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MEMBRANE PROPERTIES AND SELECTIVE CONNEXIONS OF IDENTIFIED LEECH NEURONES IN CULTURE

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SUMMARY

1. Individual, identified neurones, dissected from the central nervous system of the leech and maintained in culture for several weeks, sprouted processes and formed synaptic connexions.

2. The action potentials of isolated touch (T), pressure (P), nociceptive (N) cells and Retzius cells resembled those of their counterparts *in situ*, enabling them to be recognized unambiguously. Their input resistances were approximately 4 times greater than those of corresponding cells within the animal. In T, P and N cells trains of impulses were followed by a pronounced after-hyperpolarization, as in the animal.

3. In certain cells, notably the L motoneurones, membrane properties became altered in culture. The current–voltage relation showed novel rectification and action potentials became much larger.

4. Numerous neurites often extended for hundreds of micrometres from isolated neurones and ended in typical growth cones. Electron micrographs revealed that many fine axons were braided together to form thicker fascicles. Frequently, the processes were orientated between two neighbouring cells rather than at random. The fine structure of the cytoplasm, nucleus and organelles in cultured cells resembled those of their counterparts *in situ*. The glial cell that normally surrounds the neurones was, however, absent.

5. Pairs of Retzius cells in culture usually became coupled electrically after about 6 days. Similarly L motoneurones became coupled *in vitro*. These junctions allowed current to pass in both directions and resembled those seen in the animal.

6. Selective connexions were made by certain types of cells. Thus, P sensory neurones did not become coupled with Retzius cells but did develop electrical connexions with L motoneurones, as in the animal.

7. Novel synaptic interactions not obvious in the animal could appear in culture. Retzius and L cells became electrically coupled and, in some instances where electrical coupling between Retzius cells failed to develop, chemically mediated inhibitory potentials became apparent.

8. Isolated, identified leech neurones not only survive but regenerate processes and are capable of forming selective connexions in culture. The ability to define interactions between isolated pairs of cells offers the opportunity to explore in detail problems relating to synapse formation and cell–cell recognition.

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INTRODUCTION

Following a lesion to the central nervous system (C.N.S.) neurones of lower vertebrates and invertebrates are able to regenerate synaptic connexions with sufficient accuracy to restore function (Gaze, 1970; Anderson, Edwards & Palka, 1980). Although a number of mechanisms, such as axonal guidance and timing, may play a part in enabling the regenerating axons to find their correct targets, some form of cell–cell recognition is presumably involved in the final selection of the appropriate post-synaptic cell (Sperry, 1963).

A considerable simplification for studying the mechanism and specificity of synapse formation can be achieved by working with neural tissue in culture, where greater control is possible over the cells and their environment (Crain, 1976; Varon & Bunge, 1978; Banker & Cowan, 1979). Preparations used for such studies include explants of brain containing heterogeneous populations of neurones and glia, neurones dissected from the C.N.S., or peripheral ganglia and muscle fibres maintained in culture. In the present experiments we have dissected individual identified nerve cells from the C.N.S. of the leech and maintained them in culture. These neurones, which can be isolated by a simple mechanical procedure avoiding exposure to enzymes, offer distinct advantages for studying the selectivity of synapse formation. In the highly stereotyped ganglia of the leech a variety of sensory cells, interneurones or motoneurones have been identified, and the properties of the chemical and electrical synapses between them have been extensively studied (Muller, 1979). Thus, many different types of nerve cells can be kept alive in culture singly, in pairs or in groups and compared with their counterparts in the ganglion.

Our aims have been first to compare the membrane properties and fine structure of the isolated cells with those in the animal and second to examine the synaptic interactions that develop when cells are cultured together. Will cells form synapses in culture at random or selectively? If selective connexions are formed, how will these compare with the pattern of connexions seen in the animal? To approach these problems single identified cells can be placed repeatedly next to one another to determine the frequency and efficacy with which connexions are formed and to explore the range of compatible synaptic partners.

Isolated cultured leech neurones may also provide simplified preparations for studying the development of synapses and mechanisms of synaptic transmission. Within the C.N.S. of the leech, as in other invertebrates, experiments on these problems are complicated by the fact that synapses occur in the neuropile at a distance from the cell body. Hence, intracellular recordings made from the soma do not faithfully mirror the synaptic potentials; by the same token currents injected into the presynaptic cell body to modulate transmitter release or to spread through electrotonic junctions between coupled cells are attenuated and distorted at the terminals. In addition, the synapses are inaccessible for direct exploration by techniques such as ionophoretic application of drugs or transmitters, and sites of contact between cells are buried in a tangle of processes, complicating anatomical studies. In culture cells can be placed in direct contact and it may therefore be possible to develop synaptic connexions in which one can more fully analyse the anatomy, physiology and pharmacology of regenerated synapses between neurones.

The present study shows that isolated leech neurones survive, maintain normal resting and action potentials, sprout and form electrical and chemical connexions in culture. One example of preferential connexions has been observed. A brief account of some of these observations has been reported elsewhere (Ready & Nicholls, 1979).

METHODS

Dissection

Single cells were removed from the nervous system in a series of steps illustrated in Pl. 1 *A*. After opening the connective tissue capsule and washing away the glia (Kuffler & Potter, 1964; Nicholls & Kuffler, 1964), a fine nylon loop (Ethilon nylon monofilament, 13 μm in diameter, from Ethicon

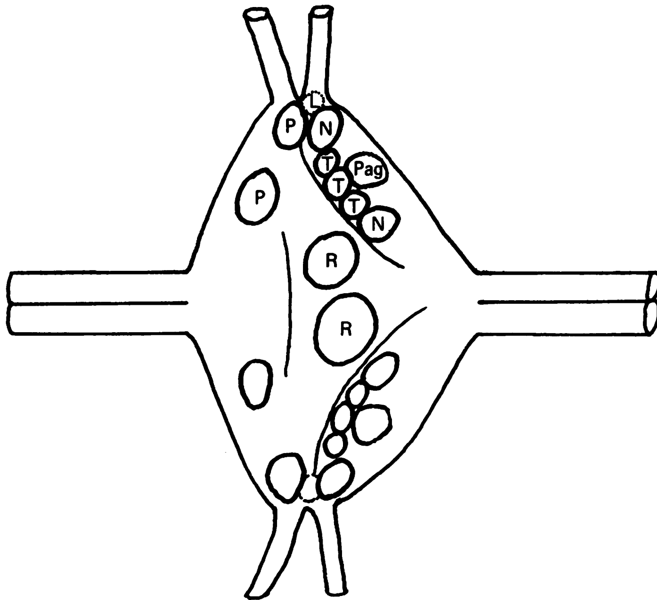


Fig. 1. Drawing of a leech ganglion in which the individual identified neurones used in these experiments are labelled. T, P, and N are the touch, pressure and nociceptive sensory cells, respectively, R is the large paired Retzius cell and L is the longitudinal motoneurone. Pag is the anterior pagoda cell, the function of which is unknown.

Corp., Somerville, N.J.) was slipped over the cell's soma, tied tightly around the initial process and the cell pulled away from the ganglion (Sargent, 1977). After 10 min the neurone was freed by shaking gently or by snapping the thread and transferred to the dish by a fine suction pipette with a constriction. After plating, the cultures were left for 1 hr at room temperature before being moved to the incubator. This gave time for cells to stick tightly to the dish bottom.

Certain cells such as the Retzius and P sensory neurones could be recognized unambiguously by visual inspection and were isolated under a dissection microscope set up in a laminar flow hood. N and T sensory cells, AE and L motoneurones and anterior pagoda cells were identified by recording intracellularly before isolation (Fig. 1). When cells were isolated under non-sterile conditions they were transferred through several drops of sterile medium before plating. Cell bodies, which were translucent before isolation, became white and opaque for a few hours before recovering their normal appearance.

Culture of single cells

Fig. 1 shows the positions in a typical leech ganglion of the various cells used for these experiments. Identified cells were cultured in 35 mm plastic tissue culture dishes (Falcon no. 3001) containing 2–3 ml. of medium (Leibowitz 15 plus 2% fetal calf serum (Gibco), 100 μg/ml. gentamycin (Schering Corp., Kenilworth, N.J.) and 0·6% glucose; 100 u./ml. mycostatin (Gibco) was added in some cases. To provide a sticky substrate, dishes were coated with rat-tail collagen (Bornstein, 1958) to which polylysine (1·0 mg/ml. in 0·1 M-sodium borate buffer, pH 8·2) was covalently bound using glutaraldehyde. Cells were positioned either as closely apposed pairs (Pl. 1 *B*) or at a distance from one another (Pl. 2*B* and Fig. 11). They usually adhered immediately. Cultures were prepared under sterile conditions in a laminar flow hood. Usually about twenty pairs of cells were placed in each dish. The cultures were maintained at 20 °C in a humid incubator and the medium was changed once a week.

Electrical recording

Glass electrodes (Haer ultratip capillary tubing) of 15–30 MΩ resistance filled with 4 M-potassium acetate were used to record from cultured cells. The recording techniques were conventional (Wallace, Adal & Nicholls, 1977).

Light microscopy

Cultured cells were photographed on a Leitz Diavert, using phase contrast optics.

Electron microscopy

The cultures were washed with leech saline adjusted with glucose to the osmolarity of Leibowitz 15 (as were all succeeding solutions: 380 m-osmole) then fixed in 0·8% glutaraldehyde (in 0·1 M-phosphate buffer, pH 7·2) for 1 hr. Cells were post-fixed in osmium tetroxide (1% in phosphate buffer) 1 to 2 hr, stained with uranyl acetate (1% in 0·2 M-acetate buffer), dehydrated and embedded in Epon. After hardening, the Epon wafer containing the cells was separated from the tissue culture dish by repeated immersion in liquid nitrogen. Small blocks of plastic containing the cell or cells of interest could then be cut out and sectioned. Sections were stained with lead, then examined and photographed on a Phillips 201 electron microscope.

RESULTS

Growth and structure of isolated neurones

Freshly isolated leech neurones adhering to the polylysine–collagen substrate appeared rounded, with no sign of the large process that had been ligated close to the cell body during the dissection. After about 5 days in culture a number of fine sprouts began to appear around the perimeter of the cells. Unlike their counterparts in the ganglion, which are unipolar, isolated leech neurones appeared to initiate neurite outgrowth at numerous points on the soma. Pl. 2*A* and *B* show the development of branches in isolated cells over several days. The neurites tended to track along each other, producing braided fascicles which grew approximately 10 to 20 μm in length per day. When two isolated cells were cultured within 50–200 μm of one another, many of the neurites spanned the area between the cells, as shown in Pl. 2*B* and Fig. 11. This characteristic appearance was often obvious by about 1 week. It is not clear whether fibres grew preferentially from cell to cell, or whether those fibres that contacted another cell were more likely to survive and acted as guides for others to follow. Single cells cultured on their own usually showed a more randomly orientated distribution of processes (Pl. 2*A*). No systematic differences were observed in the arborization of neurites produced by Retzius cells, sensory cells or motoneurones cultured with like cells or with dissimilar cells. The extent of sprouting shown by

isolated cells plated on polylysine over collagen varied unpredictably from dish to dish. In some dishes the cells survived but all of them failed to grow. In others, most of the cells would sprout, while the usual pattern was of successful sprouting by some cells but not others.

For more detailed examination of structure, Retzius cells in culture were compared with their counterparts *in situ*. Three weeks after isolation cytoplasmic components including mitochondria, rough endoplasmic reticulum, bundles of fine fibrillar material 0·5 μm in diameter and lysosomes appeared normal (Gray & Guillery, 1963; Coggeshall & Fawcett, 1964). Moreover the distribution of organelles into zones containing predominantly rough endoplasmic reticulum or mitochondria, Golgi bodies and lysosomes was preserved in cultured cells. Scattered throughout the cytoplasm were characteristic dense-core vesicles, 90 nm in diameter, resembling those described in normal Retzius cells containing serotonin (Rude, Coggeshall & Van Orden, 1969). Clear vesicles of smaller diameter (45 nm) were also found in the cytoplasm. Accumulations of both vesicle types were occasionally found in short processes that projected from the cell soma. A degenerative change occasionally seen in the cytoplasm was a roughly spherical region a few micrometres in diameter filled with irregular membranous debris, including occasional myelin figures. Moreover, the Golgi system appeared more extensive in cultured cells, perhaps in association with synthesis required for growth of processes.

A major difference between cultured and normal Retzius cells was the absence of glia. Within the ganglion, the soma of the Retzius cell is surrounded by one of the large 'packet' glial cells that invaginate the surface creating a trophospongium. During the isolation of a neurone the packet glial cell which surrounds the soma is destroyed, leaving only a coating of glial membranes and cytoplasm (Kuffler & Nicholls, 1966); in culture this was shed over the first few days, forming a small layer of debris around the cell (seen in Pl. 2). Frequently the cells cleaned this debris from a zone around their perimeter, suggesting activity by filopodia (Albrecht-Buehler, 1980). Also adherent to isolated cells were a number of small satellite cells that resembled fibroblasts and microglial cells (Gray & Guillery, 1963; Coggeshall & Fawcett, 1964). In culture some of these satellite cells often migrated a short distance from the neurone, while others remained on the cell body. They could survive for many days especially when the medium was changed frequently, but they did not divide extensively. After 3 weeks in culture the periphery of the cell was indented and irregular with intervening smooth areas. Most of the surface of the neurone was bare and in direct contact with the medium, except for regions in apposition to the occasional small satellite cells or other neurites.

The projections of Retzius cells that contacted the dish extended and branched into a profusion of neurites ranging in diameter from 0·1 to 1 μm (most being between 0·3 and 0·5 μm: Pl. 3). As shown in Pl. 3, growth cones exhibiting numerous spike-like extensions and thin membrane sheets occurred at the leading edge of outgrowing neurites. Neurites were attached to the substrate at numerous points along their length. Like the cell bodies, the processes were not ensheathed by glia (Pl. 3*C* and Pl. 4). The fine structure of the neurites appeared normal. Most contained longitudinally orientated neurotubules, as well as mitochondria and an extensive system of smooth endoplasmic reticulum. Occasionally, axons also contained fibrillar

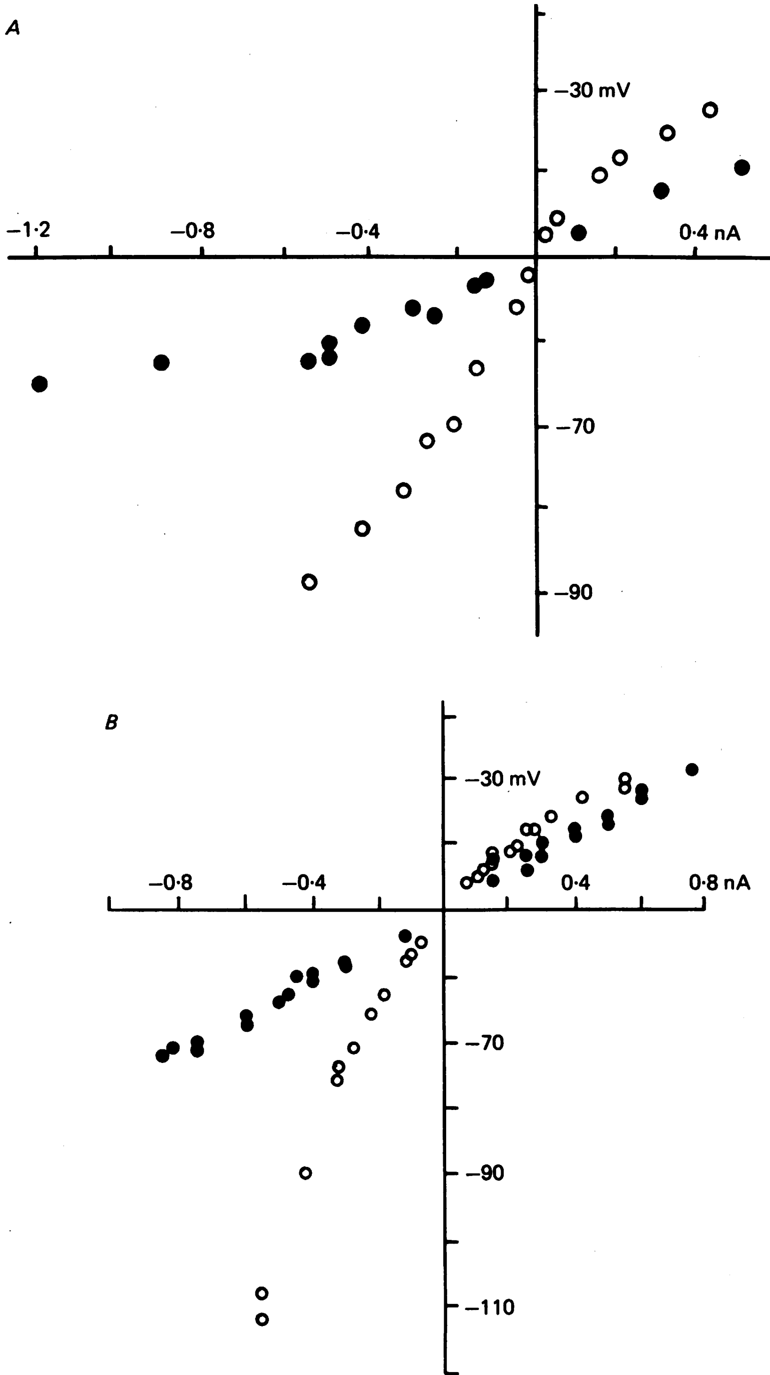


Fig. 2.

bundles up to 0·5 μm in diameter. Dense-core vesicles were dispersed throughout the length of most processes, while the smaller clear vesicles were found in numerous clusters containing from tens to hundreds of vesicles. Both types of vesicles were often observed at sites of contact with the substrate (Pl. 4).

A striking feature of Retzius cell neurites was the presence of varicosities along their length. These swellings occurred irregularly along processes at intervals of several micrometres, particularly in regions not directly in contact with the substrate. In phase contrast they appeared as dark swellings on the fascicles (Pl. 3). Typically, the varicosities were about 1 μm in diameter and 2 μm in length and contained concentrations of both clear and dense-core vesicles, as well as mitochondria and smooth endoplasmic reticulum (Pl. 4). In the animal comparable varicosities (colloquially named 'dingleberries') are normally seen on the processes of Retzius cells in the periphery and within the ganglion (Muller & McMahan, 1976; Muller, 1979).

Electrical properties of isolated neurones

Immediately after a cell had been isolated it became depolarized and the input resistance fell to a low value. Recovery of the membrane properties occurred within 1 hr. For example, in one Retzius cell the resting potential was −50 mV and the input resistance was 22 MΩ immediately before isolation. Twenty minutes after it had been tied off the resting potential was −12 mV and the input resistance only 8 MΩ. Within the next 10 min this cell recovered its normal resting potential and the input resistance had increased to 30 MΩ. After 60 min the input resistance was 72 MΩ.

The input resistance of cultured cells continued to increase for several days. In one series of experiments the average input resistance for four Retzius cells *in situ* was 20·7 ± 3·0 (S.E.M.) MΩ (normal Retzius cell input resistance can be as high as 30 MΩ). After 3 days in culture the average input resistance for these four cells was 129 ± 36 MΩ, with the highest value recorded being 240 MΩ. Since isolating a cell for culture entails removal of all its processes, one would expect such increases from the reduction in surface area alone. Furthermore, single isolated Retzius cells in culture were no longer electrically coupled to the contralateral Retzius cell as *in situ* and this could also increase the input resistance. In addition, we cannot rule out a possible increase in the specific membrane resistance of isolated cells as a factor.

The passive electrical properties of Retzius cells and sensory cells were in general

Fig. 2. *A*, voltage responses of cultured and normal Retzius cells plotted as a function of current injected through an intracellular electrode. Both cells were held at −50 mV resting potential with hyperpolarizing d.c. current. The normal Retzius cell (filled circles) was from the second ganglion of the leech nerve cord. Results from a Retzius cell isolated for 8 days are shown by open circles. The shape of the curve is similar for both cultured and normal Retzius cells, showing decreased resistance in both the depolarizing and hyperpolarizing directions. The input resistance (slope at the origin) of the normal Retzius cell was 21 MΩ, that of the cultured cell 120 MΩ. Measurements were made using two intracellular electrodes. *B*, voltage response of cultured and normal L motorneurones plotted as function of injected current. The L cell *in situ* (filled circles) showed a linear current–voltage relation; its input resistance was 25 MΩ. In culture L cells showed a marked change in the shape of the current–voltage relation, as well as an increase in input resistance. The cultured L cell (open circles) had an input resistance of 50 MΩ in the depolarizing direction, and 150 MΩ in the hyperpolarizing direction. Measurements were made using one electrode using a bridge circuit.

comparable with those recorded from each type of cell *in situ*, except for the increase in resistance. The steady-state current–voltage relationship for cultured (open circles) and normal (filled circles) Retzius cells is shown in Fig. 2*A*. Apart from a difference in slope the two relations are similar in form. L motoneurones, however, developed novel characteristics in culture (Fig. 2*B*). *In situ* the current–voltage relation was

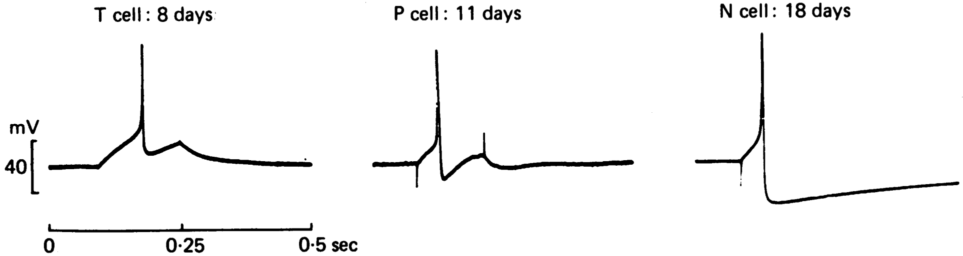


Fig. 3. Action potentials of T, P, and N cells after several days in culture. Each cell was from a different culture. The resting potential was −60 mV for the T cell, and −50 mV for the P and N cells. The characteristic shape of the action potential in each cell was similar to that recorded from homologous cells in the ganglion.

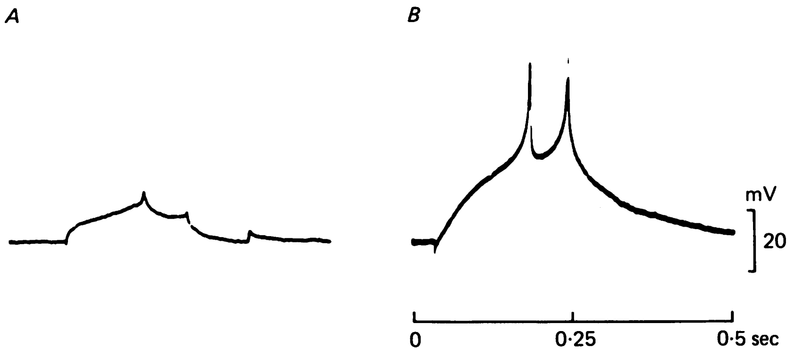


Fig. 4. Action potentials recorded from L motoneurones in the ganglion (*A*) and after culture for 10 days (*B*). Resting potential of the cultured L cell was −80 mV; the normal L cell was held at −82 mV with injected d.c. current. Depolarizing current pulses produced small (5 mV) responses in the normal L cell. Depolarization of cultured L cell produced an impulse of 30 mV, considerably larger than any responses seen in normal L cells.

linear over a large range, but in culture, in addition to the increase in slope near the origin, the curve showed a resistance increase with hyperpolarization and a resistance decrease with depolarization. This rectification was similar to that seen in T, P and N cells *in situ* (Baylor & Nicholls, 1969*a*) and in culture.

The resting potentials of various types of cultured cells were often larger than those reported for their normal counterparts *in situ*, which are usually about −40 to −50 mV. For isolated T, P and N sensory cells the values ranged from −50 to −70 mV; for cultured Retzius cells the range was −50 to −60 mV, but since the cells often fired upon penetration it was difficult to assess resting potentials accurately. The highest resting potentials were recorded from cultured L motoneurones: −80 to −100 mV, compared with −40 mV *in situ*.

The action potentials of isolated T, P and N cells were indistinguishable from those of their counterparts in the ganglion (Fig. 3). In addition, since they retained their characteristic features indefinitely in culture, the impulses provided a reliable and convenient confirmation of the identity of the cell. Similarly, Retzius cells in culture had action potentials that appeared unchanged. In contrast, cultured L cells showed changes. After about 10 days in culture the action potentials could be as large as 30 mV in amplitude, about 6 times larger than those of L cells *in situ* (Fig. 4). The ionic basis of these action potentials is not known. Presumably the soma of the islolated L cells became more excitable, since large action potentials were observed in neurones that had not sprouted.

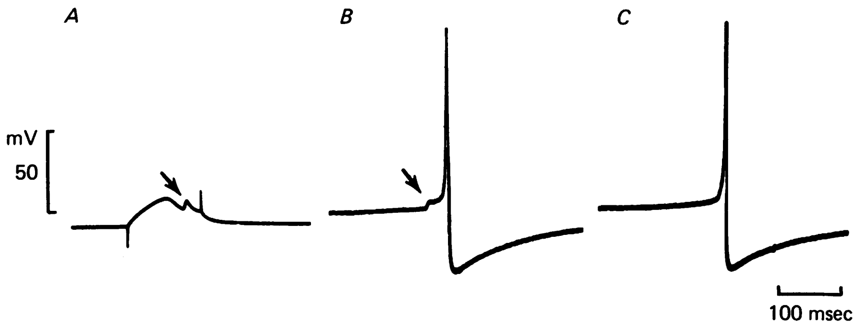


Fig. 5. Responses to depolarizing current in an N sensory cell cultured for 21 days. This cell had lengthy processes. In *A* the cell was held at −80 mV with injected current and a superimposed depolarizing pulse caused small spikes to appear, although the soma itself did not fire. In *B* the cell was allowed to depolarize to a resting level of −65 mV. Now a depolarizing current pulse elicited a full action potential from the soma, but it was preceded by a small prepotential, similar in amplitude to the 'axon' spike seen in *A*. Presumably the processes can be brought to threshold before the soma and initiate the action potential. *C* shows an impulse initiated in the soma when the N cell was further depolarized. These results suggest that, as in the ganglion, the processes are electrically excitable.

Outgrowth of processes seemed not to affect electrical excitability of the soma; the action potentials of cells which had sprouted did not obviously differ from those which had not. Indirect evidence suggested, however, that the membrane of the newly grown processes was electrically excitable. First, depolarizing pulses applied to cultured N cells that had sprouted gave rise to small, fast depolarizing potentials characteristic of axon spikes that failed to invade the soma (Fig. 5*A*). Second, d.c. depolarization of N cells and various other types of cells also gave rise on occasion to full-sized action potentials preceded by small prepotentials (resembling those seen in N cells within the ganglion). This suggested that activity in the processes had initiated the soma spike (Fig. 5*B*).

Additional evidence for the maintenance of normal concentration gradients and electrical properties by isolated cells is provided by Fig. 6. Trains of impulses in cultured sensory cells were followed by a prolonged after-hyperpolarization, resembling that seen in normal ganglia. There the mechanism underlying the potentials has been shown to be an electrogenic sodium pump and a prolonged increase in potassium conductance mediated by calcium entry (Jansen & Nicholls, 1973).

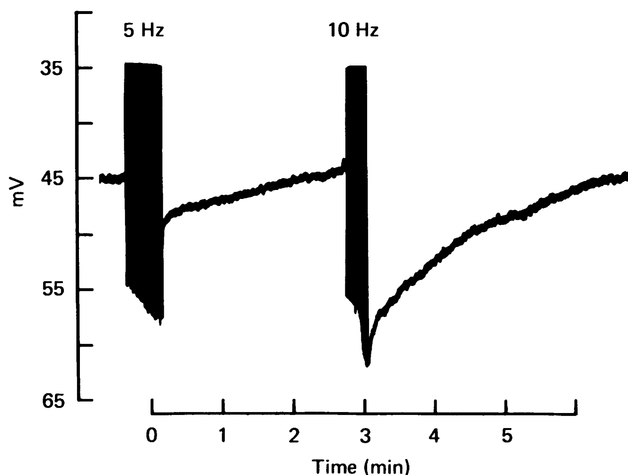


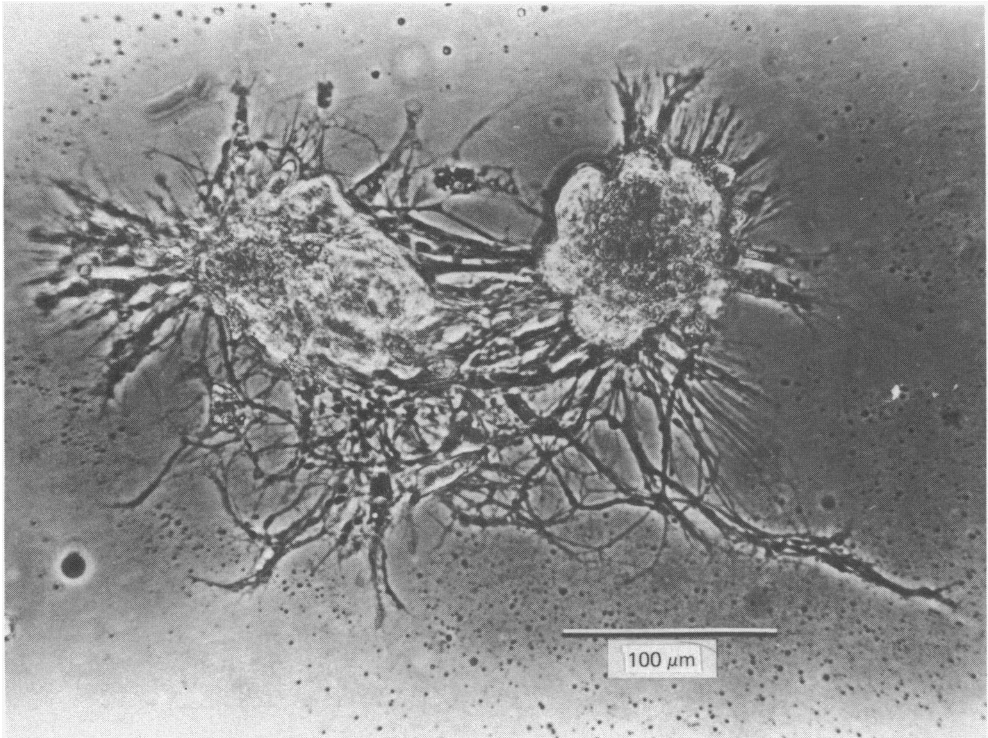
Fig. 6. After-hyperpolarization recorded from a P sensory cell maintained for 24 days in culture. Action potentials at 5 and 10 Hz were followed by a prolonged slow hyperpolarization similar to that seen *in situ.*

Electrical connexions of isolated cells

The development of electrical connexions in culture was followed by recording from various pairs of identified cells known to be coupled *in situ.* For example, the two Retzius cells in each ganglion are coupled by non-rectifying junctions, as are the two L motoneurones (Hagiwara & Morita, 1962; Stuart, 1970). The structure and location of these junctions within the neuropile are not known. In contrast, certain neurones are connected by rectifying electrical junctions. Thus, depolarization of T or P sensory neurones spreads to L motoneurones, but hyperpolarization does not (Nicholls & Purves, 1970); and in the course of these experiments (see below) similar but weak rectifying coupling was also seen between N and L cells *in situ.*

To test for coupling *in vitro,* pairs of cells were plated in direct apposition as in Pl. 1*B*. All of the cells were from an individual leech, but no attempt was made to combine cells with their original partners. Under these conditions Retzius cells usually became coupled to each other, as shown in Fig. 7. Similar results were obtained with pairs of L cells. As in the animal, both the Retzius and L cell connexions were non-rectifying (Hagiwara & Morita, 1962; Stuart, 1970). The efficacy of coupling was variable; occasionally, the coupling ratio approached 1 but in other instances the cells were weakly coupled or uncoupled (see below). Close apposition of cell pairs facilitated the formation of such connexions since pronounced outgrowth of processes did not always occur. However, cells at a distance from one another were also able to form electrical connexions by way of processes (Fig. 7).

Other identified leech nerve cells were able to form synapses in culture. The development of electrical coupling between sensory and motor neurones in culture was of particular interest since these cells are involved in reflexes that have been extensively studied in the leech. In culture, as in the animal, P sensory cells and L motor neurones became electrically coupled when placed next to one another. Moreover the spread of current showed clear rectification, resembling properties of



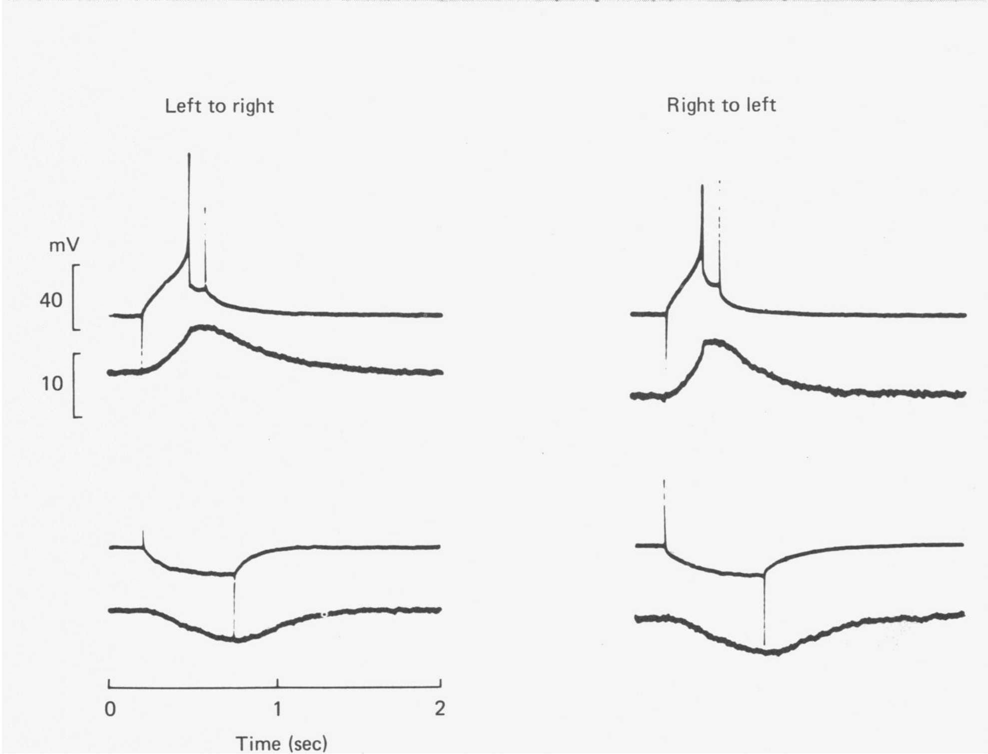


Fig. 7. Electrical coupling between a pair of Retzius cells maintained in culture for 21 days.

these connexions within the ganglion. Depolarizing current spread from the P cell to the L cell with 2 to 3 times greater efficacy than hyperpolarizing current, and hyperpolarizing current spread more effectively from the L cell to the P cell. Fig. 8 shows that N sensory cells and L motoneurones also became electrically coupled with similar rectification in culture. It was this finding that prompted re-examination of

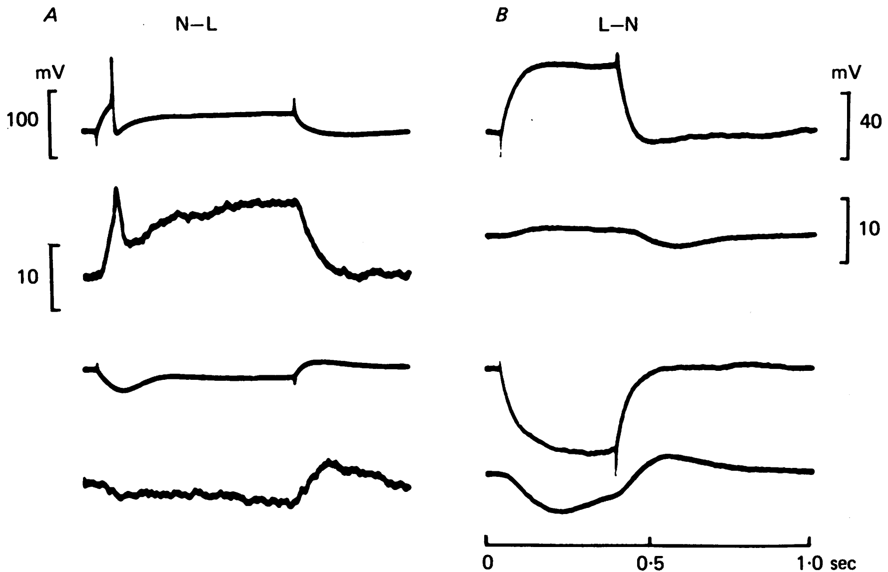


Fig. 8. Rectifying spread of current between an N cell and and L cell in culture. *A*, depolarizing current spread more effectively than hyperpolarizing current from the N cell to the L cell. *B*, conversely, hyperpolarizing current spread more effectively from the L cell to the N cell. The lower record in *A* shows that the N cell became transiently depolarized following the hyperpolarizing current pulse, giving rise to a clear depolarization of the L cell. Hyperpolarizing currents were used to maintain the resting potential of the N cell at −55 mV and that of the L cell at −100 mV. Electrical coupling between closely apposed N and L cells was usually found after about 10 days in culture. Similar rectification was observed between pairs of P and L cells that became coupled in culture.

the N to L synapse in the ganglion, where weak but definite rectifying coupling was observed for the first time, in addition to the more pronounced chemical coupling previously described by Nicholls & Purves (1970: see also Fig. 13). Such asymmetry in current flow between sensory cells and L motoneurones in culture and in the ganglion could result from rectification of the junctional membranes, the non-junctional membranes or both. (Rectification of the current–voltage relation in a cultured L cell is shown in Fig. 2*B*.)

The frequency with which electrical synapses developed varied from preparation to preparation. Out of more than 100 apposed Retzius cell pairs in which both neurones showed resting potentials of greater than −40 mV and overshooting action potentials, about 50 % became electrically coupled. It will be shown below that several pairs of the uncoupled cells showed clear signs of chemical synaptic transmission. Fewer experiments were made with L motoneurones since they were more difficult to isolate and maintain in culture. Nevertheless, seven out of nine pairs of L cells that did survive showed pronounced electrical coupling (5:1 or better). Out of twenty healthy, closely apposed N or P to L cell pairs, fifteen pairs became coupled.

Selectivity of electrical connexions formed in culture

To test whether isolated cells form connexions randomly or specifically with some targets but not others, combinations of Retzius cells, L motoneurones and P sensory cells were placed in close apposition. In one type of experiment, illustrated in Pl. 1*B*, the cells were paired as follows:

1. Retzius cells with other Retzius cells;
2. Retzius cells with P sensory cells;
3. P sensory cells with P sensory cells.

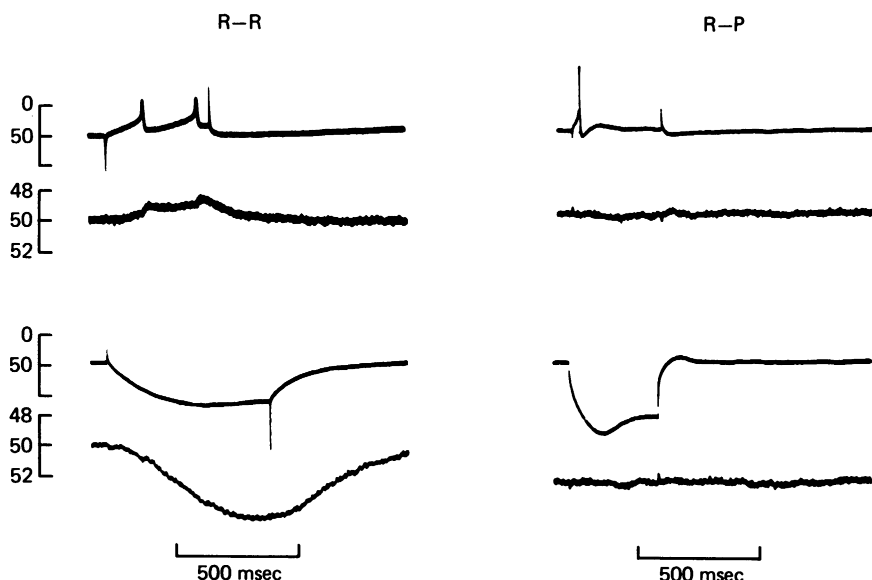


Fig. 9. Specificity of formation of electrical coupling by Retzius cells in culture. After 24 days in culture pairs of closely apposed Retzius cells (R–R) were found to be electrically coupled. In the same culture dish closely apposed pairs of Retzius and P cells (R–P) were not electrically coupled.

In five dishes maintained for 12–24 days, seventeen out of twenty-four pairs of Retzius cells became electrically coupled. Not a single instance of coupling was found between Retzius with P cells (twenty-two pairs) or P cells with P cells (eight pairs). The results of one such experiment are illustrated in Fig. 9. On the other hand, as described above, P cells did become coupled to L cells. The failure of P cells and Retzius cells to form electrical synapses therefore suggests that the development of connexions in culture is not an indiscriminate process but depends on some form of inherent cell–cell recognition.

A related but separate question is whether the connexions formed in culture mirror those seen in the animal. The results described above show that isolated cells are strikingly similar to their counterparts *in vivo*, both in the pattern of connexions and in the properties of the junctions. At the same time, certain differences become apparent in culture. For example, isolated Retzius cells became coupled to L cells (Fig. 10), a connexion one cannot discern in the ganglion by passing current. And P cells did not become coupled to P cells in culture, although there is evidence of weak

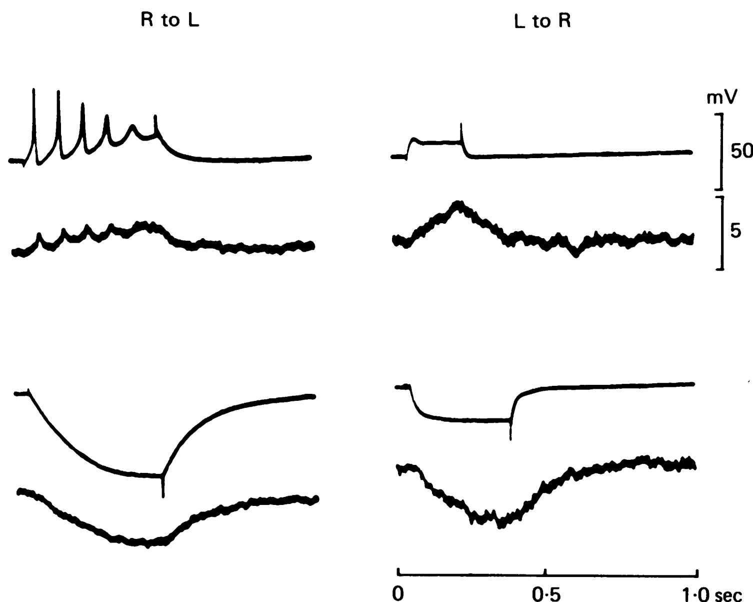


Fig. 10. Electrical coupling between a Retzius cell (R) and an L motoneurone (L) maintained in culture for 9 days. The resting potential of the Retzius cell was −50 mV, that of the L cell −40 mV. These cells were closely apposed. Coupling of this type is not readily discernible in the ganglion.

coupling in the ganglion. The possible reasons for such apparent discrepancies, which seem hardly surprising, are discussed below.

Chemically mediated synaptic interactions

Chemical interactions between cultured cells were observed far less frequently than electrical connexions. However, in certain pairs of Retzius cells, where electrical coupling was weak or absent, impulses or depolarization of one cell evoked slow hyperpolarizing potentials in the other (Fig. 11). The cells shown in Fig. 11 were some distance apart but similar responses were also seen in closely apposed cells. The long latency and time course of the hyperpolarizing potentials suggested the involvement of a chemical transmitter. This was confirmed by experiments such as that illustrated in Fig. 12, where the slow hyperpolarizing potential was reversed by hyperpolarization of the post-synaptic cell. These Retzius cells were not coupled electrically; when cells were coupled, it was not possible to determine whether inhibitory post-synaptic potentials were present, owing to the masking effect of the action potential undershoots.

In the ganglion the dominant synaptic interaction between N sensory and L motoneurones is chemical, with only a weak electrical component. In culture most N to L pairs failed to demonstrate a chemical connexion, although they became coupled electrically (see Fig. 8). In one pair of cells, however, electrical coupling was not obvious and action potentials in the N cell gave rise to clear depolarizing potentials in the L cell (Fig. 13). Unlike electrical coupling, this excitatory post-synaptic potential was labile and when thirty such responses were averaged, an 8·5

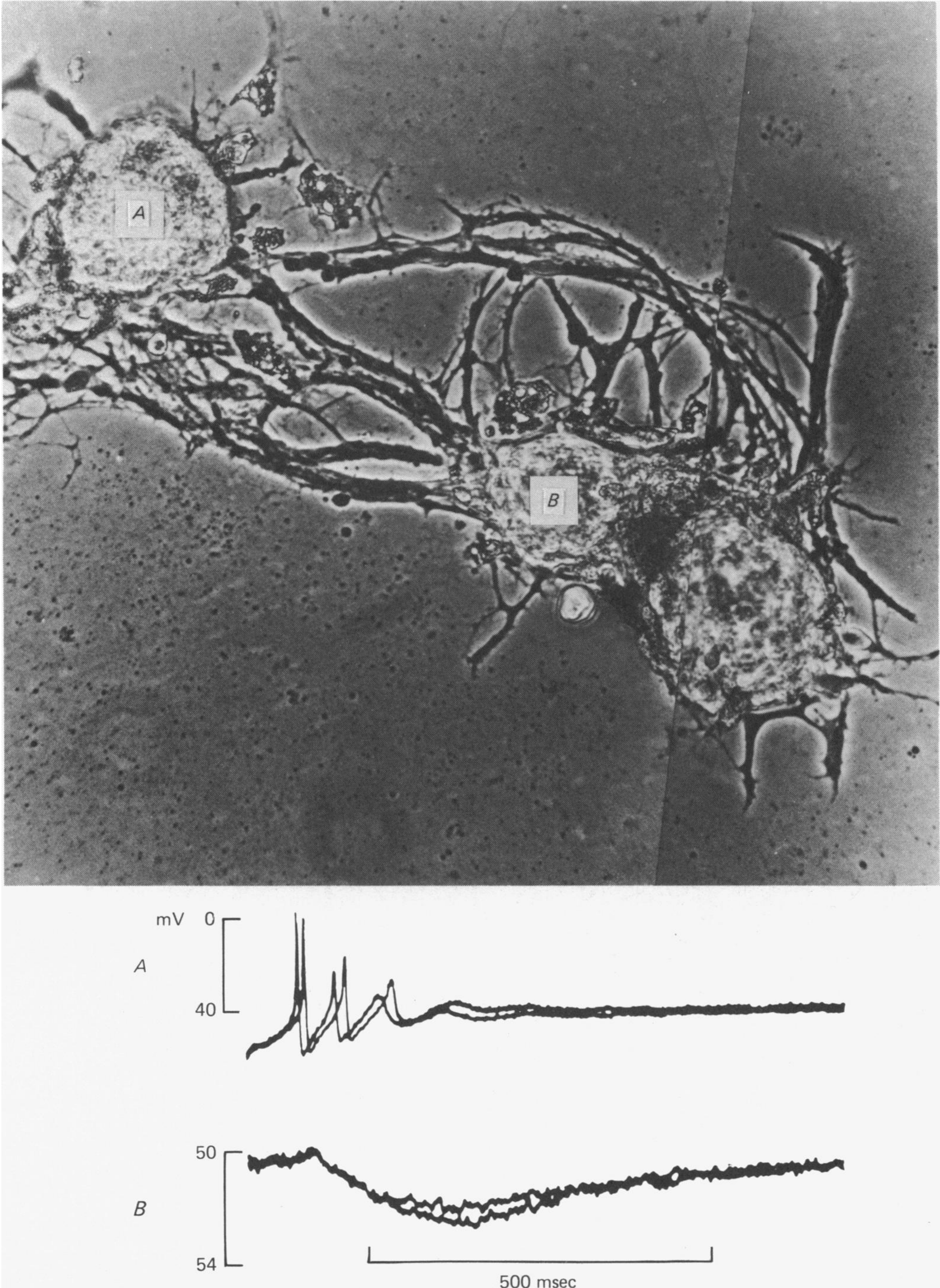


Fig. 11. Action potentials in one Retzius cell (*A*) Gave rise to hyperpolarizing responses in its neighbour (*B*) after 22 days in culture. These cells were only weakly coupled electrically. Impulses arose at the end of a hyperpolarizing pulse (not shown). Two sweeps are superimposed. Note the abundance of processes running from cell to cell, with fewer processes orientated randomly.

msec delay occurred between the peak of the presynaptic spike and the initiation of the excitatory post-synaptic potential, suggesting that this response was chemically mediated. However, without additional direct tests the mechanisms of transmission between these two cells remains less certain than the electrical connexions seen more frequently between isolated N and L cells.

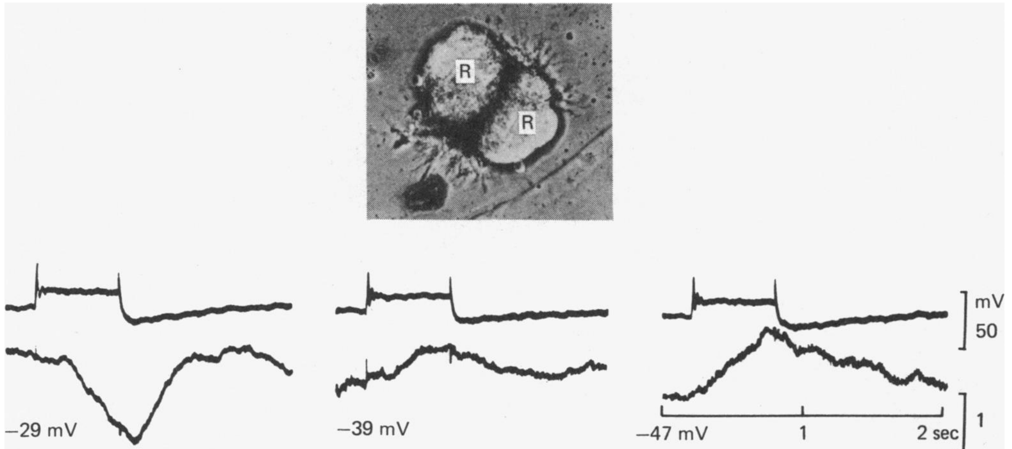


Fig. 12. Reversal of the slow chemical hyperpolarizing response in a Retzius cell after 18 days in culture. Here again prolonged depolarization of one Retzius cell gave rise to a slow hyperpolarization in its neighbour. Hyperpolarization of the post-synaptic cell caused a reversal of sign of this response. The membrane potential of the post-synaptic cell is given at the left of each trace (assessed by passing d.c. current through the bridge circuit of an intracellular electrode).

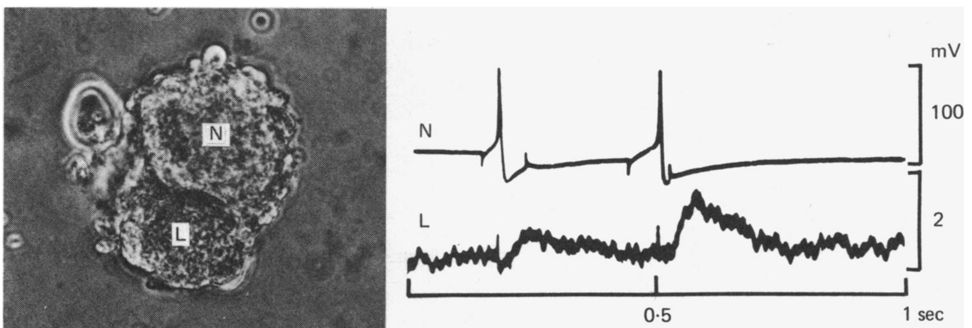


Fig. 13. After 6 days in culture action potentials in an N sensory cell gave rise to depolarizing responses in a closely apposed L motoneurone which had the appearance of chemical synaptic potentials. When thirty such responses were measured an average delay of 8·5 ± 3·7 msec from peak of the spike to the foot of the response was obtained.

DISCUSSION

Membrane properties and growth of isolated neurones

Torn from their normal surroundings and placed in a foreign environment, leech neurones survive well for several weeks. During the first days, in the absence of glia or normal blood, the neurones not only recover but sprout and form electrical and

chemical connexions. In addition, the fine structure of the isolated cells continues to appear normal, and even after several weeks in culture there is still a striking similarity between the membrane properties of isolated T, P and N sensory cells and Retzius cells and those of their counterparts *in situ*. Hence, the cultured cells preserve their unique sets of membrane conductances and maintain a roughly normal distribution of intracellular ions. Indirect evidence further suggests that for several types of cells the abundant new processes that sprout from a cell are able to generate impulses without changing the current–voltage characteristic of the cell body. This raises the possibility that newly formed membrane may have ionic conductance channels similar to those of the cell body.

In contrast, certain neurones show novel membrane properties in culture. When L motoneurones are isolated for several days, their resting potential increases, pronounced rectification with hyperpolarizing current developed and large impulses can be evoked in the soma. These changes suggest an increase in excitability of a normally inexcitable cell, similar to those observed following axotomy of insect and crayfish neurones (Pitman, Tweedle & Cohen, 1972; Goodman & Heitler, 1979; Kuwada, Hagiwara & Wine, 1979).

The increase in input resistance observed in cultured cells presumably occurs owing to the detachment of the cell from its processes and from other neurones with which it is coupled in the ganglion. The geometry of isolated leech neurones without sprouts makes it feasible to estimate the specific membrane resistance (R_m), since the soma is approximately spherical and isopotential for current–voltage measurements. For a spherical cell with an input resistance of 200 MΩ, of radius 40 μm, R_m would be 40000 Ω cm^2. Even higher values of R_m, 100000 Ω cm^2, are obtained from measurements of the prolonged membrane time constant, assuming $C_m = 1\ \mu F\ cm^{-2}$ (see Fig. 10). This discrepancy may arise because convolutions of the surface membrane seen in electron micrographs of cultured cells tend to make the effective surface area larger than that estimated from the radius. In any event, these values are considerably higher than those found for squid axons and are comparable to those in barnacle photoreceptors and *Aplysia* neurones (Graubard, 1975). At present we have no direct information about R_m of leech neurones *in situ*, but there also the length constant is large, suggesting high value (Frank, Jansen & Rinvik, 1975).

Formation of electrical connexions

The electrical synapses seen in culture resemble those in the animal in many respects. The appropriate cells can become coupled with properties that are appropriate with respect to rectification. The efficacy of Retzius to Retzius coupling in culture can be as high as 1:1, although in general coupling is less than that observed *in situ*. Synchrony of spiking, which is common in the animal, rarely occurs between pairs of cultured cells. The long time constant for coupling of cultured cells may account for some of this drastic attenuation of the action potentials. In our preparations cells are usually placed close to one another to facilitate the formation of connexions (Pl. 1 *B*). Hence the coupling ratios reflect the properties of the junction more directly than those measured in the animal, where spread of current is attenuated to a variable extent along processes within the neuropile. Presumably, then, the lower ratios observed in culture reflect a lower efficacy of coupling; this in

turn may be due to a smaller area of contact or to higher resistance junctions. The sites and structures of junctions that enable current to flow from cell to cell are not known in the animal or in culture. Although in theory specialized structures giving rise to high extracellular resistance could contribute, gap junctions or related membrane specializations seem more likely to account for coupling at the non-rectifying and rectifying synapses observed both in culture and in the animal.

We do not yet know how the time for which cells are kept in culture influences the properties and numbers of junctions. The first evidence of coupling appears at about 6 days in culture; however, transient junctions might have been formed and disrupted earlier. And at longer periods of several weeks further changes may develop.

Formation of chemical synapses in culture

We found chemical synapses between cells in culture only rarely, possibly as a result of our culture conditions. Moreover the synaptic potentials were small in view of the great increase in input resistance of isolated cells. Transmitter synthesis may be reduced or arrested, although the large numbers of normal dense-core and clear vesicles seen in electron micrographs of cultured Retzius cells suggest that transmitter synthesis continues *in vitro*. The slow hyperpolarizing potentials that developed between these cells might be the result of diffuse release rather than the formation of specialized pre- and post-synaptic structures. Another cause may be a failure to develop appropriate receptors in the membrane of regenerated neurites or to accumulate them at sites of transmitter release from the presynaptic terminal. It also seems likely that conditioning factors normally present in the blood or substrate are absent from our medium and that adding them or using higher density cultures would facilitate the formation of chemical synapses between the isolated cells that are bathed in a relatively large volume of fluid (O'Lague, MacLeish, Nurse, Claude, Furshpan & Potter, 1976).

Selectivity of connexions

One goal of the present experiments has been to develop a preparation in which questions concerning the specificity of neuronal connexions can be examined. Our first concern, therefore, has been whether isolated leech cells in culture form connexions according to fixed rules or randomly. Clearly some degree of selectivity exists for the formation of electrical synapses in culture, since certain connexions formed in culture parallel those found *in situ*. On the other hand the coupling of Retzius cells with L motoneurones and the inhibitory chemical connexions occasionally seen between Retzius cells have not been described in normal ganglia. It is possible that the strong electrical coupling between Retzius cells in the ganglion masks a weak inhibitory chemical synapse. At the same time, using electrical techniques to demonstrate connexions between neurones in the ganglion may be an indecisive criterion to apply. The electrical coupling between N sensory cells and L motoneurones is ordinarily not obvious in the face of the more pronounced chemical synaptic transmission between these cells. Conversely, it is now known that demonstrable electrical coupling between cells in the leech ganglion can be mediated not only by direct contact but also by a third interposed cell (Muller & Scott, 1979). Thus leech neurones in culture may demonstrate a pattern of cell–cell recognition which only appears to be different from that of their counterparts *in situ*.

For a number of reasons novel connexions are to be expected in culture: (1) neurones *in vitro* have been deprived of numerous cues that may influence the way in which they form connexions, such as growth along one tract or another; (2) it is reasonable to expect differences in the mechanisms used by immature cells to find their targets during development and those used by mature neurones during regeneration; (3) mechanical barriers present in the ganglion but not in culture might deny cells access to each other (for example, in the ganglion the processes of Retzius and L cells might always be kept apart by layers of glia); and (4) a cell's specific affinities may be relative rather than absolute and might change after isolation. Indeed, although leech neurones within the ganglia generally re-form their connexions with a high degree of precision (Wallace *et al.* 1977; Muller, 1979) novel interactions are observed after lesions have been made in the C.N.S. (Jansen, Muller & Nicholls, 1974) and when whole ganglia are maintained in culture (Wallace *et al.* 1977).

The selective formation of connexions by isolated neurones in our experiments must depend on inherent properties of the cells rather than on factors such as access to synaptic sites or competition. The present experiments set the stage for testing the ability of a particular cell to form synapses with a wide variety of potential targets and for defining cellular mechanisms involved in setting up a hierarchy of specificities. With two cells directly in contact and in the absence of a complex neuropile, the chemical and electrical synapses formed in culture also appear favourable for biophysical studies on intercellular movement of ions and small molecules, as well as transmitter release and chemoreception.

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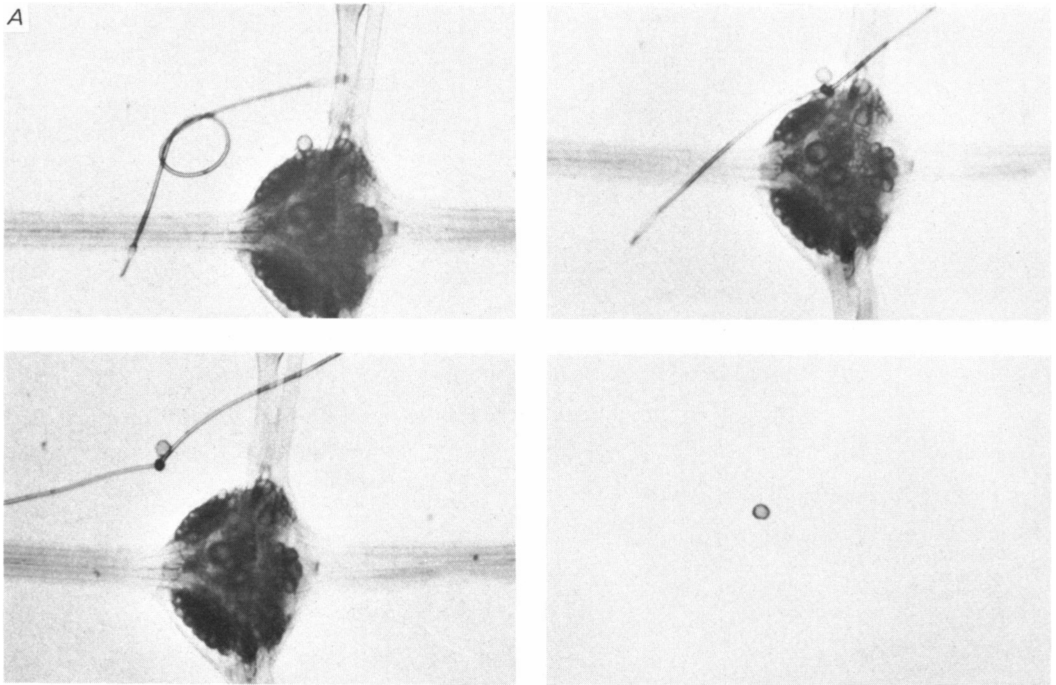

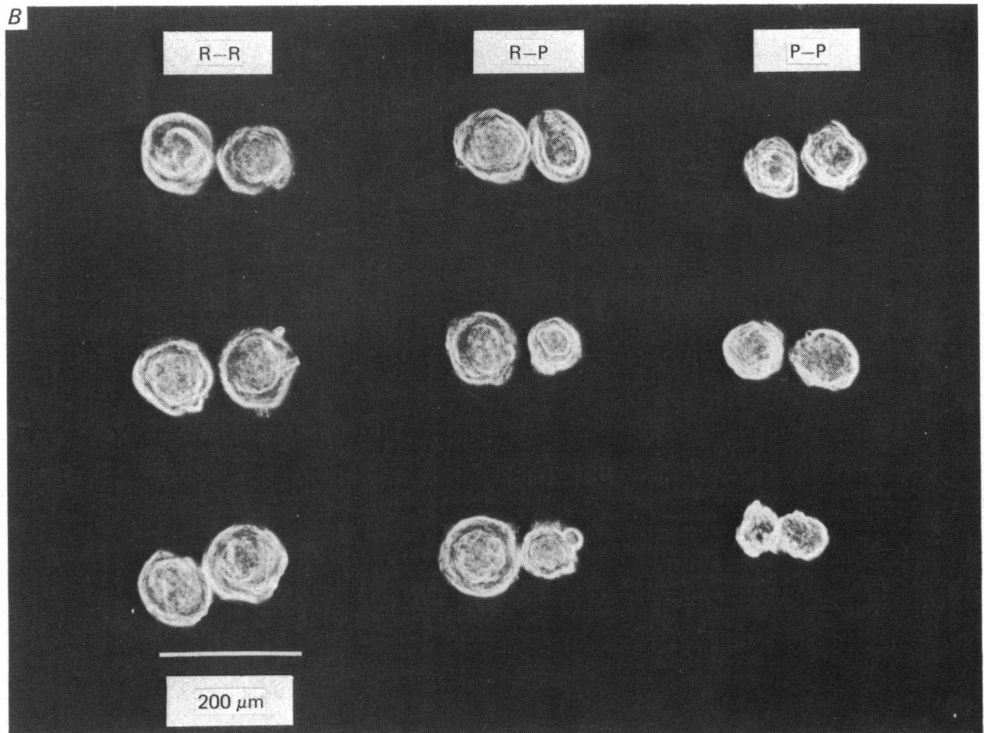

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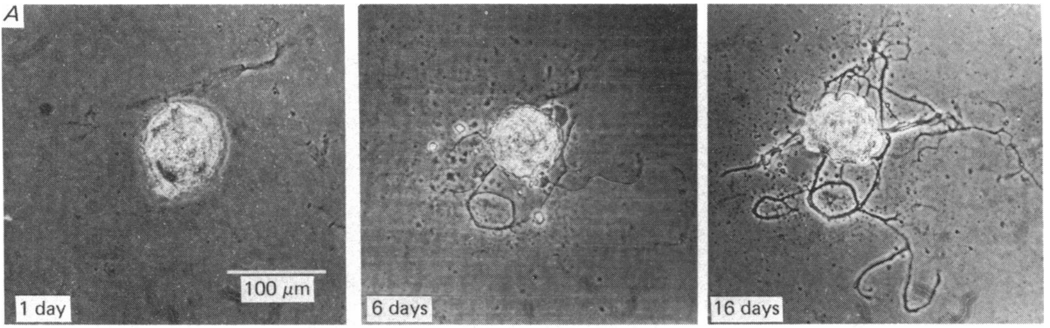

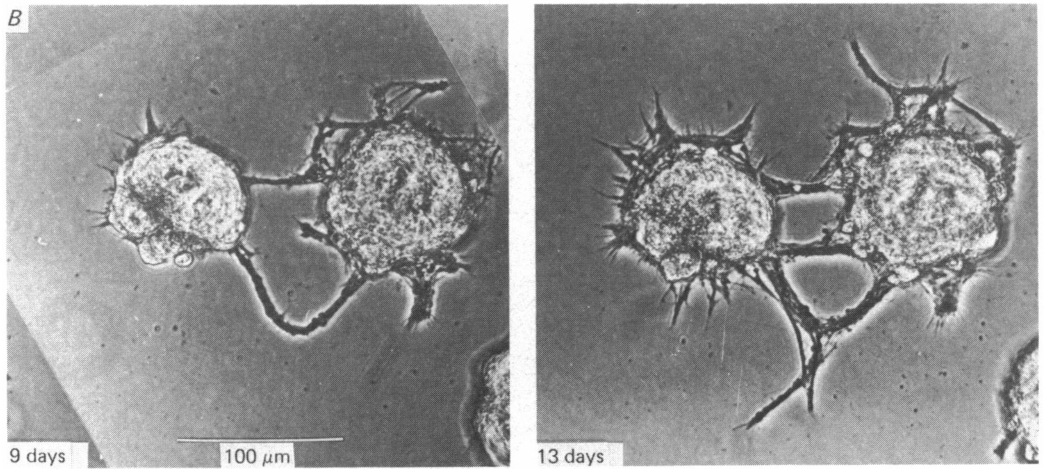

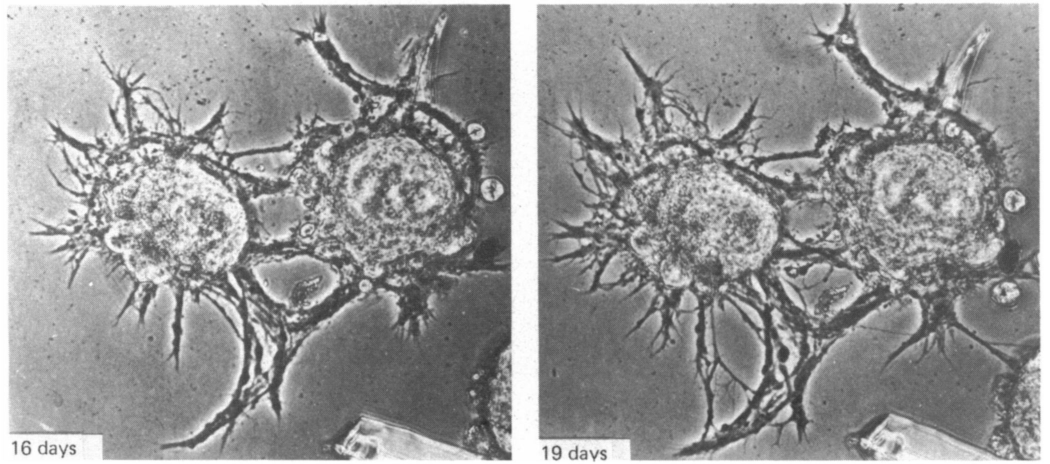

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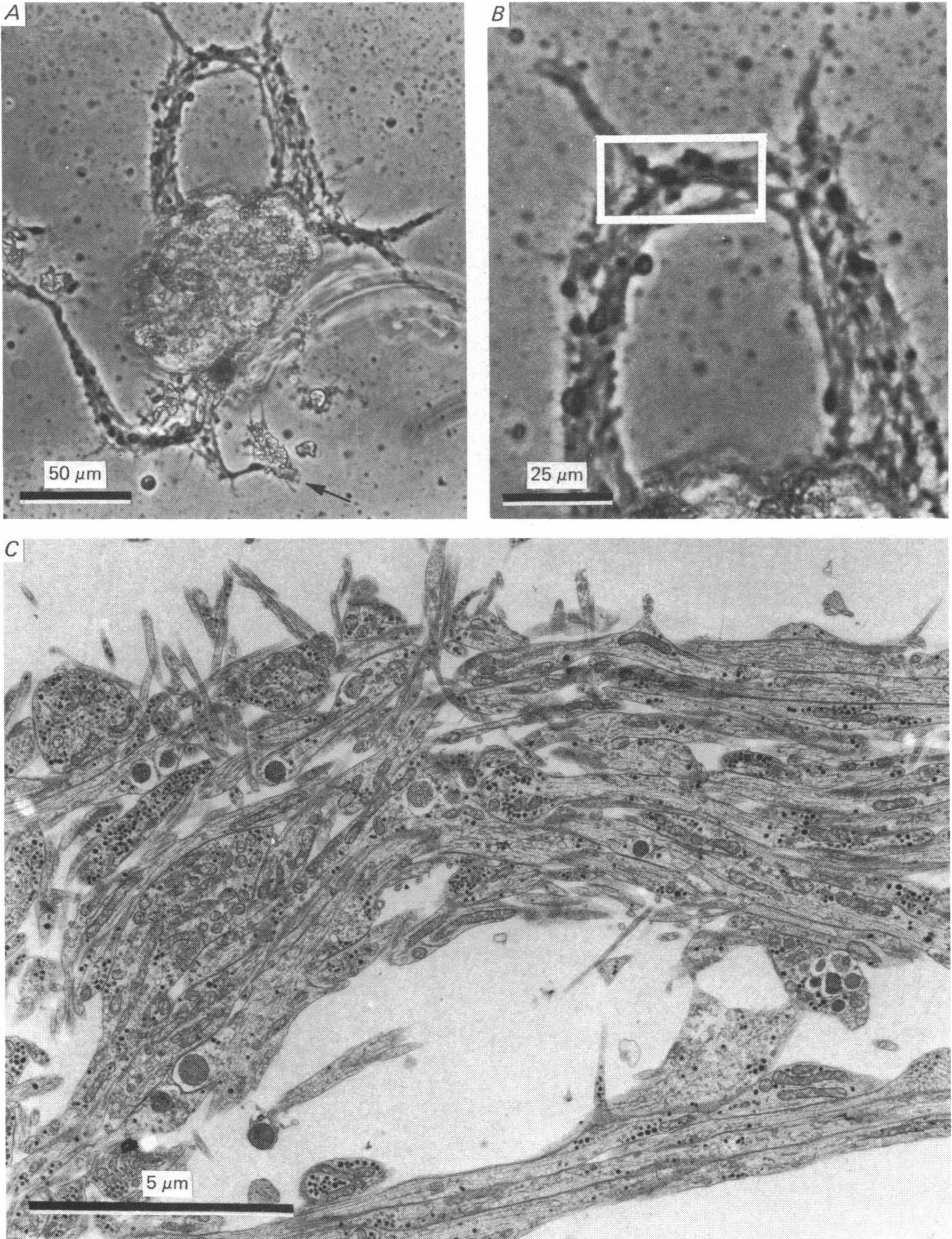

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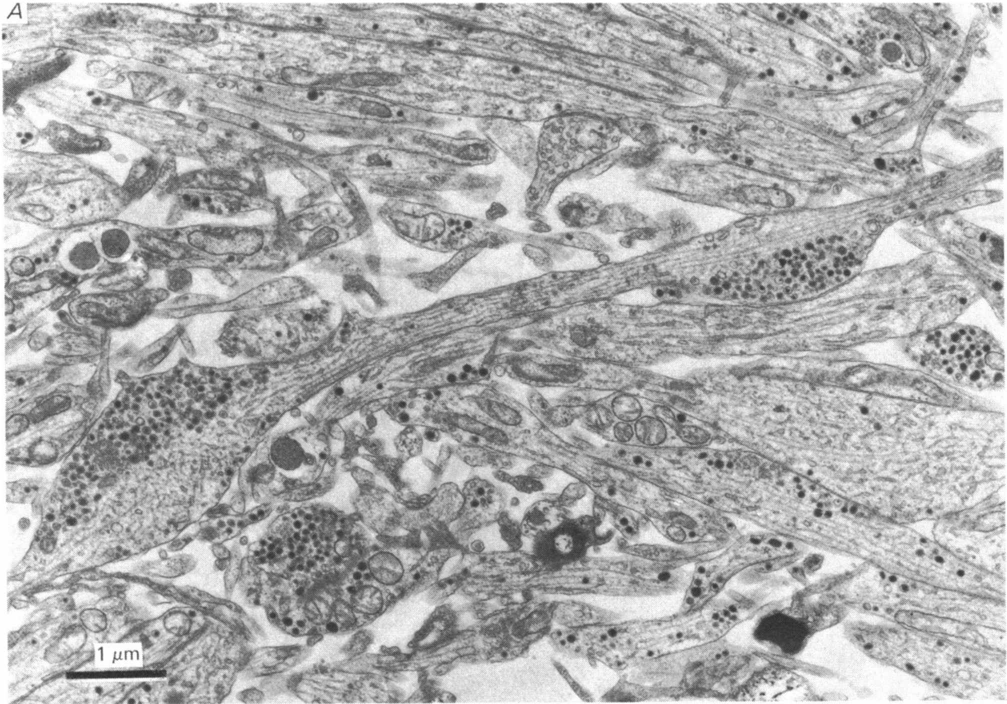

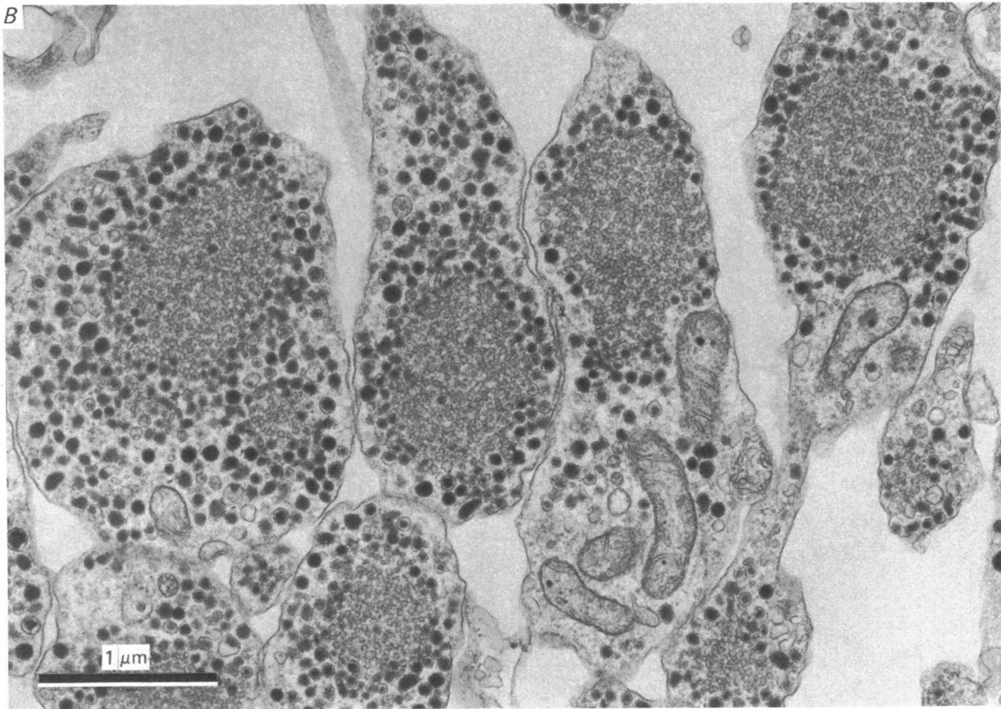

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EXPLANATION OF PLATES

PLATE 1

A, Dissection of a single N sensory cell body (diameter 60 μm) from a leech ganglion. *B*, the disposition of pairs of cells in a typical culture. Pairs of Retzius cells (R–R) were apposed in the left column, Retzius to P sensory cell pairs (R–P) in the middle column, and P to P cell pairs (P–P) in the right column. Such close apposition of the cells was often used to facilitate the formation of connexions.

PLATE 2

A, growth of an isolated anterior 'pagoda' cell over 16 days. Processes, often consisting of bundles of fine axons, could extend 20 μm per day or more, becoming thickened with time. *B*, growth of processes of two Retzius cells maintained in culture for 19 days. Processes appear to run predominantly between the two cells, rather than in all directions equally (see also Fig. 11).

PLATE 3

Light and electron micrographs of a single Retzius cell cultured for 25 days. The area of outgrown processes indicated in panel *B* was sectioned parallel to the surface of the dish shown in *C*. The arrow in *A* indicates a growth cone.

PLATE 4

A, varicosities in the processes of a Retzius cell after 25 days in culture. *B*, accumulations of clear and dense-core vesicles in projections of Retzius cell near the dish bottom, cut parallel to the surface of the dish.