SPECIFICITY OF CONNECTIONS FORMED BY NOCICEPTIVE CELLS OF THE LEECH IN TISSUE CULTURE

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SUMMARY

Individual medial and lateral nociceptive (N) cells of the leech have been paired with a variety of different target cells to determine the patterns and the properties of the connections made in culture.

1. Lateral nociceptive N cells did not make electrical or chemical connections with other N cells or with P sensory cells.

2. N cells made electrical connections with Retzius cells, anterior pagoda cells (AP) and annulus erector (AE) and L motor neurones in culture.

3. Lateral and medial N cells, which differ in the animal, exhibited consistent differences in culture. Well-established electrical junctions made by lateral N cells showed clear rectification. In contrast, medial N cells could make either non-rectifying or rectifying connections with target cells.

4. In spite of the presence of strong electrical coupling, evidence for chemically mediated synaptic interactions between N cells and other neurones was obtained on occasion.

5. These findings emphasize the specific nature of connections formed in culture. Unlike P sensory cells, N cells in culture did become electrically connected to Retzius cells and could make both rectifying and non-rectifying electrical junctions with appropriate targets.

INTRODUCTION

Isolated, identified leech neurones in culture retain their membrane properties and are able to form chemical and electrical synaptic connections with some targets but not others (Fuchs, Nicholls & Ready, 1981; Aréchiga, Chiquet, Kuffler & Nicholls, 1986). Retzius cells, anterior pagoda cells, heart motor neurones (HE cells) and L motor cells all become connected by non-rectifying electrical junctions in the dish. In contrast, P sensory cells have been shown to form electrical or chemical connections but only with some cells and not with others. Moreover, all the electrical synapses so

far observed in P cells in the dish and in the ganglion show rectification (Aréchiga
et al. 1986; Nicholls, 1987). Thus, P cells make rectifying electrical junctions with L
cells, HE cells and AP cells. They are never electrically connected to Retzius cells but
are the postsynaptic element in a chemical synapse involving 5-hydroxytryptamine
(5-HT) as a transmitter (Fuchs, Henderson & Nicholls, 1982; Henderson, 1983). On
the one hand these results demonstrate specificity; on the other hand they show that
cells in the dish are able to form connections not normally present in the ganglion.

The aim of the present series of experiments has been to extend information about
synapses formed in culture by different neurones. Interesting candidates for
exploration are the nociceptive N cells. Within the ganglia there are two N cells on
each side: one medial, one lateral. They both form excitatory chemical connections
upon the L cell and AE motor cells in the same ganglion. The connection to the L cell
exhibits a weak, rectifying electrical component (Nicholls, 1987). In addition, the
medial and lateral N cells on one side of a ganglion show differences in their
membrane properties, in their chemosensitivity and in the connections they make on
postsynaptic targets (Johansen & Kleinhaus, 1985).

From earlier work it was known that N cells retained their membrane properties
and formed mixed chemical and electrical synapses upon L cells in culture, the
electrical component showing rectification (Fuchs *et al.* 1981). We have now paired
N cells with a variety of identified cell targets to observe whether the electrical
connections are rectifying or non-rectifying, whether chemical synapses occur and
whether connections in the dish resemble those observed in the animal. The question
of how Retzius cells become connected to N cells was of interest in relation to
specificity. Would they make pure chemical synapses without an electrical com-
ponent (as on P cells) or electrical synapses or neither?

MATERIALS AND METHODS

The methods for identifying cells in leech ganglia, *Hirudo medicinalis*, removing
them and maintaining them in culture have been described previously (Fuchs *et al.*
1982). All the experiments were performed by removing cells with loops of nylon
monofilament and plating them on polylysine. Recent experiments have shown that
this procedure enhances synapse formation compared to the technique of removing
cells by suction after enzyme treatment (Dietzel, Drapeau & Nicholls, 1986). Cells
were plated on polylysine to ensure that the sprouts were short. Under these
conditions the cells are isopotential (Ross, Aréchiga & Nicholls, 1987) and current
spread from cell to cell can provide a reliable index of junctional properties (Davis,
1986). Only pairs of cells with comparable, high input resistance ($>50 M\Omega$) were
used. Culture medium consisted of L-15 with 2% foetal calf serum and $0 \cdot 1$ mg ml^{-1}
Gentamycin. The cells were cultured in microwells as closely apposed pairs.
Electrical recordings were made with microelectrodes filled with potassium acetate of
resistances $20{-}40 M\Omega$. Amplification was through Almost Perfect Electronics
amplifiers, signals being recorded on a Hewlett Packard tape recorder. The cells were
identified by visual inspection in ganglia before removal. In culture each type of cell

retains its characteristic membrane properties and action potential configuration by which it can be recognized. Occasionally T cells were isolated instead of N cells but this became apparent immediately upon recording. One source of ambiguity is that both medial and lateral N cells have very similar action potentials in culture and in the animal. Thus, the identification of these cells as medial or lateral N cells must be made in the ganglion, where their positions can show some variability. In some instances medial N cells may have been mistaken for lateral N cells.

In all experiments, except where specifically stated, the membrane potentials of both cells in the pair were held at about -50 mV by passing d.c. through the microelectrodes after balancing the bridge. (The normal resting potentials of these cells in culture range from -45 to -55 mV.) This was important since junctions with rectifying properties could appear to pass current in both directions if one cell was held at a different potential to the other (see Fig. 2; also Davis, 1986). Tests for synaptic connections were made at times ranging from 6 to 13 days. By this time the rectifying or non-rectifying properties of electrical junctions have become established (Davis, 1986).

RESULTS

Specificity of connections made by N cells in culture

The connections made by medial and lateral N cells with individual target neurones were not random. Lateral N cells never became connected by electrical or chemical synapses to each other. Action potentials, as well as large hyperpolarizing or depolarizing pulses, applied to one N cell produced no discernible effect on the other N cell in either direction (16 pairs). Similarly, N cells did not become coupled electrically or chemically to P sensory cells (eight pairs). In the following sections the connections made by lateral N cells on AP, AE and L cells are described first, followed by those made by medial N cells.

Electrical connections of lateral N cells with AP, AE and L cells

In earlier experiments by Fuchs *et al.* (1981) it was observed that lateral N cells could make rectifying electrical junctions with L cells in culture. Depolarization spread better from N to L cells, hyperpolarization from L to N cells. In the present experiments 11 closely apposed lateral N and L pairs showed similar rectification (three additional pairs showed no electrical or chemical interactions). Lateral N cells also formed rectifying electrical junctions with AP and AE cells (20 pairs). Again, the rectification was such that depolarization spread better from N cells. Fig. 1 shows that depolarizing current spread far better in one direction (from N to AP) and hyperpolarization in the other (AP to N). All junctions between lateral N cells and AP, AE and L cells continued to show clear rectification for 2 weeks, the longest time tested. Hence, the rectification does not represent a transient stage in the formation of non-rectifying junctions, such as those Davis (1986) observed for L cell pairs, AP cell pairs or Retzius cell pairs. In those experiments, current could spread in both directions by 6 days.

Apparent asymmetry of current spread between two cells could, in principle, result from damage caused by microelectrodes. In the present experiments both cells had similar high input resistances (see Materials and Methods); moreover, rectification was always in the same direction. Rectifying junctions established between P and AP cells in culture have been analysed by Davis (1986) using the voltage-clamp technique. The rectification characteristics with voltage- and with current-clamp techniques were similar and resembled those in the present experiments.

A possible pitfall in analysing current spread between pairs of cells coupled by rectifying junctions is shown in Fig. 2. As in Fig. 1, hyperpolarization spread better than depolarization from AP cell to N cell with both cells at their normal resting potential of about −50 mV. When the AP cell was hyperpolarized by steady current to −70 mV, the junctional characteristics appeared to be 'non-rectifying'. Such behaviour is inherent in the current–voltage relationship of the junctions: 'depolarization' of the AP cell from −70 to −50 mV is equivalent to a reduction in hyperpolarization by 20 mV. As expected, when both cells were held at −70 mV coupling was again rectifying. In previous experiments by Davis (1986) and Fuchs et al. (1981) both cells were always held at their normal resting potential (−50
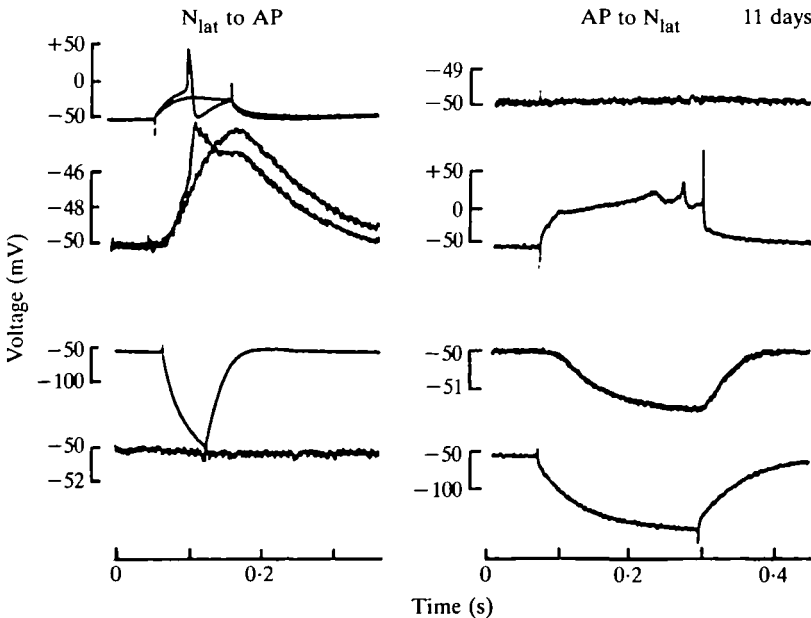

Fig. 1. A rectifying electrical synapse between a lateral N (N$_{lat}$) cell and an AP cell in culture for 11 days. In each of the paired traces the records from the N cell are above, those from the AP cell below. Low gain is used for the cell into which current is passed, high gain for the coupled cell's records. Depolarization spread better than hyperpolarization from lateral N to AP (left-hand traces), hyperpolarization better than depolarization from AP to lateral N cells (right-hand traces). Connections with similar properties were also established with AE, L and Retzius cells.
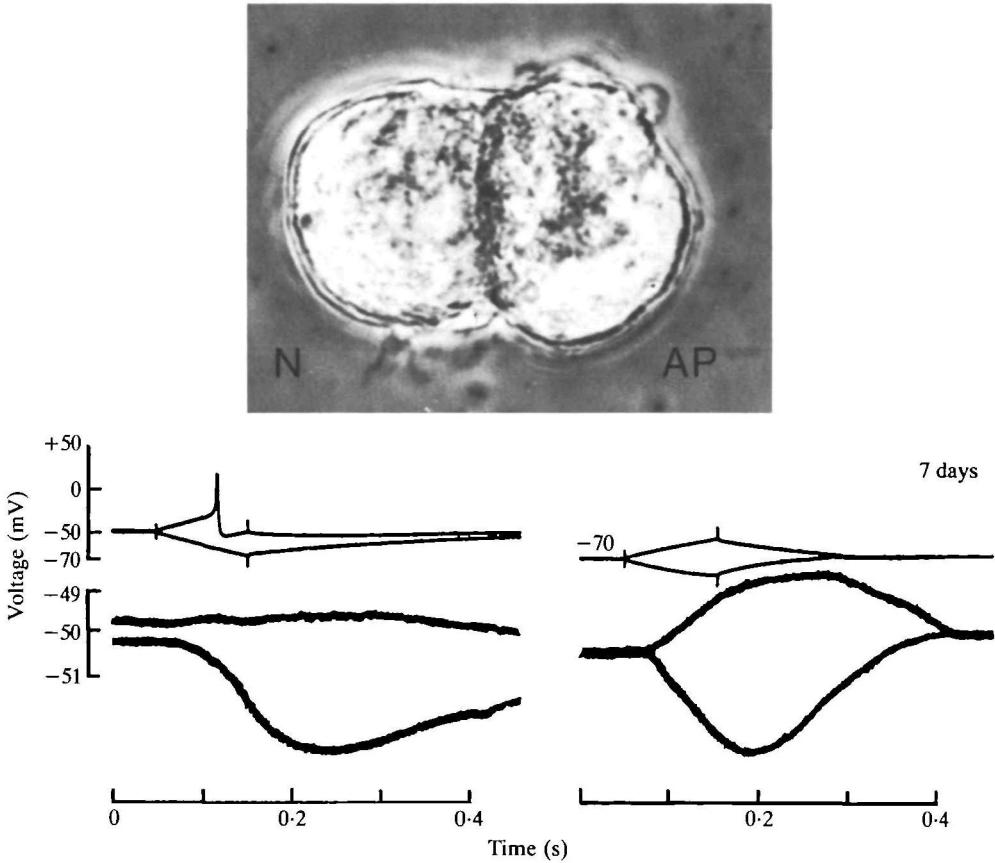
Fig. 2. Effect of membrane potential on electrical coupling between N and AP cells. Traces on the left show typical rectification for current spread from AP to lateral N cells. In both sets of records, traces from the AP cell are above (at low gain) and those from the N cell are below (high gain). For traces on the right the AP cell was hyperpolarized from −50 to −70 mV. Now 'depolarizing' potentials could spread to the lateral N cell. This result would be expected for a rectifying junction under these conditions; the 'depolarization' from −70 mV is, in fact, a reduction in hyperpolarization. The photograph shows the N cell (left) and the AP cell (right). The diameter of the N cell is 70 μm.

or −45 mV) to assess whether coupling was rectifying or non-rectifying to avoid such problems in interpretation.

Of 37 pairs of lateral N cells with various targets, 36 showed rectifying junctions. The one exception in which a non-rectifying junction was found between a lateral N cell and a Retzius cell may be due to incorrect identification of the lateral N cell during removal (see below).

Electrical connections of medial N cells with AP, AE and L cells

Unlike lateral N cells, medial N cells made non-rectifying as well as rectifying connections with various targets. Figs 3, 4 show examples of both types of junctions

made between medial N and AE cells in culture. The rectifying or non-rectifying properties of these junctions were not influenced by the duration of the time in culture beyond 6 days. The presence of rectification or non-rectification was not associated with the general condition of the cells as assessed by input resistance, electrical characteristics or microscopic appearance. Out of 12 medial N cells paired with AP and AE cells, four showed non-rectifying junctions and eight showed rectification. It seems likely that the single example of a non-rectifying synapse made by a lateral N cell in our experiments was due to an error in identification during or after removal and that this cell was in fact a medial N cell. Such errors can occur on occasion for N cells, whose position in the ganglion may vary.

Connections of Retzius and N cells

Retzius cells in hundreds of trials have been shown never to make electrical connections with P sensory cells. Even large voltage-clamp pulses to the Retzius cells produce no coupling potentials in P cells (Dietzel *et al.* 1986). Instead, Retzius cells make chemical inhibitory synapses upon P cells with 5-hydroxytryptamine as the
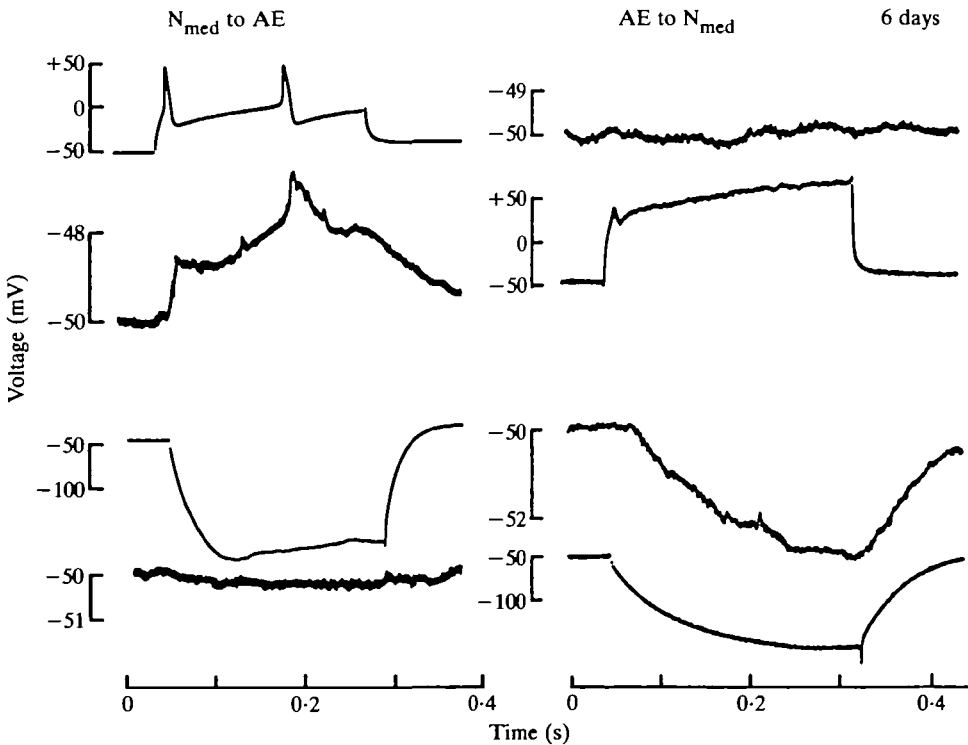

Fig. 3. Rectifying electrical connections of a medial N (N_{med}) cell with an AE cell at 6 days in culture. Records are arranged as in Fig. 1. Records for current injection into N cells (low gain, upper trace) and responses in AE cells (high gain) are shown in the panels on the left. Right-hand panels show effects of injecting currents into AE cells (low gain, lower traces) and responses in the N cells (high gain, upper traces). As for lateral N cells, depolarization spreads better in one direction, hyperpolarization in the other.

transmitter. In contrast, electrical connections were observed in 11 out of 11 pairs of Retzius and lateral N cells. In two other pairs, in which Retzius and medial N cells were used, no rectification was observed, as shown in Fig. 5.

Chemically mediated synaptic interactions

Only rarely, in five pairs of cells out of all the trials we made of N cells, did we observe interactions that appeared to be chemically mediated. Fig. 6 shows synaptic potentials in a pair of N and L cells in which coupling was weak and in which there was a clear synaptic delay. In one other pair there was evidence of facilitation. However, depression was a more obvious feature and this, together with the usual strong electrical coupling, precluded a detailed analysis of chemically mediated potentials.

DISCUSSION

That N cells can make either rectifying or non-rectifying electrical junctions is an unexpected finding. Previous work in the animal and in culture had suggested that certain leech neurones could make only rectifying electrical junctions. For example,
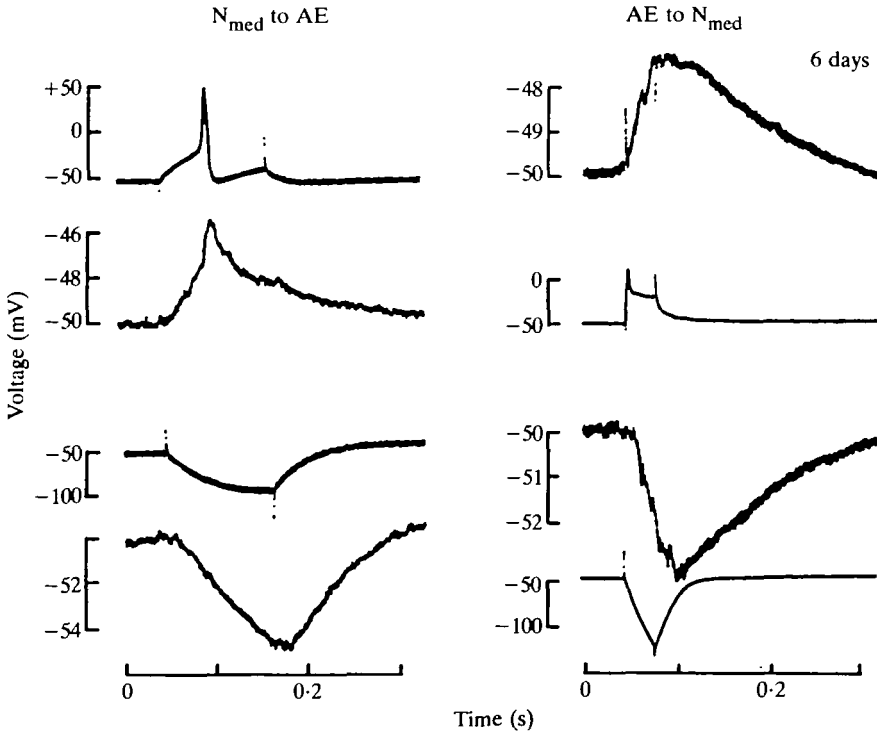

Fig. 4. Non-rectifying electrical connection of a medial N (N$_{med}$) cell with an AE cell at 6 days in culture. For this pair of cells, current spread in both directions. Records arranged as in Figs 1 and 3.
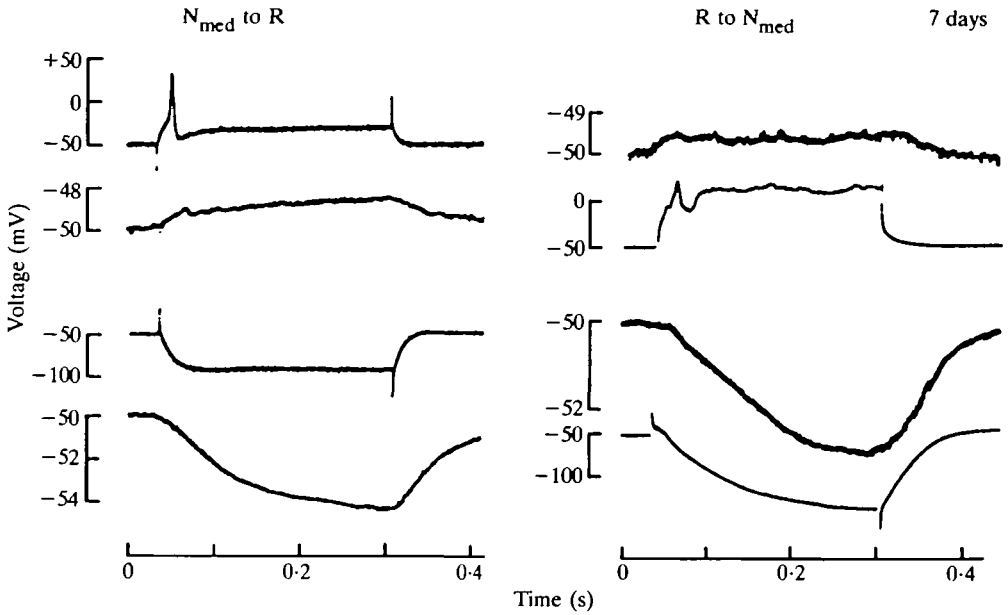
Fig. 5. Electrical connections of a medial N (N_{med}) cell with a Retzius cell at 7 days. In this pair of cells current spread in both directions. In other pairs rectification did occur and was similar to that shown in Fig. 3. Lateral N cells also made rectifying electrical junctions with Retzius cells. N cells differ from P cells which never make electrical connections with Retzius cells.
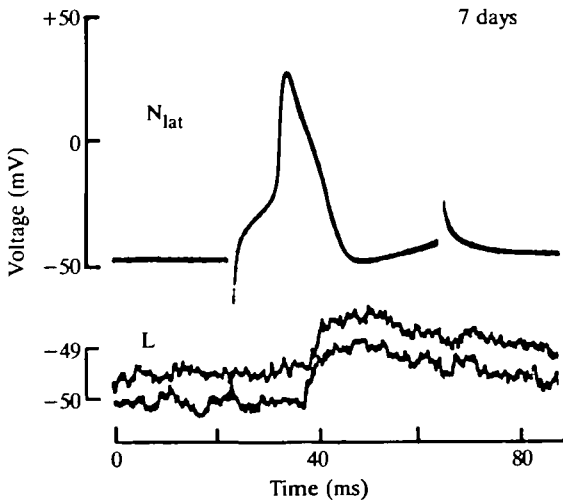

Fig. 6. Synaptic interactions between a lateral N (N_{lat}) and L cell in culture for 7 days. In this pair of cells coupling was weak and the synaptic potentials arose after a delay. With repeated stimulation the synaptic potentials first facilitated and then became depressed, eventually disappearing. These results suggest a chemical origin for the synaptic potentials.

T, P and N sensory cells in the ganglion all make rectifying junctions with L motor neurones, such that depolarization spreads better from sensory cell to motor neurone. In the ganglion, T cells are interconnected by doubly rectifying junctions – depolarization spreads in both directions, hyperpolarization in neither (Nicholls, 1987).

A consistent picture has emerged from studies made on cultured neurones: whenever P cells make an electrical connection it is in the same direction whatever the target (Aréchiga *et al.* 1986). Thus, P cells always make similar, rectifying connections with AP, AE, HE and L cells. In contrast, these cells all make non-rectifying junctions with each other and with Retzius cells in culture.

From these results it seemed that some types of cells (such as P, N or T cells) could make only rectifying junctions; others that made non-rectifying junctions could also receive depolarizing signals from the rectifying cell type. Additional findings that extended these conclusions were obtained from experiments on pairs of cells in culture at different stages (Davis, 1986). At the earlier stages, P cell junctions with AP cells showed rectifying properties qualitatively similar to those at more mature, stronger junctions. In contrast, pairs of cells that make non-rectifying junctions (e.g. L–L, AP–AP, Retzius–Retzius, etc.) at early stages showed asymmetrical current flow. For example, identical L cells at 2 days showed depolarization spreading better in one direction, hyperpolarization in the other. However, by 6 days in the same pair of cells current spread symmetrically in both directions.

As shown in Figs 3, 4, medial N cells differ from lateral N cells and constitute a first exception: medial N cells can make both rectifying and non-rectifying junctions with the same target. Lateral and medial N cells had previously been shown to differ in their connections in the ganglion, in their electrical properties and in their antibody staining (Johansen & Kleinhaus, 1985; Johansen, Hockfield & McKay, 1984).

What determines whether a medial N cell in culture makes non-rectifying or rectifying junctions with its targets? Time does not seem to be a critical variable, neither do the membrane potentials nor the passive electrical properties of the cells in culture. One possible source of error that we cannot rule out is that the medial N cells from different leeches and from different segments were pooled in these experiments and they may vary in their properties. To remove identified medial N cells from ganglia is technically difficult; and we did not keep track of the segmental origin of each cell. This has not been a significant variable for other connections studied in culture. It would be of interest, albeit a daunting task, to make such experiments. Another approach would be to arrange cells in triplets. For example, one medial N cell could be placed between an AP cell and a Retzius cell. Would both connections be non-rectifying, rectifying or mixed?

Only occasionally were signs obtained of chemically mediated transmission with N cells as pre- or postsynaptic elements. The presence of electrical coupling would make small EPSPs or IPSPs hard to discern. Electron micrographs of N cells paired with L cells (D. Kuffler, unpublished data) have revealed morphological specializations resembling synapses with a widened cleft and accumulations of clear vesicles

apposed to the presynaptic membrane. The frequency and extent of these structures have not yet been established.

Unlike P cells, N cells became coupled electrically to Retzius cells. This result further emphasizes the specificity of connections formed in culture. Somehow Retzius cells and P cells are provided with cues that enable chemical but not electrical synapses to form. The cues for Retzius and N cells do allow them to make electrical synapses. With more detailed information about such hard and fast ground-rules, it may become possible to search, using modern techniques, for underlying mechanisms involved in cell–cell recognition in this system.

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