Steps in the development of chemical and electrical synapses by pairs of identified leech neurons in culture

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Experiments have been made to follow the development of chemical and electrical transmission between pairs of leech neurons in culture.

1. The cell bodies of identified neurons were isolated from the CNS by suction after mild enzyme treatment, together with a length of the initial segment (or 'stump'). The neurons tested were Retzius cells (R), annulus erector motoneurons (AE), Anterior pagoda cells (AP) and pressure sensory cells (P). Pairs of cells were placed together in various configurations, with different sites on their surfaces making contact.

2. When pairs of Retzius cells were apposed with their stumps touching, serotonergic, chemically mediated synaptic transmission became apparent before electrical transmission. By 2.5 h impulses in either of the two Retzius cells produced hyperpolarizing inhibitory potentials in the other. These potentials were reversed by raised intracellular Cl and showed clear facilitation. The strength of chemical transmission between Retzius cells increased over the next 72 h.

3. After chemical transmission had been established, weak non-rectifying electrical transmission became apparent between Retzius cells at about 24-72 h. By 4 days coupling became stronger and tended to obscure chemically evoked synaptic potentials.

4. When pairs of Retzius cells were aligned in culture with the tip of one cell stump touching the soma of the other, chemical transmission also developed rapidly. Transmission was, however, in one direction, from stump to soma. At later stages non-rectifying electrical coupling developed as with stump-stump configuration. With the cell bodies of two Retzius cells apposed, electrical coupling developed after several days, before chemical transmission could be observed.

5. When Retzius and P cells were cultured with their stumps in contact, inhibitory chemical synaptic transmission developed within 24 h. Transmission was always in one direction, from Retzius to P cell. Electrical coupling of Retzius and P cells never occurred whatever the spatial relations of the cells to one another.

6. Annulus erector motoneurons, which contain ACh and a peptide resembling FMRFamide, first developed electrical coupling when the two stumps were in contact and then, later, bi-directional chemical transmission. Anterior Pagoda pairs placed stump-to-stump showed electrical connections.
7. Electronmicrographs revealed the presence of synaptic structures within 24 h after Retzius-Retzius, Retzius-P or AE-AE stumps were apposed.

8. The specificity of connections between cultured cells was similar to that observed in earlier experiments. A marked difference was in the speed and reliability with which chemical synapses developed when stumps were in contact. The results show that the tip of a neuron represents a preferential site for the formation of chemical synapses.

**Introduction**

A principal aim of the present experiments has been to follow steps that occur during the formation of chemical and electrical synapses. Much information is now available about molecules that regulate the formation of active zones and the clustering of ACh receptors at neuromuscular junctions (Wallace 1986). Changes in presynaptic transmitter release and in postsynaptic receptor properties can be followed during development and regeneration (Xie & Poo 1986; Schuetze & Role 1987). At the same time many questions remain concerning specificity and mechanisms of synapse formation. For example, factors that determine which types of neurons can make connections with one another are largely unknown. Similarly, it is not clear whether particular sites on neurons preferentially make chemical or electrical synapses; or which type of transmission is established first at mixed chemical and electrical synapses.

Distinct advantages for studying such problems are provided by invertebrate neurons maintained in tissue culture. Pairs of identified cells from *Aplysia* (Rayport & Schacher 1986), *Helisoma* (Haydon et al. 1987; Haydon 1988) and the leech, *Hirudo medicinalis*, form chemical, electrical and mixed synapses in culture. For example, with leech neurons it has been possible to make detailed measurements of transmitter release and transmitter action at chemical synapses and to characterize the properties of rectifying and non-rectifying electrical junctions (Dietzel et al. 1986; Aréchiga et al. 1986; Vyklicky & Nicholls 1988). In particular, the chemical synapses that Retzius cells (modulatory neurons) and pressure sensory neurons (P cells) form in culture have been studied in detail.

The starting point of the present experiments was a series of observations that suggested an inhomogeneity of membrane properties in isolated leech neurons. When a cell is pulled out of the CNS of the leech a variable length of the initial process or ‘stump’ is removed together with it. This stump has distinctive properties. It is more sensitive than the soma to certain chemical transmitters; Na\(^+\), K\(^+\) and Ca\(^{2+}\) currents are greater at the stump than elsewhere and sprouting starts preferentially from it rather than from the soma (Y. Liu, unpublished data; Bookman et al. 1987; Chiquet & Nicholls 1987; Ross et al. 1987). The experiments presented here now show that the stump represents a preferred site for synapse formation. Transmission becomes established more rapidly (within hours) and more reliably (with almost 100% success) when cells are carefully plated in culture so that their stumps are touching.

Under these conditions it has been possible to study electrophysiologically events occurring during synapse formation. The main emphasis has been on the
mixed chemical electrical synapse that forms between Retzius cells (Fuchs et al. 1981). In earlier experiments Retzius cells were routinely cultured together in random configurations. Non-rectifying electrical transmission was observed at about 2 days, becoming strong by 6 days. By this time chemically mediated inhibitory synaptic interactions were occasionally observed accompanied by morphological signs of reciprocal chemical synapses in electron micrographs. Retzius cells in culture have been shown to synthesize, store and secrete 5-hydroxytryptamine (5-HT) in quantal packets as their transmitter (Henderson 1983; Henderson et al. 1983). The results presented in this paper show that in certain pairs of cells (Retzius–Retzius) chemical transmission precedes electrical transmission whereas in others (AE motoneurons) electrical precedes chemical. Moreover, the regions of the two cells that come into contact dramatically influence the type of transmission that will be established, as well as the speed of formation.

**Methods**

The techniques for removing cells from leech ganglia, for culture and for recording electrically have been described elsewhere (Dietzel et al. 1986; Chiquet & Nicholls 1987). In brief, identified nerve cells were isolated by suction after mild enzyme treatment of desheathed ganglia by collagenase–dispase (2 mg ml\(^{-1}\)) for 1 h. The culture medium was Leibovitz 15 medium with foetal calf serum (FCS) (2% by volume), 0.6% glucose and garamycin (0.6% by volume; 10 μg ml\(^{-1}\)). An essential procedure in these experiments was to ensure that a long stretch of the initial process was sucked out together with the soma. Of key importance for obtaining long, clean stumps was the cell sucker. The opening of the glass tube had to fit snugly over the cell. Cells were routinely left overnight to allow their surfaces to become cleared of debris. Selected healthy pairs of cells were then plated in microwells, coated with concanavalin A (Con A) or in a few experiments with polylysine or cell-free extract of leech extracellular matrix (Chiquet & Acklin 1986). The microwells were first filled with L15 medium without foetal calf serum to facilitate cell adhesion to substrate. Later, 4% foetal calf serum was added. A technically difficult but essential step was to orientate the two cells in one of three configurations (see figure 1, plate 1): stump touching stump, stump touching soma or soma to soma (this being the configuration used in all experiments on leech neurons in culture before these). In a few experiments neurons were placed with their stumps not quite touching. In others, the cell and its stump was removed in the first place by lassoing it with nylon monofilament. This obviates the need for enzymes during isolation and also allows tests to be made with different lengths of stumps.

Intracellular recordings were made with microelectrodes (20–30 MΩ resistance) filled with 3 m KCl or 4 m potassium acetate (KAc). In a series of experiments it has been shown that recordings of synaptic potentials in P cells and Retzius cells are remarkably stable for long periods when KCl electrodes are used. The sizes of depolarizing, inverted inhibitory postsynaptic potentials (IPSPs) provide a convenient index of the amount of 5-HT released by the Retzius cell. The quantal unit size of approximately 300 μV varies little from P cell to P cell (Dietzel et al.
The Ca concentration in L15 FCS solution was increased to 12 mM to enhance chemically mediated synaptic potentials. With care to prevent contamination, the same pair of cells could be recorded from more than once over the first three days in culture. In such experiments the fluid was changed after each recording. Electrometers and stimulators made by Almost Perfect Electronics, Basle, were used and the results displayed on a Tektronix oscilloscope. Traces were recorded on a videocassette recorder.

**Results**

*Development of connections between Retzius cells*

*Synapse formation with stump-to-stump contact of Retzius cells*

After stumps were placed in contact, the first signs of transmission between Retzius cells appeared in about 2.5 h. Times shorter than this were not explored in detail for technical reasons. Transmission in the earlier stages was always chemical and from the outset it was bi-directional in most pairs of cells (48 out of 56 pairs in one series of experiments). Usually, however, transmission was stronger in one direction, as in figure 2, which shows two Retzius cells with their stumps touching at 25 h and recordings made by intracellular electrodes filled with 4 mM KAc. Transmission in one direction (figure 2a), was stronger than the other; in figure 2b a train of impulses was required for hyperpolarizing ipsps to become apparent. These inhibitory potentials, like those seen in earlier experiments with Retzius–Retzius or Retzius–P cells placed in culture in random configurations, were reversed by hyperpolarizing currents and by impaling the cells with KCl electrodes (figure 2c, d).

Over the first two days, chemically mediated transmission became progressively stronger before electrical coupling developed. With care it was possible to record from the same pair of cells more than once without damage or infection of the culture (figure 3) so as to follow changes in the strength of transmission directly. Between 7 and 56 h the synaptic potentials in figure 3 grew from approximately 1.3 to 13 mV.

The timecourse of synapse formation was notably slower for electrical than for chemical synapses. Figures 2 and 3 show that electrical coupling if present at 2.5–24 h was too weak to be detected. At later stages at approximately 36 h electrical transmission started to be evident. By four days maintained hyperpolarization and depolarization spread directly from one cell to the other with no sign of rectification (figure 4).

A remarkable feature of these experiments was the success rate. In a series of 66 Retzius cell pairs with their stumps apposed (figure 5a, b) almost every cell evoked chemical ipsps in its partner within one day and at later stages showed non-rectifying electrical coupling. Tests were made to determine whether chemically mediated transmission persisted after the development of electrical transmission which tended to predominate at later stages. With KAc-filled electrodes ipsps could still be recorded although electrical coupling was present. For example, in the four-day-old pair of Retzius cells shown in figure 6, clear hyperpolarizing potentials were evoked by each presynaptic action potential; in the same pair of
Figure 2. Chemically mediated synaptic connections between a pair of Retzius cells plated with stumps in close apposition. The photograph above shows two Retzius cells plated on Con A for 25 h. (a, b) Intracellular recordings made with electrodes containing KAc. In (a) the presynaptic action potential evokes a clear IPSP in the other cell. Transmission in the opposite direction (b) is weaker. Lower records (c, d) show reversal of IPSPs by Cl injection from KCl-filled microelectrodes.

Figure 3. Development of chemically mediated synaptic transmission between a pair of Retzius cells plated with their stumps closely apposed on Con A at 7 h and later at 56 h (same pair of cells). Note the large increase in amplitude of the synaptic potential (KCl electrodes.) Similar results were obtained with successive penetrations of 15 pairs of cells using KCl- or KAc-filled electrodes.
Electrical coupling between a pair of Retzius cells plated on Con A for four days with stumps closely apposed. Depolarizing or hyperpolarizing pulses applied to one cell spread to the other equally well in both directions. Intracellular recordings made with KAc electrodes; chemically mediated synaptic transmission is obscured by the electrical coupling (see below).

![Graphs showing timecourse of formation of chemical (filled circles) and electrical (open circles) synapses between Retzius cells in culture with stumps apposed.](image)

(a) Virtually 100% of 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 at one time. In (b) the mean values are shown; s.e. 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 bi-directional, usually stronger in one direction than the other. Electrical transmission was non-rectifying.

Cells steady hyperpolarizing potential spread through the electrical junctions. Results such as these indicated that chemical transmission continued to function as electrical coupling developed. Another method for demonstrating persistence of chemically mediated transmission was to stimulate the presynaptic cell with a brief pulse. The impulse initiated an IPSP but the electrotonic pulse being short was attenuated.
Physiological properties of inhibitory potentials in Retzius cells connected by their stumps

Clear facilitation of chemically mediated synaptic transmission was observed between Retzius cells with their stumps apposed. Facilitating inhibitory synaptic potentials were reversed by KCl injection into the postsynaptic Retzius cell. Similar facilitation has been seen at Retzius–P synapses in culture where it has been shown to be presynaptic in origin and to result from increased quantal release of 5-HT from Retzius cells (see Henderson et al. 1983).

Tests were made to determine whether 5-HT release was quantal at early stages in preparations with stump–stump apposition. In principle the synaptic potentials observed could arise from: (a) focal release from specialized presynaptic endings apposed to the postsynaptic cell; (b) release from distant sites, diffusing indiscriminately to postsynaptic receptors; (c) a mixture of both mechanisms. If miniature potentials could be observed with times to peak similar to those of evoked synaptic potentials, this would suggest the presence of closely apposed synaptic structures. In earlier experiments made with Retzius–Retzius synapses established in culture without stump–stump apposition for three days or longer, spontaneous miniature potentials approximately 300 μV in amplitude were recorded; structural specializations were observed by electronmicroscopy (Henderson et al. 1983; Kuffler et al. 1987). With stump–stump apposition of Retzius cells, miniature potentials appeared as early as a few hours and were of similar amplitude to those observed in the earlier experiments. The spontaneous miniature potentials seen in a postsynaptic Retzius cell had rise times strikingly
similar to those of the full size synaptic potentials. For example, in one series the half-time to peak was $36 \pm 8$ ms (s.d.) for miniature potentials compared with $35 \pm 6$ ms for evoked potentials that were approximately 20 times larger in amplitude (see figure 7a, b). Miniature potentials occurred irregularly at low frequency with occasional ‘giants’ and were particularly obvious after stimulation of the presynaptic cell. A description of the morphology of synapses formed between stumps is given below (see figure 11, plate 2).

\[ \begin{array}{c}
\text{(a)} \\
\text{mV} \\
0 \quad 1 \quad 2 \quad \text{time/s} \\
\end{array} \]

\[ \begin{array}{c}
\text{(b)} \\
\text{mV} \\
0.5 \\
\end{array} \]

**Figure 7.** Synaptic potentials and miniature potentials recorded from a pair of Retzius cells plated on Con A with stumps apposed for 24 h. (a) An impulse in one Retzius cell evoked a large synaptic potential in the other Retzius cell. Note miniature potentials appearing late in trace. (b) Spontaneous miniature potentials recorded from the postsynaptic Retzius cell. The unit size appears to be approximately $300 \mu$V, similar to that previously observed at Retzius–P synapses in culture. Note the similarity in rise times of miniature potentials to the rise-time of the large evoked potential. Measurements in this experiment and in earlier experiments of quantal release were made with KCl-filled microelectrodes (see Methods). From preparation to preparation the synaptic potentials recorded in this way are remarkably constant.

**Influence of substrates and stump length on synapse formation**

Tests were made to assess the influence of a number of variables on the development of chemical and electrical synapses with stump–stump apposition of Retzius cells. Although the molecular composition of the substrate plays a key role in sprouting and in the distribution of Ca$^{2+}$ channels (Chiquet & Nicholls 1987; Ross *et al.* 1988), it had no effect on synapse formation. Thus chemical transmission appeared before electrical coupling with similar reliability within a few hours: (a) on polylysine on which cells did not sprout at all at early stages (13 pairs); (b) on Con A on which they sprouted rapidly with curved, branched processes (76 pairs);
and (c) on laminin-containing extract made from leech extracellular matrix upon which they also sprouted rapidly but with a different pattern (three pairs). The results were also similar with cells taken from one leech or paired from different leeches.

In another series of experiments Retzius cells were removed from ganglia by lassoing them with nylon monofilament. With this procedure the use of enzymes is avoided and the stump is broken off closer to the soma. In such cells (nine pairs) the sequence of events was the same as when enzyme was used even though different regions on the stump were in contact. When enzyme-treated Retzius cells (13 pairs) were placed with their stumps close together (approximately 20 μm apart) but not quite touching, similar results were obtained except that transmission developed later and synaptic potentials were smaller. Retzius cells were also allowed to remain in medium for five days before plating them with their stumps touching; as usual, chemical transmission developed in a few hours to be followed by electrical transmission.

**Synapse development with stump to soma or soma to soma apposition of Retzius cells**

With stump to soma apposition (see figure 1) the results were in certain respects similar to those described for stump–stump configuration: chemically mediated transmission first appeared after about 6 h with high reliability (20 out of 22 pairs), followed by non-rectifying electrical transmission. A major difference is shown in figure 8. In the initial stages up to six days chemical transmission was unidirectional from stump to soma, with no sign of transmission in the opposite direction (17 out of 17 pairs). Chemical transmission in the opposite direction appeared at later stages (six days or longer) but it was difficult to detect because by then electrical transmission was strong.

With soma to soma apposition of Retzius cells (figure 1), chemical transmission developed even more slowly and with greater variability. This was the configuration used in earlier experiments on leech neurons in culture. In only a few pairs (3 out of 15) could weak signs of chemical transmission be detected after three days. Electrical coupling of Retzius cells is observed regularly with soma–soma contacts (Fuchs et al. 1981). These results indicate that a specific region on the neuronal surface of a Retzius cell, the stump, acts as a preferred site for forming presynaptic chemical specializations. The soma, by contrast, can receive synaptic input as readily as the stump, but is less favourable for developing release sites.

**Retzius to P connections with stumps apposed**

The chemical synapse that has till now been most intensively studied in culture is that between Retzius and P cells. This offered the advantage that no electrical component was ever detected in literally thousands of pairs. Transmission was always from Retzius to P cell and was shown to be mediated by quantal release of 5-HT. As for Retzius–Retzius synapses, the ipsp's were reversed by raised intracellular Cl. Clearly defined synaptic structures were seen in electronmicrographs (see Henderson et al. 1983; Kuffler et al. 1987). In such experiments, transmission at Retzius–P synapses developed by about three days, becoming
**Figure 8.** Synaptic interactions between Retzius cells plated on Con A for 36 h with stump in contact with soma (KAc electrodes). Chemical transmission between such cells developed rapidly but was always in only one direction: from stump to soma, not from soma to stump (see also figure 1).

strongest at seven to ten days. However, many pairs looped or sucked out of the ganglion failed to form synapses. We could not identify a single primary factor that would influence the success rate. We attributed this variability to the vagaries of tissue culture.

Accordingly it was of interest to determine what connections would form between Retzius and P cells with stump–stump apposition. Figure 9 shows a Retzius and a P cell with their stumps in contact at 25 h. An impulse in the Retzius cell evoked a large inhibitory synaptic potential in the P cell that was reversed by Cl injection. The properties of such synapses were identical to those of R–P cells in culture described previously, with two important differences: they appeared in hours instead of days and in every healthy pair (8 out of 8), reliably instead of capriciously. Unlike Retzius–Retzius synapses no signs of electrical coupling appeared at any stage. These results indicate that the specificity of connections is maintained when stumps of different types of neuron are apposed.

**Connections of AE motoneurons and AP cells**

In culture, AE motoneurons and AP cells have been found to make non-rectifying electrical connections between each other and from cell type to cell type. Electrical synapses were detected at two days and became stronger by six days.
Rapid synapse formation

0 0.5 1.0

Figure 9. Chemical transmission between a Retzius and a P cell plated on Con A with stumps in close apposition for 25 h. An impulse in the Retzius cell (lower trace) evoked a large synaptic potential in the P cell (upper trace). An impulse in the P cell (right upper trace) had no effect on the Retzius cell (right lower trace). Recordings were made with KCl-filled electrodes. These results are in agreement with experiments made on Retzius and P cells plated in random configurations. The synaptic potentials under those circumstances developed only after three or more days, were far less reliable and often of smaller amplitude.

Such cells were paired in close apposition with random configurations. For AE cells, occasionally hints of weak chemical inhibitory interactions were observed but they could not be analysed because of strong electrical coupling (Aréchiga et al. 1986). Chemical synapses would be of pharmacological and physiological interest because both AE and AP cells are known to be cholinergic and to contain a peptide resembling FMRFamide. This has been observed by antibody staining to be present in dense core vesicles (Aréchiga et al. 1986).

AE motoneurons in culture with their tips apposed could develop chemical interactions within 48 h. Figure 10 shows rIPSPs evoked in one AE motoneuron by stimulation of the other at five days. Chemical transmission was often apparent in both directions. From earlier experiments it was known that ACh applied ionophoretically to the AE cell in the ganglion and in culture gives rise to hyperpolarizing potentials reversed by Cl (Sargent et al. 1977; Y. Liu, unpublished data). A clear difference from Retzius pairs and Retzius–P pairs was that electrical coupling between AE cells usually preceded chemical transmission, appearing in
FIGURE 10. Inhibitory synaptic interactions between AE motoneurons. The photograph above shows a pair of AE cells plated on Con A for five days with stumps apposed. An impulse in one AE cell (lower trace) evoked a large hyperpolarizing potential in the other; recorded with KAc-filled electrodes. Such chemical synaptic potentials often coexisted with electrical coupling. Although chemical transmission could be initiated from either cell, it was usually stronger in one.

the first 24 h. AP cells with stumps in contact also developed electrical coupling rapidly in a few hours. Chemical transmission, if present at later stages, was weak and masked by the strong coupling.

Morphology of stump contacts

Electronmicrographs have been made of pairs of cells that had their stumps in contact and that showed chemically mediated transmission. An example is shown

DESCRIPTION OF PLATE 1

FIGURE 1. Retzius cells plated on Con A with their stumps 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 was 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.
Proc. R. Soc. Lond. B, volume 236

Liu & Nicholls, plate 1

Figure 1. For description see opposite.
Figure 11. For description see opposite.
in figure 11, plate 2, in which the junctions between a Retzius and a P cell are shown.

The synaptic potentials recorded in this P cell were large (2.5 mV in amplitude recorded with KCl electrode). By 25 h structures similar to those observed at synapses in situ and in culture were apparent. In Retzius cells clear vesicles were clustered in close apposition to active zones with occasional dense core vesicles. These presynaptic structures were situated directly opposite postsynaptic membranes. The presynaptic specializations were only found in Retzius cells, never in P cells. The differences in cytoplasm and intracellular organelles make identification of these two cells unambiguous in the electronmicroscope. Earlier studies by Kuffler et al. (1987) have shown that P cells and R cells contain distinctively different dense core vesicles. In serial sections fine processes can not only be recognized but traced to the cell of origin. Synapses with similar characteristics were observed in pairs of Retzius cells in which synaptic potentials had been recorded. Such synapses were, however, reciprocal, with presynaptic release sites observed in both cells, often opposite one another.

AE cells in which chemical transmission had been demonstrated physiologically also exhibited characteristic synaptic structures. Morphological concomitants of electrical coupling have not yet been observed by electronmicroscopy. A fuller description of these morphological features in relation to the timing of synapse formation and vesicle turnover is in preparation (D. P. Kuffler, personal communication). These results indicate that synaptic structures develop between stumps of cells at the same time as chemical transmission becomes apparent.

**Discussion**

A useful feature of the leech neuron preparation is that both pre- and postsynaptic cells can be impaled with electrodes allowing direct tests to be made of the mechanism of transmission at every stage during synapse formation. This is not usually possible in other preparations having small presynaptic terminals, such as motor nerve endings or synapses established by fine processes at a distance from the cell bodies. That chemical transmission should be established before electrical coupling represents a novel and somewhat unexpected finding. Thus dye-coupling, electrical transmission and gap junctions can develop in minutes in certain types of cell (Loewenstein 1981). In contrast, the structural elements required for the formation of a chemical synapse seem so elaborate that one might

**Description of Plate 2**

*Figure 11.* Electronmicrographs showing synaptic structures developing between Retzius (R) and P cells in culture for 24 h. In both pairs of cells 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 by this and by other criteria. Presynaptic specializations are closely apposed to the postsynaptic membranes. Transmission at these synapses is serotonergic and always from R to P. These electronmicrographs were kindly prepared and photographed by Dr D. P. Kuffler.
perhaps have expected them to develop more slowly (see also Haydon 1988). In certain preparations signs of chemical transmission do appear rapidly as receptors on a target cell respond to transmitter released from nerve terminals (Xie & Poo 1986). Similarly, at about 2.5 h the first chemically mediated synaptic potentials observed in Retzius cells were small, slow and could have arisen through diffusion from distant release sites with no true synaptic specializations. By 12 h or so virtually all pairs of cells showed strong chemical interactions with rapidly rising miniature potentials and normal physiological properties. In those preparations examined by electronmicroscopy well-defined synaptic structures were apparent at this stage; yet there was no sign of electrical coupling.

The sequence (chemical transmission before electrical) was not always followed but depended critically on several variables. When the same two Retzius cells were placed with their somata in contact the electrical coupling became apparent at about the same time as when the stumps touched; but chemical transmission now took several days instead of hours to develop. With different types of cell, for example AE cells with their tips in contact, chemical and electrical transmission often appeared together. Variables such as substrate, which have dramatic influences on growth and the distribution of Ca channels (Ross 1988), had no effect on the formation of chemical synapses. Many synapses formed in culture between different types of neuron show combined chemical and electrical transmission (Haydon 1988; Rayport & Schacher 1986). It is not yet known, however, whether the sequence of steps is similar. Nor are the steps occurring during embryonic development or regeneration known.

With cultured leech cells different regions of the neuronal surface can be tested in detail for their propensity to form chemical or electrical connections. A key finding was that the initial segment or stump of a unipolar leech neuron is the preferred site for developing presynaptic specializations. This region of the cell is particularly sensitive to chemical transmitters; lower concentrations are required to elicit responses here than on the soma (Y. Liu, unpublished observations). The Ca\textsuperscript{2+}, Na\textsuperscript{+} and K\textsuperscript{+} currents measured by loose patch-clamp electrodes are greater in the stump than on the soma in both acutely removed and chronically cultured cells (Bookman et al. 1987). Additional evidence for higher concentrations of calcium channels in the stump has been provided by studies in which Ca transients were measured optically by the indicator arsenazo III (Ross et al. 1987). Growth in culture has been observed to start from the stump. However, sprouts can also grow out from the soma and do so in profusion when neurons are removed without a stump (Chiquet & Nicholls 1987). Presumably the initial process represents a region in which constituents required for synapse formation are already present.

It is tempting to speculate further that channels and receptor proteins synthesized in the cell body accumulate at the stump before being transported to growing neurites. Numerous questions remain unanswered. It is not clear why chemical synapses should be formed before electrical junctions at some synapses but not others and why the sites of contact should so strongly influence the sequence of events. (In all previous experiments electrical synapses developed before chemical synapses when somata of Retzius cells were apposed.) Also obscure are the mechanisms by which cells recognize their partners so as to form the
appropriate chemical and electrical connections in the appropriate sequence. An obvious long-term goal of studies involving these isolated identified cells in tissue culture is to approach such questions by molecular biological techniques.

**Advantages of new procedures for establishing synapses**

The rapid and reliable development of chemically mediated synaptic interactions represents a significant technical advance for culturing leech cells. So far, synapse formation has been slow (three days or more) and the success rate has been inconsistent from day to day and leech to leech. Moreover, in *Aplysia*, snail and leech neurons electrical transmission often appears with chemical transmission (Rayport & Schacher 1986; Haydon 1988) and this seriously handicaps detailed analysis of synaptic mechanisms.

The new technique of placing Retzius or AE cells with their stumps touching has to a large extent taken care of these problems. Provided the cells are healthy, clean and placed correctly, chemical transmission always appears within a day or two. This short time and the extraordinarily high success rate shorten the feedback loop for the experimenter between removal and electrical testing and provide a consistent baseline for testing various factors such as media and substrates.

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