latency periods of the discharges between clusters reflect slow propagation of impulses in immature neurites and complex polysynaptic delays. After the addition of strychnine, however, synchronization was greatly enhanced and even some of the small clusters containing only a few neuron perikaryons then showed patterned, long-lasting, repetitive-spike bursts concomitant with more complex discharges in larger clusters.

Since these dissociated neurons from the CNS can now be studied with cytologic and electrophysiologic techniques during the entire period of regeneration and aggregation in culture (*10*), this method should facilitate analysis of the



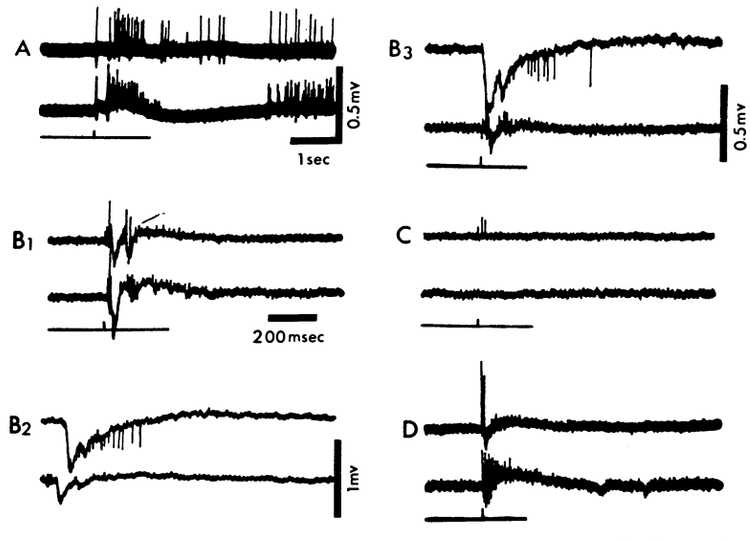


Fig. 3 (left). Photomicrograph obtained during electrophysiologic recording from this culture of aggregates of cells from the cerebral cortex of fetal mouse, 2 weeks after dissociation. (White line through axis of each micropipette; cluster r_1 not visible at this low magnification due to optical distortion produced by pipettes dipping into bath fluid). Scale, 1 mm. Fig. 4 (right). Organotypic spike barrages and oscillatory afterdischarges in culture seen in Fig. 3. (A) Long-lasting, intermittent bursts of repetitive spikes in two clusters of reaggregated neurons (r_1 and r_2) (2 mm apart) evoked by single stimulus applied to intervening clusters. (B_1) After addition of strychnine to the medium (10 μg/ml), slow-wave components are enhanced in amplitude, and rhythmic positive potentials (~ 15 per second) appear during long-lasting negativity. (B_2) Similar repetitive sequences also occur spontaneously, and synchronously, between the two clusters. There is a sequential increase in amplitude of spikes during spontaneous and evoked discharges (B_2 and B_3). (C) After increasing the concentration of Mg^{2+} from 1 m*M* to 5 m*M*, all complex discharges were blocked, and only brief spikes with short latent periods could be evoked. (D) Restoration of complex discharges after the concentration of Mg^{2+} was returned to 1 m*M*.

role of each cell of these synaptic networks in generating organotypic bioelectric discharges as a model of CNS activity.

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Freezing Resistance in Polar Fishes

Abstract. *Arctic and antarctic fishes, living in contact with sea ice at — 1.9°C, have plasma equilibrium freezing points near — 1.2°C which are dependent on salt concentrations. These supercooled fishes have plasma protein concentrations much higher than other polar animals have, and the proteins impede ice propagation at temperatures down to — 2°C. Plasma protein concentration increases as environmental water temperature decreases.*

Polar fishes living near shore in −1.9°C ice-covered sea water are exposed to conditions that would normally cause death by freezing in nonacclimated fishes. Fishes in the Labrador fjords were the first to be investigated for the presence of antifreeze in their blood (*1, 2*). I now compare freezing resistance in arctic and antarctic fishes and seek to illuminate the freeze-resistant properties of their blood.

Eleginus gracilis and *Myoxocephalus scorpioides*, two arctic species living in −1.8° and −1.4°C ice-covered sea water, respectively, were caught by a hook and line at a depth of 2 m near Nunivak Island, Alaska. Two antarctic shallow-water fishes (*Notothenia corriceps* and *Notothenia gibberifrons*) were caught in 2°C sea water near shore at Anvers Island, Antarctica. Two antarctic hemoglobin-free icefishes (*Chaenocephalus aceratus* and *Pseudochaenichthys georgianus*) were caught in deeper 2°C sea waters off Anvers Island, Antarctica.

Blood samples were usually taken from the posterior end of the caudal vein or by heart puncture. Measurements of colloid osmotic pressure were made by a stretch dialyzing membrane technique (*3*). The anticoagulant heparin was used for most samples, and its small contribution to the pressure was subtracted. Total plasma protein concentration was determined by a micro-Kjeldahl method (*4*). Plasma melting points, freezing points, temperatures of initial ice propagation, and rates of ice propagation were determined by a field technique modified from Scholander *et al.* (*1*). Modifications included a viewing port on the side of a copper box insulated with Styrofoam. Adjustment of the bath temperature was accomplished using heating wires connected to a variable transformer. With zero heat input, bath temperature remained constant at −4°C. Ice crystals were observed with a 20-power microscope. A 5-mm^3 plasma sample was frozen with "Spra-Freeze" (Laboratory Supplies Co.); then melting and freezing points were determined by observing the last small upward-floating ice crystal.

The melting point, determined to within 0.01°C, was the temperature at which the last small upward-floating crystal began to blur. The freezing point, measured with equal precision, was the temperature at which the edges of this last crystal began to sharpen. In NaCl solutions the melting and freezing points show a disparity of approximately 0.01°C when this apparatus is used (see Table 1). On the other hand, this disparity can be several times greater in a concentrated protein solution. Moreover, one must be careful to distinguish the equilibrium freezing point from the temperature of initial ice propagation in plasma. The former is 0.01° to 0.09°C below the equilibrium melting point, whereas the latter can be 1.00°C below the equilibrium melting point (Table 1).

Studies of ice growth in the plasmas of polar animals necessitated a trichotomy of ice propagative characteristics (Table 1). In the control group of animals, birds and mammals, ice propagation in plasma began at temperatures just below the equilibrium freezing point and the propagation rate was linearly dependent on how far the bath temperature was below this point. Ice growth occurred by bulk freezing and took the form of feathery plumes advancing through the plasma solution. This freezing behavior was also evident in solutions of NaCl, but in these macromolecule-free solutions ice began to propagate at the equilibrium freezing point. A second group of animals, polar bottom fishes which live supercooled at −1.8°C but never contact sea ice or anchor ice, showed a slightly larger disparity between the equilibrium freezing point and the temperature of initial ice propagation. Growing ice crystals took a dendritic form in this second group also. A third group, the nearshore fishes that live in contact with sea ice at −1.8° to −1.9°C, had strikingly different ice propagative behavior in their plasmas. When the bath temperature was dropped slightly below the plasma equilibrium freezing point in these fishes (noted by the sharpened ice crystal edge), a small amount of crystal growth occurred but then suddenly stopped. Upon further lowering of bath temperature (from 0.8°