J. exp. Biol. 153, 141–154 (1990)
Printed in Great Britain

THE SPECIFICITY OF SYNAPSE FORMATION BY IDENTIFIED LEECH NEURONES IN CULTURE

BY J. G. NICHOLLS, Y. LIU*, B. W. PAYTON† AND D. P. KUFFLER‡

Department of Pharmacology, Biocenter, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

Summary

The physiological and fine structural events accompanying synapse formation have been followed while identified neurones of known function make contact in tissue culture. Particular pairs of identified neurones isolated from the central nervous system (CNS) of the leech form chemical synapses; other pairs of cells form nonrectifying electrical junctions, rectifying electrical junctions, mixed chemical and electrical synapses or no synapses at all, depending upon the partners that have been paired. Moreover, certain specific regions on the cell surface (such as the soma, initial cell segment or axon tips) preferentially develop chemical or electrical synapses. Of particular interest are the large, serotonergic Retzius cells that form mixed chemical and electrical synapses in culture, as in the animal. When these cells are juxtaposed at their initial segments, it has been shown that chemical synapses can develop reliably within 6 h of contact in culture. Shortly after transmission can be detected physiologically, the principal features of synaptic structure are evident. The physiological and morphological characteristics resemble those of mature synapses studied within the central nervous system. Only at later times, after the chemical synapses have been formed, do electrical connections appear. By contrast, when other specialized regions of the Retzius cells are apposed (the tips of their axons), electrical synapses appear earlier. By comparing the connections that different types of serotonergic neurones make in culture we have been able to assess the role played by the transmitter in determining specificity: the results show that the transmitter does not determine what type of synapse is made on a particular partner. For example, Retzius cells make purely chemical synapses upon the sensory P neurone in culture; other serotonergic neurones (known as DL and VL) make purely electrical connections on this same pressure sensory neurone. Together, these results demonstrate that highly specific cell–cell recognition is a necessary feature of synapse formation after neurones have grown to their appropriate destinations.

* Present address: Department of Neurobiology and Behavior, SUNY at Stony Brook, Stony Brook, NY 11794, USA.

† Present address: Faculty of Medicine, The Health Sciences Center, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1B 3V6.

‡ Present address: Institute of Neurobiology, University of Puerto Rico, Old San Juan, PR 00901, USA.

Key words: leech, tissue culture, chemical synapses, electrical synapses.

Introduction

As neurones send out processes towards their targets and form synapses during development or regeneration, they execute a complex sequence of steps. In the initial stages, trophic factors and substratum molecules provide stimuli for neurite extension and cues for guidance. As a last step, the right cells must form the correct connections with each other if neuronal circuits are to generate performance that is functionally useful. In addition, the physiological properties of these synapses must be appropriately tuned. Thus, a particular synapse between particular types of neurones in the CNS has a defined set of properties that depend upon the specialized pre- and postsynaptic structures: the synapse is characteristically excitatory or inhibitory, chemical or electrical or both, rectifying or nonrectifying, strong or weak, facilitating or depressing with trains of impulses, and subject or not subject to modulation.

Numerous questions arise concerning the way in which newly formed synapses acquire this assembly of properties that make up specificity (Vidal-Sanz *et al.* 1987). These include: (1) how specialized pre- and postsynaptic structures are formed on appropriate regions of the cells, (2) the mechanisms that influence the choice of establishment of a chemical or an electrical synapse and (3) the possible role of the transmitter itself in determining synapse formation. (For example, do all neurones containing serotonin have the ability to connect in the same way with the same type of target cell?) In the present series of experiments we have approached these and related questions that bear on cell recognition and synapse formation between pairs of identified neurones maintained in tissue culture.

For technical reasons it is usually not possible to record physiologically from synapses as they form within the CNS of a vertebrate or an invertebrate. The synapses at which transmission actually occurs are inaccessible and the exact timing and mechanism of synapse formation cannot be measured directly. The sequence of changes in fine structural appearance is hard to define. Moreover, it is usually not possible to record simultaneously with microelectrodes from the two individual cells that constitute the pre- and the postsynaptic elements. Such difficulties arise largely from the geometry of the neurones and the profusion of cells within the CNS.

An idealized preparation would be far simpler and would consist of two identified nerve cells of known function *in vitro*, with known membrane properties and transmitters. It would be important for synapses to form rapidly, within hours, at well-defined sites close to the soma from which electrical recordings could be made. Since 1979 (Ready and Nicholls, 1979), when identified leech neurones were first isolated from the leech CNS and placed in culture, numerous invertebrates have provided preparations that fulfil these criteria for following synapse formation. For example, neurones isolated from leeches, snails and *Aplysia* can re-establish chemical or electrical synapses, often with properties resembling those normally formed during development in the CNS of the animal (Fuchs *et al.* 1982; Liu and Nicholls, 1989; Bodmer *et al.* 1988; Haydon *et al.* 1987; Rayport and Schacher, 1986). The experiments described here show that the

connections between the isolated leech neurones can form rapidly and reliably, are highly specific and can be used to follow in detail the sequence of steps as chemical and electrical synapses are formed.

Experimental procedures for isolation and culture of leech neurones

The particular cell that is to be isolated can usually be recognized by its shape, size and position in the leech ganglion. Particularly easy to identify without electrical recording are the sensory P, N and T cells, the 5-hydroxytryptamine (5-HT)-containing Retzius cells, the L and the AE motoneurones and the AP cells of unknown function (Nicholls, 1987). Other cells such as the tiny DL or VL serotonergic cells cannot be found simply by visual inspection of the ganglion: they must be recorded from electrically or stained selectively before removal to identify them (Acklin and Nicholls, 1990; Stuart *et al.* 1974). The cell is pulled out either by lassoing with fine nylon monofilament or by suction into a mouth-held pipette after mild enzyme treatment of the ganglion with collagenase–dispase. Neurones can now be extracted together with a variable length of the axonal arborization or without processes as rounded cell-bodies.

The isolated neurones survive for up to 3 weeks in a suitable medium consisting of Leibowitz-15 with 2% foetal calf serum and garamycin (Dietzel *et al.* 1986; Fuchs *et al.* 1982). A convenient feature is that N, P, T, Retzius and AP cells maintain their distinctive action potential configurations in culture. Hence, in a pair of cells that has made a synapse it is always possible to be certain which cell is which. In spite of such constancy, measurable changes do occur in the distribution over the cell surfaces of ion channels for sodium and potassium with time in culture. For example, immediately after a Retzius cell has been removed, the sodium channels are present in highest concentration in the axons, especially at their broken tips. As outgrowth proceeds, the sodium channels appear in the growth cones and also in the soma (Garcia *et al.* 1990).

For the establishment of synapses, pairs of identified neurones are placed close together in various configurations (see Fig. 1 and below). Neurones will also form synapses when placed at a distance from one another after process growth has occurred. Such synapses are, however, less favourable for physiological and morphological analysis since the sites of transmission cannot be recognized by simple inspection within the profusion of neuronal processes.

Specificity of synapse formation

The presence or absence of a synaptic connection, as well as the mode of transmission, depends on which two cells have been apposed in culture. The results obtained so far with leech neurones are shown in Table 1. The following are a few examples. (a) Purely electrical synapses without rectification form between pairs of L cells. (b) Rectifying electrical synapses develop when an L cell is paired with a P sensory cell. (In this case depolarization of the P cell spreads well to the L

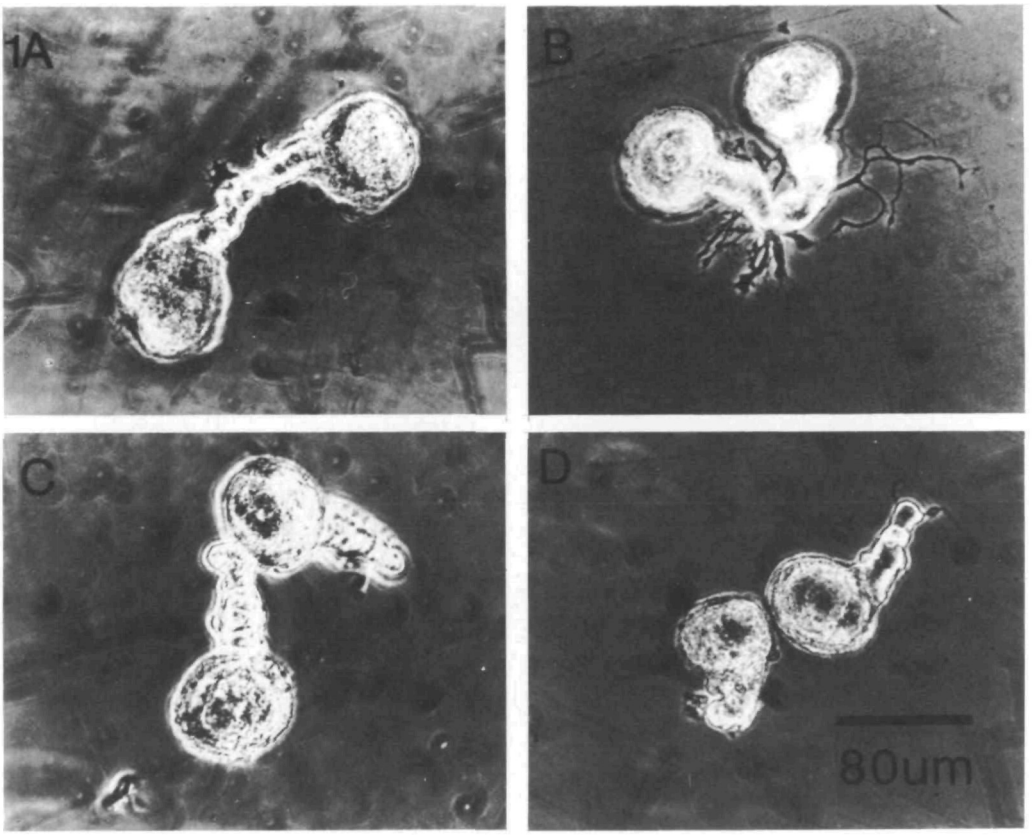


Fig. 1. Pairs of Retzius cells isolated from the leech CNS and maintained in culture, on a substratum of concanavalin A. The cells were plated with their initial segments in various configurations. (A, B) Cells with their stumps in close apposition (12 h and 25 h: chemical transmission in both directions had been established between these cells). (C) Retzius cells with stump apposed to soma at 24 h. Transmission between these cells were chemical and in only one direction from stump to soma. (D) Soma–soma contact between Retzius cells at 17 h. No transmission was apparent between these two cells (from Liu and Nicholls, 1989). In all experiments before 1989 soma–soma contact had been used. The pattern of specificity was the same but synapses developed much more slowly (4–12 days) and less reliably. Only in preparations in which electrical transmission was weak could chemical transmission be demonstrated. In electron micrographs chemical synapses were observed after about 3 days. Scale bar, 80 μm.

cell, but hyperpolarization does not. Conversely, hyperpolarization but not depolarization spreads from L to P.) No signs of chemically mediated interactions occur at these electrical synapses. (c) By contrast, purely chemical transmission with 5-HT as the transmitter develops when Retzius cells are paired with P cells: no sign of electrical transmission can be detected. (d) When a Retzius cell is paired with another Retzius cell, mixed chemical and electrical synapses develop (without

Table 1. *Specific connections of identified neurones in culture*

Presynaptic cell	Postsynaptic cell: Retzius	Postsynaptic cell: P
Retzius	Mixed: chemical (bidirectional) and electrical	Pure chemical (unidirectional)
DL VL	Electrical, nonrectifying	Electrical, nonrectifying
L	Electrical, nonrectifying	Electrical, rectifying
N lateral	Electrical, rectifying	No connection
N medial	Electrical, rectifying or nonrectifying	No connection
P	None	None

See Vyklicky and Nicholls (1988) and Acklin and Nicholls (1990).

rectification). (e) Touch sensory (T) cells have not been seen to establish connections in culture. Numerous other connections are shown in Table 1 (see Fuchs *et al.* 1982; Aréchiga *et al.* 1986; Liu and Nicholls, 1989).

What these connections demonstrate is orderliness and regularity. The same types of cells always form the same types of connections. One exception to this rule is provided by the N cells. These two cells, medial and lateral in their locations in the ganglion, are sensory for nociception and have similar receptive fields. They differ in their membrane properties and connections, and a specific antibody has been found that binds to the medial but not the lateral N cell. These two N cells also make distinctive connections in culture. Like the P cell, the lateral N cell, when paired with Retzius, L, AE or AP cells, always makes a rectifying electrical connection (such that depolarization spreads better than hyperpolarization from the lateral N cell to its partner and *vice versa*). The medial N cell, however, behaves differently. It can make a rectifying or a nonrectifying synapse on the same partner. In some cultures the medial N cell forms a nonrectifying junction with the Retzius cell while in others the junction shows rectification (Vycklicky and Nicholls, 1988). The factors that influence this choice by the medial N cell are not known.

Some but not all of the connections made in culture resemble those seen in the ganglion: Retzius to Retzius, Retzius to P, P to L and L to L connections seen in the dish are virtually identical to those occurring in the animal. At the same time, certain connections fail to reappear between these cells. Although Retzius cells form chemical connections on P cells in the ganglion and in culture, the normal

strong chemical connection from P to Retzius cells is not reproduced in culture, nor is the strong chemical connection from P to L. We cannot at present explain these failures. A reasonable assumption would be that certain factors conducive to synapse formation are present in embryos but lacking in the dish.

In addition, novel types of connections not seen in the ganglion can form in culture. The strong electrical coupling between Retzius and L cells and AE cells provide examples (Aréchiga *et al.* 1986). Again, this result, though not explained, is hardly surprising. Presumably cell–cell recognition is only one of many factors that determine the patterns of connections that develop during embryogenesis. One can imagine that neurones that have the potential for forming synapses never do so because they never come into contact within the nervous system. In culture the same two neurones can be directly apposed and contact one another.

Together these results emphasize the importance of cell–cell recognition as the final step in synapse formation. By mechanisms that are not known, a neurone, such as the Retzius or the P cell, can decide what type of synapse it will form, and whether it will be the pre- or the postsynaptic element, depending solely on the partner it encounters, without reference to the fluid environment or the substratum.

Role of the transmitter in determining specificity

Do all cells containing the same transmitter make similar connections? This question has been answered by isolating DL and VL cells (Acklin and Nicholls, 1990). These two small neurones, like Retzius cells, contain and secrete 5-HT as their transmitter. Yet, as shown in Table 1, they make quite different connections from those of Retzius cells. They form purely electrical synapses on the P cell with no sign of chemical transmission (Fig. 2). Hence the type of transmitter that a neurone liberates does not on its own determine how its target cell is selected.

Properties of synapses formed in culture

To what extent do synapses in culture resemble those in the ganglion? First, in their physiological properties the newly formed synapses display normal characteristics. Thus, the nonrectifying synapses established between Retzius, L or AP cells are indistinguishable from those *in situ*. Similarly, the rectifying P to L or N to L junctions show normal voltage sensitivity. Chemically mediated transmission is more flexible, with marked facilitation and a slow modulation that depends on the holding potential in the presynaptic cell. Quantal release of 5-HT has been demonstrated by measuring miniature potentials and by statistical analysis of fluctuations in the amplitudes of evoked synaptic potentials (Henderson *et al.* 1983). These experiments, together with voltage-clamp studies, show that changes in release rather than postsynaptic mechanisms account for facilitation and modulation (Stewart *et al.* 1989). As soon as the synapses form in culture they are endowed with these dynamic characteristics (see below).

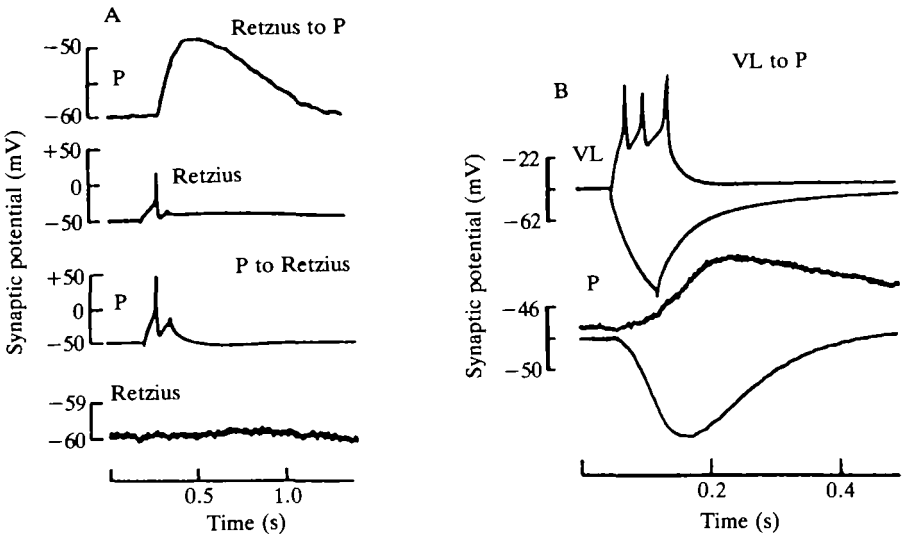


Fig. 2. Retzius cells and VL cells make different synaptic connections on the P cell in culture. Both Retzius and VL are serotonergic. (A) Chemical synapse between Retzius and P after 25 h in culture with initial segments in apposition. No sign of electrical coupling could be detected. The chemically mediated potential showed facilitation and was blocked by raised Mg^{2+} level. Transmission was exclusively in the direction Retzius to P. (B) VL cells made purely electrical connections on P cells without rectification, 3 days in culture. (A after Liu and Nicholls, 1989; B after Acklin and Nicholls, 1990.)

The electron microscopy of synapses in culture reveals fine structural features similar to those *in situ*. A problem for making such comparisons is that the chemical synapses most intensively studied *in vitro*, the serotonergic Retzius to P and Retzius to Retzius connections, have not been identified by electron microscopy in the leech central nervous system. Nevertheless, the principal characteristics shown in Fig. 3 are typical for 5-HT synapses in general. Dense-cored vesicles form a ring around clusters of clear vesicles that are apposed to active zones in the presynaptic Retzius cell (Kuffler *et al.* 1987). At these sites the pre- and postsynaptic membranes are thickened; the extracellular space is widened and contains densely staining material. In the postsynaptic P cell cisternae are evident. The chemical synapses formed by Retzius cells on one another exhibit similar morphological characteristics. Many but not all of these features appear within hours as the synapses form in culture (see below).

The dense-cored and clear vesicles turn over after prolonged stimulation. Markers such as horseradish peroxidase, ferritin and colloidal gold in the external fluid are taken up and transferred from coated vesicles, cisternae and vesicles (Kuffler *et al.* 1987). As expected, only the presynaptic Retzius cell takes up label after stimulation of both cells with a high external K^+ concentration (50 mmol l^{-1}). Colloidal gold particles have been observed in both dense-cored and clear vesicles.

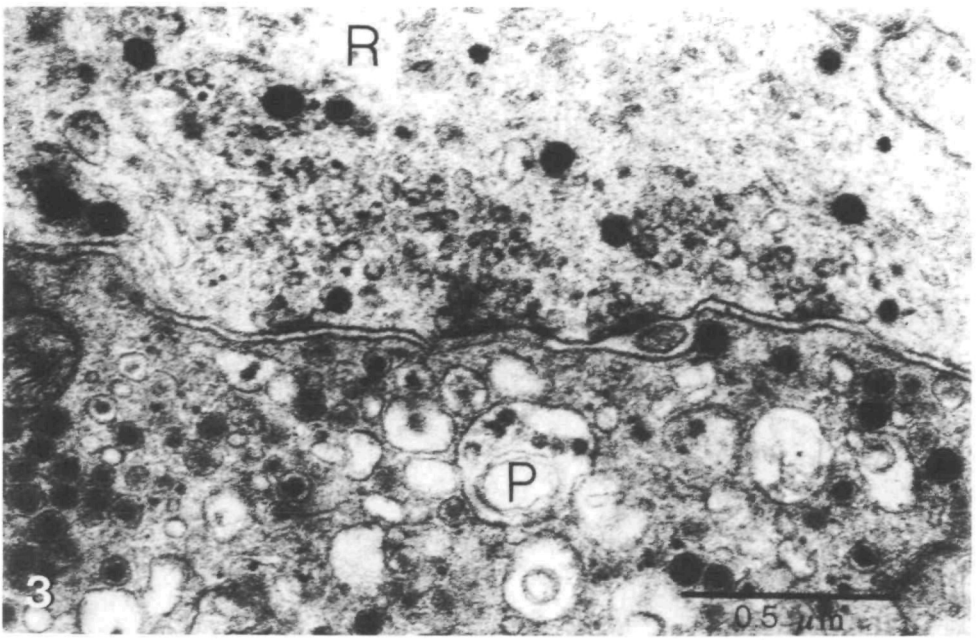


Fig. 3. Electron micrograph showing synaptic structures developing between Retzius (R) and P cells in culture for 24 h. Electrical recordings showed synaptic potentials approximately 2.5 mV in amplitude. Clear vesicles in the R cell are apposed to presynaptic thickenings. Occasional dense-core vesicles are apparent in both R and P cells. Such dense-core vesicles have been shown to have different characteristics in the two types of cells, allowing them to be recognized unambiguously in thin sections. Presynaptic specializations are closely apposed to the postsynaptic membranes. Transmission at these synapses is serotonergic and always from R to P.

We do not yet know which type of vesicle corresponds to the quantum of release. Gap junctions have not yet been sought or identified at electrical synapses formed by leech neurones in culture.

Preferred sites on the neuronal surface for synapse formation

The experiments illustrated in Fig. 4 and Table 2 were made to determine whether the properties and speed of synapse formation are influenced by the particular sites on the surfaces of the two cells that come into contact. Neuronal cell bodies within the ganglion of the leech are virtually free of synapses (see, however, French and Muller, 1986) and all contacts are made at a distance in the neuropile. Are cell bodies, initial segments and axons equivalent in their ability to form pre- and postsynaptic structures, as well as chemical or electrical synapses?

Neurones were apposed in various configurations: (1) with their somata touching (this was the site of contact in all earlier experiments); (2) with initial segment touching initial segment; (3) with initial segment touching soma and (4) with axon touching axon. The differences were dramatic. For convenience the

Table 2. *Preferred sites for formation of chemical and electrical synapses between pairs of Retzius cells**

	A –OO–	B O–+–O	C O–O–	D O–◇–O
Initial connection	Electrical (2–3 days)	Chemical (6–8 h), bidirectional	Chemical (6–8 h), unidirectional	Electrical (12 h)
Delayed connection	Chemical (3–8 days), bidirectional	Electrical (24 h)	Electrical (24 h)	Chemical (24 h), bidirectional

* From Liu and Nicholls (1989); Y. Liu (unpublished results).

results are summarized in Table 2 for the mixed chemical and electrical synapses that develop between pairs of Retzius cells in culture (Liu and Nicholls, 1989).

With soma to soma contact between Retzius cells (A), electrical transmission appeared first, after about 3 days, becoming stronger over the next 6 days. Chemical transmission invariably developed later at about 3–8 days, but was often masked by the strong electrical coupling (alas! no method has yet been found for uncoupling leech electrical junctions). With initial segment to initial segment apposition (B), the sequence and reliability were quite different. Within 6–8 h bidirectional, chemically mediated transmission appeared. Often chemical transmission in one direction was stronger than in the other. These chemical synapses became stronger hour by hour and showed clear facilitation and depression. Their fine structure examined at 12–24 h resembled that observed in mature synapses in culture. The site could be easily identified at the tips of the apposed initial segment. Only after a prolonged delay of 24 h was there evidence of weak electrical transmission (which is easier to detect, by physiological recording, than chemical transmission). Unlike soma to soma contact, the sequence, timing and reliability of synapse formation were precise. That the tip of the initial segment represented a preferential site for chemical synapse formation was demonstrated by apposing cells with the tip of one Retzius cell initial segment touching the soma of another (C). Again, chemical synaptic transmission appeared in 6–8 h, became stronger and was followed after about 24 h by electrical transmission. But, with this configuration the chemical transmission was in only one direction – from initial segment to soma, never in the reverse direction. Y. Liu (in preparation) has further studied synapse formation by the same cells with their axon tips touching (D). For those experiments the Retzius cell was pulled out with a length of axon intact. The broken tips of the axons were apposed in culture, a technically difficult manoeuvre. With the Retzius neurones in this configuration electrical transmission appeared before chemical transmission.

The appeal of these experiments is that they set the stage for examining cellular and molecular events in synapse formation. At what other synapses can one follow the sequence of steps in such detail and so directly? Experiments are now in

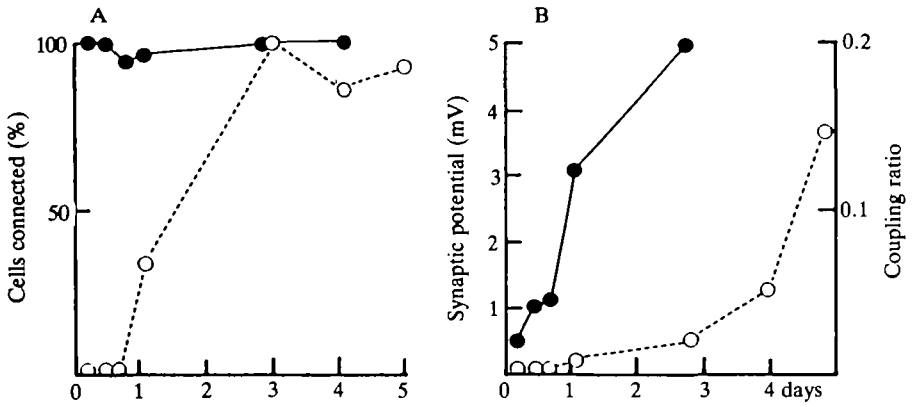


Fig. 4. Graphs showing time course of formation of chemical (filled circles) and electrical (open circles) synapses between Retzius cells in culture with initial segments apposed (as in Fig. 1A,B). (A) Virtually all the Retzius pairs became connected within the first 24 h. The earliest time of appearance was 2.5 h (see text). In the same cells, electrical connections started to appear after a delay. (B) Increased strength of chemical (filled circles, millivolts) and electrical (open circles, 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. In B the mean values are shown; S.E.M. (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 nonrectifying. By contrast, when a length of axon beyond the initial segment was removed with each Retzius cell and placed in contact, axon to axon, electrical transmission appeared earlier (not shown). From Liu and Nicholls, (1989); Y. Liu (unpublished results).

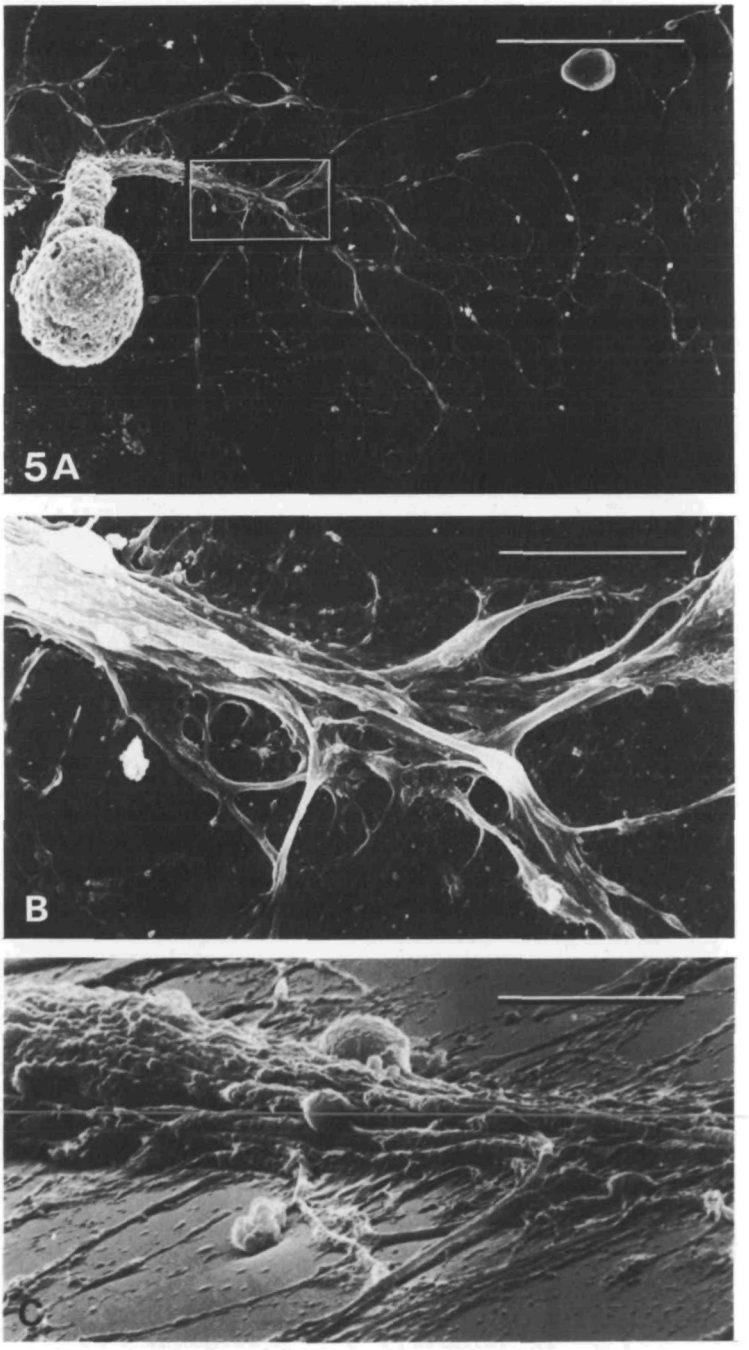
progress to examine the cytoskeleton during synapse formation at predetermined sites.

Influence of substrata on neurite outgrowth

The molecular composition of the substratum upon which a leech neurone is plated determines the extent and pattern of its outgrowth, as well as the distribution of voltage-sensitive calcium channels in the newly grown processes. Of particular interest is a laminin-like molecule that can be extracted from the CNS

Fig. 5. Retzius cell with profuse branches grown on concanavalin A for 4 days. Scanning electron micrographs after fixation and coating with gold. Each type of leech neurone displays a characteristic fingerprint on concanavalin A and a different pattern on laminin substratum. (A) Low power view showing initial segment, growth cones and processes. Scale bar, 50 μm. (B) Area delineated by rectangle in A at higher magnification. Processes can be seen to form bundles and to grow over each other. Scale bar, 10 μm. (C) Same cell as in A and B observed after tilting the preparation further to show relationship of processes to substratum. Scale bar, 5 μm.

(Chiquet *et al.* 1988; Masuda-Nakagawa *et al.* 1988). With this high molecular weight protein as a substratum, certain neurones such as the Retzius cell, start to grow within 30 min and send out long slender processes with few branches. The processes contain large numbers of Ca^{2+} channels: stimulation leads to Ca^{2+} entry



that can be measured optically using the Ca^{2+}-sensitive dye Arsenazo III (Ross *et al.* 1987, 1988). The location of the laminin molecule can be studied in leech ganglia and connectives using a specific monoclonal antibody. In the adult nervous system, the laminin is restricted to the basal lamina surrounding ganglia and connectives, and is not in direct contact with axons or cell bodies. In developing leech embryos, however, or after injury, the laminin is distributed differently. Laminin is observed at sites in the connectives that link ganglia along which axons will grow during development or regenerate after injury (L. Masuda-Nakagawa, unpublished observations).

A second substratum upon which leech neurones grow profusely is the plant lectin concanavalin A. Retzius cells plated on concanavalin A rapidly send out processes after about 30 min. But, unlike those on laminin, the Retzius cell processes become curved, thick and show marked fasciculation (see Fig. 5). Such processes contain relatively few calcium channels compared to those grown on laminin. Thus, different substrata can have markedly different effects on outgrowth rates, patterns and Ca^{2+} channel distribution. This result raises the possibility that certain other substratum molecules might be located at key sites within the CNS or even on particular regions of neuronal surfaces. In principle, such molecules could facilitate or inhibit synapse formation by mechanisms analogous to those used by agrin at the skeletal neuromuscular junction. As yet the molecules that influence synapse formation by leech neurones in culture have not been identified.

Conclusions

The formation of a synapse represents the end-point of a series of decisions made by two neurones. The principal focus of the present experiments was the demonstration of cell–cell recognition in the absence of external cues, using the formation of (or failure to form) chemical or electrical synapses as indicators. With two identified cells close together in culture one can monitor with microelectrodes the mechanism of transmission from outset to maturity (something that cannot usually be achieved within the CNS). That chemically mediated transmission, with its vastly more complex structural requirements, could precede the formation of electrical junctions was a surprise, as was the finding of regional preferences over different regions of the cell surface. We are, however, still far from understanding molecular mechanisms and cytoskeletal rearrangements that cause outgrowth, pattern formation, cessation of growth, the formation of specialized pre- and postsynaptic endings or the selection of the chemical or electrical mode of transmission. How does the nature of the partner determine these events? It seems that simple adherence could not on its own provide an explanation.

For nervous systems consisting of millions of cells rather than hundreds or thousands it seems that the same principles might apply but for large populations rather than for single cells. The simple leech, with only 14 sensory neurones in each ganglion to tell it where a body segment is being stroked, pressed or bitten,

has presumably to specify each single cell to a far greater extent to obtain information about the outside world and to regulate its behaviour.

We wish to thank our colleagues at the Biocenter in the Department of Pharmacology, particularly Drs W. B. Adams, L. Masuda-Nakagawa and R. R. Stewart. We also wish to thank Mr P. Bättig and Ms J. Wittker for their excellent technical assistance and secretarial help. Dr R. Guggenheim and Mr D. Mathys kindly provided assistance at the Electron Microscopy Laboratory of the Geology Institute. This work was supported by grants from the Swiss National Fonds to JGN (3.556–0.86) and to DPK (3.516–0.86).

References

ACKLIN, S. E. AND NICHOLLS, J. G. (1990). Intrinsic and extrinsic factors influencing properties and growth patterns of identified leech neurons in culture. *J. Neurosci.* (in press).

ARÉCHIGA, H., CHIQUET, M., KUFFLER, D. AND NICHOLLS, J. G. (1986). Formation of specific connexions in culture by identified leech neurons containing serotonin, acetylcholine and peptide transmitters. *J. exp. Biol.* **126**, 15–31.

BODMER, R., VERSELIS, V., LEVITAN, I. B. AND SPRAY, D. C. (1988). Electrotonic synapses between *Aplysia* neurons *in situ* and in culture: aspects of regulation and measurements of permeability. *J. Neurosci.* **8**, 1656–1670.

CHIQUET, M., MASUDA-NAKAGAWA, L. AND BECK, K. (1988). Attachment to an endogenous laminin-like protein initiates sprouting by leech neurons. *J. Cell Biol.* **107**, 1189–1198.

DIETZEL, I. D., DRAPEAU, P. AND NICHOLLS, J. G. (1986). Voltage dependence of 5-hydroxytryptamine release at a synapse between identified leech neurones in culture. *J. Physiol., Lond.* **372**, 91–205.

FRENCH, K. A. AND MULLER, K. J. (1986). Regeneration of a distinctive set of axosomatic contacts in the leech central nervous system. *J. Neurosci.* **6**, 318–324.

FUCHS, P. A., HENDERSON, L. P. AND NICHOLLS, J. G. (1982). Chemical transmission between individual Retzius and sensory neurones of the leech in culture. *J. Physiol., Lond.* **323**, 195–210.

GARCIA, U., GRUMBACHER-REINERT, S., BOOKMAN, R. J. AND REUTER, H. (1990). Distribution of Na^+ and K^+ currents in soma, axons and growth cones of leech Retzius neurones in culture. *J. exp. Biol.* **150**, 1–17.

HAYDON, P. G., MCCOBB, D. P. AND KATER, S. B. (1987). The regulation of neurite outgrowth, growth cone motility, and electrical synaptogenesis by serotonin. *J. Neurobiol.* **18**, 197–215.

HENDERSON, L. P., KUFFLER, D., NICHOLLS, J. G. AND ZHANG, R. J. (1983). Structural and functional analysis of synaptic transmission between identified neurones in culture. *J. Physiol., Lond.* **340**, 347–358.

KUFFLER, D. P., NICHOLLS, J. G. AND DRAPEAU, P. (1987). Transmitter localization and vesicle turnover at a serotoninergic synapse between identified leech neurons in culture. *J. comp. Neurol.* **256**, 516–526.

LIU, Y. AND NICHOLLS, J. G. (1989). Steps in the development of chemical and electrical synapses by pairs of identified leech neurones in culture. *Proc. R. Soc.* B **236**, 253–268.

MASUDA-NAKAGAWA, L., BECK, K. AND CHIQUET, M. (1988). Identification of molecules in leech extracellular matrix that promote neurite outgrowth. *Proc. R. Soc.* B **235**, 247–257.

NICHOLLS, J. G. (1987). The search for connections: Studies of regeneration in the nervous system of the leech. *Magnes Lecture Series* **2**, 1–86. MA: Sinauer Associates.

RAYPORT, S. G. AND SCHACHER, S. (1986). Synaptic plasticity *in vitro*: cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J. Neurosci.* **6**, 759–763.

READY, D. F. AND NICHOLLS, J. G. (1979). Identified neurones isolated from leech CNS make selective connections in culture. *Nature, Lond.* **281**, 68–69.

ROSS, W. N., ARÉCHIGA, H. AND NICHOLLS, J. G. (1987). Optical recording of calcium and

voltage transients following impulses in cell bodies and processes of identified leech neurons in culture. *J. Neurosci.* **7**, 3877–3887.

ROSS, W. N., ARÉCHIGA, H. AND NICHOLLS, J. G. (1988). Influence of substrate on the distribution of calcium channels in identified leech neurons in culture. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4075–4078.

STEWART, R. R., ADAMS, W. B. AND NICHOLLS, J. G. (1989). Presynaptic calcium currents and facilitation of serotonin release at synapses between cultured leech neurones. *J. exp. Biol.* **144**, 1–12.

STUART, A. E., HUDSPETH, A. J. AND HALL, Z. W. (1974). Vital staining of monoamine-containing cells in the leech nervous system. *Cell Tissue Res.* **153**, 55–61.

VIDAL-SANZ, M., BRAY, G. M., VILLEGAS-PEREZ, M. P., THANOS, S. AND AGUAYO, A. J. (1987). Axonal regeneration and synapse formation in the superior colliculus by retinal ganglion cells in the adult rat. *J. Neurosci.* **7**, 2894–2909.

VYKLICKY, L. AND NICHOLLS, J. G. (1988). Specificity of connections formed by nociceptive cells of the leech in tissue culture. *J. exp. Biol.* **134**, 17–26.