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GROWTH AND SYNAPSE FORMATION BY IDENTIFIED LEECH NEURONES IN CULTURE: A REVIEW

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SUMMARY

Within hours after identified neurones have been isolated from the CNS of the leech, they begin to sprout and to form synapses. Electrical recordings made by loose-patch clamp show that the tip of the isolated neurone has distinct properties with a high density of sodium channels. Neurites grow out from this tip after about 30 min and continue to grow for the next few days. The extent of growth, the branching pattern and the distribution of calcium channels all depend critically upon the molecular composition of the substrate. The tip of the neurone also represents a preferred region for synapse formation. For example when the tips of two serotonin-containing neuromodulatory neurones, the Retzius cells, are placed in contact, chemical synapses develop within about 6 h. These chemical synapses are bidirectional and become stronger over the next 2 days. Electrical synapses between the two Retzius cells develop more slowly and appear only after about 20 h. When the tip of one Retzius cell is apposed to the soma of another, chemical transmission develops more slowly. When other regions of these same cells are placed in contact, electrical transmission can appear before chemical. Together these results show that specialized areas of neuronal membrane are involved in neurite extension and in the formation of specific synaptic connections.

INTRODUCTION

During development and regeneration a neurone can extend processes over long distances. Once at the destination the axon branches extensively to form synaptic terminals or specialized endings on the appropriate target cells. In this review we describe recent experiments made in our laboratory to analyse growth and synapse formation with particular emphasis on cellular mechanisms and the way in which ion channels are distributed in different regions of the neuronal surface.

Suitable preparations for investigating these problems are provided by neurones, isolated from the CNS of the leech, growing on defined substrates in defined medium in tissue culture (Nicholls, 1987). With such cells it becomes possible (1) to measure the properties and distribution of Na^+, K^+ and Ca^{2+} channels in different regions such as the soma, the initial segment, the axons and the newly grown processes, (2) to determine whether specific regions on the cell surface represent preferred areas for new outgrowth and synapse formation, (3) to establish what part is played by the molecular composition of the substrate in determining ion channel distribution and growth, and (4) to analyse the steps that occur during the formation of chemical and electrical synapses – which type of synapse is established first, chemical or electrical?

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WHY USE IDENTIFIED LEECH NEURONES IN CULTURE?

Ready & Nicholls (1979) devised techniques for removing identified neurones from ganglia of the leech CNS. Such cells were shown to survive in culture, to maintain certain membrane properties, to sprout and to form chemical as well as electrical synapses with specific targets (for review see Nicholls, 1987). Since then, comparable studies have been made on isolated neurones of other invertebrates including *Aplysia*, snails and *Drosophila* (see Wu, Suzuki & Poo, 1983; Schacher, Rayport & Ambron, 1985; Haydon, McCobb & Kater, 1987; Lin & Levitan, 1987).

The use of identified neurones allows one to compare a particular cell *in situ* with its counterpart in culture. We have concentrated on the Retzius (R) cell which is a modulatory neurone that contains and secretes the transmitter serotonin. The paired Retzius cells in each ganglion modulate the swimming behaviour of the leech. Each Retzius cell is connected to the other Retzius cell in the ganglion by mixed chemical and electrical synapses and to the P sensory neurone by a purely chemical synapse. These connections are faithfully reproduced by Retzius cells and Retzius and P cells in culture (Fuchs, Henderson & Nicholls, 1982). The chemical synapses formed in culture exhibit properties normally seen in leech ganglia such as quantal release and facilitation. Such phenomena can, however, be analysed more directly and more fully in the single cells in culture than in the ganglion where cells have an elaborate geometry, are surrounded by glia and make their connections within a complex neuropile. It is simply not possible at present to measure directly how ion channels are distributed in different parts of a neurone *in situ*.

HOW ARE ION CHANNELS DISTRIBUTED IN RETZIUS CELLS AS THEY GROW IN CULTURE?

A variety of different techniques can now be used to assess the properties of Na^+, K^+ and Ca^{2+} channels and to measure their concentrations in different regions of the cell. Cells are isolated from ganglia by suction after mild enzyme treatment with collagenase dispase (2 mg/ml). Enzyme can be avoided if the cells are removed by lassoing with fine nylon monofilament. The isolated cell consists of a soma and an initial segment (called the stump). For Retzius cells it is now possible by a new technique to remove an additional length of axon that divides into two secondary axons (Fig. 1). It is from the stump or from the tips of the secondary axons that growing processes emerge. Growth always begins preferentially at the point of transection.

Currents of Na^+, K^+ and Ca^{2+} observed with a two-electrode voltage clamp can be isolated by their properties and by pharmacological tools (Stewart, Nicholls & Adams, 1989). In Retzius cells the Na^+ currents are abolished by reducing external sodium concentration (unfortunately tetrodotoxin does not block leech Na^+ channels); rapidly activating and inactivating K^+ channels are blocked by 4-aminopyridine and slow K^+ channels are blocked by tetraethylammonium (TEA) (Fig. 2). Calcium channels can be blocked by cadmium or manganese.

Fig. 1. Growth of an isolated Retzius cell on substrate consisting of ConA. Immediately after removal two axons and an initial segment can be discerned. By 15 min neurites started to grow out preferentially from the tips. In earlier experiments only the initial segment could be removed with the soma. The new procedure involves treatment of the desheathed ganglion with enzyme followed by removal by suction of all the small cells surrounding the Retzius cell. After a second treatment with enzyme, the Retzius cell can be sucked out together with its axons.

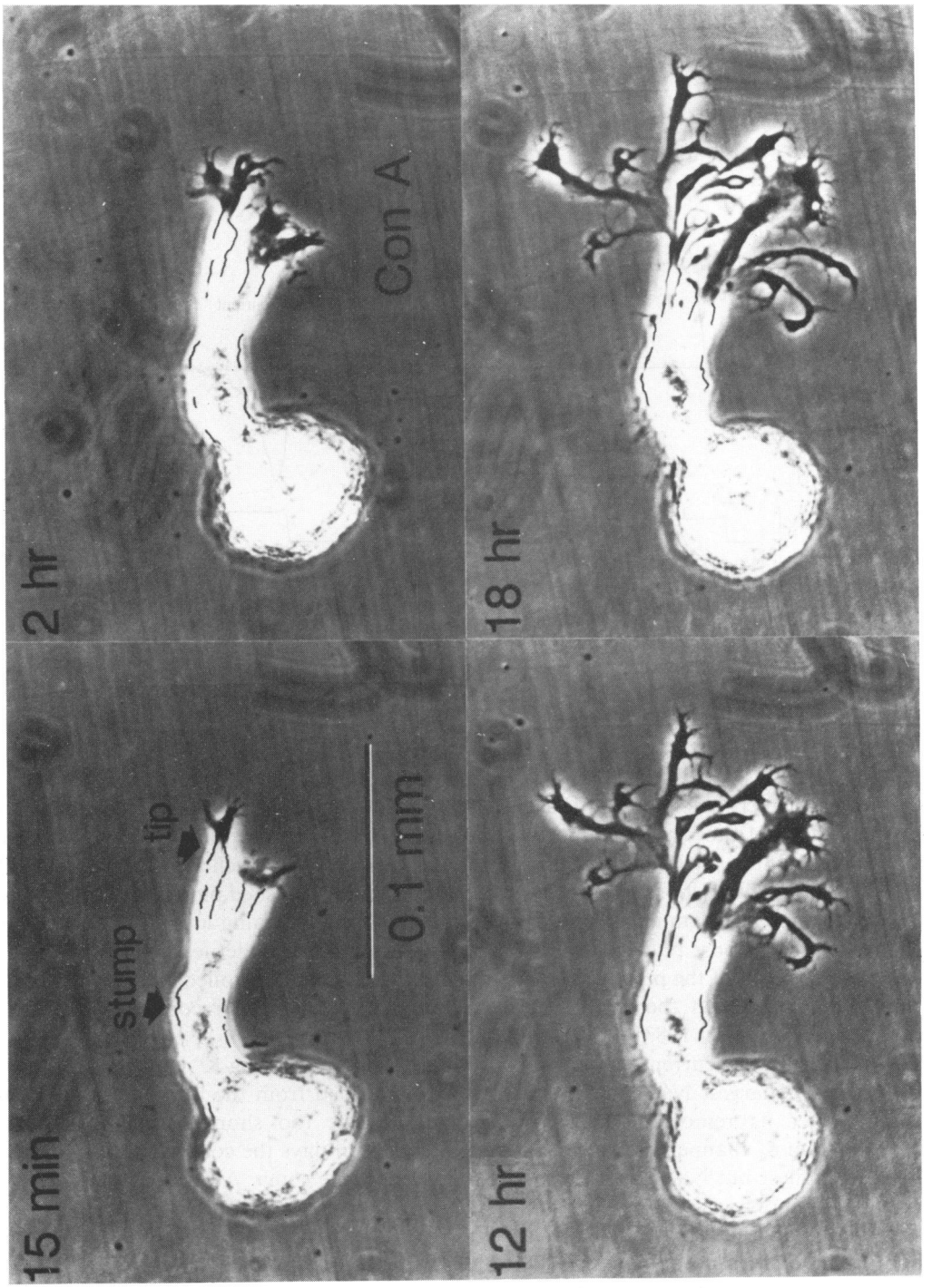

Fig. 1. For legend see facing page.

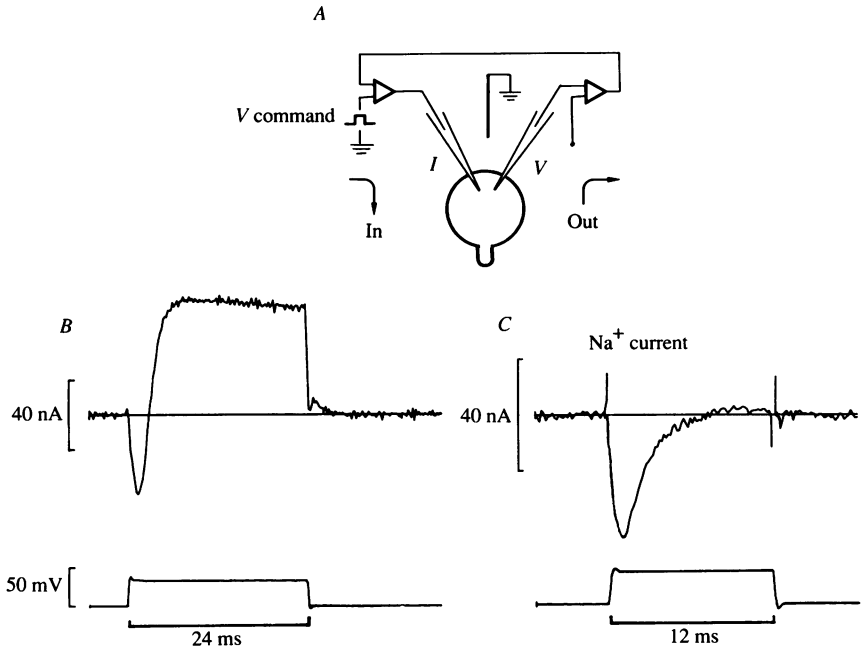

Fig. 2. Two-electrode voltage clamp recordings of ionic current in an isolated Retzius cell at 4 days. *A* shows the arrangement of recording (*V*) and current passing (*I*) microelectrodes. Fluid of different ionic composition was perfused past the cell. *B*, a depolarizing pulse of 40 mV (from −50 mV, the resting potential, to −10 mV lower trace) evoked inward and outward membrane currents in the Retzius cell bathed in normal L-15 medium (upper trace). *C*, after having blocked potassium currents with 120 mM-TEA and 5 mM-4-aminopyridine and calcium currents with 2 mM-manganese a rapidly inactivating inward sodium current remained. Although such records provide evidence about the characteristics of ion channels in the cell, they fail to reveal inhomogeniety of distribution in different regions of the Retzius cell (after Stewart *et al.* 1989; reproduced with permission).

Although micropatch electrodes with tight seals can be used to record from single channels it is hard to make quantitative estimates of their distribution in this way. Instead Bookman, Reuter, Nicholls & Adams (1987) and Garcia, Grumbacher-Reinert & Nicholls (1989) have used the 'loose-patch' clamp technique. A fine-polished pipette with a diameter of approximately 10 μm is brought close to the membrane forming a seal of about 5 MΩ (hence the term 'loose patch'). A discrete patch of membrane underneath is bathed by the solution in the tip of the pipette. The potential of the patch is controlled by current passing though the pipette at the same time as membrane currents are recorded (see Almers, Stanfield & Stühmer, 1983).

Examples of Na^+ currents observed by loose patch after blocking K^+ currents are shown in Fig. 3. The largest Na^+ current is consistently recorded from the tip of the cell's axon 20 min after its removal. This presumably represents too short a time for drastic translocation of channels to have occurred. After several days the concentration at the tip remains highest but Na^+ currents are also recorded at the soma. At whatever site the cut is made upon removal (along the stump, the major axon or the secondary axon) that place has the highest density of Na^+ channels. For K^+ channels the distribution is more homogeneous with no obvious concentration at the cut end.

An alternative method for measuring channel distribution is to make use of optical recording methods (Grinvald, Frostig, Lieke & Hildesheim, 1988; Ross, Aréchiga & Nicholls, 1987, 1988). The indicator dye Arsenazo III provides a reliable measure of changes

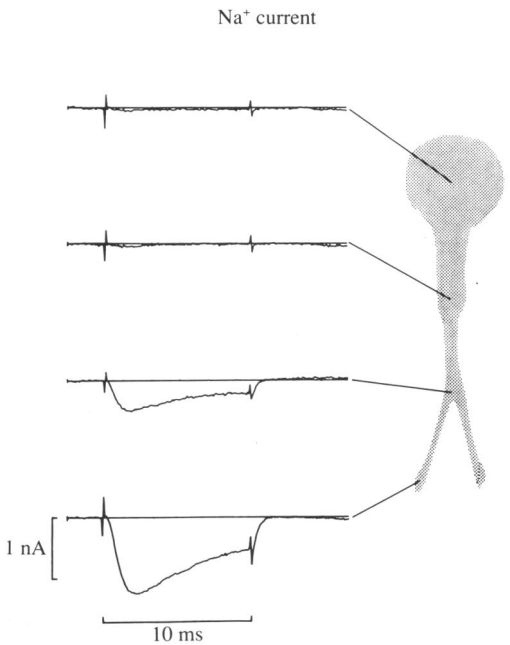

Fig. 3. Sodium currents recorded by loose patch from the tip (bottom trace) and bifurcation of a Retzius cell 20 min after removal from the ganglion. No sodium currents could be measured in the soma (top) or stump. Records of sodium currents were taken in solutions containing 20 mM-TEA and 5 mM-4-aminopyridine to block potassium currents together with 2 mM-cadmium to block calcium currents. Invariably the highest density of sodium channels was found in the tips from which growth started. Even though the secondary axon was slender (approximately 5 μm diameter) recordings could be made close to the tip. After a few days sodium currents could also be recorded from the soma and stump (after Garcia *et al.* 1989; reproduced with permission).

in calcium concentration by changing its absorbance. Figure 4 shows the distribution of calcium entry in two Retzius cells growing on different substrates. In both cells the largest signals corresponding to the highest density of calcium channels were recorded from the stump (these cells were removed without primary or secondary axons). The Retzius cells growing on a substrate consisting of the plant lectin concanavalin A (ConA) had very different branches from those growing on an extract of an extracellular matrix (ECM) containing leech laminin. Neurites on ConA were thicker, more branched, and more curved than those on ECM which were straight and slender (see Masuda-Nakagawa, Beck & Chiquet, 1988). Large calcium signals were evident on the thin processes growing on ECM laminin; by contrast calcium signals could not be recorded from the stout flat processes growing on ConA even though the conditions were more favourable for optical recording. Experiments are now in progress to make comparable measurements of calcium channels on growing processes with the loose-patch technique (U. Garcia and S. Grumbacher-Reinert, unpublished).

Together these results show that: (1) the severed tip of a neurone has the highest density of sodium channels. It is from here that the sprouts start to grow; into these new processes sodium channels are presumably incorporated so as to permit impulse conduction; and (2) the molecular composition of the substrate can influence the distribution of ion channels in growing processes. A question that arises is whether the tip of a growing neurone acts as a specialized site for synapse formation.

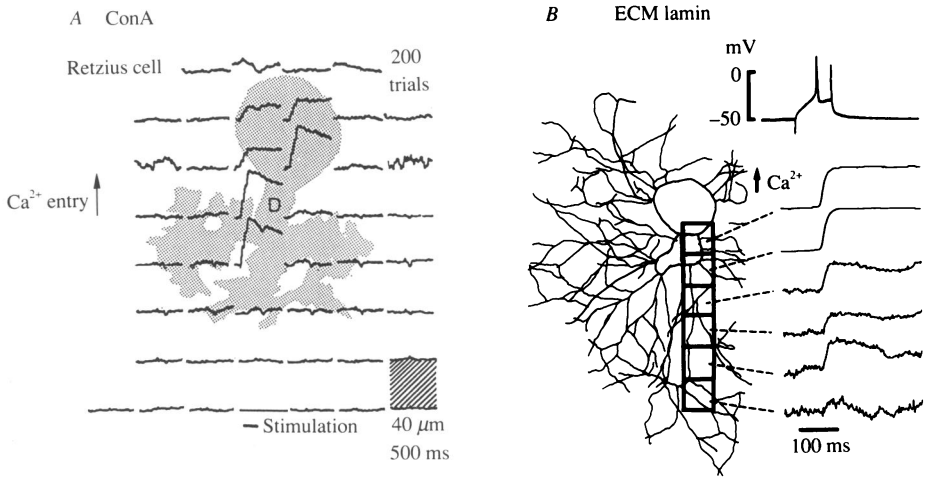

Fig. 4. Optical records made with Arsenazo III of calcium entry into different regions of Retzius cells growing on ConA (*A*, left) and ECM containing leech laminin (*B*, right). The processes of the Retzius cell are different on the two substrates, slenderer and less branched on ECM laminin. Each trace is the averaged record from one photodetector which covers an area $40 \times 40\ \mu m^2$. This area is shown hatched at the bottom right corner of *A* and as rectangles in *B*. On ConA clear calcium transients were evident in the cell body and stump (D) but not over the broad processes. By contrast, *B* shows that impulses in the Retzius cell on ECM laminin gave rise to clear calcium signals over the very fine processes. These results indicate that substrate can influence the distribution of calcium channels in growing processes (after Ross *et al.* 1987, 1988; reproduced with permission).

DO CHEMICAL AND ELECTRICAL SYNAPSES FORM BETWEEN SPECIALIZED AREAS OF MEMBRANE SURFACE?

During development or regeneration of the nervous system synapses form between appropriate regions of the appropriate cells. Moreover the type of synapse that one cell makes on its target is highly specific – chemical or electrical, excitatory or inhibitory, rectifying or non-rectifying. In culture as in the animal, leech neurones form connections that are specific.

Thus, cultured Retzius cells have been shown to form chemical and non-rectifying electrical junctions with one another; Retzius cells form purely chemical synapses upon P cells; and P cells form rectifying electrical connections with L motor cells. These connections in culture resemble those occurring in the animal (see Nicholls, 1987).

The following experiments by Liu & Nicholls (1989) show that different types of connections were made when Retzius cells were paired in close apposition in a variety of configurations, with different areas of membrane in contact (Fig. 5). Of particular interest were the stumps and the tips of secondary axons in which sodium channels are present at highest density and from which growth starts. When two Retzius cells were placed with their stumps touching, chemically mediated transmission became apparent within 2–6 h, becoming progressively stronger over the next 2 days (Liu & Nicholls, 1989). With this configuration chemical transmission was bidirectional; the reliability of chemical synapse formation was virtually 100% in healthy pairs of Retzius cells plated in this way (Fig. 6). Electrical transmission developed more slowly and only after a delay. When the stump of one Retzius cell was apposed to the soma of the other the results were in some respects similar – chemically mediated transmission developed rapidly and was followed after a

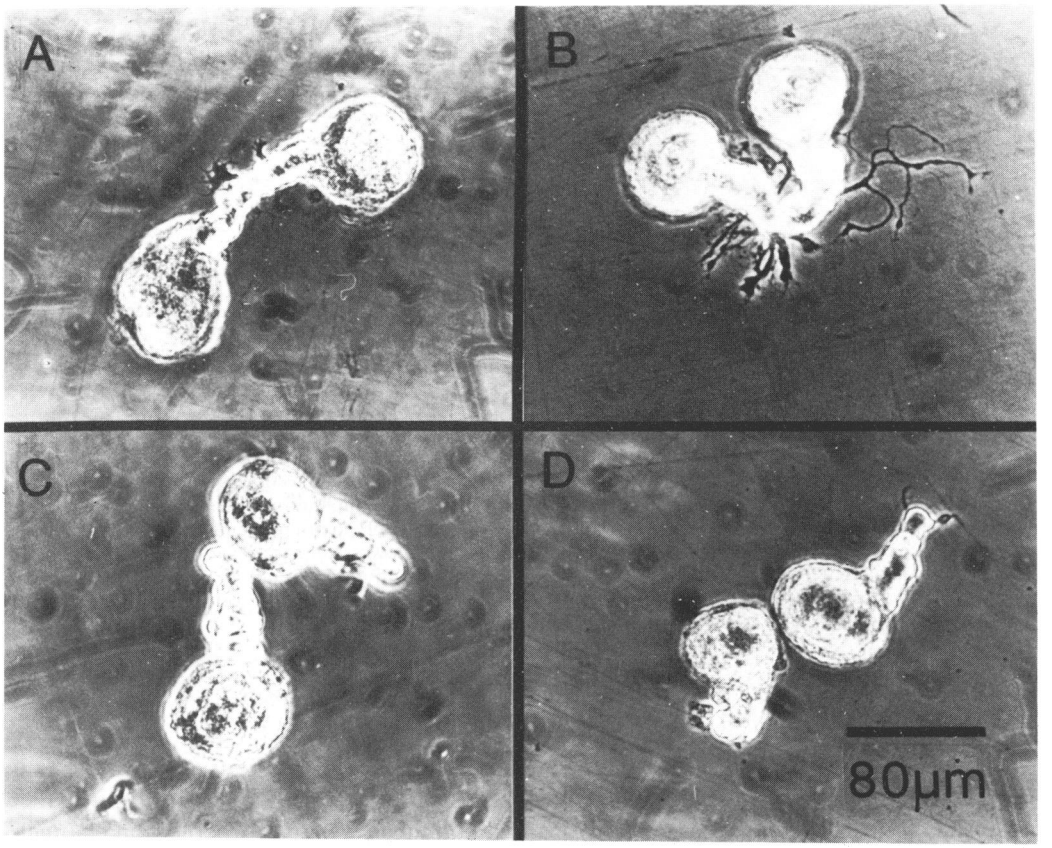

Fig. 5. Retzius cells plated on ConA with their stumps in various configurations. *A* and *B* show cells with their stumps in close apposition (12 and 25 h respectively; chemical transmission in both directions had been established between these cells). *C*, Retzius cells with stump apposed to soma at 24 h. Transmission between these cells was chemical and in only one direction from stump to soma. *D*, soma-to-soma contact between Retzius cells at 17 h. No transmission was apparent between these two cells. With this configuration transmission developed after several days, electrical transmission often appearing first (after Liu & Nicholls, 1989; reproduced with permission).

delay by electrical transmission. But with this configuration the chemical transmission was always exclusively in one direction – from stump to soma. When soma was apposed to soma the results were again different. Electrical transmission developed as before after 2 days but chemical transmission developed more slowly, less reliably and only after a delay of several days.

A technically difficult experiment was to appose the tips of secondary axons instead of the stumps (Y. Liu, unpublished). With tips touching, chemically and electrically mediated transmission appeared at about the same time during the first day. Thus, electrical synapses formed more rapidly from this region of the cell than from the stump. These results show that the cut end of a neurone acts as a preferred site for synapse formation. Moreover different regions of the neuronal surface appear specialized for generating electrical or chemical connections or both.

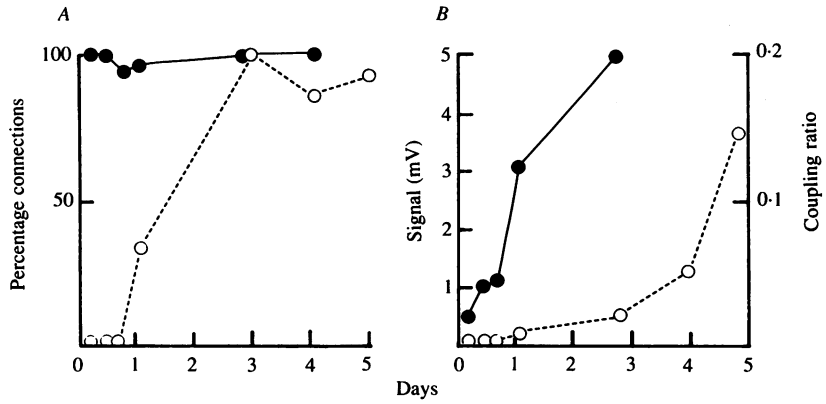

Fig. 6. Time course of formation of chemical (●) and electrical (○) synapses between Retzius cells in culture with stumps apposed. *A*, virtually 100% of Retzius pairs became connected by chemical synapses within the first 24 h. The earliest time of appearance was 2·5 h. In the same cells electrical connections started to appear after a delay. *B*, increased strength of chemical (●, mV) and electrical (○, coupling ratio) connections in the same Retzius cells as shown in *A*. Sixty-six pairs of cells were examined. Each point represents results obtained from at least four pairs of cells at one time. In *B* the mean values are shown. Standard error of mean (not shown) was approximately 0·5 mV or less; for coupling ratios 0·03 or less. In most but not all pairs of cells chemical transmission was bidirectional, usually stronger in one direction than the other. Electrical transmission was non-rectifying (after Liu & Nicholls, 1989; reproduced with permission).

CONCLUSIONS

The appeal of isolated leech cells is that one can ask direct questions in a way that is impracticable for neurones within the CNS. By using these cells one can ask whether chemical or electrical transmission develops first at a mixed synapse, whether ion channels are concentrated in particular regions of a neurone and whether these regions have special physiological properties. It is tempting to speculate that the growing tip of a neurone contains the membrane channels and specialized machinery for growth and synapse formation. The substrate with which it comes into contact can determine, in addition, the branching pattern, the growth rate and the distribution of calcium channels. The target presumably plays a further role in determining what type of synapses shall be formed. At present we have little or no information about molecular mechanisms involved in these processes. It will be of interest to explore further the synthesis of new ion channels and the way in which they move along the cell and become redistributed so as to facilitate the formation of new connections.

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