NEURITE OUTGROWTH AND SYNAPSE FORMATION BY IDENTIFIED LEECH NEURONES IN CULTURE

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SUMMARY

After injury, neurones in the central nervous system (CNS) of the leech regenerate with a high degree of specificity. The aim of our experiments has been to study the sequential steps involved in neurite growth and synapse formation using isolated identified neurones in culture. An important requirement for sprouting of leech neurones is the substrate. Neurites grow only slowly and sparsely on polylysine or vertebrate laminin. The extracellular matrix of leech ganglion capsules contains a protease-sensitive factor which can be extracted with urea. With this material as substrate, growth proceeds rapidly in defined medium. Another neurite-promoting substrate is provided by the plant lectin concanavalin A (Con A). The activity of Con A, but not of the capsule matrix factor, is blocked by the Con A-specific hapten methyl α-D-mannoside. The morphology and branching pattern of the neurites in culture depend on the specific substrate and on the type of neurone. During stimulation, less Ca^{2+} uptake occurs into growth cones than in cell bodies. The mechanism of neurite growth seems not to depend on activity-mediated Ca^{2+} influx or on interactions between neuronal cell surfaces. However, even without profuse outgrowth, electrical and chemical synapses develop between neighbouring neurones. The type of synapse depends predictably on the types of neurones within the cell pair. Since the development of a synapse can be followed with time in culture, the sequential events can each be studied separately for this multi-step process.

INTRODUCTION

Neurones within the central nervous system of the leech (*Hirudo medicinalis*) have a high capacity to regenerate functional connections after injury. The phenomenon of accurate regeneration has been demonstrated *in situ*, in operated animals and in isolated nerve cords maintained in culture (Muller & Nicholls, 1981). Experiments in which certain cellular elements were selectively injured or killed made it possible to determine what the axotomized neurone, the distal axonal stump, the glial cells and the target neurones contribute to the process of regeneration (Muller & Nicholls, 1981).

A key question concerns the cellular and molecular machinery that is essential for regeneration. By what mechanism does axotomy induce neuronal growth? How do neurites interact with the extracellular substrate, other cell surfaces and soluble

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factors to sustain growth? How is a neurite growth cone guided towards a potential
target, and what causes it to stop growing and form a synapse? To tackle such
questions, we start with single isolated leech neurones in culture. Such a cell plated
on a specific substrate in defined medium can be confronted with a variety of target
cells. Thus, the exact function of each factor contributing to neurite outgrowth and
synapse regeneration can be studied separately (Nicholls, 1987). An advantage of the
leech is that identified neurones of known function can be used, and that the
regenerative response of every neurone type in culture can be compared with the
performance of the same cell *in situ*.

This article starts with a brief review of experiments previously made on
regeneration of leech neurones in the animal (Nicholls, 1987). Next, we demonstrate
(1) that isolated neurones can be reimplanted into foreign ganglia, where they sprout
at ectopic sites and form synapses with host neurones, (2) that attachment to a
specific substrate is essential to initiate the sprouting of leech neurones, and (3) that
the molecular composition of the substrate has profound effects on the morphology
and branching pattern of growing neurites. Later sections deal with the role of other
external factors (e.g. divalent cations or the presence of other cells) in modulating
neurite growth in culture. The last section provides evidence that specific synapse
formation between cultured neurones can be uncoupled from profuse neurite
growth, and seems to be based on cell–cell recognition which is independent of the
culture conditions.

REGENERATION *IN SITU*

Leech segmental ganglia are linked by a bundle of three nerves: the two
connectives and Faivre's nerve (Payton, 1981). A cut through the whole nerve bundle
physically separates the stumps and makes regeneration between ganglia impossible.
Cutting only one connective, however, generates a V-shaped wound which can be
bridged by regenerating axons (Baylor & Nicholls, 1971). Sprouting is initiated at
the cut ends of damaged axons within 1 day after axotomy (Mason & Muller, 1982);
the cellular mechanism is not known. [However, undamaged leech axons can be
induced to sprout by injury to other processes and target removal (Scott & Muller,
1980), or by killing of homologous neurones (Blackshaw, Nicholls & Parnas, 1982) or
of ensheathing glial cells (Elliott & Muller, 1983).] Scavenger microglial cells
accumulate at the site of a lesion (Morgese, Elliott & Muller, 1983), and a cut is filled
with connective tissue (Baylor & Nicholls, 1971). The wound-healing process allows
profuse neurite growth, leading to the formation of an abnormal neuropile at the site
of injury (Wallace, Adal & Nicholls, 1977). Specific cell surface recognition can occur
within this irregular pseudoneuropile: sprouting axons can find their original stumps
and form synapses or even fuse with them, thereby shortcutting regeneration to the
target cell (Muller & Carbonetto, 1979; DeRiemer, Elliott, Macagno & Muller,
1983). Alternatively, sprouts which leave the pseudoneuropile at a site close to the
original axon tract can grow all the way to the next ganglion and reconnect precisely
with the original target neurone (Wallace *et al.* 1977; Macagno, Muller & DeRiemer,
1985).

Regenerating axons can occasionally form contacts with novel, aberrant targets, but not at random, thus suggesting again specific cell–cell recognition (Macagno *et al.* 1985; French & Muller, 1986).

To elucidate the role of cellular elements during regeneration, they have been selectively eliminated by intracellular injection of pronase (Parnas & Bowling, 1977). In one example, killing of the target neurone did not affect the ability of a regenerating axon to grow back to its old synaptic site and to stop there (Muller & Scott, 1979). In this case, cues along the pathway rather than the target determined the accuracy of regeneration. However, killing of the connective macroglial cell severely disturbed the normal anatomy of the axonal pathways and many aberrant axon branches formed during regeneration (Elliott & Muller, 1983). Nevertheless, cut axons could still grow all the way back to the next ganglion and make functional synapses with normal targets (Elliott & Muller, 1983). Whether the surviving axonal stumps could also guide the regenerating axons in this case has not been determined, but it seems evident that regenerating leech neurones can reach their original targets by aberrant pathways, in the absence of the macroglial cell.

While much is now known about the different routes and the accuracy of neuronal regeneration in the leech CNS, no information is available from experiments made in animals on how the surfaces of regenerating axons interact with the substrate (i.e. other cell surfaces and extracellular matrix) on which they travel in search for connections. In a newly formed wound neuropile, all the necessary components for axonal sprouting and specific cell–cell recognition seem to be present. With single cultured identified cells, it is possible to study the process of regeneration step by step, and to dissect it into a sequence of specific molecular interactions between a growing axon and its environment.

ISOLATED NEURONES IMPLANTED INTO GANGLIA CAN REGENERATE

During axonal regeneration *in situ*, the cell body of a neurone maintains its normal anatomical position and its interactions with surrounding cells; its former target may be available. To test the regenerative capacity of *isolated* neurones under optimal conditions, identified nerve cells were removed from one ganglion and reimplanted into a second one at an ectopic site. After several days, their physiology and anatomy were examined. Leech ganglia were pinned on Sylgard-coated dishes in standard culture medium (Leibovitz-15 medium containing 2% foetal calf serum and antibiotics; Fuchs, Nicholls & Ready, 1981). The ganglion capsule was opened with forceps, and a loop of fine thread tied around an identified neurone (a Retzius or a P cell) which was then pulled out of the ganglion. A small slit was made in the capsule of another ganglion, into which the isolated neurone was to be inserted, by means of a fire-polished glass capillary (Zhang & Nicholls, 1983). The operation damaged the connective tissue and the glial cell, but not the neurones of the host ganglion. After maintaining operated ganglia in tissue culture for a few days, intracellular recordings were made and the cells filled with peroxidase (Muller, Nicholls & Stent, 1981).

The implanted neurones continued to exhibit normal membrane resting and action potentials. They started sprouting at their axonal stumps and showed extensive arborizations after a few days. Neurite growth was not random: consistently, processes found their way through the neuropile to enter the roots and connectives, extending over distances of several millimetres (Fig. 1). Stimulation of roots or connectives with suction electrodes evoked postsynaptic potentials in the implanted cells, demonstrating that they received synaptic input from host neurones. When two Retzius neurones were implanted into a host ganglion, they became electrically coupled as in a normal ganglion (Zhang & Nicholls, 1983).

These experiments showed that leech neurones are able to regenerate axons and synapses after isolation from their normal anatomical context and in the absence of skin and muscle. The host ganglion in this case was permissive for the regeneration of a supernumerary neurone and provided all the necessary cues. Neither axonal growth nor formation of connections appeared random, although the rules and the basis for specificity were not investigated.

ATTACHMENT TO SPECIFIC SUBSTRATES IS ESSENTIAL FOR NEURITE GROWTH

The experiments described so far do not address the question of how growth is initiated and maintained. In culture one can identify the molecular components essential for neurite growth. It turns out that the substrate to which leech neurones attach is the most important factor.

Retzius and anterior pagoda neurones were isolated as described above or by a modified procedure in which ganglia with opened capsules were treated with $2\,mg\,ml^{-1}$ collagenase/dispase for 1 h, and the neurones then aspirated with a fine glass capillary (Dietzel, Drapeau & Nicholls, 1986). When such cells are plated onto a polylysine-coated substrate, they begin to sprout slowly after a lag of a few days (Chiquet & Acklin, 1986). This might indicate that the neurones have to 'condition' this artificial substrate (by the deposition of extracellular material) before they are able to grow. Experiments were made with S. Acklin to search for substrates promoting rapid growth. Only two were found: one non-physiological, the plant lectin Con A, and one physiological, a factor extracted from leech ganglion capsule extracellular matrix (ECM) (Chiquet & Acklin, 1986).

Single neurones sprouted at a fast rate within a few hours after plating on plastic coated with $2\,mg\,ml^{-1}$ Con A. The growth rate was the same in serum-containing standard medium (see previous paragraph) as in serum-free, defined Leibovitz-15 medium (in which the survival time was, however, diminished) (Fig. 2). This result demonstrates clearly that neurite growth by leech neurones does not depend on soluble growth factors or the presence of other cells.

The neurite growth is mediated by a specific interaction between mannosyl-containing cell surface receptors and the oligosaccharide binding sites of substrate-bound Con A. First it was shown that soluble Con A in the medium did not promote sprouting (Chiquet & Acklin, 1986). Second, lectins with a sugar specificity
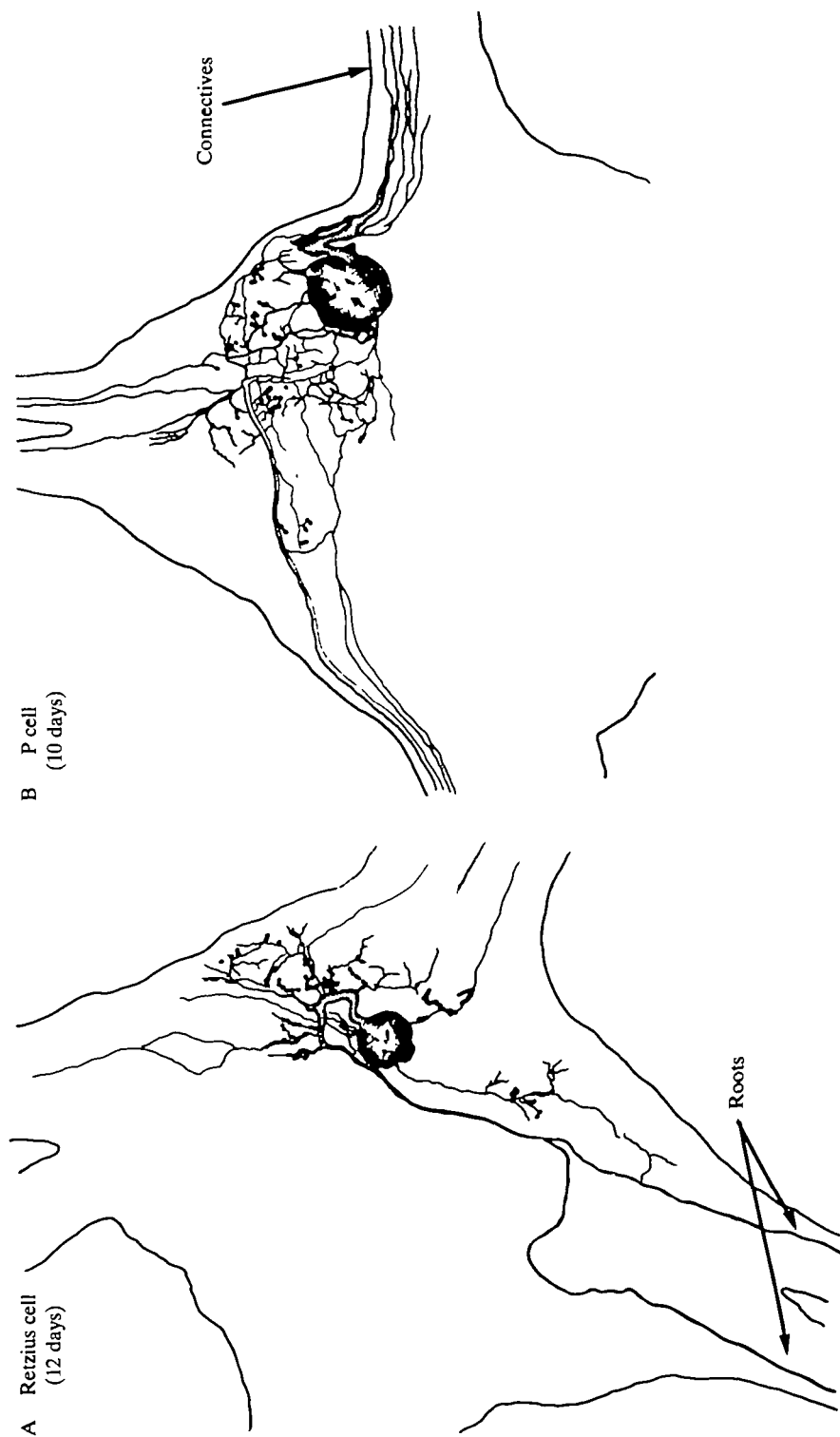
Fig. 1. Sprouting by individual neurones into normal ganglia in culture. *Camera lucida* drawings of a Retzius cell implanted for 12 days (A) and a P neurone implanted for 10 days (B). Both the Retzius and the P neurone sent processes through roots and connectives and were able to form connections within the ganglion. The diameter of the Retzius cell is approx. 60 μm.

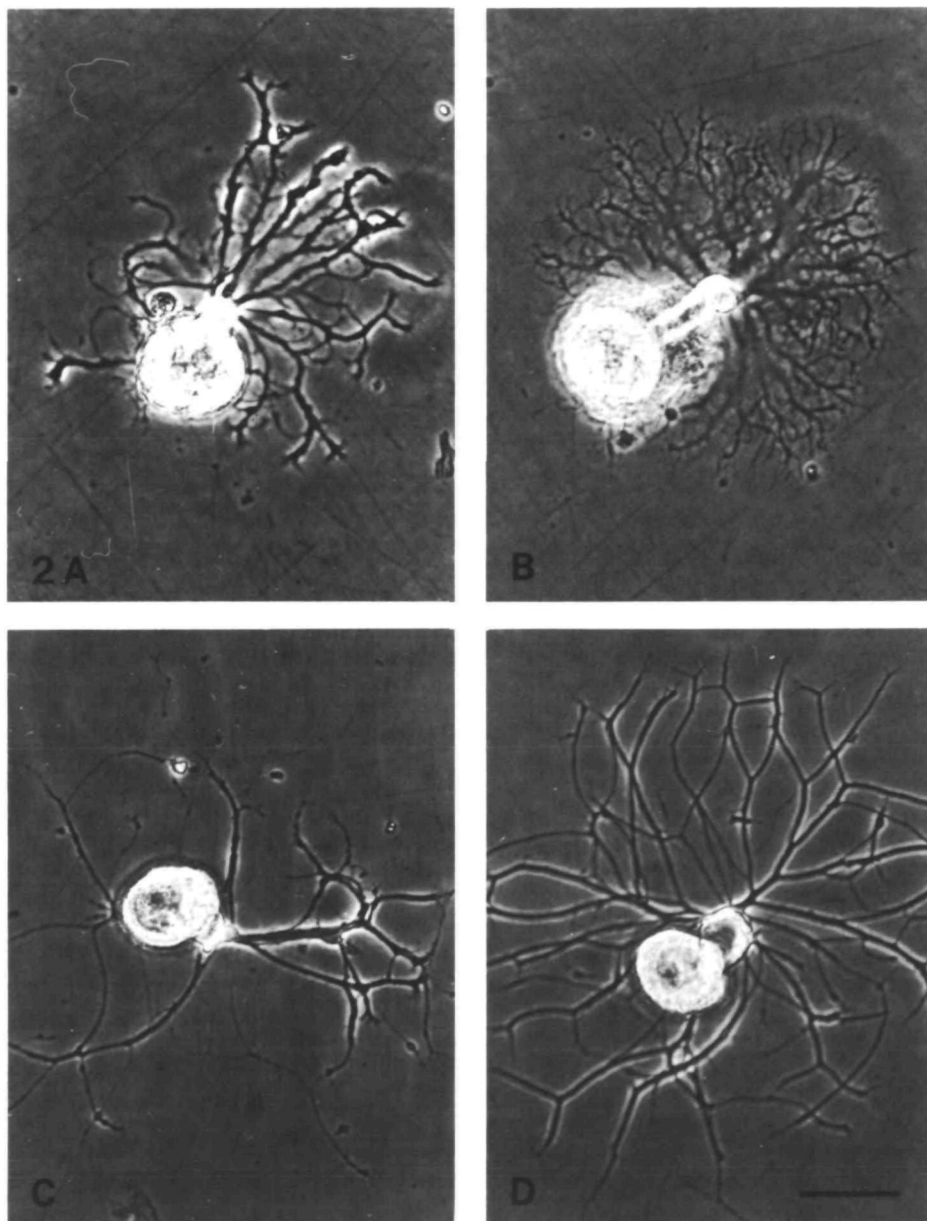Fig. 2. Neurite growth by identified neurones cultured for 1 day on Con A substrate (A,B) or for 2 days on dishes coated with urea extract of ganglion capsule extracellular matrix (C,D). Retzius neurones are shown in A and C and anterior pagoda neurones in B and D. Note that the neurite morphology varies between cell types, and, for a given cell type, between substrates. The anterior pagoda cell on Con A (B) was grown in defined, serum-free medium. This procedure did not change neurite growth and morphology during the first 2 days in culture. Scale bar, 100 μm.

different from Con A (e.g. wheat germ agglutinin and phytohaemagglutinin) were not active as substrates. Third, the Con A-specific inhibitory sugar methyl α-D-mannoside (at a concentration of $100 \, \text{mmol} \, l^{-1}$ in osmolarity-adjusted medium) inhibited neurite growth on Con A substrates (Table 1). Interestingly, low concentrations (10 and $20 \, \text{mmol} \, l^{-1}$) of methyl α-D-mannoside in the medium *stimulated* growth on Con A (Table 1). It is therefore possible that a Con A substrate allows optimal adhesion, but not optimal growth of neurones. Low concentrations of specific inhibitor might facilitate the dynamic interaction between the substrate-bound lectin and its receptors on the neurite surface, thereby increasing the growth rate. At this stage, however, we cannot exclude an indirect metabolic effect of methyl α-D-mannoside on the cells.

It was of obvious interest to find an endogenous substance promoting neurite growth and to test whether it acted by a similar or different mechanism from Con A. Because the connective tissue is thought to be important for wound healing and regeneration *in situ* (Baylor & Nicholls, 1971), cell-free extracellular matrix (ECM) was prepared from leech ganglion capsules. Ganglia were extracted extensively in 2 % Triton X-100 containing $10 \, \text{mmol} \, l^{-1}$ Tris-HCl, pH 7·4, and protease inhibitors (Chiquet & Acklin, 1986). The remaining insoluble matrix was dried onto culture dishes and used as a substrate for cultured neurones. Again, isolated Retzius or anterior pagoda cells started sprouting immediately after plating, at a rate comparable to that on Con A (Fig. 3). From the capsule ECM, the activity could be extracted with $4 \, \text{mol} \, l^{-1}$ urea as described (Chiquet & Acklin, 1986). After dialysis against $10 \, \text{mmol} \, l^{-1}$ Tris-HCl, such extracts could be used to coat culture dishes (by drying down $2 \, \mu l$ of extract per well, or by incubating polylysine-coated wells with extract solution). On wells coated with ECM extract, leech neurones grew as profusely and rapidly as on the ECM itself (Fig. 2).

Table 1. *Effect of methyl α-D-mannoside on sprouting mediated by Con A substrate*

Medium supplement*	Total neurite length per cell† (μm)
none	800 ± 140
$100 \, \text{mmol} \, l^{-1}$ α-D-glucose	730 ± 190
$10 \, \text{mmol} \, l^{-1}$ methyl α-D-mannoside	1280 ± 170
$20 \, \text{mmol} \, l^{-1}$ methyl α-D-mannoside	2340 ± 480
$50 \, \text{mmol} \, l^{-1}$ methyl α-D-mannoside	780 ± 240
$100 \, \text{mmol} \, l^{-1}$ methyl α-D-mannoside	390 ± 130

* The culture medium used in this experiment was Leibovitz-15 medium containing 2 % foetal calf serum, but no glucose supplement (none). The other media were prepared by adding isotonic ($0·3 \, \text{mol} \, l^{-1}$) sugar solutions to basic medium up to the concentrations indicated.
† Isolated Retzius and anterior pagoda neurones were cultured as single cells in the different media on Con A-coated dishes, and the total neurite length per cell was determined from photographs taken after 5 days. Data are expressed as the mean ± S.E.M. of the total neurite length per cell; sample sizes were 9 or 10 cells per medium tested.

What is the molecular nature of the neurite-promoting factor in the leech ECM? As reported (Chiquet & Acklin, 1986), the factor is sensitive to boiling and to digestion with pronase and trypsin, but not with collagenase and hyaluronidase. Recent attempts to purify the activity (M. Chiquet & L. Nakagawa, in preparation) revealed that its apparent native molecular weight is about 10^6 (judged from molecular sieve chromatography on a Biogel 1·5 m column run in 0·5 mol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, pH 7·4). Upon sodium dodecyl sulphate (SDS) gel electrophoresis, active fractions contained major polypeptides of M_r 350 000 and 220 000. It seems quite likely that the leech tissue contains a laminin-like molecule (Edgar, Timpl & Thoenen, 1984). At the same time, vertebrate ECM components, including embryonic chick lens capsules and purified mouse EHS tumour laminin, were found to be inactive in promoting the sprouting of leech neurones. Anti-mouse laminin antisera do not crossreact with leech ECM.

Neurite growth on leech capsule ECM or ECM extracts could be inhibited by an antiserum against capsule ECM, but not by 100 mmol l^{-1} methyl α-D-mannoside in the medium. Besides showing that methyl α-D-mannoside is not toxic to leech neurones, this result provides evidence that sprouting on capsule ECM is based on a different type of neurite–substrate interaction than sprouting on Con A. This physiological interaction is likely to be based on specific molecules, since the activity is enriched in ganglion capsules compared to other tissues, and since it can be further purified biochemically.

NEURONE TYPE AND SPECIFIC SUBSTRATE AFFECT NEURITE MORPHOLOGY

Working with isolated leech neurones, the growth characteristics of different identified cell types can be compared under identical culture conditions. For example, anterior pagoda neurones always form more neurites and branch more frequently than Retzius cells on a permissive substrate (Fig. 2). Thus, part of the regenerative pattern of a particular neurone is due to intrinsic characteristics that are unknown.

Another important factor that influences neurite morphology *in vitro* is the substrate. On dishes coated with Con A, neurites of a given cell type are flat, crooked and have many branches. On the capsule ECM factor, however, the same neurone produces neurites which are straighter, longer and have fewer branches. Thus, every combination of a certain neurone type with a permissive substrate gives rise to a distinct neurite pattern (Fig. 2).

One might argue that the differences in neurite morphology on Con A *versus* ECM factor are due to differences in the 'adhesiveness' between the two substrates. This concept was put forward by Letourneau (1975a), who measured neurone adhesion to artificial substrates. Using patterned substrates, he then demonstrated that neurites would always grow on the most adhesive surface if given a choice. He was thereby able to guide neurites and influence their morphology (Letourneau, 1975b).

Experiments analogous to those of Letourneau (1975b) produced a different, remarkable result. When a leech neurone was given the choice between Con A and

ECM, it did *not* prefer one over the other, but grew neurites which crossed the borders easily (Chiquet & Acklin, 1986). By doing so, neurites changed their morphology. They grew straight for long distances on ECM, but became more spread out and branched more frequently once they encountered the Con A-coated substrate (Fig. 3).

It is all but impossible to explain this result by differential adhesiveness of the two substrates, since the neurites adhered equally well on both substrates and since the overall growth rates were very similar (Chiquet & Acklin, 1986). Moreover, the Con A specific hapten sugar inhibited sprouting on Con A, but not on ECM (see previous section). Thus the adhesion of the neurites to the two substrates is not based on a quantitative difference of the same kind, but rather on two different (and specific) types of molecular interactions which, in turn, might affect neurite morphology.

One question is whether different neurite-promoting ECM components with different effects on neurite morphology exist *in vivo*. If they do, one could hypothesize that neurones possess multiple specific adhesion systems which are
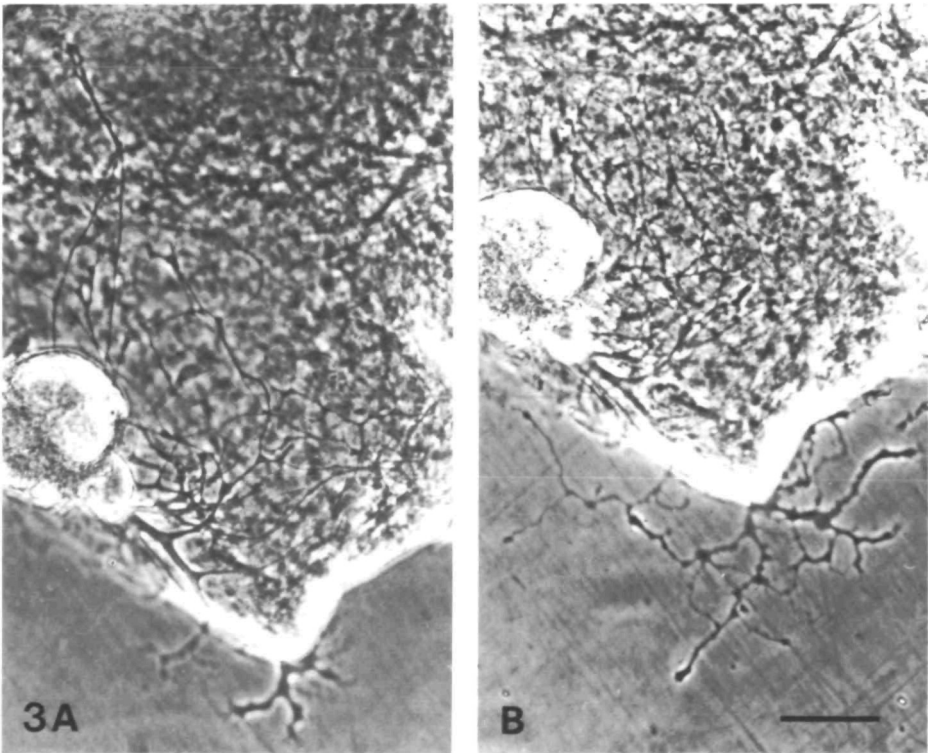

Fig. 3. Growing neurites can cross the border between extracellular matrix and Con A substrate, assuming a different morphology. A single Retzius neurone was plated at the interface between ganglion capsule matrix and Con A-coated plastic and photographed after 1 day (A; focused onto the matrix surface) and after 2 days (B; focused onto the Con A-coated plastic). Scale bar, $100 \mu m$.

activated by encountering a certain ECM component. Different neurone types might have different sets of surface receptors for these components. As shown, different leech neurone types exhibit different sprouting patterns on a given substrate (Fig. 2). If this hypothesis is correct, one might expect to find cases where a certain neurone type grows straight processes on one substrate and branches on another, while another neurone type behaves in the opposite way. This would provide a model for how different neurone classes form and maintain different morphologies in an identical environment. This hypothesis can be tested by presenting identified leech neurones with patterned culture substrates.

DIVALENT CATIONS AND NEURITE GROWTH *IN VITRO*

The experiments described so far demonstrated that attachment of isolated leech neurones to a specific substrate is sufficient to initiate neurite growth in defined, protein-free medium. Can exogenous soluble factors in the culture also modulate neurite growth?

Sprouting by cultured neurones could be initiated and maintained for at least 1 day in leech Ringer (115 mmol l^{-1} NaCl, 1·8 mmol l^{-1} CaCl$_2$, 4 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-maleate, pH 7·4; Muller *et al.* 1981). Other components present in Leibovitz-15 medium and foetal calf serum do not seem to be essential for neurite growth by freshly isolated leech neurones, but are necessary for their survival in culture. (Glucose and glutamate are likely to be important.)

Of special interest is the role of divalent cations for neurite growth. Ca^{2+} is an important intracellular messenger activating cytoskeletal movements (Adelstein, 1982) and exocytosis (Miledi, 1973), two processes essential for neurite growth (Johnston & Wessels, 1980). It has been suggested that neurotransmitters derived from target neurones might modulate neurite growth, e.g. by changing the Ca^{2+} conductance through growth cone membranes (Haydon, McCobb & Kater, 1984). Whether and how much external Ca^{2+} is required for neurite growth is still questioned, however. Neurite extension by PC12 cells depends on Mg^{2+} but not Ca^{2+} in the medium (Koike, 1983; Turner, Flier & Carbonetto, 1987). In leech neurones, we found that complexing the Ca^{2+} in the culture medium with EGTA slowed down, but did not abolish, neurite growth. (*Vice versa*, sprouting was initiated in Ca^{2+}-containing, Mg^{2+}-free Ringer solution, as mentioned above.)

Another line of evidence suggested that, in leech neurones, the influx of Ca^{2+} into growing neurites is small. Voltage- and Ca^{2+}-sensitive dyes have been used to measure electrical characteristics and Ca^{2+} conductance of cultured neurones (Ross, Arechiga & Nicholls, 1987). A single, growing cell in culture was injected with the Ca^{2+}-sensitive indicator Arsenazo III. RH 155, a voltage-sensitive dye, in the solution measured membrane potential changes in the soma and the fine processes. By these techniques it was shown that growing neurites are isopotential with the soma, i.e. voltage changes are not drastically attenuated along processes. Neverthe less, Ca^{2+} influx differed in various regions of neurone surface; with the Ca^{2+}-sensitive dye, the biggest signals were recorded over axon stumps and cell bodies,

whereas Ca^{2+} signals over the neurites and growth cones (normalized to the voltage signals) were much smaller (Fig. 4). Large Ca^{2+} signals were, however, recorded from neuronal processes *in vivo*. Thus, since the density of Ca^{2+} channels on growing neurites seems to be low, this does not support the idea that Ca^{2+} influx following impulses is important in regulating the sprouting of leech neurones.

THE EFFECTS OF CELLULAR CONTACTS ON NEURITE GROWTH

The results so far deal with single cells in culture. Does the presence of other cells modulate neurite formation? One observes regularly that neurites from neighbouring neurones in culture will meet at random, fasciculate (i.e. grow along each other), and thereby reinforce connections between cells. Under our culture conditions, we have no clear-cut evidence for directed growth between neurones (Kuffler, 1986), or of growth inhibition between neurites from neighbouring cells (Kapfhammer, Grünewald & Raper, 1986).
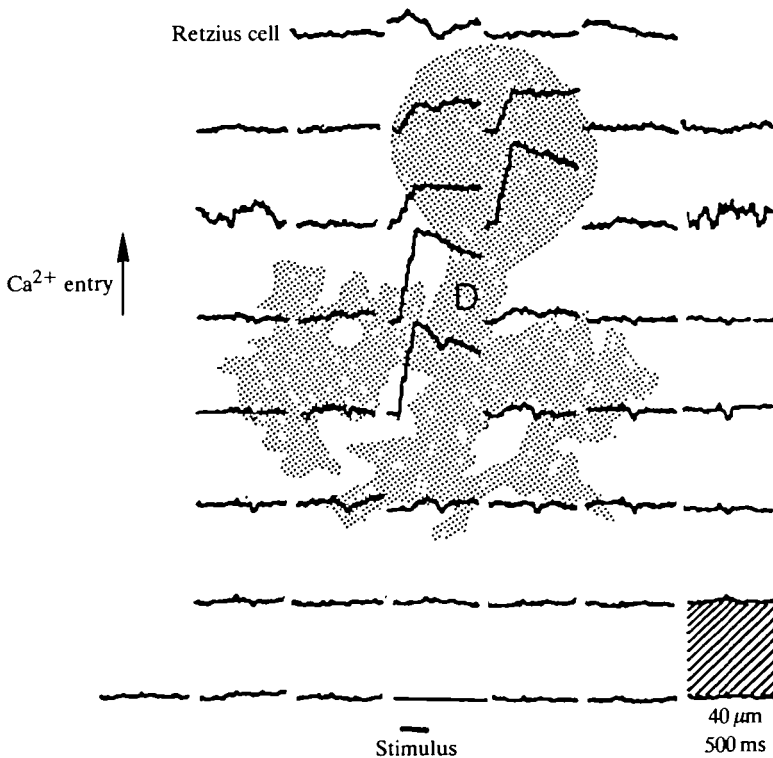

Fig. 4. Ca^{2+} signals following impulses in the soma and growing processes of a cultured Retzius cell (2 days) filled with Arsenazo III. Each trace shows the absorbance change during 500 ms in a field $40 \times 40 \, \mu m^2$ (cross-hatched area at bottom right). The cell was stimulated to fire three action potentials (bar at bottom); 200 sweeps are averaged; signals low-pass filtered at 60 Hz. The vertical scale is arbitrary but the same for all traces. Large absorbance changes were detected over the soma and initial segment (D) but not over growth cones.
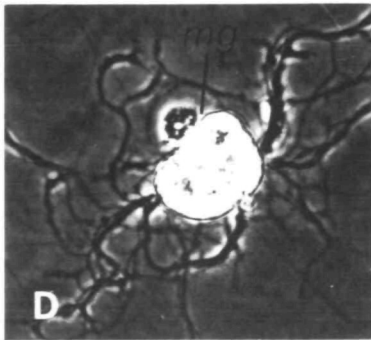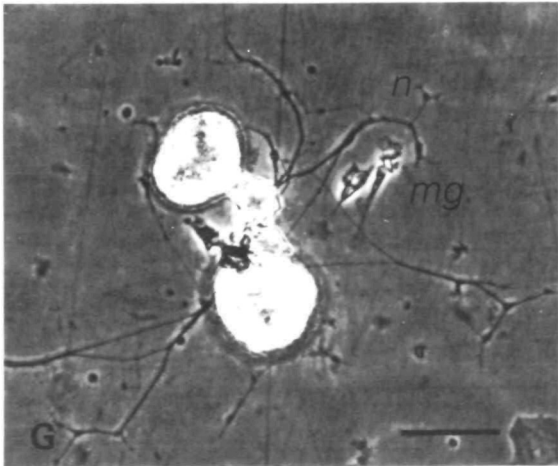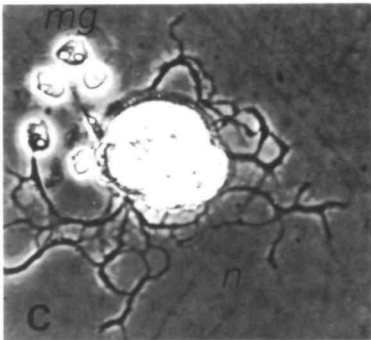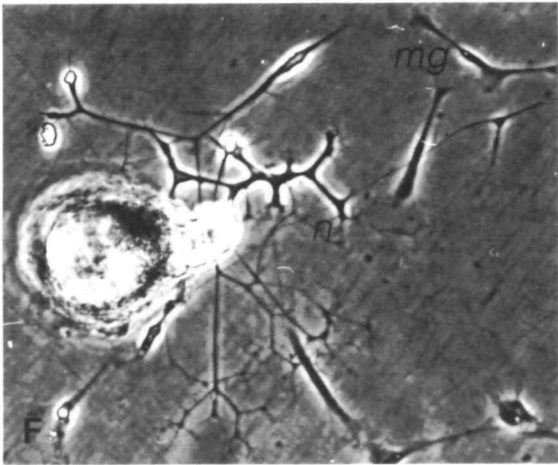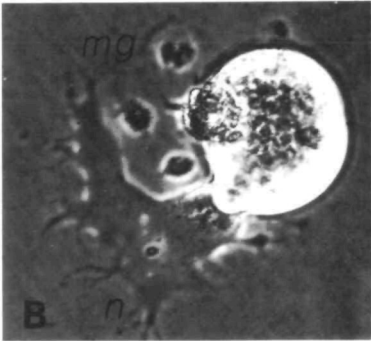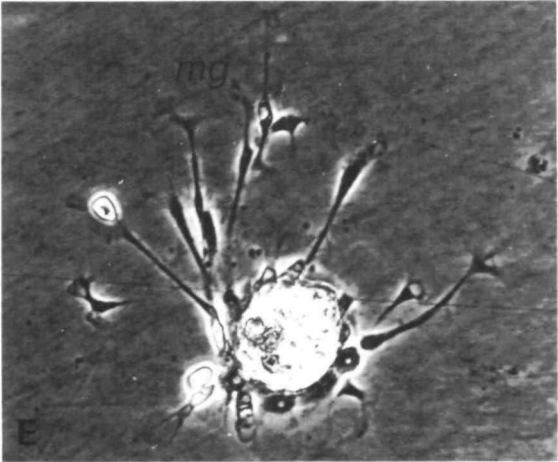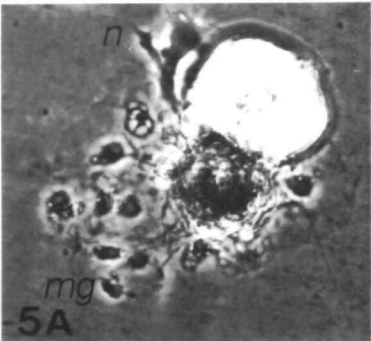
One observation bears on the question of what role the microglial cells play during regeneration (Morgese *et al.* 1983). Depending on the isolation conditions and the leech batch, neurones were sometimes isolated with a variable number (up to a dozen) of these small satellite cells attached. On different substrates, these cells assumed different morphologies: on Con A-coated dishes, they attached very firmly and became flat and saucer-shaped. Time-lapse video films revealed intensely ruffling membranes at the cell margins; however, net movement of microglial cells was small on Con A, and they tended to form a 'necklace' around the big neurone (Fig. 5). In contrast, microglial cells on the ECM substrate became spindle-shaped, formed a pronounced leading edge, and migrated away from the neurone quickly (Fig. 5).

Microglial cells consistently inhibited neurite growth by neurones cultured on Con A. Thus, sprouting only occurred from a position on the neuronal cell body not facing microglia (Fig. 5). Neurites grew around the microglia, giving the impression of avoiding their membrane surfaces (Fig. 5).

On ECM substrate, the inhibitory effect of microglia upon neurite growth was less pronounced. The onset of neurite growth seemed to be delayed, however, until the microglial cells had migrated away from the neuronal cell body. Neurite growth cones seemed to avoid the flat ruffling membranes of microglial cells, but were found to cross their elongated cell bodies (Fig. 5).

Based on time-lapse video studies, inhibition of neurite growth by microglial cells appears to depend on short-range interactions between the two cell surfaces, similar to the repulsion described between chick sympathetic and retinal neurites (Kapfhammer *et al.* 1986) or between rat neurones and a certain type of CNS oligodendrocytes (Schwab & Caroni, 1986). This seems to be paradoxical in view of the reported accumulation of microglial cells at sites of crushes *in situ* (Morgese *et al.* 1983), where neurite formation is very active. Microglia are known to phagocytose debris at a wound and later to ensheath the regenerated axons (Elliott & Muller, 1981). During active neurite growth, however, they might restrict sprouting to certain directions rather than actually promoting it.

SELECTIVE SYNAPSE FORMATION BETWEEN CULTURED NEURONES

During regeneration in the leech CNS, neurite growth is a prerequisite for synapse formation between a neurone and its former target simply because of the distance separating them. In contrast, isolated leech neurones in culture can be presented with closely apposed targets: any combination of paired cells can be plated onto an

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Fig. 5. Inhibitory effect of microglial cells on neurite outgrowth. Neurones with attached microglial cells were plated onto dishes coated with Con A (A–D) or with extracellular matrix extract (E–G). Photographs were taken after 3 h (E), 1 day (A,B,F,G) or 4 days (C,D). On Con A substrate, microglial cells (*mg*) flatten around the neurone; neurite (*n*) outgrowth occurs only in places not occupied by microglia (A–D). On matrix extract, microglial cells elongate and migrate away from the neurone within a few hours (E). After that, outgrowth occurs normally (F), but growth cones often travel around microglia (G). Scale bar, 100 μm.

adhesive substrate with the cell bodies touching each other. This allows one to ask whether neurite growth is indeed required for synapse formation, and whether it influences the selectivity of regenerating connections. Under these artificial conditions, any selectivity observed in the process of synapse formation is likely to be based on specific cell–cell recognition, rather than on neurite growth guided by pathways and targets. We will summarize the evidence that leech neurones paired with targets in culture do make synapses between cell bodies (i.e. without profuse neurite growth) and that the characteristics of a new synapse depend predictably on the cell types within the pair.

When a Retzius neurone and a pressure-sensitive (P) neurone are paired in culture, an inhibitory serotoninergic synapse develops within a week from the Retzius onto the P cell (Fuchs, Henderson & Nicholls, 1982). This connection is an ideal preparation for studying the mechanism of chemical synaptic transmission (Dietzel *et al.* 1986). As long as the cell bodies of the paired neurones touched each other, this same type of synapse developed whether the cells were plated on a substrate permissive for neurite growth (Con A) or not (polylysine). The frequency of synapse formation was even higher on polylysine than on Con A substrate. It has been shown before that cells paired on polylysine form short processes on each other's cell bodies; these might favour synapse formation (Fuchs *et al.* 1982). Nevertheless, it became clear that profuse neurite outgrowth on the substrate was not required for, and did not influence the selectivity of, synapse formation between pairs of cultured leech neurones in this and other cases examined. For cultured snail neurones, it has been reported that inhibition of growth cone mobility also inhibits synaptogenesis (Haydon *et al.* 1984).

Taking Retzius and P neurones as examples, the selectivity of synapse formation in culture could be clearly demonstrated. Thus, the serotoninergic synapse was the only one developing when these two cells were paired. P cells were never presynaptic to Retzius cells in culture, but could be presynaptic to anterior pagoda (AP) neurones (Arechiga *et al.* 1986). Retzius cells never became coupled electrically to P cells, but formed nonrectifying electrical junctions with certain other neurone types (including other Retzius cells). P cells, however, were never found to be involved in nonrectifying electrical coupling: all electrical synapses recorded between P cells and certain other neurone types (not Retzius cells; see above) rectified (Arechiga *et al.* 1986).

Some of the synapses formed between cell pairs *in vitro* resembled those found in the ganglion between the same neurones. Other connections which are prominent *in vivo* could not be demonstrated in culture; however, some connections formed only *in vitro*. Thus, the rules by which specific synapse types develop between cell pairs *in vitro* are not the same as *in vivo*. The result obtained *in vitro* can, nevertheless, be predicted from the neurone types within the cell pair. This is in accordance with the hypothesis that the cultured preparation assays for specific cell–cell recognition independently of other factors which are equally important for synapse formation *in vivo*, e.g. guided neurite growth, developmental timing and competition between neurones.

CONCLUDING REMARKS

Problems concerning the specificity of neuronal repair are being investigated in many different preparations including vertebrates. In recent years, much progress has been made in the isolation and identification of molecules important for the growth of vertebrate neurones, including target-derived growth factors (Levi-Montalcini, 1975), specific extracellular matrix components (Edgar *et al.* 1974), and cell–cell adhesion molecules (Edelman, 1986). Less is known about comparable molecules used by invertebrates such as *Aplysia*, snails and the leech. These preparations do, however, offer certain advantages. Thus single neurones of known function can be isolated and cultured, singly, in pairs, or as multiples (Haydon *et al.* 1984). The present results show that different types of identified leech neurones produce different neurite patterns in defined medium on a specific substrate, and make specific synapses among each other. At the level of single neurones with known function, one can study the molecules that are involved in the interaction between the neurone surface and the environment during regeneration.

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