ISOLATION AND MAINTENANCE IN CULTURE OF IDENTIFIED NEURONES FROM *HELIX ASPERSA*

BRIAN POWELL

DECLARATIONS

I, Brian Powell, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

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ABBREVIATIONS

ACh	Acetylcholine
ATP	Adenosine-triphosphate
cAMP	cyclic-Adenosine monophosphate
Cell A	Anterior
Cell M	Median
Cell P	Posterior
C1	Cerebral ganglion cell 1
C3	Cerebral ganglion cell 3
Con A	Concanavalin A
epsp	Excitatory postsynaptic potential
f.c.	Fibroblast-like cell
FMRFamide	Phe-met-arg-phe-NH
GSN	Giant Serotonin Neurone
g.c.	Growth cone
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
5-HT	5-Hydroxytryptamine or Serotonin
L-15	Leibovitz-15 Medium
MAP	Microtubule-associated protein
MT	Microtubule
NCAM	Neural cell adhesion molecule
RGD	Arginine-glycine-aspartate

UNITS

cm	Centimeter
g	Gram
hr	Hour
mg	Milligram
ml	Millilitre
mm	Millimeter
mM	Millimolar
mV	Millivolts
min	Minutes
M	Molar
nA	Nanoamps
N	Normal
sec	Seconds
ug	Microgram
uM	Micromolar

SUMMARY

(1) Isolated, identified Helix neurones were maintained in culture conditions for up to two weeks. The neurones developed large lamellipodia from which an extensive neuritic tree developed.

(2)The isolated neurones maintained their electrical activity. The neurones showed action potentials both spontaneously and when stimulated .

(3) Intact ganglia from Helix were maintained in culture conditions for up to four weeks.

(4)The neurones within the intact ganglion maintained electrical activity, exhibiting spontaneous action potentials and excitatory post synaptic potentials.

(5) Identified neurones from the buccal ganglion showed the same response to the neurotransmitter 5-hydroxytryptamine *in vitro* as was seen *in situ*.

(6) Helix neurones attached readily to Concanavalin A and would attach to poly-l-lysine but did not attach to fibronectin, laminin or collagen.

(7)The C3 neurone isolated in culture conditions continued to produce a FMRFamide-like substance. The presence of the FMRFamide-like substance was shown in the soma, neurites and growth cones using immunohistochemical techniques.

(8) The shape of growth cones from the C3 neurone and the C1 neurone showed distinct characteristics which were related to the type of neurone.

(9) The morphology of neuritic development from the C3 neurone and the

C1 neurone was altered if the axon was retained. When the axon was absent there was equal distribution of neuritic sprouting from the perimeter of the both neurones. In the presence of the axon the C3 soma produced few neurites but the C1 soma produced profuse neuritic growth.

(10) The neurites and growth cones of neurones from Helix were enlarged when these neurones were seeded onto fibroblast-like cells. Neurones in the same culture dish but not in contact with fibroblast-like cells would maintain their normal morphology.

(11) The tentacle muscle contained a small cell which displayed a morphological characteristic of a neurone i.e. these cells developed long narrow neurites. The neurites from the C3 neurone appeared to make connections with this type of cell.

(12) Factor(s) in the tentacle muscle extract promoted increased neuritic length and the development of fine neuritic structures which were not seen when the C3 neurones were cultured without the muscle extract.

(13) Electrical synapses were formed and there was limited evidence that chemical synapses were formed.

(14) Resting membrane potentials were lower in Helix neurones maintained in culture when compared to the neurones in situ.

(15) There was indirect evidence for microtubule assembly in the axon of the C1 neurone.

INTRODUCTION

INTRODUCTION

1.1 The isolation and maintenance of certain identified neurones in the central ganglia of the mollusc, Helix aspersa offer major experimental advantages for addressing several important questions relating to the actions of contained transmitters, axonal growth and synatogenesis. The C1 neurone (serotonin and Ach containing) and C3 neurone (Ach and FRMFamide containing) have been characterised morphologically and electrophysiologically (Cottrell 1981), and for the transmitter and peptide present (Cottrell, Schot and Docherty 1983; Cottrell and Davies and Green 1984). The Anterior, Medial and Posterior neurones which receive a synaptic input from the C1 neurones, occur in the buccal ganglia and can be clearly identified (Cottrell 1988).

Morphological characteristics, of the developing neurones in culture, have specific advantages for studying organelle transport which is of particular importance where peptide-containing vesicles are believed to be produced in the soma and transported to the synapse. The isolation of cells means that the effects of drugs and other stimuli can be studied on these specific cells and co-cultures without the possible influence of cells remote but synapsing onto them.

Workers, mainly in the USA, have made advances developing techniques for culturing isolated neurones from Helisoma (Wong, Hadley, Kater and Huaser 1981), Aplysia (Schacher and Proshansky 1983) and leech (Ready and Nicholls 1979), but, hitherto, I am unaware of any reports on the successful isolation and maintenance of Helix neurones. For these reasons I conceived this project to isolate and maintain in culture neurones taken from the central ganglia and periphery of Helix aspersa (Methods: figures

2,3 and 4). A major part of the work centred on experiments with specific identified neurones, for the reasons outlined above. In introducing this work I have described many of the external and internal influences and requirements related to the development of neurones in culture.

(If any reader is unfamilar with the morphological structures of a neurone developing in culture a brief description has been included at the beginning of the results section.)

1.2 CELL ADHESION FACTORS (SUBSTRATE)

Many types of cells will attach easily to the plastic or glass surface of culture vessels, but others will either not attach or will not develop processes when they do. These cells require an intermediate layer of substrate on which to develop. The use of such substrates also serves the purpose of retaining endogenous growth promoting substances,or conditioning factors (see section on Endogenous Factors).

A series of substrates have been used by different workers. These include poly-l-lysine (of varying molecular weights), fibronectin, collagen, concanavalin A and laminin. The type of substrate can affect the density and direction of neuritic growth (Letourneau 1975). The degree of adhesiveness increases the neuritic initiation, branching and length of neurites. Some substrates which have been used are naturally occurring as constituents of the cell membrane and the extracellular matrix. This matrix fills the intercellular spaces and consists of glycoproteins, collagens, proteoglycans, hyaluronic acid and elastin.

1.2.1 Collagen

Collagen makes up the largest proportion of proteins in the

extracellular matrix. It is a fibrous protein and there are several types with varying proportions of carbohydrates . The important structural feature of collagens is the winding together of three polypeptide chains to form a rope-like molecule. Although a dozen types exist, there are five main groups. Groups 1,11 and 111 are the most common in the connective tissue, 1 making up 90% of collagen in the tissue. Groups 1,11 and 111 make well ordered fibrils, which are long and thin and grouped into fibres. Groups 1V and V do not make fibrils and their precise arrangement in tissue at present is uncertain (Alberts, Bray, Lewis, Raff, Roberts and Watson 1984).

1.2.2 **Concanavalin A**

Concanavalin A (isolated from the Jack Bean Concanavalin masiformis) comes from a group of glycoproteins known as lectins. These were first found in large concentrations in plant seeds and in bacteria. Of several lectins tested with leech neurones Concanavalin A (Con A) was the only one shown to have been effective as a substrate. The site of interaction between cell and substrate is a specific one between mannosyl-containing cell surface receptors and the oligosaccharide bindind sites of the substrate bound Concanavalin A (Chiquet and Nicholls 1987).

Gund (1977) had shown eight classes of glycoprotein receptors in the isolated synaptic membrane from the cerebral cortex of adult rats. The membranes showed receptor activity for the lectins Concanavalin A, wheatgerm agglutinin (WGA), Lens culinaris phytohaemagglutinin (LCH) and Ricinus communus agglutinin (RCA). The four lectins, when reacted with the receptors in increasing concentrations, reached a maximum response, filling available sites. However this plateau was different for each, which

suggested the lectins were reacting with different populations of receptors.

Neuritic sprouting was very extensive with Con A in isolated leech neurones, within a few hours. This was in marked contrast to the slow response on other substrates e.g.glycoproteins, polylysine and other lectins (Chiquet and Acklin 1986).

Of the various substrates Con A is best known for having effects on synapse formation. The pattern of chemical synapses in identified, electrophysiologically characterised cells in Aplysia is changed when Con A is added to the culture medium, causing more than 75% of cells that had formed chemical synapses to form electrical synapses (Lin and Levitan 1987). Con A inhibited the desensitisation of glutamate receptors in locust skeletal muscle (Mathers and Usherwood 1976). The opening of novel potassium channels in Aplysia cell cultures was greatly increased by the presence of Con A(Lin, Dagan and Levitan 1989). These affects were in response to Con A in the medium or in the intracellular electrodes and not as a surface bound substrate. Although the specific mechanism of the action of Con A is not known one possibility is that it could act as a nerve growth factor (NGF), based on its observed ability to increase neuritic and lamellapodial outgrowths. NGF can influence the levels of cAMP. Increased intracellular levels of cAMP or its analogues can initiate neuritic outgrowths in many mammalian cells(Greene and Shooter 1980).

1.2.3 **Fibronectin**

Fibronectin is another type of fibre-forming, but non-collagenous, glycoprotein which is mainly produced by fibroblasts. Fibronectin was first shown to have cell adhesive promoting qualities when added to

transformed fibroblasts which produced little fibronectin (Alberts et al 1984). These cells which hitherto adhered poorly, quickly adhered and flattened when exposed to fibronectin.

It is now known that there are at least two types of fibronectin: one type is soluble in the plasma and body fluids, the other is cellular and associated with other glycoproteins in the basement membrane and the cytoskeleton (Hynes and Yamada 1982). Hynes and Yamada showed that the loss of cytoskeletal organisation associated with transformed cells can be reversed by the addition of fibronectin. They also show that it increases migration of cells in culture, is important in cell migration in vivo and is associated with phagocytosis and the regulation of differential pathways.

Akizama and Johson (1983) using radioimmunoassay carried out an evolutionary survey for the presence of fibronectin. It had previously been shown in mammals, birds, fish and amphibia. They extended the list to include several invertebrates eg. sponges, sea snails and crustaceans. Although it was shown in very simple organism e.g. sponges, the evolutionary distribution was not complete as it could not be found in earthworms and some bacteria.

In the specific instance of Helisoma Mattson and Kater (1988) showed a fibronectin-like immunofluorescent staining in neurones, non-neuronal cells and substrates treated with conditioning factor. Low concentrations of fibronectin antiserum increased neuritic outgrowth but inhibited it at higher concentrations. Filopodia and lammellapodia extensions were inhibited by increasing concentrations.

1.2.4 The Arginine-Glycine-Aspartate (RGD) Tripeptide

In fibronectin and other cell attachment glycoproteins (eg.Laminin) a group of aminoacids, the arginine-glycine-aspartate (RGD) tripeptide has shown to be the essential cell surface recognition point (Ruoslahti and Peirschbacher 1986). While synthesised RGD in solution inhibits the attachment of cells to fibronectin, as an insoluble substrate it will cause attachment. RGD displays a versatile configuration, the orientation differing for individual adhesive proteins.

The ability to recognise specific ligands is brought about by the receptors or integrins (Ruoslahti and Peirschbacher 1987), of which ten have been isolated from cell extracts. The structure of integrins can consist of one or two polypeptides with an extracellular amino-terminal for attachment, a transmembrane segment and a carboxl group in the cytoplasmic domain. The integrins require magnesium or calcium to bind to their ligands. The significance of these two structures, RGD and integrins, is far reaching in cell activity. How a cell attaches to others could determine: the cell shape in a tissue ; its position, as a cell will move up a gradient of concentrations until it reaches a point of maximal adhesion.

1.2.5 **Laminin**

Timpl and Rhode (1979) isolated a non-collagenous glycoprotein from tumours in mouse to which they could not attribute a clear role. Fodairt, Bec, Yarr, Rennard, Gullino, Martin and Katz (1980) showed laminin to be formed between the collagen (Type IV) in the basement membrane and the surface of the cell. Malinoff and Wacha(1983) isolated a surface receptor protein for laminin from fibrosarcoma cells in mice. Laminin has separate binding sites to attach to cell surfaces; collagen(Type IV) and

glycoaminoglycans heparine sulphate (Hopkins and Agranoff 1987). These authors plated retinal explants from goldfish onto laminin-coated and polylysine-coated surfaces. The explants developed extensive neuritic outgrowths. Heparin and heparan sulphate (which attaches tightly to heparin sites) were added to each culture. The neuritic growth was abolished on poly-lysine surfaces but not on laminin surfaces showing that neuritic growth was not dependent on the heparin binding site.

In a comparison of peripheral and central nervous tissue from the chick embryonic dorsal root ganglia Rogers, Letourneau, Palm, McCarthy and Furcht (1983) found that cells from both regions developed neurites on laminin but in the same study only peripheral neurones developed on fibronectin. Pathways of laminin 7-10um wide could direct neurites and growth cones from neurones in the chick dendritic root ganglia, sympathetic ganglia, brain and spinal cord. Non-neuronal cells (mouse fibrosarcoma and Schwannanoma cells) showed the same response to these pathways. (Hammerback, MacCarthy, Palm, Furcht and Letourneau 1988). The method used to lay down the pathways involved irradiating with ultraviolet light a laminin substrate on which a grid had been laid. Lengthening the time of exposure to ultraviolet increased the effectiveness of the pathways in guiding neurites; this was also the case when the initial concentration of the substrate was increased.

The same treatment of fibronectin, although clearly producing pathways, did not show the guidance effect on the cells. The exception was fibrosarcoma cells, which initially orientated along the paths but began to move into non-irradiated areas after a few hours. The ability of these pathways to guide could be blocked by anti-laminin antibodies but not by anti-fibronectin antibodies.

1.2.6 **Poly-l-lysine**

Poly-l-lysine (a positively charged polymer)of molecular weights 14000 to 300,000 was used. The low molecular weight had been effective in vertebrate cells (Barker 1982) but the large molecular weight 300,000 was required for the invertebrate Helisoma (Kater 1985).

1.3 INFLUENCE OF THE SUBSTRATE ON NEURITIC DEVELOPMENT

The substrate is much more than an inert surface to which cells will adhere. Although its first and most obvious effect is on the morphology of regenerating cells, neuritic networks and growth cone shape, the substrate can influence neuronal activity and membrane channel openings.

Letourneau(1975) did much to show the effect of substrates, and their increasing order of adhesiveness, on axonal initiation, elongation and branching. A system of air blasting was used to determine the adherence of growth cones to the substrate. A blast of air was delivered from a syringe over a $4mm^2$ area of the culture dish. The removal of growth cones was inversly proportional to the adherence to the substrate and it was concluded that with increasing adherence there was increase in the number of axons formed, rate of elongation and branching. Cells grown on collagen developed thin, straight, narrow neurites attached only at the growth cone with very little branching and only at large angles. However on poly-l-lysine the neurites were firmly attached along their length,thicker, many branched and of a wandering nature(Wong,Hadley,Kater and Hauser 1981).

Leech neuronal cells on Con A and a laminin-like extract from the leech nervous system exhibited considerable differences. The new processes on Con A were thicker and the growth cones large and flat compared to the

laminin extract. Electrical pulses could be detected throughout the processes on both substrates and an optical dye,RH 155, which indicates the presence of calcium in response to volatage changes showed that calcium distribution was greatly reduced in cells grown on Con A. The cells on this substrate displayed calcium signals on the cell body and in the first segments of the neurites but never in the fine processes, where as on laminin calcium was universally detected. However this applied to one cell type only, in others weak calcium signals could be detected in the endings(Ross,Arechiga and Nicholls 1988).

These authors also showed that neurons grown on a substrate e.g. poly-l-lysine will continue to exhibit axonal elongation in the presence of an actin disrupting chemical, cytochalasin. However, under the same conditions but without the substrate, the neuritic extension and growth cone motility was inhibited.

1.4 ENDOGENOUS GROWTH PROMOTING FACTORS (CONDITIONING FACTORS)

1.4.1 Conditioning Factors

Isolated Helisoma neurones in culture, although adhering to the substrate and shown to be viable through electrophysiology, do not sprout neuritic processes(Wong et al 1981). However they do sprout processes on Con A. This was changed dramatically by the addition of factors extracted from the whole ganglia, or when cells were placed in co-culture with whole ganglia. Sprouting Helisoma cells in a control medium had development of 1% of cells after 4 days while those in conditioning medium was 17% in the same time. Electrical excitability of the cells was the same with or without the presence of conditioning factor. The factors were absorbed on to the substrate and, in a series of experiments, it was demonstrated that

treating half of a collagen coated plate with C.F. caused to cells develop in that half and that the C.F. would remain active, even if transferred serially to several plates.

1.4.2 **Properties of Conditioning Factor**

Although little is known about the active factors in conditioning medium, Wong, Martel and Kater(1983) found it was not species specific, the factor from one snail species could promote neuritic growth in another. Its actions could be separated into two areas of metabolism and growth (Barker, Wong and Kater 1982). The presence of conditioning factors were found to increase the incorporation of tritiated choline into acetylcholine and phosphorylcholine, and lipids and aminoacids into proteins. The growth promoting factors could be absorbed onto a polylysine surface without reduction in metabolic activity. However when a protein synthesis inhibitor,anisomycin, was added the metabolic component was abolished but the effect of the growth factor was reduced by a third.

1.4.3 **The Haemolymph**

The fluid taken from the body cavity of invertebrates described as haemolymph and made up of the circulating blood, is shown to increase the sprouting efficiency of isolated neurones from Aplysia (Schacher and Proshansky 1983). Cells in solutions of L-15 medium, supplemented with 5-50% haemolymph had a rise in the number showing neuritic initiation (judged by any cell showing a neurite 20um long) and increased growth rate (daily measurment of the three longest neurites per cell). The varying precentages of haemolymph, although showing similar efficiency in producing regrowth , as measured above,and becoming almost identical in effect on the growth rate after 4 days, were an order of 10 times more effective than in

L-15 medium alone. The morphology was also changed, the lower percentage of haemolymph showed fewer, thicker neurites, and the higher percentage produced increased numbers of neurites but with the individual neurite diameter reduced.

1.5 CELL RECOGNITION FACTORS

1.5.1 The ability of a neurone to recognise and connect with target cells was deemed by Sperry(1965) to be based on chemotaxis. From many experiments on the anatomy of regenerating nerve pathways, made from 1943 onwards he developed the chemoaffinity theory. This hypothesis relates that as the brain develops and arranges itself through genetically controlled chemical codes each cell acquires a chemical label by which it is recognised from all other cells.

Gottleib and Glaser(1981) suggested that the limitation of anatomical studies should be overcome by studying the effects of the molecular components of regeneration and synapsing. They suggest that the type of molecules should be identified and that the disruption of these components would alter the direction and form of the connections.

Some of the molecular components that affect cell recognition have been described under cell adhesion factors i.e. the glycoproteins of the extra-cellular matrix and below i.e. neural cell adhesion molecules. Others are proteins involved in synapse formation.

Embryonic vertebrate studies on cell recognition have demonstrated very specific direction finding. Motor neurone axons of the developing limb of chick go without searching to the appropriate muscle(Landmesser and Morris 1975). Further the developing retina of chick

was partially excised and the remaining developing nerves made the correct connections ignoring the sites that were appropriate for the missing retinal portion(Crossland et al 1974). In invertebrates, Chiquet and Nicholls(1987) found that leech neurones would reconnect to their target cells. Edwards and Polka(1971) delayed development of the cercal afferent in the house cricket but they still connected to the central neurones.

1.5.2 **Neural Cell Adhesion Molecules**

The ability of the processes of cells to follow existing axons and navigate without obvious physical constraints on their direction suggests that there are cell surface clues that cells produce and express. Some of these clues are substances which have been isolated and identified as the cell adhesion molecules(CAMs). An essential factor in neural development is the ability of the growth cones to grow and connect to a specific target neurone or muscle cell(Rutishauser 1984, Obrink 1986). The directing factor, the neural cell adhesion molecules (NCAM's) have been found in embryo and adult vertebrates and in an adult mollusc. The NCAM's were not found in the adults of other invertebrate species, insect , crustacean and nematode but were briefly found during the early development of flies and worms (Hall and Rutishauser 1985). In arthropods and annelids Rutishauser's test of reaggregation (i.e. cells with NCAM's will stick together when mixed), had shown that cells remain dispersed, but the guide post cells of grasshoppers (Bentley and Keshishian 1982) do show cell surface molecules that suggest a CAM-like substance at work. The known biochemistry of CAMs (Edelman 1983, Rutishauser 1984) shows an amino terminal, sialic acid containing carbohydrate and a carboxyl terminal in the cell membrane. CAM is homophilic ligand and its ability to bind varies inversely with the sialic content of the carbohydrate component.

The work of Garner (1984) suggests that CAM's are transported with the fast component down the axon and respond to different conditions, being produced as required. The presence of CAMs is shown using immunofluorescence techniques on the neuronal cell soma, neuritic processes and growth cones and also on glial and muscle cells (Rutishauser 1985). The role of neural cell adhesion molecules in making synaptic connections received strong support in experiments co-culturing skeletal muscle myotubules and spinal cord explants. When the regenerating cord axons connected with a myotubule they preferentially grew on it with many neurites growing rapidly from one myotubule to another. Synapses formed quickly. The addition of anti-NCAMS to the cultures showed the neurities adhering to the substrates and growing past the myotubules (Rutishauser 1983).

1.6 SYNAPTIC CONNECTIONS FORMED IN CULTURE

1.6.1 In Helisoma the ability to form synapses was dependent on neurites growing from both of paired, identified neurones. Regeneration of these cells (designated neurone 5) was achieved by isolating and maintaining in culture or by crushing the connecting nerve(Hadley and Kater 1983), or selective destruction of the axon by irradiating in ultra-violet light after injecting Lucifer Yellow (Hadley, Kater and Cohen 1983). Electrical synapses did not form if either of the cells had reached their stable state, which was usually after four days. Stability was judged by lack of neuritic extension or mobility of the growth cone. The ablity to make electrical connections declined with distance, the highest incidence of connection being at 200um apart, but lessened steadily towards 1000um (Hadley,Bodnan and Kater 1985).

Isolated identified neurones from Aplysia which normally make chemical synapses will re-form these connections when maintained in culture. Such connections are specific, for when the identified neurones were presented with cells that respond to the same transmitter released by these neurones they would not make synapses. When the identified neurones were given a choice between their normal follower cells and the above cells, the former was chosen (Camardo,Proshansky and Schacher 1983). Chemical connections formed when paired adult neurones in Aplysia were grown in culture with juvenile paired neurones (Schacher and Flaster 1986). Paired adult cells alone would form synapses in 15% of cases whereas juvenile pairs formed synapses in 85% of cases. If adult cells were grown in co-culture with juvenile target and non target-cells the incidence of the adult neurones making synapses was greatly increased. The critical factors supplied by the juvenile cells has not been identified, but Ambron, Dou and Schacher (1985) showed that levels of certain proteins associated with synaptic formation were increased in juvenile Aplysia cells.

Schacher (1985) presented evidence that two branches from a single axon of the metacerebral cell in Aplysia showed different responses to follower cells in co-culture. Follower cells which were normally contacted by one these branches were presented to each of the branches. In the branch that served the follower cells 90% of contacts formed synapses and in the other branch 20% formed synapses. The rate of neuritic growth in the non-preferred branch was reduced when a follower cell was placed next to the preferred branch, but the reverse was not true.

1.6.2 **Synaptogenesis**

Ambron et al (1985) examined the number of newly synthesised proteins

in Aplysia neurones before and after synapses were formed. More than 300 polypeptides incorporated isotope-labelled methionine and were distributed throughout the regenerating processes. A large number were taken into the cytoskeleton and 30 that could be found in the cell body were missing or reduced in the neurites. Two proteins, as yet only identified by molecular weight, were increased by synaptogenesis.

A clue to the transformation of growth cone to synapse can be found in the appearance of synaptic proteins in embryos. Mason(1986) considered the development of embryonic mouse cerebellum and at what stage a phosphoprotein, synapsin 1, was found. Synapsin 1 is seen in the last quarter of pre-natal development and is widely distributed along the neurites , but by the third day after birth, when the endings have become distinct synaptic boutons and are carrying out synaptic functions, synapsin 1 is restricted to these structures. This neurone-specific protein is involved in the grouping of synaptic vesicles at pre-synaptic terminals. When it is phosphorylated it causes the movement of the vesicles to the release sites (Baehler and Greengard 1987). At the synapse other proteins are also involved in the release in transmitters from synaptic vesicles. Synaptophysin is a calcium binding protein of the synaptic vesicle membrane(Rehm,Weidenmann and Betz 1986). It is always associated with vesicles containing classical transmitters but not with vesicles containing the co-existing peptidergic transmitters (Trimble and Scheller 1988).

1.7 FACTORS ALTERING THE DEVELOPMENT OF NEURONAL PROCESSES

There are many influences that affect the cell and its patterns of growth. Action potentials within the neurone; electrical and field stimulation of the neurone and its processes and ; transmitters and second

messengers.

1.7.1 **Neuronal Activity**

The influence of neuronal activity, the presence of action potentials or their blockage has a profound effect on the pattern of axonal growth and direction finding of growth cones. The individual skeletal muscle cells of newborn rats are known to have polyneuronal innervation which is lost in the weeks after birth, becoming mononeuronal in adults (Thomson, Kuffler and Jansen 1979). However if the muscles are paralysed by prolonged by exposure to tetrodotoxin (TTX), which reversibly blocks the impulses to the muscle, the polyneuronal connections are maintained.

Intraocular injection of the sodium channel blocker, TTX, abolishes action potentials in the retinal ganglion cells of a neonatal vertebrate. The effect was established over a period of weeks and although no morphological effect could be seen in the cells or optic fibres the target area of the brain, the lateral genticulate network in the visual cortex, had undergone gross changes. The layers of cells were reduced in thickness and individual cells reduced in size (Archer, Dubin and Stark 1982). Two mechanisms were considered: inhibition of formation of appropriate synaptic patterns, from the first indiscriminate connections in the first weeks after birth or correct synaptic already existing, but without electrical imput new neuritic sprouting occurs which disorganises normal development.

1.7.2 **Electrical Activity**

Stimulation of Helisoma isolated cells caused cessation of neuritic outgrowth. Cohan and Kater (1986) have described three conditions to determine this; (1) the growth cones' movement can be quantitatively

assessed while the cell is stimulated, (2) the cell should be available to stimulation for periods of several hours and (3) the growth cone movement should not be restricted by the recording or stimulating electrodes. For stimulation patch electrodes were used, as intracellular electrodes stop neuritic growth. After an hour control period, cells were stimulated. Neurite elongation ceased within 15 min. All growth cones recovered in times varying from 15 mins to 2 hours. The growth cone structure also changed. The number of filopodia decreased and the cones, normally broad, flat and phase dark became rounded and phase bright. These changes were also reversed when stimulation ended.

The mechanisms underlying these effects are as yet unexplained. But Cohan and Kater (1986) have shown that action potentials cause a large influx of calcium into growth cones.(The effect of calcium is considered elsewhere).

1.7.3 **Electrical Fields**

Patel and Poo (1984) using Xenopus embryo neurones applied D.C. currents to the neurites either through uniform electrical fields via two silver-silver chloride electrodes across a culture chamber or focal fields via a glass electrode close to the growth cone.

Amplitude and frequency for the uniform field was 2.5V/cm at 10 Hz and 4 pA/m^2 at 10 Hz for the pulsed field, which they calculated was similar to that in the synaptic cleft during synaptic activity. In the uniform field the neurites and area of sprouting was directed to the cathode electrode. In the focal field growth cones moved towards the electrode during negative polarity and away during positive polarity. Response usually occurred within minutes.

Although the mechanism of cellular change could not be explained some possibilities were suggested: (1) The glycoprotein involved in cell adhesion to the substrate could be laterally realigned (as was shown in the Con A receptor redistribution in embryonic Xenopus muscle,(Poo and Robinson 1977), (2) change in the membrane potential or enzymes may lead to build up of cytoplasmic messengers required for the filopodia to be formed. However, they did not argue that the electrical field is a primary force in guidance in vivo as many types of cells throughout a number of animal studies responded similarly. Further nerves have been shown to grow to their targets without impulse activity, without the original target cell and with blocked post-synaptic ion channels.

1.7.4 Role of Transmitters

1.7.4.1 Serotonin

Haydon and Kater (1987) established growth cone morphology specific to individual, identified cells in the snail Helisoma. Serotonin at threshold level of 10^{-9}M caused complete cessation of growth cone movement in one cell (number B19) but not in another (number B5). In the affected cell (B19) the appearance of the growth cone changed, the flattened cone retracted its surrounding veil and filopodia to leave a smooth club-shaped ending. These effects were dependent on the dose and could be reversed.

Precursors or metabolites of serotonin affected the cell B19 but not the cell B5. High concentrations of serotonin had no effect on B5, showing the cell specificity of the action. The isolated cell had numerous neurites and growth cones but only the cone to which the serotonin was applied was affected. Serotonin had no effect on growth cones when applied

to neurites or cell body. The effects were confirmed in vivo. Further experiments whereby a serotonergic cell innervating the affected cell had its serotonin content lowered by treatment with an analogue (5,7-dihydroxytryptamine) caused substantial increase in the neurite outgrowth, pointing to the possibility of a growth regulatory role. Goldberg and Kater (1989) also showed that serotonin was central to the development of the Helisoma nervous system during embryogenesis. Serotonin containing neurones appeared at different stages in a pattern specific to each ganglion. If the levels of serotonin were lowered during certain stages the morphology of specific neurones was greatly altered.

1.7.4.2 **Dopamine and Acetylcholine**

Dopamine (at concentrations from 10^{-7} to 10^{-5}M) had a similar effect on the same cells, however acetylcholine had no such effect on the morphology of the Helisoma B19 and B5 cells but it did alter the effect of serotonin on these cells. The inhibitory effect of serotonin could be stopped or completely reversed (McCobb and Kater 1986).

1.7.4.3 **Glutamate**

Glutamate, an excitatory neurotransmitter in the central nervous system, controls outgrowth from isolated hippocampal pyramidal neurones in rat foetuses (Mattson, Dou and Kater 1988), causing regression of the dendrites, while the axon continued to grow. Cell death increased with higher concentrations of glutamate but the presence of cobalt, a calcium channel blocker, could stop this effect. The effect on the dendrites could not be easily reversed by simply washing out the glutamate but if a glutamate receptor antagonist e.g. y-D-glutamyl glycine is employed, dendrites continue to develop. Kudo and Ohura (1986) found that in

embryonic rat hippocampal cells the increase in the intracellular levels of calcium, in response to glutamate, was dose dependent.

The inhibitory and neurotoxic effects of glutamate in hippocampal neurons is in complete contrast to those on identified neurones of Helisoma (Jones and Bullock 1988). Here glutamate at the neurotoxic levels shown above promote neuritic growth. However as this was in an axotomised cell in an intact ganglion, it is not known whether it was a direct effect on the cell or the effect of neurotropic factors released from surrounding cells.

1.7.5 **Second Messengers**

1.7.5.1 **Calcium**

Gunderson and Barrett (1980) showed that when calcium or its ionophore A23187 was presented to the growth cones of chick dorsal root ganglia using a drug gradient, they had no effect on turning response. The gradient was produced by placing a pipette 25um from the growth cone and a flow of 25ml/hr maintained by perfusion. However when the calcium ionophore A23187 which allows calcium to flow into the growth cone, was added to the medium the cone did turn towards the calcium gradient. Kostenko, Musienko and Smolikhina (1983), in the snail Lymnea stagnalis, found that increase of intracellular concentration of calcium in the presence of ionophore A23187 inhibited the formation of neurites and caused retraction of those already formed.

The use of fluorescent calcium indicator Fura-2, which can cross cell membranes but is then transformed and so trapped inside a cell, allowed Cohen et al (1987) to quantify amounts of calcium in a growth cone and even

within different regions of a neurone. Action potentials evoked an increase in the intracellular free calcium in growth cones of identified cells of Helisoma. Serotonin also caused an influx of calcium into the growth cone of a serotonergic cell and subsequent cessation of growth. Comparison of motile growth cones showed a difference in resting levels of calcium content of an average of 2-3 times. Calcium in the soma and neurites was low when compared to the growth cone.

A23187 stopped neurite growth in all Helisoma buccal neurons but lithium, a known calcium channel blocker, caused increased neurite growth even if the known inhibitory transmitter serotonin was added (Mattson and Kater 1987, Mattson et al 1988).

1.7.5.2 **Effect of Light on Calcium Sequestration**

Exposure of the large pigmented neuronal cells in Aplysia to a normal white light source causes hyperpolarisation of the membrane. Henkart (1975) suggested that this was brought about by the release of calcium into the cytoplasm from the pigment granules. The effect was reproduced by injecting calcium chloride into the cell and the response to light was abolished by injecting the calcium chelator, EGTA. Considerable morphological differences were observed between cells kept in darkness and those in light. The granules displayed the capability of accumulating divalent ions when kept in saline and where strontium was substituted for calcium there was strontium in the granules.

Brown, Baur and Tuley (1975), Baur, Brown, Rogers and Brower (1977) confirmed this and showed that the 0.2-5um granules (or ,as designated by their content, lipochondria), which containing lipids and a pigment B-carotene, went through morphological changes after 30 sec. Their

explanation was that there is a photoisomerisation of the pigment, which changes the molecular structure of the granule membrane. The resultant increased permeability in turn releases sequestered calcium into the cytoplasm which increases potassium conductance of the cell membrane and hyperpolarises it. Sugaya and Orozuka (1978) found that the pigmented neurones of a Japanese land snail exhibited the same morphological changes and the same release of calcium and consequent neuronal impulse activity.

1.7.5.3 **Cyclic-AMP**

Gunderson and Barrett (1980) in looking at the effect of nerve growth factors in the turning response of neurites, and acting on previous work that showed NGF increased the internal levels of cAMP in some cells, examined the direct action of cAMP on the growth cones. Although cAMP alone had no effect, neurites would turn towards lipid-soluble cAMP analogues and, if phosphodiesterase inhibitors were present in the medium or pipette, a turning response towards a cAMP source was elicited. Phosphodiesterases inactivates cAMP.

Examining the transport of organelles (see following section) in the cytoskeleton of Aplysia growth cones and the possible role of cAMP in controlling their movement Forscher, Kaczamerek, Buchanan and Smith (1987) found several effects. Forskolin and anti-phosphodiesterases increased levels of cAMP in the cone causing expansion of the area of organelle movement (see central and peripheral domains;the cytoskeleton) but only when used in combination as forskolin alone had little effect. In Helisoma, cAMP suppresses neuritic growth and the inhibitory effect of forskolin is dose-dependent, rapid and reversible (Mattson et al 1988).

1.8 THE CO-EXISTENCE OF CLASSICAL TRANSMITTER AND PEPTIDERGIC

TRANSMITTER

Evidence for the existence of two transmitters in a single neurone has been shown in the invertebrates: Ach and 5-HT or octopamine in identified Aplysia cells (Brownstein, Saavedra, Axelrod, Zeman and Carpenter 1974); 5-HT and dopamine in flat worms (Kerkut, Seddon and Walker 1967); Ach and 5-HT in Helix (Cottrell 1976). Filagamo and Marchisio(1971) showed that undifferentiated neuroblasts of chick embryos possessed the ability to produce Ach although they might later develop into adrenergic or cholinergic neurones.

Lunberg and Hokfelt(1983) reviewed the evidence for another relationship, that of classical transmitters and the neuropeptides. They suggest that the neuropeptides and neurotransmitters have different storage sites, different synthesis and replacement mechanisms. The neuropeptides require a greater level of stimulation for release and cause a long lasting response. Classical transmitter causes short, quick responses. The neuropeptides are not synthesised and recycled at the synapse but are produced in the cell body and transported to the site of action. Consequently frequent stimulation of the cell may lead to depletion of releasable transmitter (Swanson 1983).

Iverson(1983) lists up to thirty neuropeptides giving a four-fold increase in possible neurotransmitters in the mammalian system. In invertebrates, the molluscs, annelids and arthropods have represented the main areas for study of peptidergic action (O'Shea and Scaffer 1985). Greenberg et al(1988) showed molluscs to have up to ten FMRFamide-like peptides. One of the cells under study, the C3 which innervates the tenatacle muscle in Helix has been shown to contain Ach and

FMRFamide(Xu, Bewick and Cottrell, 1989). Lehman and Greenberg(1987) suggested a model for the action of FMRFamide in Helix. In the active snail the tentacle is extended and the tentacular retractor muscle is relaxed, but when it is required to retract the peptide-containing neurones fire releasing FMRFamide, causing the tentacle to withdraw in proportion to the firing rate.

1.9 THE CYTOSKELETON

Essential to any cell, established or regenerating, is a system of transport for the materials required for construction and maintenance of connections with other cells or tissue types. The structure of this system is formed by the cytoskeleton, the main components of which are microtubules, actinfilaments and neurofilaments. The microtubules and neurofilaments and actin filaments can be rapidly assembled and disassembled from proteins in the cell (Alberts et al 1983).

1.9.1.1 Cytoskeletal Assembly

A problem to be resolved is whether these proteins remain disassembled until reaching the distal end of the axon or neurite and joined to the cytoskeleton there or added at the proximal end. By labelling these proteins with (^{35}S)methionine and measuring the amount remaining soluble Black,Keyser and Sobel(1986) found that after two hours (^{35}S) methionine was incorporated in the microtubules and neurofilaments. The proteins of neurofilaments and microtubules are transported at the slow axonal rate of 0.1-2mm/day and it was suggested that two hours was to short for the assembly point to be distal.

The point of assembly during development was examined by Banburg, Bray

and Chapman (1986).Chick dorsal root ganglia in culture were treated with drugs known to disrupt growth cone advance: colcemid and nocodazole preventing polymerisation of tubulin, taxol which causes growth cessation through uncontrolled microtuble assembly. These drugs, when applied topically to the cell body neurite and growth cone were found to be 100 times more active on the growth cone, leading to the conclusion that the main point of assembly in the developing cell is at the growth cone.

Solomon (1986) offered another explanation for these observations, suggesting that Banburg's method of measuring extension of neuritic length is not an adequate criterion for whether assembly or disassembly is occurring. Other cells have systems in which microtubules are transported from the cell body fully assembled then disassembled at the growth cone. It is thought that this system is the one affected. Banburg et al (1986) countered this view by reasserting that colchicine and related drugs (colcemid) are extremely powerful antimiotic drugs, easily entering cell bodies. The growth cones have high sensitivity compared with other regions of the cell, which indicates that they are the primary site for assembly.

The action of cytochalasin, which is known to disrupt actin filaments and thereby the formation of filopodia, illuminates their possible importance in the direction finding of growth cones. Neuronal growth in embryonic grasshoppers was treated with Cytochalasin B (Bentley and Taroian-Raymond 1986), inhibiting the the formation of growth cones. The growth cones were still apparent, but simply as an extension of the axon ending. The axon continued to extend but a large number of cones became disorientated.

Gordon-Weeks(1987) in work on the isolated growth cones in neonatal

rats found that studies using conventional fixatives, e.g. osmium tetroxide, for electron microscopy had no neurofilaments or microtubules in the growth cone. The addition of lysine to the fixative(this protects microfilaments from disruption by a mechanism as yet not understood) showed microfilaments to be present. The addition of calcium chelators does not lead to the appearance of microtubules(calcium is known to disassemble microtubules). Adding taxol, which increases microtubule formation does, suggesting tubulin is present in the growth cone but not in its polymerised state.

A substance (bb'-immodiproprionitrile, DPN) known to slow down the transport of neurofilament components (Parhad, Griffin, Hoffman and Koves 1986) was used to test the connection of microtubules and neurofilaments. It is believed that although they form separate bundles in the axonal cytoplasm, they are connected by short filaments and trasported together, the microtubules carrying the neurofilaments. The movement of neurofilament bundles were impeded but not the microtubules, indicating a weak link between them.

1.9.1.2 **MAPS**

The microtubule-associated proteins (MAPS), identified as co-polymers with tubulin forming microtubules, change during development. Marek, Fellous, Francon and Nunez(1980) examined these changes in the developing rat brain (post-natally at 3 and 35 days). In the young and the old rats two of the proteins (MAP1 and MAP2) were present in equal amounts. However when a third protein the tau complex, was examined in the young preparations by gel electrophoresis, there were half the number of bands present in the old. The ability of the old MAPs to assemble tubulin was

shown to be markedly greater than the young. They proposed a model for the purpose of the changes in the MAPs in the development of the brain. Large amounts of tubulin would be accumulated to produce the cytoskeleton of undifferentiated neuronal cells. As these cells began to differentiate other proteins e.g the tau complex, would be produced to allow the rapid manufacture of microtubules for axonal growth.

1.9.2 **Regional Differences**

A neurone exhibits considerable regional differences in its cytoskeleton. These differences exist in the neurone, axon, dendrite, neurite and growth cone. Comparisons of proteins within the cell body and the axons of Aplysia (Drake and Lasek 1984) show that two distinct cytoskeletons exist, one in the cell body and one in the axon. Although actin and tubulin are present in similar quantities in the cell and axon there were five times as many neurofilaments in the axon as in the cell body. The nucleus has actin in abundance and tubulin is almost exclusively in the cytoplasm. When the protein components of the cytoskeleton were tested for stability i.e. remaining associated with the cytoskeleton and not being removed when the cell and axon were solubilised, the neurofilament proteins were equally stable. The actin and tubulin were nearly twice as stable in the cell as in the axon.

Drake and Lasek speculate that two mechanisms account for the regional differences. The first is that there are two separate areas within the cell body where the proteins are synthesised or put together. The second is that there is differential metabolism, whereby there is a single method of cytoskeletal production within the cell and the axonal components are assembled after they have left the cell body.

1.9.3 **Organelle Movement**

Bridgeman, Kachar and Reese (1986) defined the domains in the cytoplasm of cultured fibroblasts and epithelial cells from Xenopus laevis in an attempt to explain the movement of organelles through the cells and their extensions. Organelles move in a saltatory fashion i.e. moving for a distance then stopping, this repeating as transportation continues. Small organelles move smoothly along the axon.

Two domains were defined. A central(C) one with many microtubules and small particles showing Brownian motion, and smooth movement of organelles. A peripheral(P) domain, containing mainly actin and virtually devoid of microtubules. These domains were shown in the cell body, C domain around the nucleus and P domain at the cell edges. Organelles of less than 500 nm moved smoothly in one direction, larger organelles and mitochndria moved intermittently , and back and forth. In the P domain organelles always moved intermittently.

Organelle movement within the growth cones of Aplysia (Forscher, Kaczmarek, Buchanan and Smith 1987) showed the characteristics of the C and P domain, with microtubules continuing in straight lines into the proximal growth cone area but greatly decreased in density and organisation in the lamellae. The organelles i.e. secretory granules and mitochondria will attempt to move from the C domain into the P domain but they are quickly reversed. Filopodia formed from microfilaments appear to connect to the microtubule endings in the growth cone area.

However it should be noted that these definitions of domains do not appear to apply to all cells. Axons and growth cones in cultures of dorsal root ganglia cells of chick embryos were shown with a central region of

neurofilaments and surrounded by microtubules (Yamada, Spooner and Wessels 1971). The difference in the arrangement of the microtubules and neurofilaments found by Yamada *et al* as compared to that found by the preceding authors is as yet unexplained. The three groups used different animal species and cell types at different developmental stages. Forscher *et al* and Bridgman *et al* used adult *Aplysia*(sea hare) neurones and embryo *Xenopus*(toad) fibroblasts respectively and Yamada *et al* used chick embryo neurones.

1.10 The Aims of the Project

The advantages of using isolated, identified neurones were described at the beginning of this introduction and following from this the broad aims of the project were to:

(1) Establish the conditions in which the isolated *Helix* neurones could be maintained in culture.

(2) To examine the morphology of the development of the neurones in these conditions.

(3) To test if connections were formed between the neurones and if such connections were electrical or chemical.

METHODS

METHODS

2.1 Sources of Helix aspersa

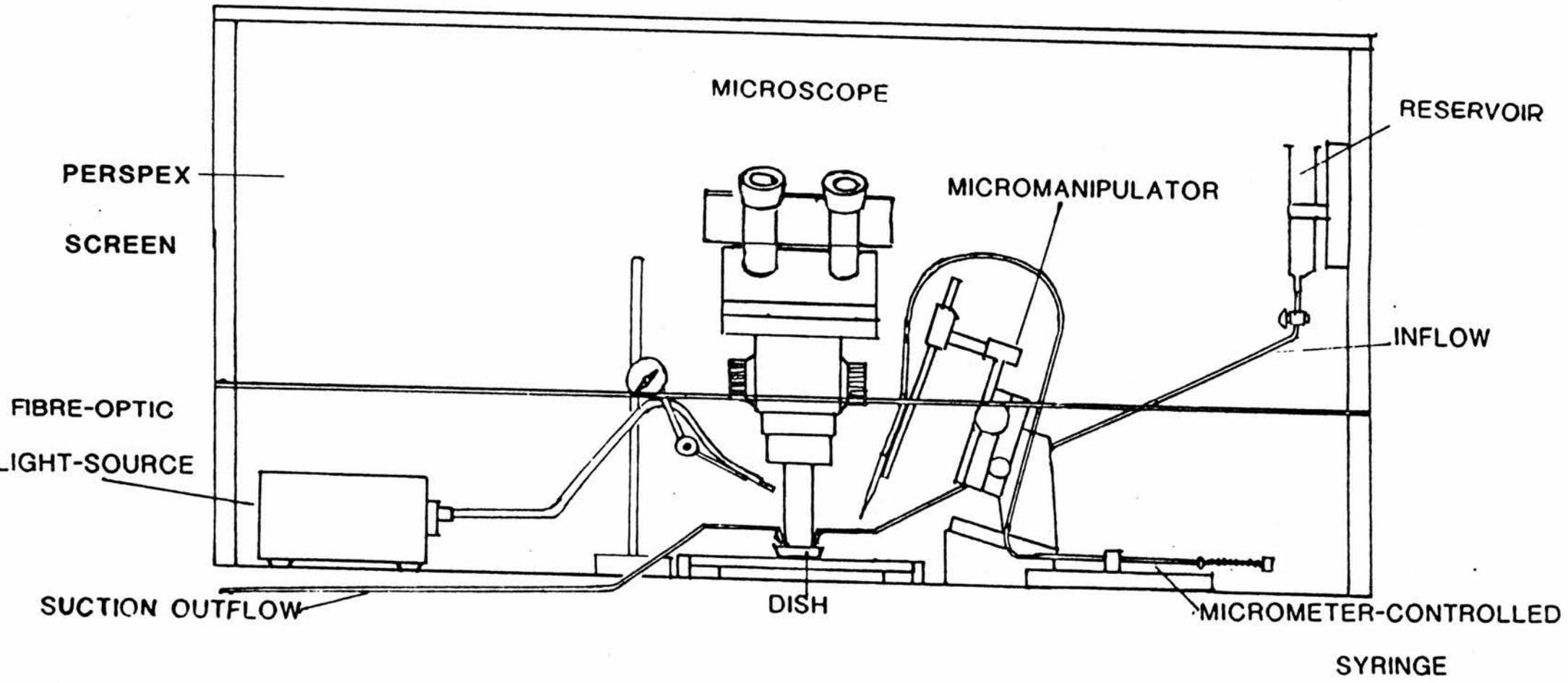
Adult Helix aspersa(6-8 gm weight, 2-3cm diameter) were collected locally and kept in the laboratory for up to three weeks. The snails were used throughout the year. They were most active from about April to September. In the months of March and October snails could be found in various stages of hibernation i.e. dependent on the temperature they might seal their shells but would become active again if there was a rise in temperature. The snails used during the hibernation period had the shell sealant or operculum removed 24hr before dissection and were placed in warm water (approx. 30°C) until they were moving actively.

2.2 General Dissection

(Any solution not described here are shown in detail under Solutions). Dissection was carried out under a purpose built hood(figure 1) and the enclosed area was washed with 70% ethyl alcohol. The dissecting instruments were also stored in 70% ethyl alcohol. All dissections were carrried out under a Zeiss stereoscopic microscope and illuminated by a Flexilux fibre optic light source. An adaptor was fitted to concentrate the area of illumination to 3mm.

The snails were deshelled and soaked in a solution of 25% Listerine/Helix saline for 15 min and then in fortified Helix saline for 20 min. The sterilised snails were pinned through the head and foot in a wax dish and cut along the dorsal midline. The circumoesophageal ring, under the conditions of this dissection, is in a posterior position to the buccal mass. However, it can expand to allow the buccal mass to move through it.

Figure 1 The area for sterile dissection was enclosed in a aluminium hood with a hinged front. A Perspex platform (22x22x1.5cm) over the dissecting area of the microscope serves as a stable base. The culture dishes can be moved across its surface with the minimum of disturbance to the neurones. These are left for 18-24hr to allow attachment to the substrate.



1. APPARATUS FOR STERILE DISSECTION

The buccal ganglia are at the junction of the buccal mass and oesophagus(figure 2).

The oesophagus was cut and the ganglia (circumoesophageal ring and buccal ganglia) removed with lengths of nerve attached to facilitate pinning down i.e. pins put through the nerves to avoid damage to the ganglia. The ganglia were stored in fortified Helix saline until a sufficient number were collected to be pinned out for removal of neurones. Ganglia were pinned in a Sylgard(Dow Corning) filled dish either dorsal or ventral side upwards(the C3 is on the dorsal surface and the C1 is on the ventral surface of the cerebral ganglia). The buccal ganglia would be dorsal side up. The outer layers of connective tissue sheath were stripped away leaving a single layer through which the cells could be seen.

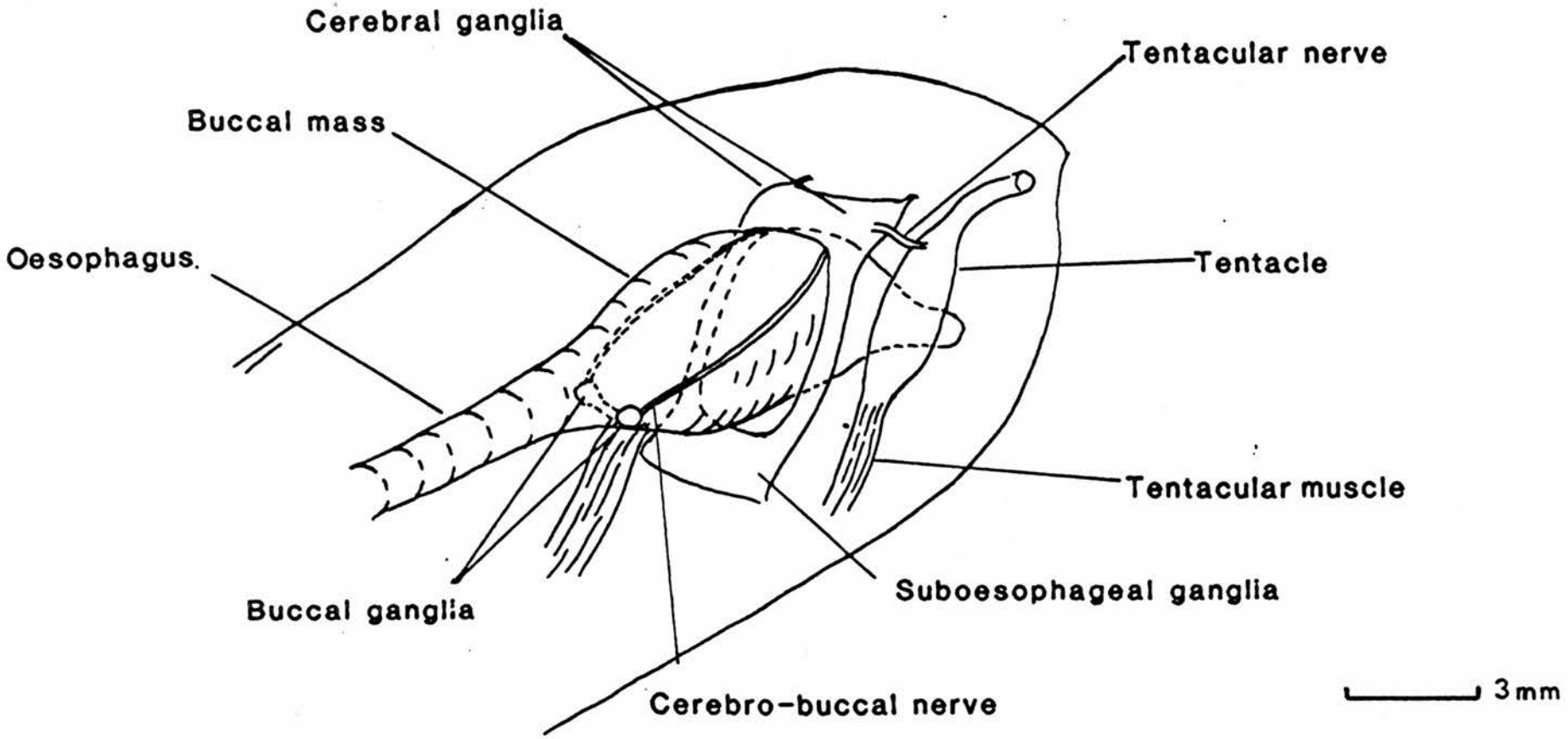
The solutions around the ganglia were replaced by gravity inflow and suction removal. 30ml of Helix saline was routinely flushed through to remove the digestive enzymes being applied to the ganglia. Care was taken that when solutions were being removed the level of liquid did not fall to that which would expose the cells to the tension of the miniscus and damage the cells.

2.3 **Enzyme Treatment of the Connective Tissue and Ganglia**

The connective tissue was exposed to 0.5% Pronase(BDH)in Leibovitz-15 medium for periods of 2min, 20min,40min and 60min. The enzyme-softened, final layer of connective was teased apart using sharpened No 5 stainless steel forceps. Trypsin(GIBCO) at 0.1% in L-15 was applied to the exposed neurones and left for periods of 30 min, 60min and 90min. As the time for the most effective action of pronase and trypsin was established a routine of a single period was set. Trypsin inhibitor(Sigma) at 0.1% in Helix

Figure 2

This simple drawing shows the circumoesophageal ring in position in the head of the snail. The C3 neurone is on the dorsal surface and the C1 is on the ventral surface of the cerebral ganglia. The visceral and parietal ganglia are on the dorsal surface of the suboesophageal complex which contains 7 ganglia.



2. A simple drawing showing the position of the ganglia in Helix aspersa.

Tentacle is shown withdrawn and only one is included.

saline was added for 30min then washed out with 30mls of Helix saline.

2.4 Dissociation of Identified, Single Neurones

Neurones were identified by position, size and colour. The position of the neurones are shown in figure 3a and b. The C1 is the only large cell (100-150um diameter) on the ventral surface of each cerebral ganglia and has red pigmentation. The C3 is on the dorsal side of each cerebral ganglion and is 90-120um diameter. The buccal cells(A,M and P, 100-120um diameter) were clearly seen at the extreme edges of these ganglia.

Only cells that appeared clear and with a defined nucleus were taken, cells that were opaque or milky in colour were not used. The non-clear cells when examined electrophysiologically in situ had no resting potential and in culture would not adhere to any surface. Nicholls (1982) found that leech neuronal cells which appeared milky would later clear and adhere. This was not the case however with Helix cells.

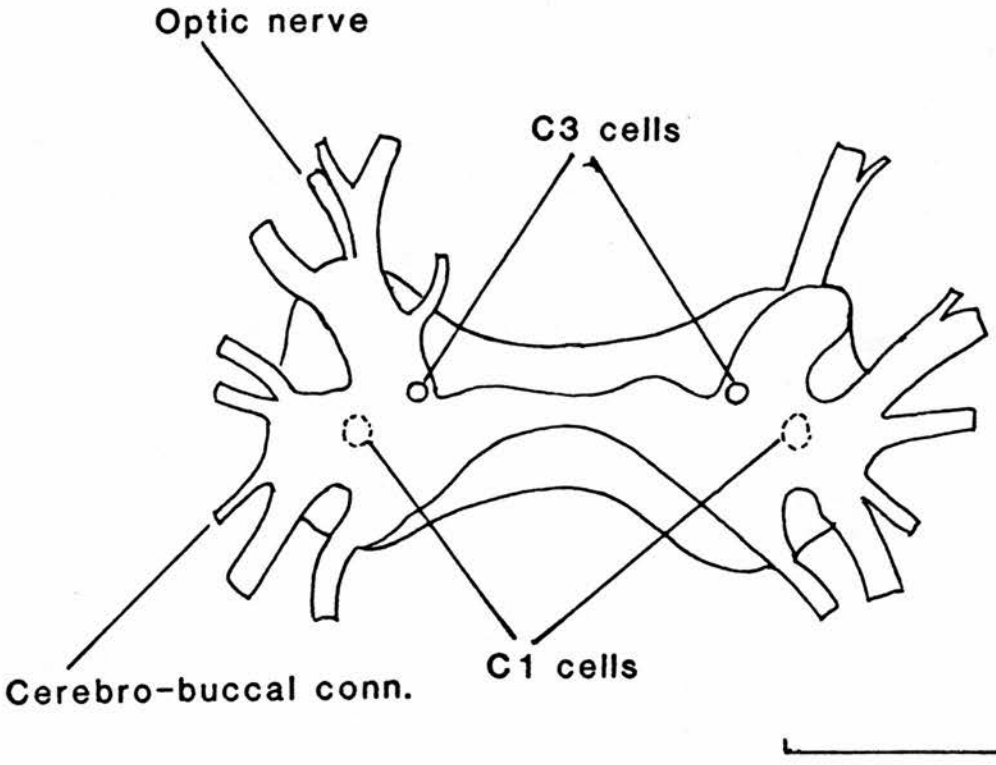
To remove the neurone perikaryon a cut was made into the ganglia with Zeiss iridectomy scissors to severe the axon. Surrounding cells were teased away with forceps and knives made from steel dissecting pins(A1,Watkins and Doncaster) embedded in glass handles.

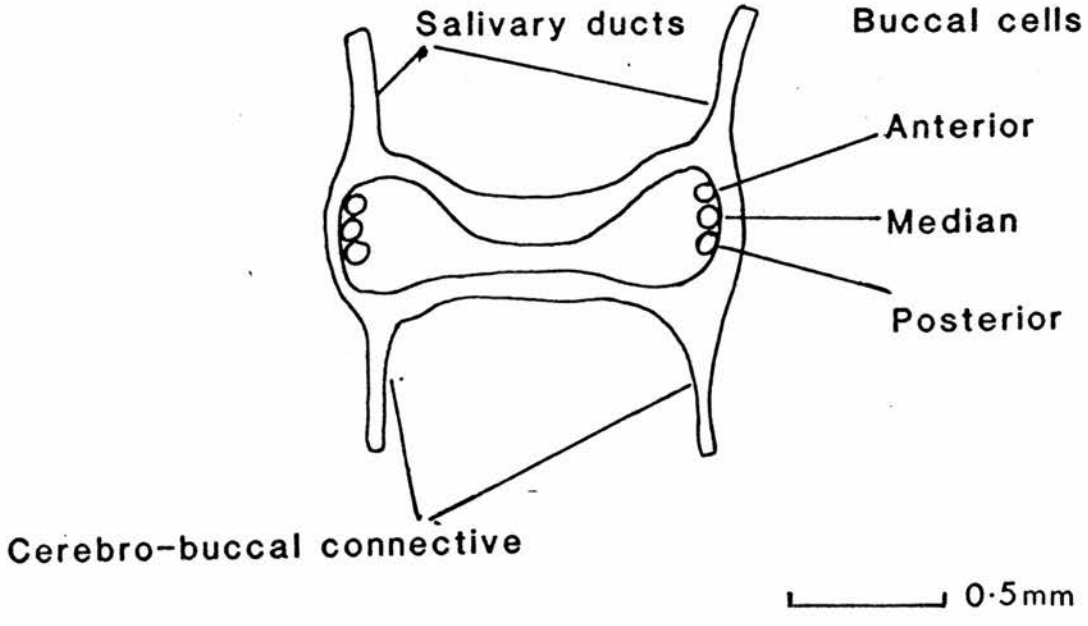
An alternative method of removing the cells was to loop a monofilament thread(Ethicon nylon, 13um diamter, Ethicon Edinburgh)around the axon and when this was pulled tight the axon was cut. The cell would either float free or could be gently shaken free of the thread(Nicholls 1982).

2.4.1Dissociation of Neurones from the Suboesophageal Ganglia

The cell mass of the visceral and right parietal ganglion was removed

3a. Cerebral ganglia





3b. Buccal ganglia

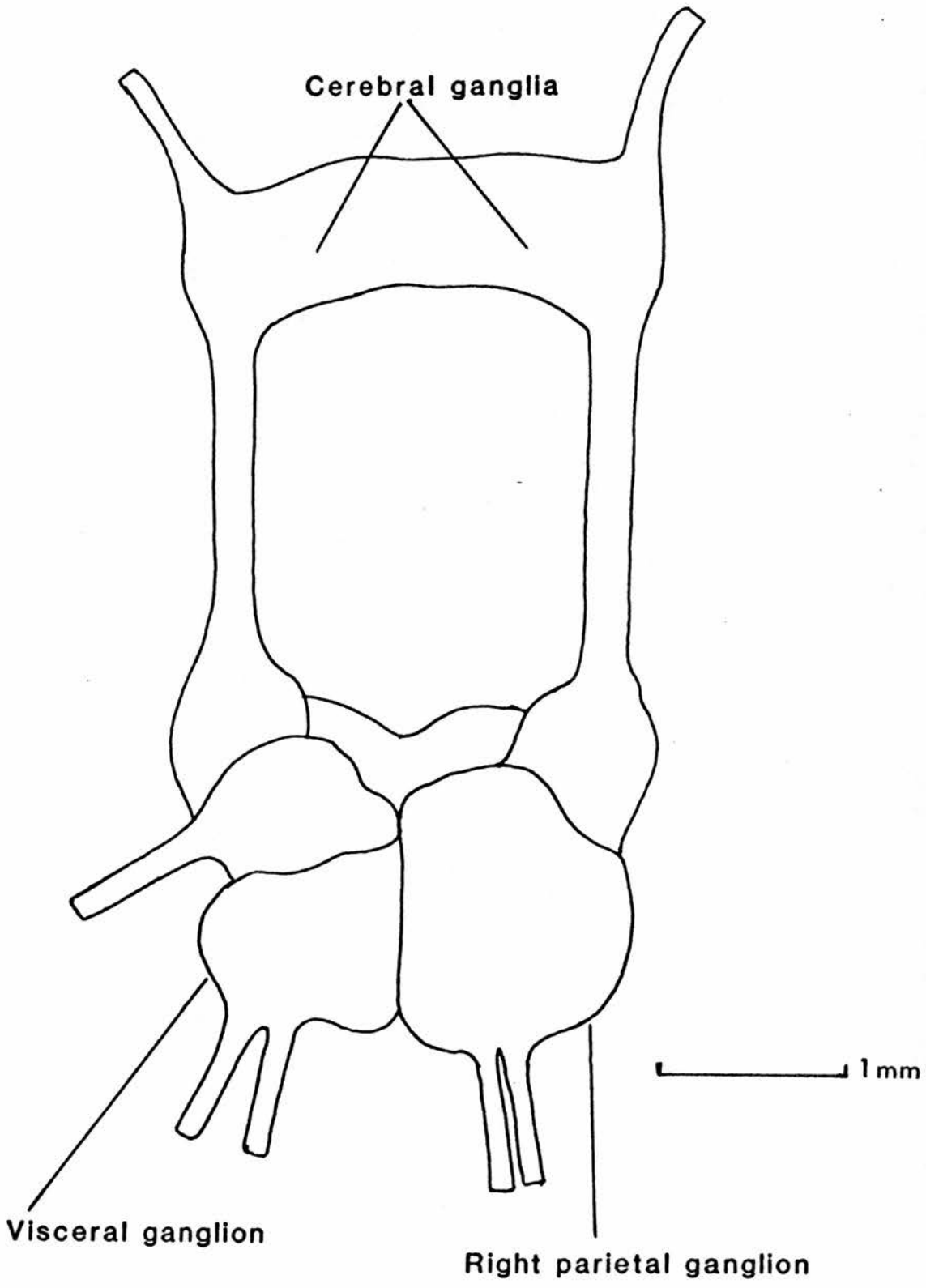
intact from the connective envelope (figure 4). The cells were transferred to a prepared culture dish using a fire-polished Pasteur pipette. The dissociation was produced by either drawing the clumps of neurones in and out of the pipette or by crushing the axons against the bottom of the dish with a fine steel pin. The neurones could also be separated by flicking the clumps with a fine steel pin, however this produced a lower yield of viable neurones.

2.5 **Cell Transfer**

Glass tubing of external 0.5mm diameter was pulled to a tip of approximately 250um diameter and the tip fire-polished. The tapered tube was attached to a suction line and the tip was allowed to fill with disssecting fluid by capillary action. Each cell was drawn into the first 1mm of the pipette from the preparation and transferred to a prepared culture dish.

A refinment of the removal procedure was developed using a syringe where the volume was controlled by a screw -thread movement. The syringe was connected to the pipette by polythene tubing and the pipette was accurately directed by attaching it to a Prior micromanipulator. A small movement of the thread allows the cell to be moved into the pipette and held there. The positioning of the cell in the culture dish in relation to other cells can be achieved with greater accuracy. The manipulation and positioning of cells could also be achieved by connecting a microelectrode, such as those used for intracellular recording, to handheld, air-controlled flexible tubing which was part filled with saline. Positive pressure projected a stream of fluid onto the cells pushing them into proximal positions.

Figure 4 The circum-oesophageal ring showing the position of the visceral and right parietal ganglia in relation to the other ganglia. All of these ganglia are contained within an envelope of connective tissue. Dorsal veiw.



4. Circum-oesophageal ring

2.6 Cell Culture

2.6.1 Culture Dish Preparation

Culture dishes(NUNC plastic) of 30 mm diameter were prepared in three ways: (a) a plain plastic dish (b) a dish with 22mm No 1 coverslip free in the bottom (c) a dish with 2cm hole drilled in the base and replaced with a round coverslip, cemented onto the bottom of the dish with Ambersil 151 to create a well.

The coverslips were taken through the cleaning procedure of alternating alkaline with acid solutions, thoroughly washed after each stage in distilled water then stored in 95% ethyl alcohol. The coverslips were placed in the culture dishes and allowed to dry for 12hr.

The dishes and coverslips were coated with:

(1) Poly-L-Lysine of the following molecular weights, 14,000, 28,000(Sigma) at a concentration of 2mg/ml, 300,000(Cellcult) at a concentration of 10ug/ml. After 5min the dishes were washed with sterile distilled water and air-dried.

(2) Collagen(Calbiochem) at a concentration of 2mg/ml. Air dried.

(3) Concanavalin A(Sigma) at a concentration of 2mg/ml. Exposed for 2hr, washed with distilled water and air dried.

(4) Fibronectin(Sigma) at a concentration of 5ug/ml for 30min then air-dryed. Washed three times with distilled water and air-dried again.

2.6.2 Conditioning the Dishes

All dishes were then treated with conditioning medium for 24hr before

being used. The plates were washed with L-15 medium to remove any remaining conditioning medium. 1.5ml of L-15 medium, containing 100 units Penicillin, 100ug Streptomycin per ml and 0.75ug/ml of Fungizone, was added to each plate.

After dissection, the plated cells were left undisturbed in the hood for 18hr at a room temperature of 20°C. The cells might then be examined under a phase contrast, inverted microscope(Olympus), however they were usually placed in a humidified incubator for a futher 4 hr before examination. When the cells of the visceral and right parietal ganglia were used in culture, these ganglia were removed intact from the connective sheath, transferred to a dish in a fire polished pasteur pipette and dissociated by flicking the cells free of the ganglia with a fine glass electrode or by trituration with a Pasteur pipette.

2.7 **Co-cultures with Tentacle Muscle Cells**

The whole of the tentacle muscle of a Helix aspersa (figure 2)was removed and the cells dissociated using two methods.

The first was that of Ishii and Takahashi(1982) whereby the muscle was torn into three strips in Helix saline treated for 90-120 min in 0.5% trypsin/0.01% collagenase followed by 4x60min in 0.05% collagenase. During this procedure the tissue was gently shaken in a water bath at 30°C.

At each change of solution, the muscle extract was centrifuged at 100g for 2min, the supernatant removed and replaced by fresh enzyme solution. Prior to centrifugation an aliquot of 0.1ml was taken for a visual estimate of the cell yield. At the final change the extract was washed and centrifuged three times in L-15 to remove any enzymes remaining and then

resuspended in a volume of 0.2ml for addition to the culture plates.

The second method used(Ishii,Takakuwa and Takahashi 1986) was developed to remove any tryptic activity after the first 90-120min. The mixture consisted of 0.15% collagenase(Sigma Type 1), 0.1% trypsin inhibitor(Sigma Type I-S) and 1ug/ml leupeptin(Sigma), dissolved in Helix saline.(Leupeptin was added to inhibit non-specific protease activity, possibly in the collagenase or from damaged cells).

2.8 Solutions for Cell Culture

Water was double distilled and filtered through a 0.22um Millipore filter.

2.8.1 Leibovitz-15(L-15) Medium

The medium is based on the original L-15 (Leibovitz 1963) but made as a special order by GIBCO without organic salts and without L-glutamine. All other constituents i.e. vitamins,aminoacids and galactose are included at half the normal concentration. The salts that make up Helix saline were added to the modified Leibovitz solution, but the sodium chloride content was reduced by one fifth to compensate for the contribution to the osmalality from the sodium in the aminoacids. In the incubator the L-15 medium was in an atmosphere of air, a combination designed for sympathetic neurone cultures which selected for the survival of neurones only. An L15/CO_2 mixture allowed non-neuronal cells to survive (Hawrot and Patterson 1979).

2.8.2 Helix Saline

Helix saline (from Meech and Thomas 1977), NaCl 64mM, KCl 5mM, $CaCl_2$

7mM, $MgCl_2$ 5mM, HEPES buffer 20mM. This saline was buffered to pH 7.4 with 10N sodium hydroxide and filtered through a 0.22um Millipore filter. The complete solution was stored in an incubator at 22° C until required. (At the time of use 300mM L-glutamine(GIBCO) was added).

2.8.3 **Fortified Helix Saline**

This solution consists of Helix saline (with NaCl at the normal level of 80mM) to which was added 500units of penicillin, 500ug streptomycin and 1.25ug Fungizone per ml.

2.8.4 **Cell Maintenance Medium**

The medium required to maintain the cells was replaced every three days and was made up from Leibovitz-15 medium to which was added 100units penicillin, 100ug streptomycin and 0.75ug Fungizone per ml.

2.8.5 **Preparation of Endogenous Factors(Conditioning Factor)**

Method A: Helix aspersa were taken through the sterilising procedure and the circumoesophageal ganglia removed into cell maintenance medium (4ganglia/ml). The connective tissue was carefully torn away exposing the cells in both the cerebral and suboesophageal ganglia. The ganglia were incubated at 22 C for 72hr. The resulting solution was filtered through a 0.22um Gelman filter (Millipore filters were not used as the endogenous growth promoting factors released during the above procedure were absorbed onto these filters, Wong et al 1981). The solution was stored in a deep freeze at -20° C until required.

Method B: Complete circumoesophageal ganglionic rings were placed in prepared dishes (2/dish) and the connective tissue carefully pulled aside

to expose the cells. The ganglia were left in the plate for 72hr then removed and the medium replaced with fresh. The exogenous factors produced by the ganglia and large rafts of fibroblast like cells attached to the plate.

2.9 **Immunohistochemical Localisation of FMRFamide**

Cells were plated in dishes with wells, described above. The well greatly reduced the risk of dislodging the neurones when solutions were changed. The method used for processing the cells for immunohistochemistry was adapted from Cuello(1983).

(a) The isolated cells were fixed in Zamboni's solution at 4^{o} C for 1hr.

(b) They were washed in 80% ethyl alcohol three times for 2min each time until the yellow colour of the picric acid was removed.

(c) The FMRFamide antiserum(a gift from Professor Dockray, University of Liverpool) was diluted 1:800 in phosphate buffered saline(PBS). The cells were exposed to this solution overnight at room temperature in a humidified, sealed container. The humidifying solution contained 0.1% of the antibacterial agent, sodium azide.

(d) The cells were washed three times for 5min in PBS

(e) They were then exposed to the secondary antiserum(fluorescein isothiocyanate conjugated to rabbit immunoglobulin, GAR-FITC) at a dilution of 1:240 in PBS for 1hr in the humidified chamber.

(f) They were washed three times for 5min in PBS

(g) The coverslip was removed from the dish and mounted on a slide in carbonated buffer:glycerol(1:2) and examined under ultraviolet light.

2.10 Solutions for Immunohistochemistry

Zamboni's Tissue Fixative, pH 7.3

20g para-formaldehde

150ml double filtered saturated solution of picric acid

2.5% NaOH solution

0.2M phosphate buffer(pH 7.0)

The p-formaldehyde and the picric acid solutions were mixed and heated to 60 C. The heated solution was made alkaline with drops of the NaOH. It was then filtered and made up to 1 litre with the phosphate buffer.

Phosphate Buffer(0.2M, pH 7.0)

For 500ml:

Na2HPO42H2O 5.43g

NaH2PO42H2O 3.04g

Phosphate Buffered Saline(PBS, 0.01M, pH 7.1)

Nacl 8.5g

Na2HPO4 1.07g

NaH2PO42H2O 0.39g

The solution was made up to 1 litre and the pH adjusted with 0.2M

Na2HPO4

Carbonate-Buffered Glycerol

Carbonate buffer:

Solution A: 0.5M Na2CO3 (pH approx. 11.5)

Solution B: 0.5M NaHCO3 (pH approx. 8.3)

Take 100ml of solution B and adjust to pH 8.6 using solution A. Mix with glycerol (2:1)

2.11 Injection of Isolated Neurones with Lucifer Yellow

To identify the processes of the cells in culture the cell body was injected with the fluorescent dye, Lucifer Yellow. This dye has the advantages of spreading quickly through the cytoplasm, is non-toxic, fluoresces brightly and is not easily removed by fixation. The main disadvantages are it will precipitate on contact with potassium(potassium chloride is used as solution for intracellular recording electrodes) either in the electrode or at the electrode tip when it is introduced into the cell. It also fades under ultraviolet light(Warner and Bate 1987). A glass recording electrode was filled for the first 0.5cm with 5% Lucifer Yellow and the remainder filled, without mixing, with 1M lithium chloride.The identified neurone was impaled and given 500 millisecond hyperpolarising pulses of up to 10 nA at 1 Hertz for 30 min and then left at 4° C overnight. The cell was then fixed in 4% paraformaldehyde (BDH) for 1hr and taken through 20, 40, 60, 80 and 100% ethyl alcohol solutions (5min for the first three and 2x5min for the last two). The coverslip with the cell attached was mounted on a slide with methyl salicylate and veiwed on a

Ortholux microscope under ultraviolet light.

2.12 **Photography and Microscopy**

The photographs of the cells used in this study were taken on three different systems. The film used for 1 and 2 was Kodak Panatomic X(ASA 32) and for 3, Kodak T Max(ASA 400).

(1) Photomicrographs taken under phase contrast optics used a Leitz Diavert inverted microscope with Wild MPS 45 photographic system and a Zeiss IM 35 inverted microscope with attached Contax camera. The second of these was also where some of the electrophysiological studies were made.

(2)Differential interference contrast(or Nomarski) optics were used on a Zeiss Universal microscope.

(3)Fluorescing neurones under ultraviolet light were photographed on a Leitz Ortholux microscope using a Leitz vertical illuminator with an excitation turret(excitation filter 490-495 nanometres, beam-splitting mirror K 540nm, suppression filter K 515nm).

2.13 **Electrophysiological Recording Methods**

2.13.1 Recording Conditions: Recordings of the electrical activity of the cells were made in the culture dishes. Flow through of solutions was visually monitored for any disturbance of the cells. The high profile of the large, isolated neurones increased their susceptibility to movement of solutions in the dish. A system was devised whereby the isolated cells could be illuminated from below and above. Lighting from below meant that cells and their processes could be seen without the need for phase contrast optics.

2.13.2 Recording system: The signal from the cell was passed from the electrode through a silver/silver chloride wire to a Neurolog DC preamplifier(NL 102) and was monitored on a Nicolet 3091 oscilloscope and simultaneously recorded on a Racal 4DS tape recorder from which hard copies of the recordings could be made on a Gould 220 chart recorder.

2.13.3 Electrodes: Recordings were made with thin-walled capillary glass with inner filament(Kwik-fil,1.5mm outside diameter, 15cm long). The electrodes were pulled on laboratory built puller and back-filled with 2M potassium acetate. Electrodes of 2-5 meg-ohms were used.

RESULTS

Figure 5

Brief descriptions of cell morphological structures seen in culture conditions.

The composite drawing opposite shows many of the structures found in the following photographs.

Cell lamellipodium- This is a thickened area extending out from the neurone in the early stages in culture conditions, from which growth cones might develop.

Dissociated neurone- This could either be a neurone with all connections removed or a neurone with an axon or axons attached.

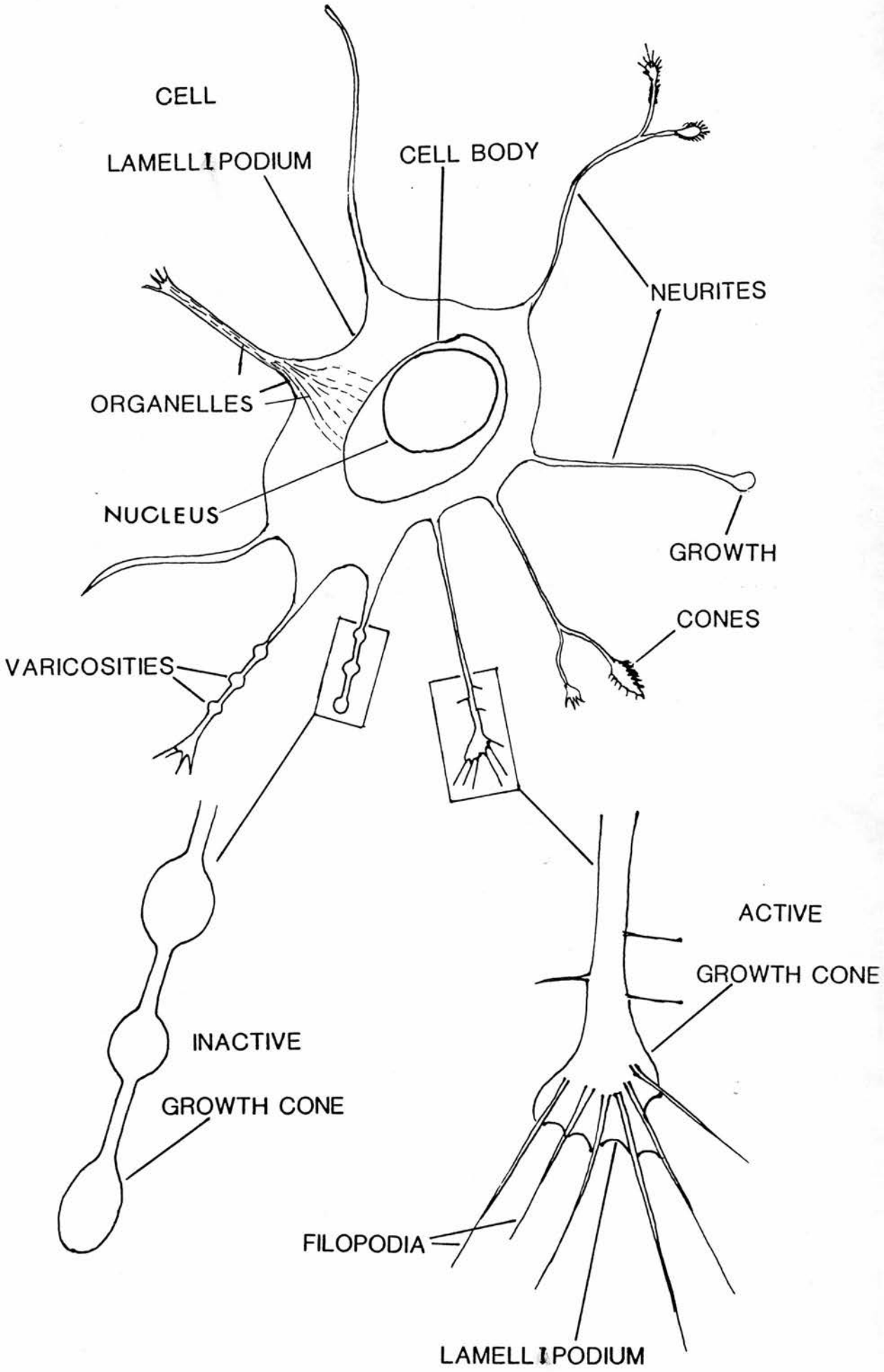
Growth cone- The enlarged areas at the distal end of a neurite which can take several forms: (1) Bulbous and clublike when growth has ceased (2) Flat and fanshaped with extended filopodia when growth is in progress (3) Long and clublike with filopodia along the edges,also a growing stage.

Growth cone lamellipodium- The thin membrane between the filopodia.

Neurite- The newly developed, threadlike extensions from the neurone or its axon lead by a growth cone.

Organelles- Elongated structures seen spread through the cell lamellipodiun.

Varicosities- Bulbous structures along the length of the neurite.


CELL
LAMELLIPODIUM
CELL BODY
NEURITES
ORGANELLES
NUCLEUS
GROWTH
CONES
VARICOSITIES
ACTIVE
GROWTH CONE
INACTIVE
GROWTH CONE
FILOPODIA
LAMELLIPODIUM

RESULTS

3.1 Long-term Maintenance of Ganglia in Leibovitz-15

3.1.1 Early attempts to maintain isolated cell cultures had very limited success. In those experiments only small cells(15-25um) adhered to the substrate. They produced few neurites. Consequently the ability of the medium to support Helix neurones in situ was tested. Buccal ganglia were maintained in culture with Leibovitz-15 medium for up to 16 days. The electrical activity of identified neurones was recorded after 1 day, 7 days and 16 days (figure 6). The connective tissue had been removed after treatment with 0.5% Pronase to make the recordings. The connective tissue had grown back over the exposed neurones 7 days after it was first removed.

3.1.2 Effect of 5-HT on an Identified Buccal Neurone In Situ

The anterior neurone, in the buccal ganglion which had been characterised, electrophysiologically , and was known to respond to the neurotransmitter 5-HT, were tested. 5-HT was iontophoresed onto an A neurone after 6 days in culture. 5-HT caused an increase in spiking (figure 7), a similar response to that seen in situ.

3.2 Periods of Exposure to Pronase

The connective capsule held cells under tension in the ganglion and removal of this layer, without pre-treatment with digestive enzymes, resulted in a rupture through which cells were forced. The damage could either be seen immediately i.e. the cells became white in colour, or, when plated out, the cells were granular in appearance and showed no neuritic growth. To minimise this problem the connective layer was softened by

exposure to Pronase (0.5 %).

2 min exposure to Pronase (0.5%), although effective for the ganglionic connective tissue in snails such as Helisoma had no useful effect in Helix. After 20min exposure, The connective tissue was still taught and movement of the underlying neurones could be seen when the connective was manipulated. This indicated the connective tissue and the neurones were still closely connected. 40min exposure produced softening of the connective such that the suboesophageal ganglia , containing many neurones, would produce a reasonable number that were viable. In the cerebral ganglia, however, where the C1 and the C3 neurones were lying immediately below the connective, 60min exposure was required to minimise the mechanical damage to these neurones. The connective tissue could be pulled away without visible movement of the neurones below.

3.3 **Periods of Exposure to Trypsin**

The period of time required for trypsin to be most effective differed for individual ganglion. The C1 and C3 neurones in the cerebral ganglion were tightly embedded amongst very small cells. Under the conditions of microscopy for dissection these small cells appeared as a single smooth surface. The population of neurones seen in the suboesophageal and buccal ganglion were generally large (50-250 um) and more readily separated. The time required in trypsin for neurones from the suboesophageal ganglion to be separated was 30min. The time for the C1 and C3 neurones was 60min. The identified buccal neurones were also given 60min to ensure they were easily removed.

Figure 6

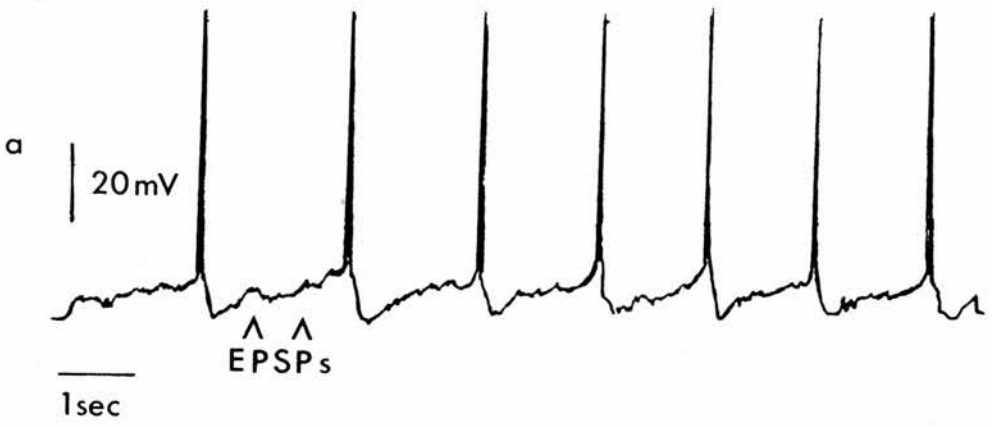
Response of identified neurones in the buccal ganglia maintained in L-15 medium from 1 day to 16 days.

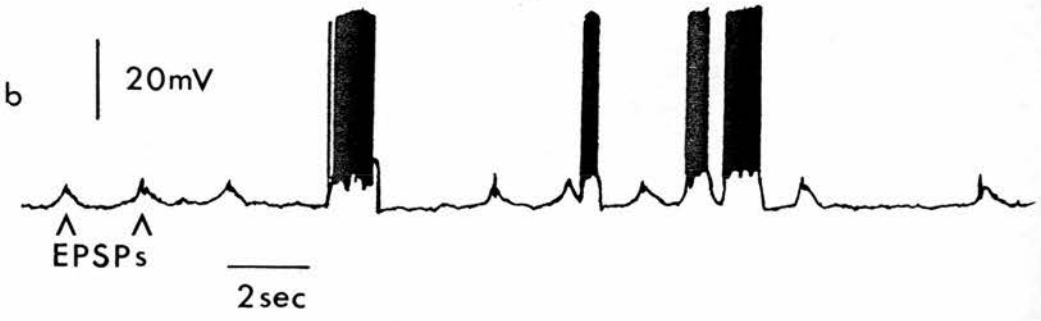
All neurones had resting potentials

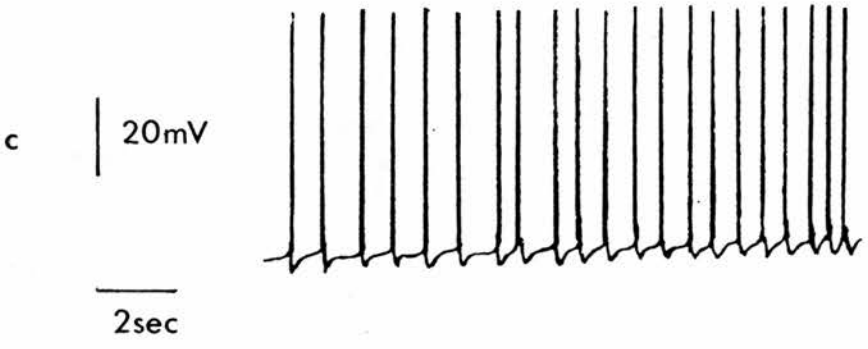
(a) 1 day in culture. Middle neurone. This showed "spontaneous" impulse activity which may have resulted from synaptic imput (note small potentials, possibly excitatory post synaptic potentials, EPSPs). Membrane potential -35mV.

(b) 7 days in culture, Anterior neurone. This has evidence of burst of EPSPs and when current was injected through the electrode the neurone fired a burst of impulses. Membrane potential -40mV.

(c) 16 days in culture, Middle neurone. The rate of impulse activity increased by artificially depolarising the neurone. Membrane potential -38mV.



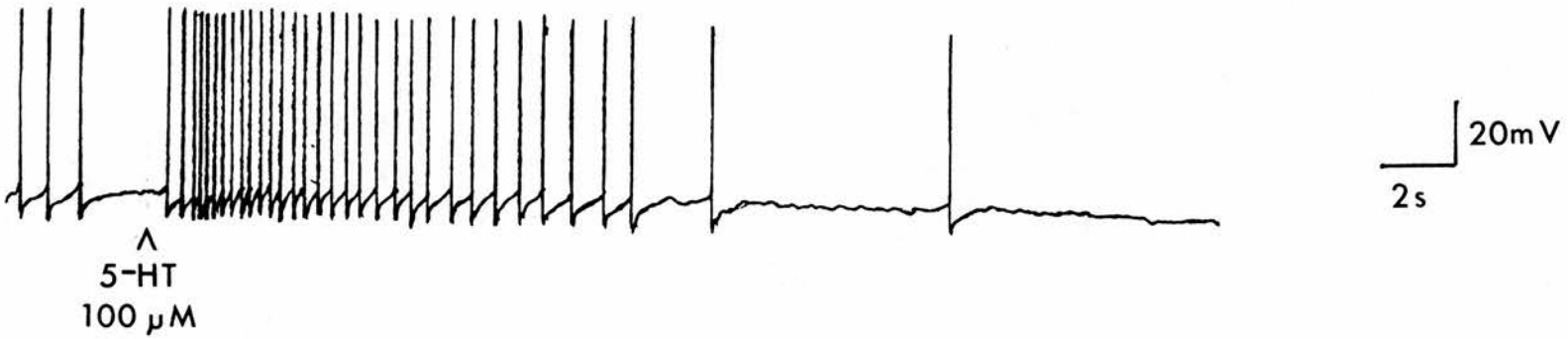




6.

Figure 7

Effect of 5-HT on an identified A neurone from one of the buccal ganglia maintained in L-15 for 6 days. 5-HT(concentration in the pipette, 100uM) locally applied increased firing rate. Membrane potential -38mV.



7.

3.4 **Substrates: The Attachment Factors**

The influence of the attachment subtrate was examined and the effect shown in Tables 1 and 2.

The cells from two suboesophageal ganglia were plated onto a glass coverslip (22x22mm) which had been coated with the substrates below and treated with conditioning factor. All the cells larger than 10um from three random areas (800um diameter) from three dissections were counted. Cells smaller than 10um could be non-neuronal or glial. The count was made 24hr after plating and the medium was replaced to remove any cell not attached. Any unattached cells that might have remained in the dish were revealed by tapping the microscope stage.

The neurones shown in figures 8 to 36 and 39 to 43 were plated on Concanavalin A. The others those maintained on other substrates and surfaces, as described in the text and figure legends.

Table 1: Percentage of Suboesophageal Neurones that Attached and Developed Neurites

Substrate	No. Cells Counted	No. Cells Attached	% Cells Attached	No. Cells Developed	% Cells Developed
Con A	91	76	83	70	77
Collagen	109	3	2.7	3	2.7
Fibronectin	52	2	3.8	2	3.8
Poly-L-Lysine					
MW 14,000	80	0	0	0	0
MW 28,000	59	0	0	0	0
MW 300,000	52	12	23	12	23

Table 2: Percentage of C1,C3 and Buccal Neurones(A,M and P) that Attached and Developed Neurites on Concanavalin A

Cell	No of Cells Plated	No of Cells Attached	% of Cells Attached	Cells Developed	% Developed
C1	58	27	46	18	31
C3	39	17	43	13	33
Buccal	18	14	77	13	72

On poly-L-Lysine only the C1 neurones attached and developed neurites (3 neurones out of 10 plated). None of the isolated, identified neurones attached to the other substrates used.

3.5 **Cell Cultures of Dissociated Neurones from the Suboesophageal** Ganglia

Neurones from the visceral and right parietal ganglia (figure 8) were used to assess different procedures for maintaining dissociated neurones in culture. These ganglia were used because they are the most accessible and they contained neurones whose perikarya range from 20-250um in diameter. These neurones served as a control for attachment and viability in the culture conditions and were taken at each dissection of identified neurones. Such neurones were also tested electrophysiologically. Many of the neurones readily sprouted showing dense patterns of neuritic growth within 18hr after dissociation. The neurites that developed were long and narrow, and emerged directly from the cell body i.e. they rarely formed a lamellipodium from which neurites developed, a characteristic of the identified neurones (see below). This was so even if the suboesophageal cells were in large or small groups or isolated as single neurones.

Figures 8a,b,c,d and e

Unidentified neurones from the visceral and right parietal ganglia 4 days in culture. Neurone somata, with neuritic processes, varied in size from 30um to 120um. Many of these neurones exhibited polar forms of development i.e. the neurites grew from specific points of the neurones and did not have the radiating neuritic growth shown by the large identified neurones. The majority of neurones did not have distinguishable growth cones but formed a network of neurites that appeared to be connected. However the neurone (MP) in the top righthand corner of (d) did show short neurites with growth cones. All the large cells shown in these figures are neuronal suboesophageal cells. Phase contrast optics.

a,b,c,d and e scale bar=100um

BC=Background cells, which did not develop processes

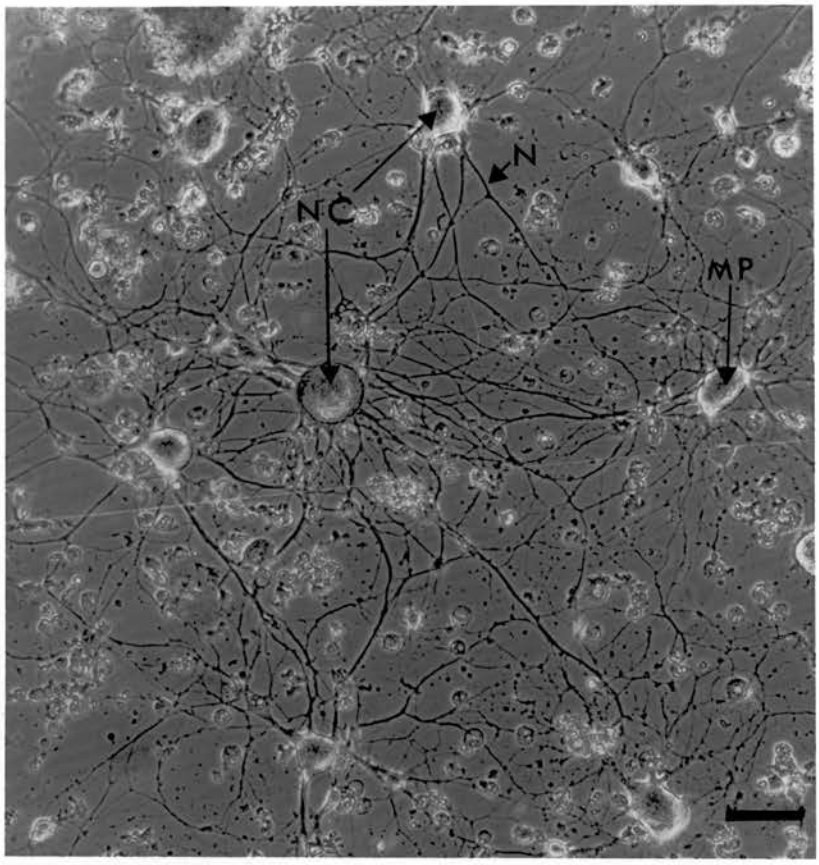
GC=Growth cone

MP=Multipolar neurones

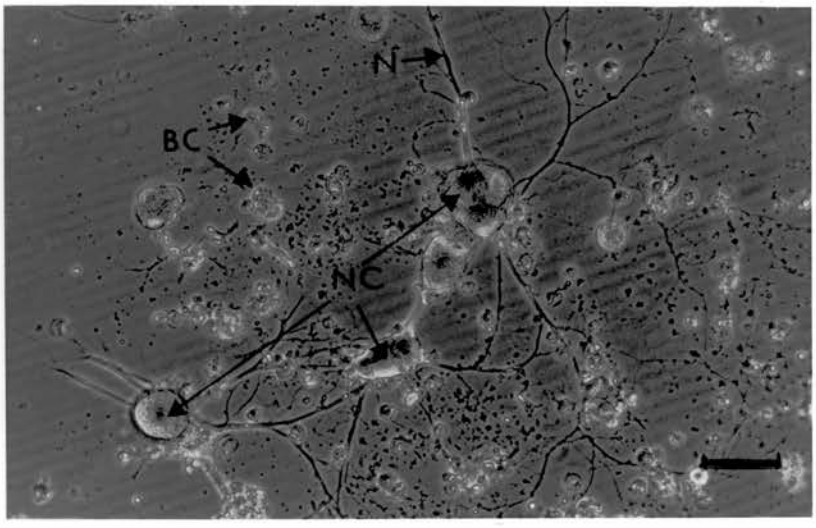
N=Neurites

NC=Neuronal cell

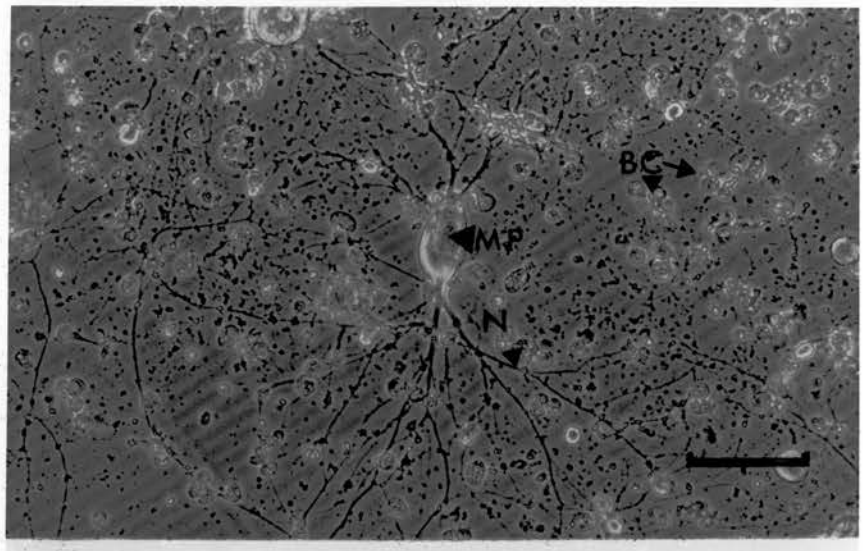
a



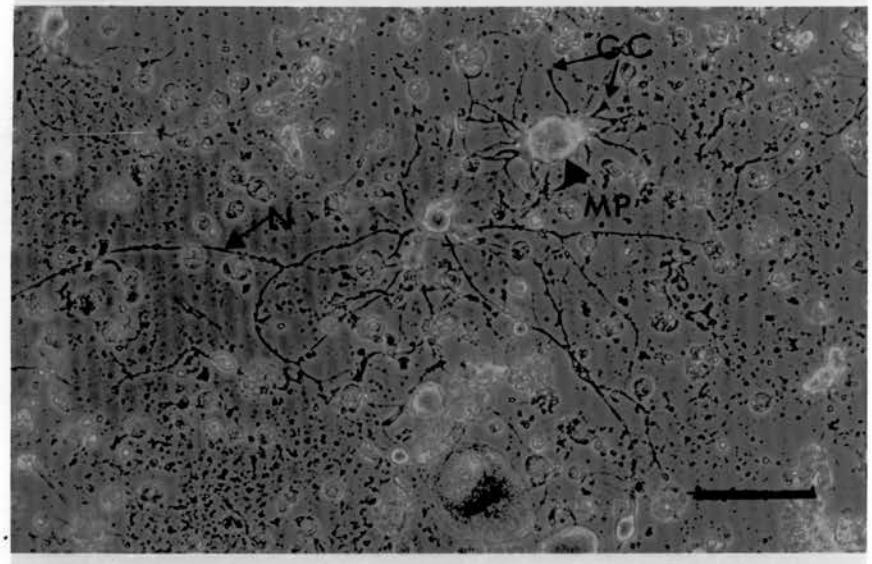
b



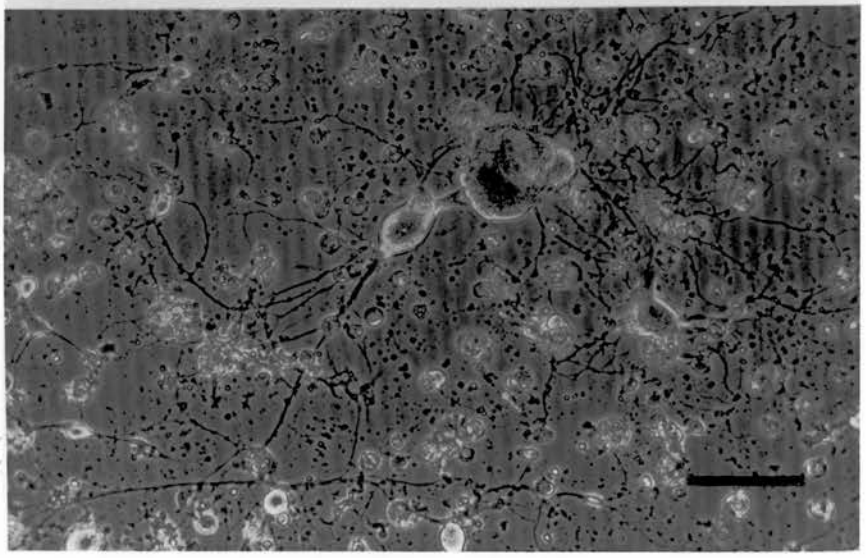
c



d



e



3.6 The Identified Cells: The C3 Neurones

3.6.1 Morphology of the C3 Neurone

The majority of redeveloping C3 neurones showed an enlarged, flattened growth or lamellipodium (LP) from which growth cones and neurites appeared to sprout (figure 9). The distance from the cell body to where the LP ceased to extend and the growth cones and neurites become the exclusive advancing form varied widely e.g. in figure 9b from 25-120um. The edge of the lamellipodium in figure 9b and, too a lesser extent, in 9c showed a distinctive thickened border with short spikelets along much of its length. This border became much less distinct where growth cones began to form. Examination of the lamellipodium in greater detail under Nomarski optics (figure 11) shows thread-like structures running through it. Such structures converged to form the central area of the neurites and extended into the growth cones. The long, spindle shapes of these structures suggested they could be mitochondria.

3.6.2 Growth Cones

Three forms of growth cones could be seen extending from the lamellipodium (figure 10a): (1) The predominant form shown at the centre of figure 10b was rounded form with many long filopodia and a narrow neurite; (2) At the extreme right edge of figure 10a is a flat cone with with a wide peripheral domain, short spiky extrusions and a broad neurite. Where filopodia could be seen on the second form these were also long (right side of figure 11a); (3) A smooth, rounded cone with no filopodia and long neurites (N2 in figures 10a and 10b).

3.6.3 Growth Cones and Cell-Cell Contact

Growth cones from C3 neurones appear to avoid some cells with which they have made contact. In figures 12a and 12b the growth cones from the C3 neurone had investigated the obstructing cells with their filopodia. In figure 12a a gap is seen between the g.c.s and the unidentified cells. Filopodia extended into this gap but on the opposite side of the growth cone no filopodia are evident (see also the drawing oppsite figure 12a). However as seen above figures 10a and 10c showed neurites from the C3 neurone which had developed across the lamellipodium, both from the same neurone. The growth cones had not avoided contact.

Figure 9 a,b and c

C3 neurone, showing developing cell lamellipodium (a)24hr (b)40hr (c)60hr. Phase contrast optics.

a,b and c scale bar=100um

ARE=Advancing ruffled edge

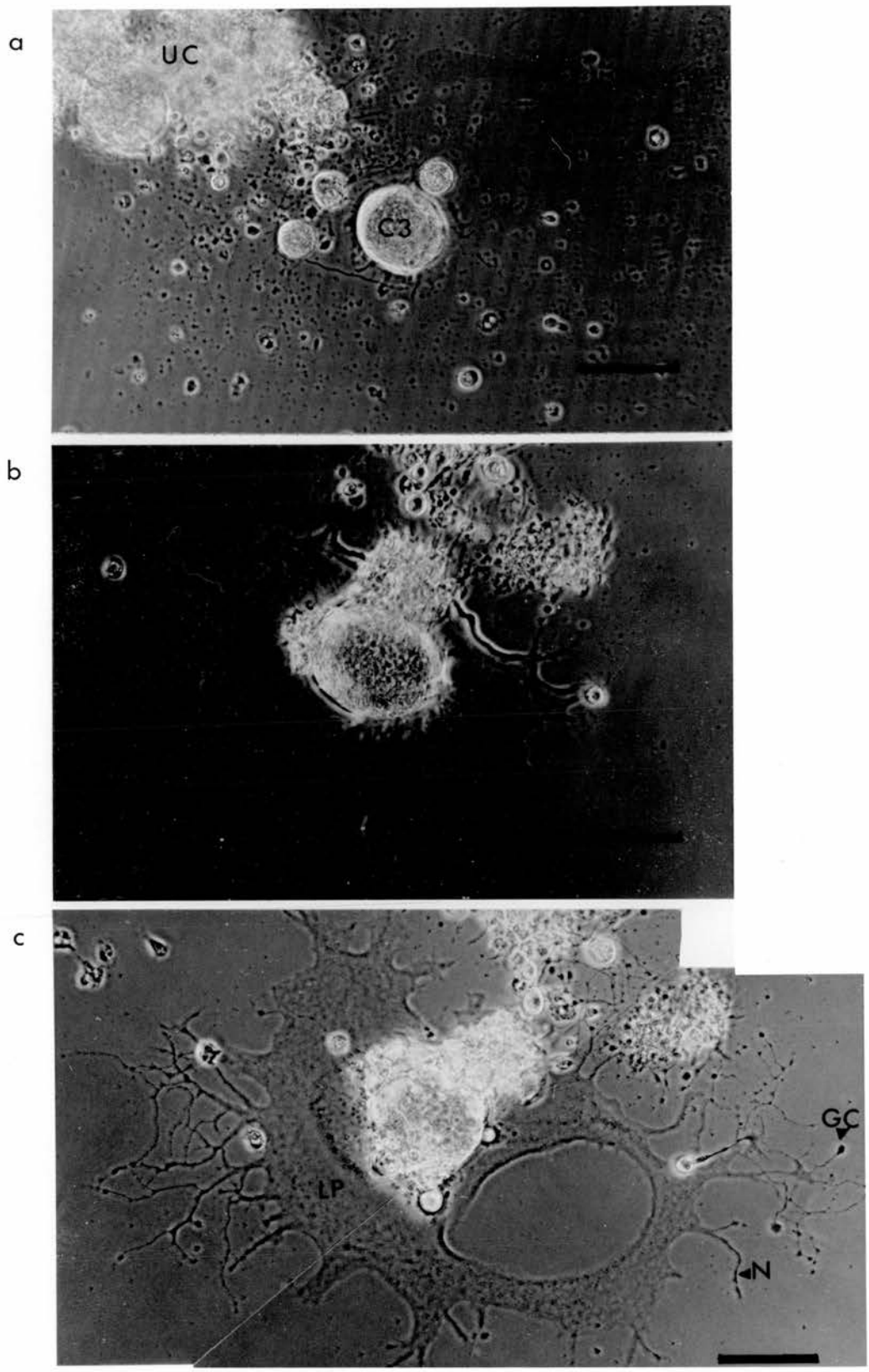
C3=Identified neurone

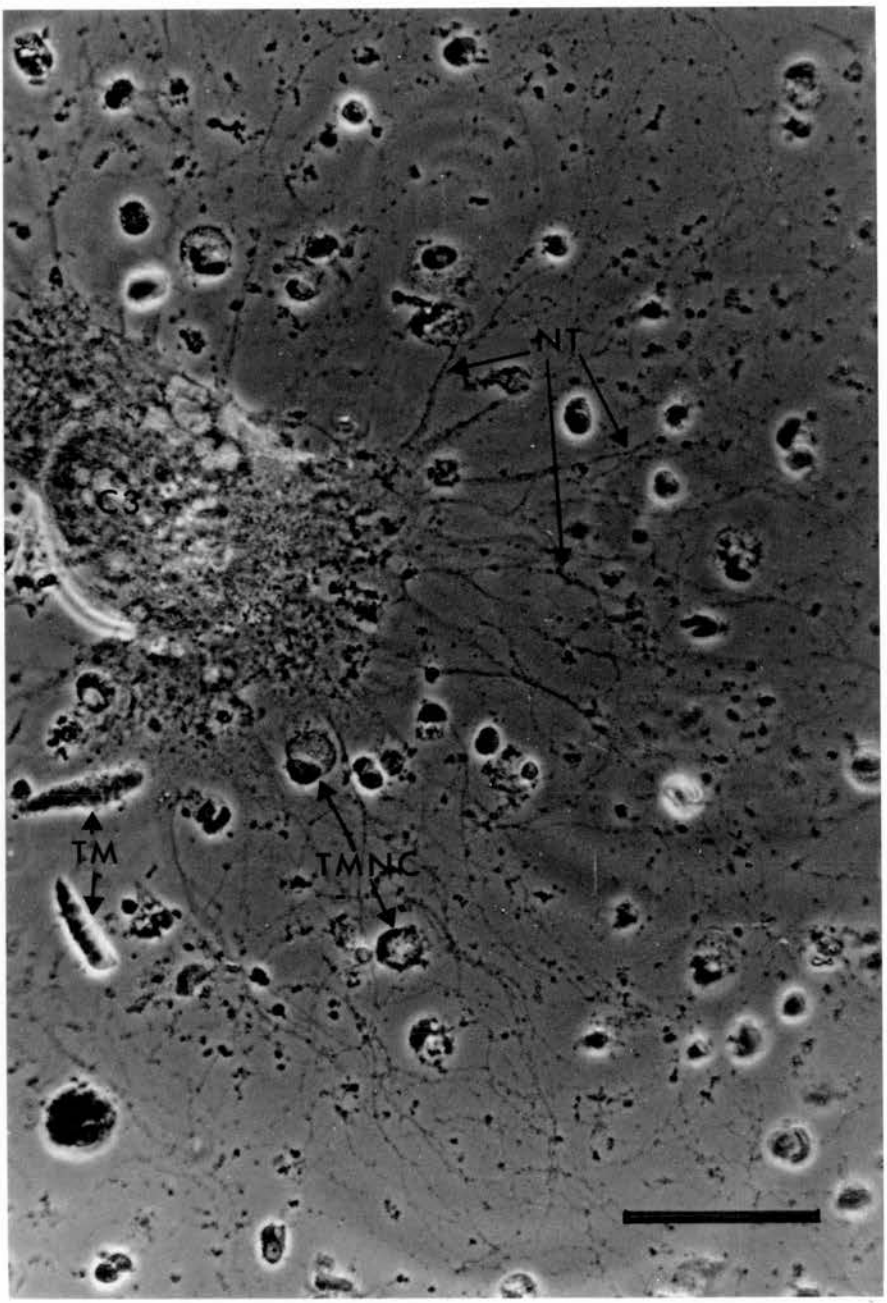
GC=Growth cone

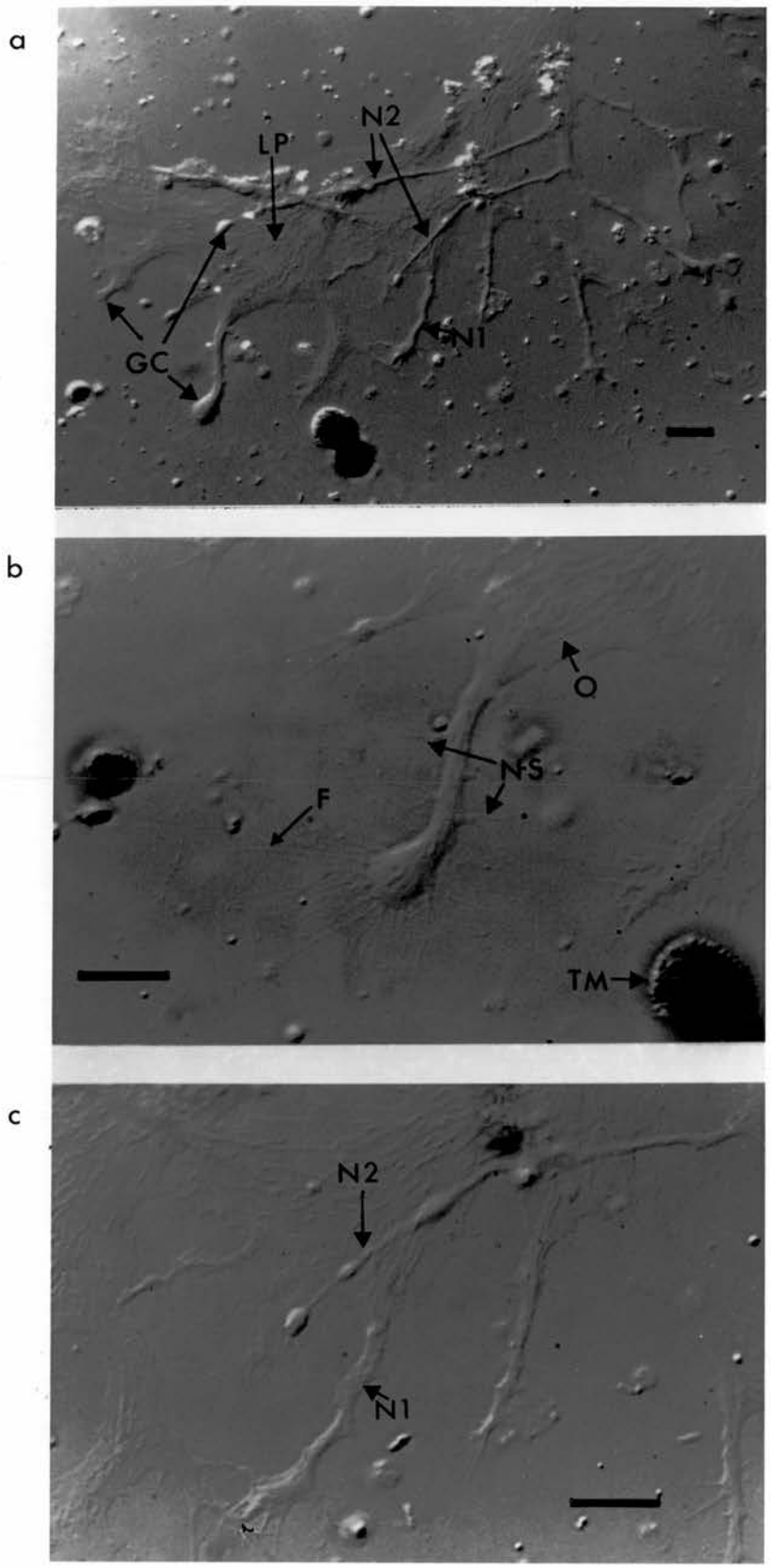
LP=Lamellipodium

N=Neurite

UC=Unidentified cells taken out with the C3 neurone during dissection


a
UC
C3
b
c
GC
LP
N


NT
C3
TM
TMNC


a
LP
N2
GC
N1
b
O
NS
F
TM
c
N2
N1

Figures 11

The LP of the C3 neurone in more detail showing evidence of organelle transport.The LP had a border with no organelles in it. The neurites also had a central area containing organelle-like structures and an empty border area .Nomarski optics.

Scale bar=10um

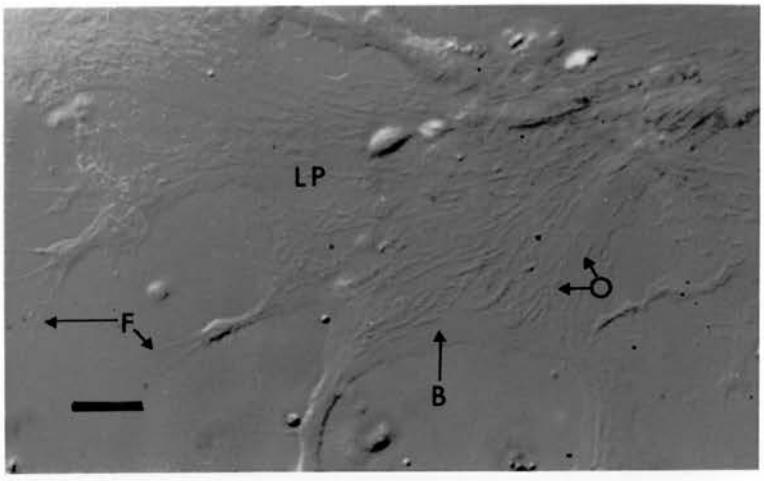
B=Border

F=Filopodia

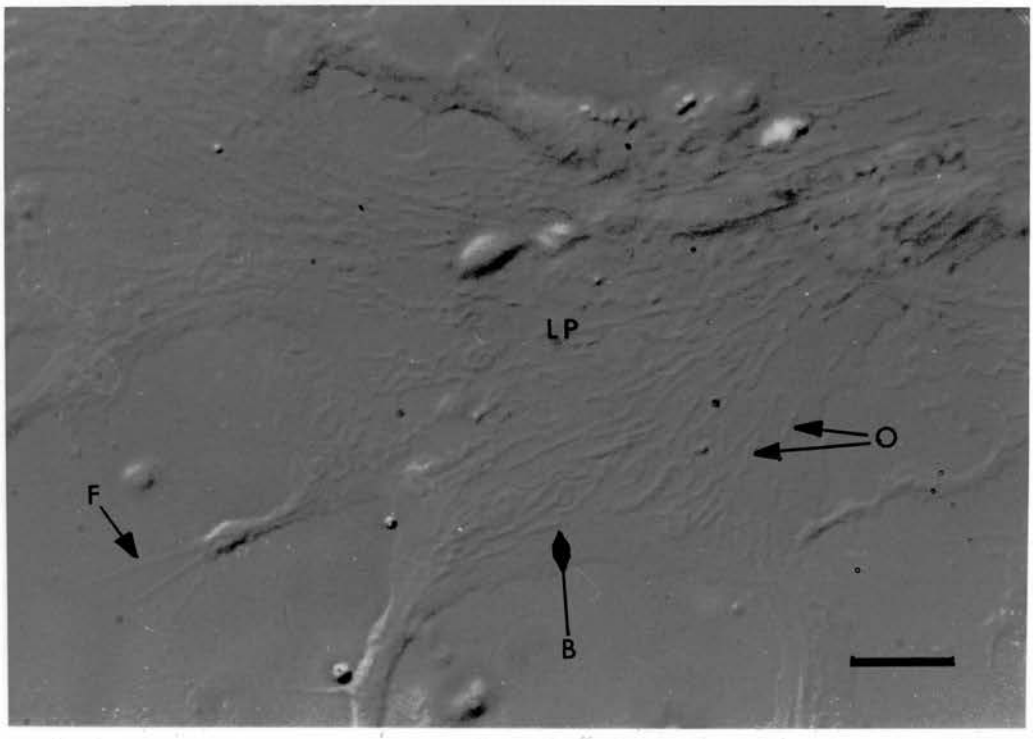
LP=Lamellipodium

O=Organelle

a



b



Figures 12a and b

C3 growth cones navigating past obstructing cells, this occured in areas 1 and 2. The drawing of the growth cone 1 in figure (a) was a view in the microscope at the time the photograph was taken and shows more clearly the filopodia touching the cell. The edge away from the cell had no filopodia. Phase contrast optics.

18, scale bar=50um

19, scale bar=100um

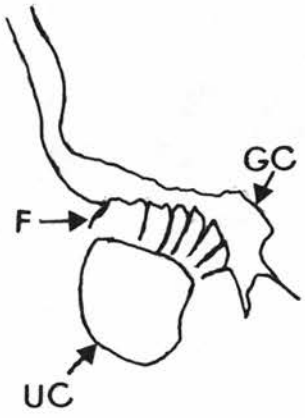
C1=Identified neurone (serotonin containing)

C3=Identified neurone

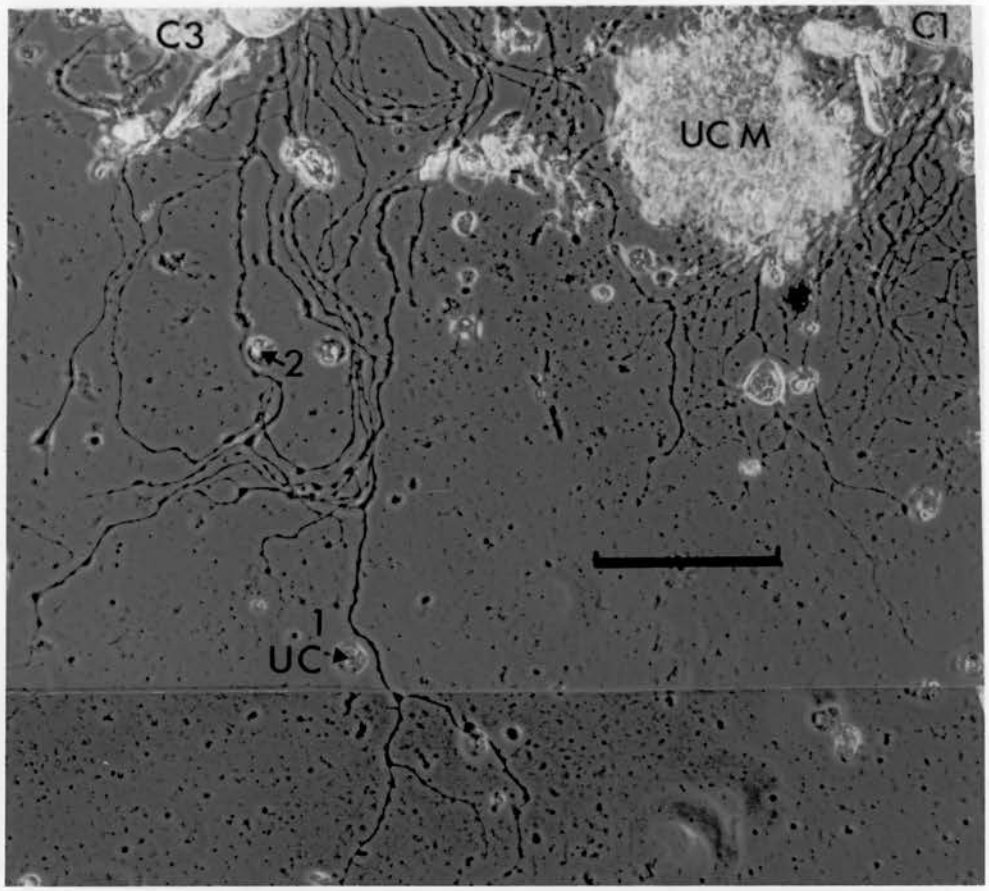
GC=Growth cone

UC=Unidentified cell

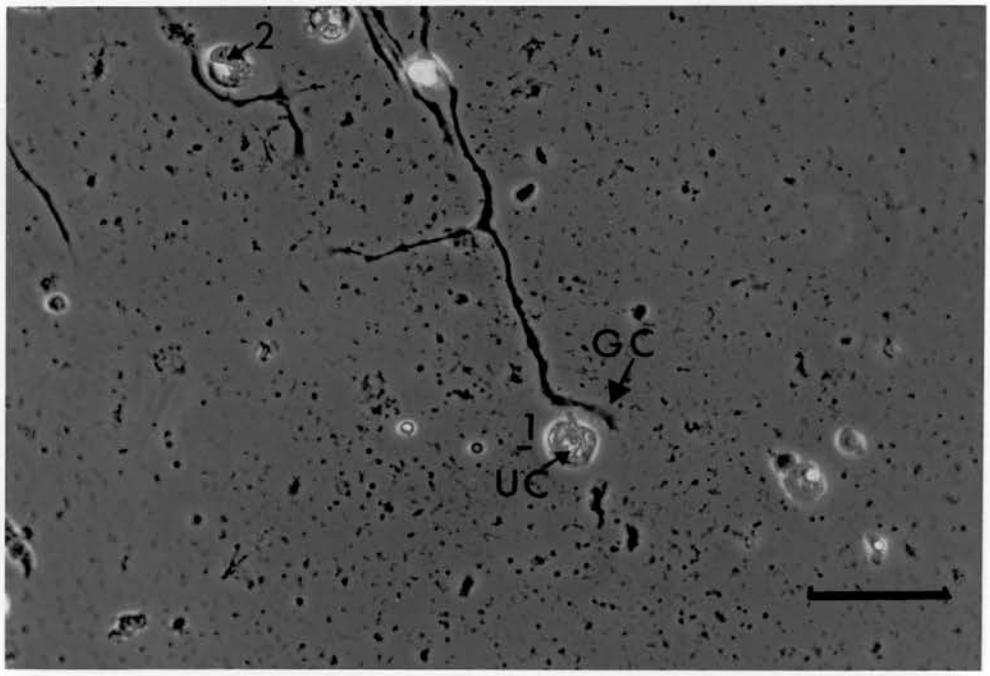
UCM=Unidentified cell mass


GC
F
UC

b



a



3.7 C3 Neurone in Tentacle Muscle Extract

The dissociation of the tentacle muscle produced a solution containing muscle fragments, small cells and other small particles. This solution was called the tentacle muscle extract. This extract was placed in the culture dish first and the muscle fragments etc adhered to the substrate. The C3 neurone was added to the dish. The behaviour of processes of the redeveloping C3 neurones when exposed to tentacle muscle extract leads to the observation of motile activity at a finer level than that of filopodial exploration. A C3 neurone exposed to material of the digested tentacle muscle developed its normal system of growth cones and neurites (figure 13). However, after 3 days, the areas which had been covered by the tentacle muscle extract were cleared of material. The cleared area was larger than that normally associated with filopodial searching (which is 2-3 times the length of the growth cone). The growth cones, the neuritic processes and the central point of redevelopment from the axon had become obscured by the extract material being drawn back onto their surfaces.

Examination under Nomarski optics at higher power revealed longer processes extending from the ends of growth cones. However, the single filopodium did not seem capable of pulling in such extensive areas of extract. A more detailed study showed a very fine network of "sub-filopodia" extending to the particulate material obtained from the tentacle (figure 15). In figure 16, a C3 neurone, without an axon, is shown at a later stage of development in co-culture with tentacular material. Closer to the cell body the same pattern of gathering had occurred but it was the very long processes (up to 1500um) that were important in this instance.

Microscopical examination of C3 neurones developed without tentacular particulate extract but with other cellular material around them showed no such pattern of "sub-filopodia" (c.f. figure 12). This suggested that in these conditions factors were released from the material of the normal target cell of the C3 neurone. These factors altered the morphology of the neurone, heightening the seeking behaviour of the filopodia and the development of longer neurites.

Shown below in the section on cells associated with the C3 neurone are small cells which were found in the tentacle muscle extract but it should be noted here the apparent connection between one of these small cells and the neurite of the C3 (insert, figure 16).

3.8 **Dendritic Redevelopment**

The axon of the C3 neurone has a section of its length which contains dendritic connections (figure 17). This area, as in figure 22, showed intense redevelopment along its length. Axons would normally only sprout neurites from their cut end or, if damaged, from the point where the damage had occured (figure 32, area A3 on the axon).

Figure 13

C3 neurone with the axon attached after three days in culture. When first added to the culture dish the C3 consisted of the cell body and axon alone, shown by the overlay, all other developments were new. Note the clear areas around the axon and processes, and that the endings of the processes are obscured by material from the tentacular muscle extract. The overlay shows the original cell body and axon. Phase contrast optics.

Scale bar=100um

A=Axon

C3=Identified neurone

CA=Cleared area

D=shows the neurite and area seen in more detail in figure 15

GC=Growth cone

N=Neurites

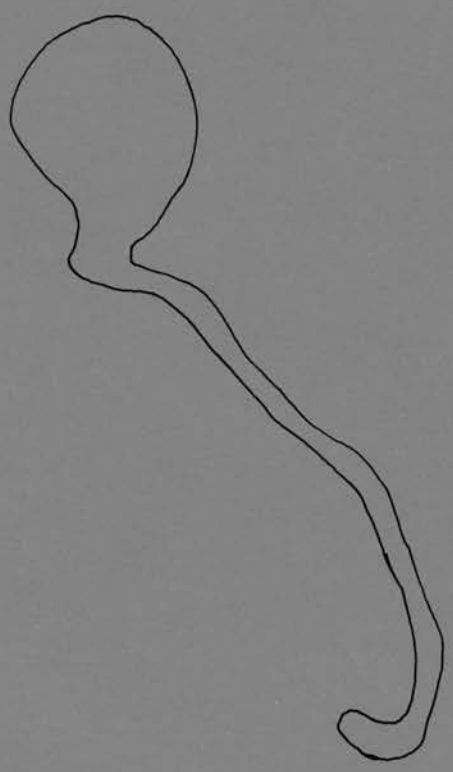
TME=Tentacular muscle extract

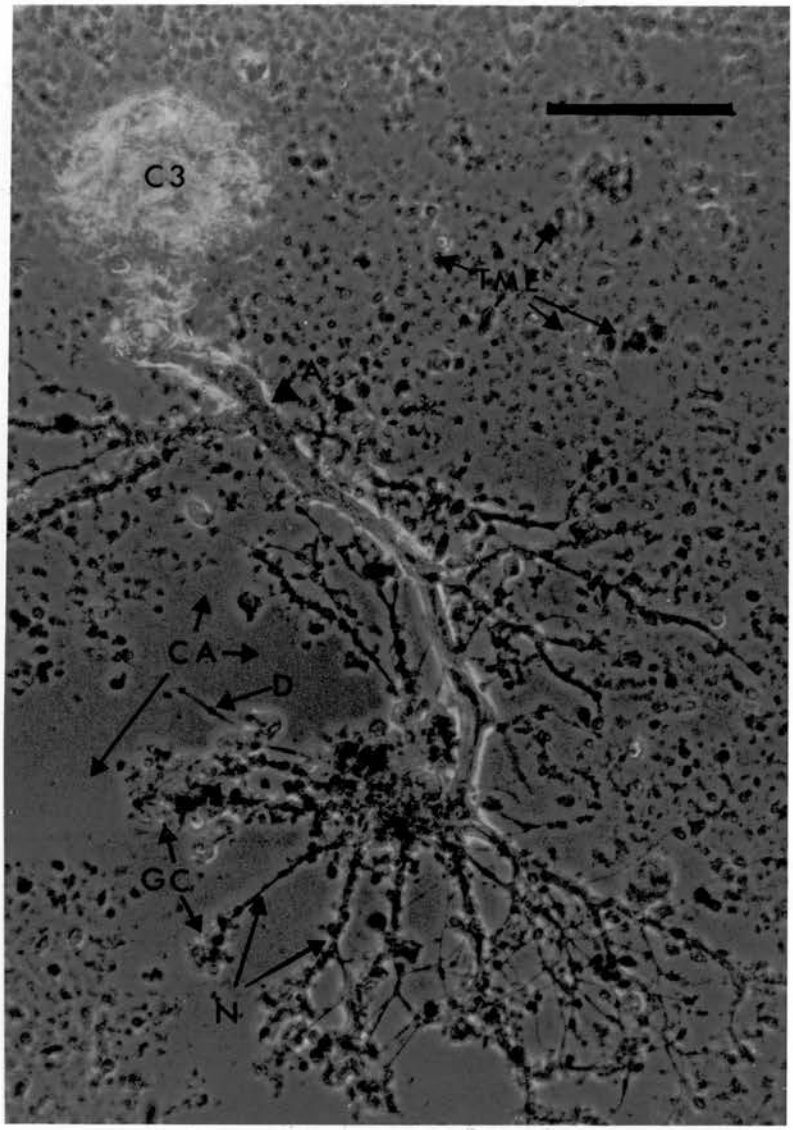
Figure 14

C3 neurone as in figure 13 but using Nomarski optics.

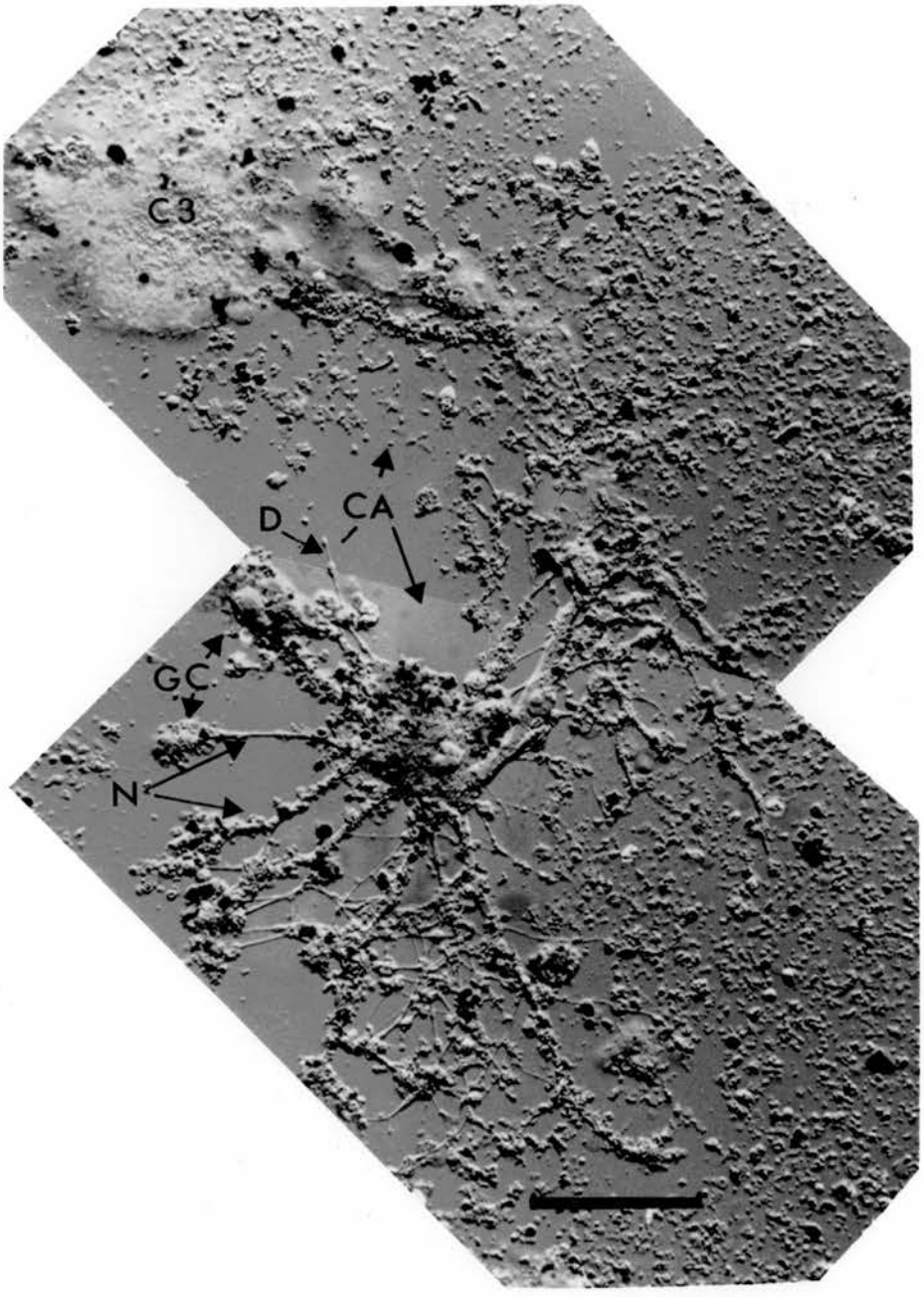
Abbreviations are as in figure 13.

Scale bar=100um





13


C3
D
CA
GC
N

no

Figure 15

A detail from figure 13 showing the fine network of processes from t growth cones, stretching long distances into the surrounding tentacul extract. The lines drawn on the overlay indicate the position a direction of some of the "sub-filopodia". Nomarski optics.

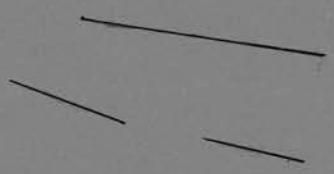
Scale bar=50um

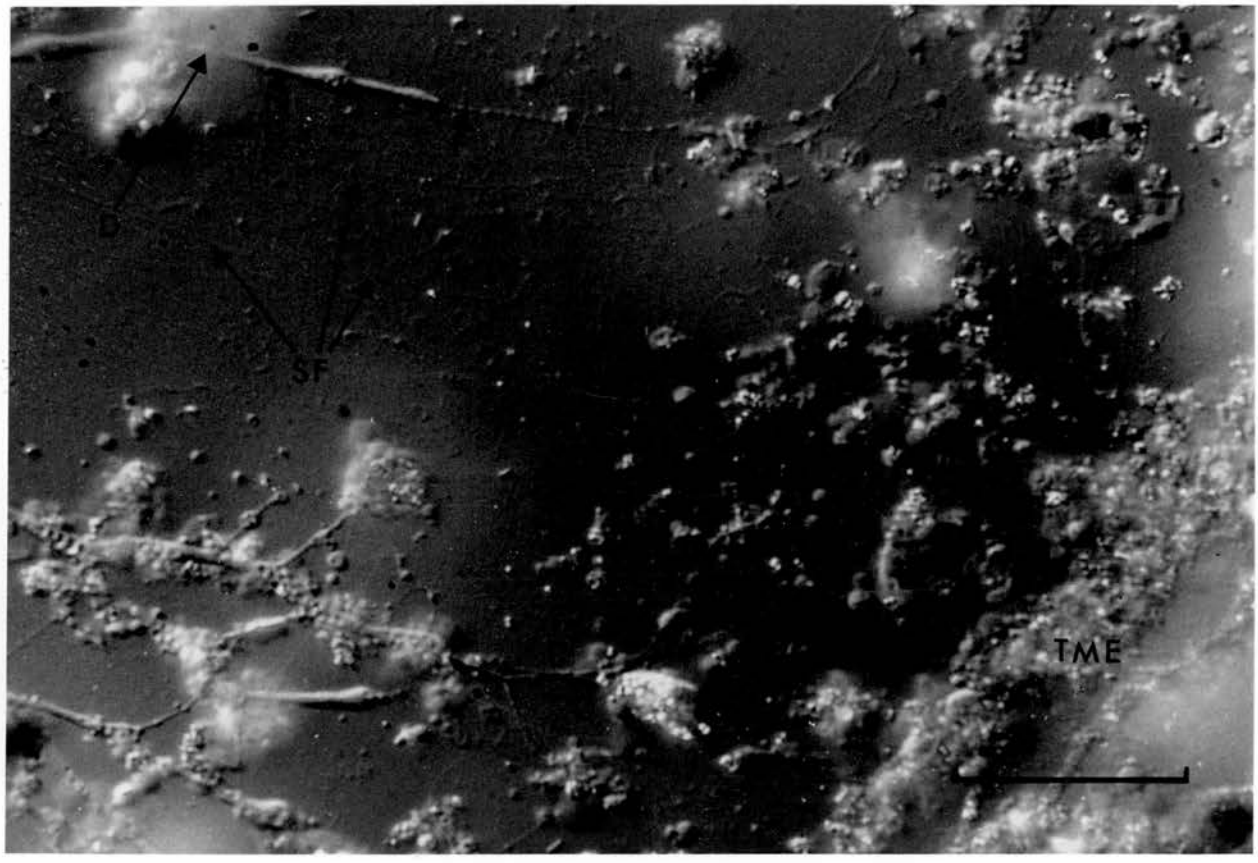
F=Filopodia

N=Neurite

SF=Sub-filopodia

TME=Tentacular muscle cell extract





15

Figure 16

C3 neurone in co-culture with tentacle muscle extract showing the extended neuritic growth produced after five days in culture. The insert shows neurites which appeared to have made connections with a small tentacular "neurone". Nomarski optics.

C3 and neurites, scale bar=100um

Insert, scale bar=20um

C3=Identified neurone

N=Neurite

TMNC=Tentacle muscle "neuronal" cell

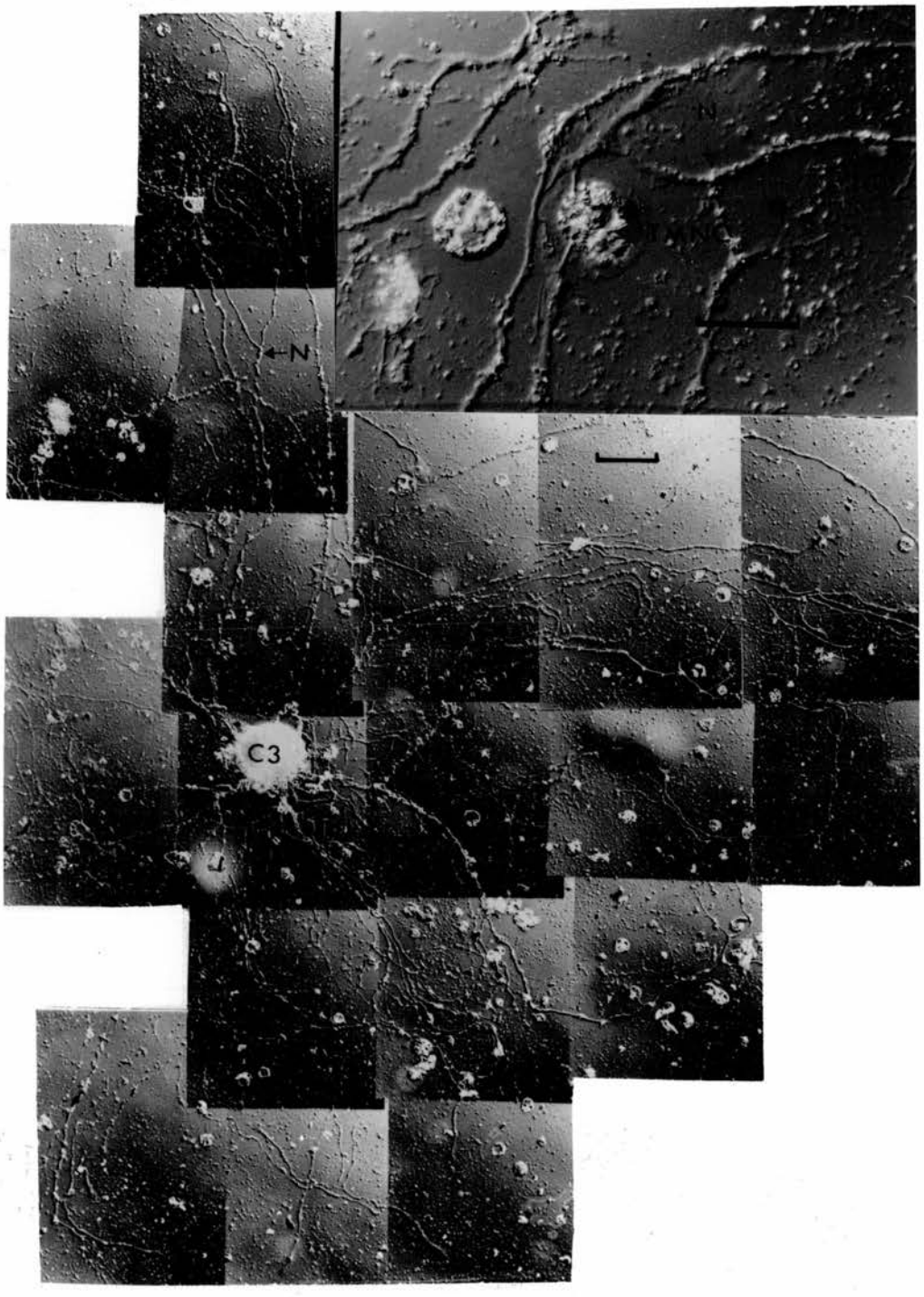

N
N
C3

Figure 17

C3 neurone with axon attached, 18 hr in culture. This shows the area of dendritic processes which were attached to the axon when the neurone was dissected. Figure 22 shows a neurone which also had dendritic processes attached and developed intense neuritic growth from this area. Phase contrast optics.

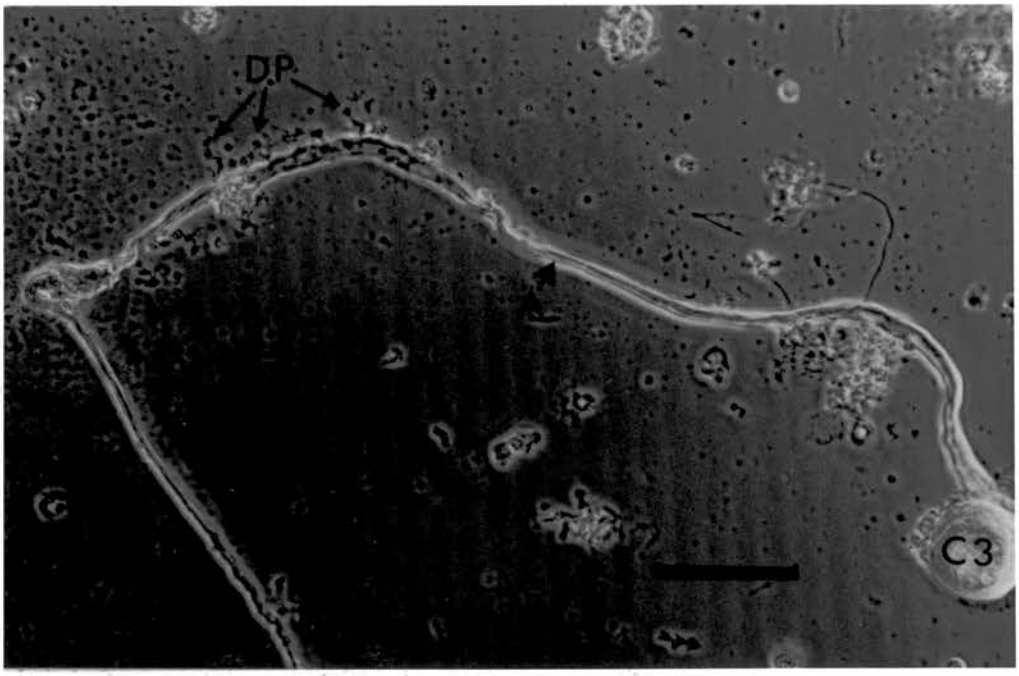
Scale bars=90um

C3 Neurone

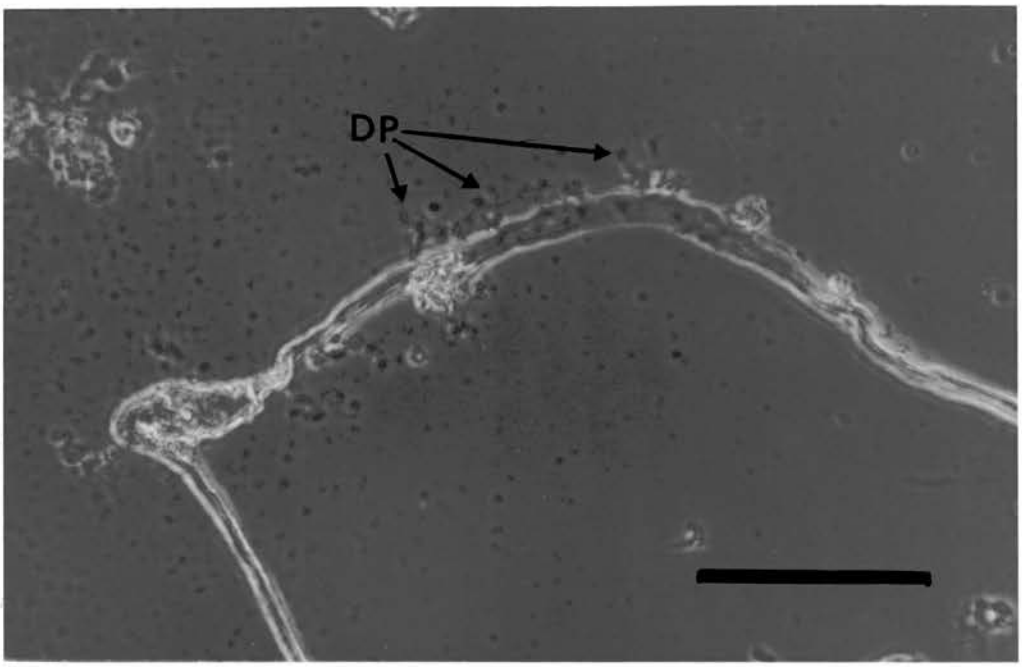
A=Axon

DP=Dendritic processes

a



b



3.9 **FMRFamide-like Immunoreactivity in the C3 Neurone**

The C3 neurone *in vivo* had shown FMRFamide-like immunoreactivity. The continuing expression of a FMRFamide-like substance in the neurone throughout isolation and subsequent redevelopment was examined. Neurones at three stages of development were used.

(1) A neurone 1 day old in culture, with no descernible neuritic growth (figure 18). When the fluorescence had faded focusing through the neurone showed a non-fluorescing nucleus and the cytoplasm filled with discreet granules (figure 19).

(2) A neurone 4 days old with well developed neuritic growth from the cell body (figure 20). This demonstrated fluorescence in the neurone and throughout the neuritic network. (figure 21a and b). Where intact muscle cells are shown (figure 20) in contact with neurites no obvious connection could be seen at 1000x magnification. The neurites travelled past the muscle cells and showed no break in continuity.

(3) A neurone 4 days old with an intact axon and neuritic growth from the axon and cell body (figure 22). The development from this neurone first showed the axon end flattened from which a large lamellipodium extended. The dendritic area developed simultaneously and in the last 24hr the soma developed a limited neuritic tree. Fluorescence could be seen in the cell body, lamellipodium, neurites and growth cones (figures 23a and b).

Figure 18

C3 neurone (24hr in culture).

(a)The C3 neurone with phase optics. The other material in the background are cells and muscle fragments from co-cultured dissociated tentacle muscle.

(b) The above C3 neurone showing immunohistochemical staining for a FMRFamide-like substance. The clump of cells attached to the C3 were removed with it at the time of dissection. These cells were dull orange in colour and the C3 neurone was bright yellow. Fluorescence optics.

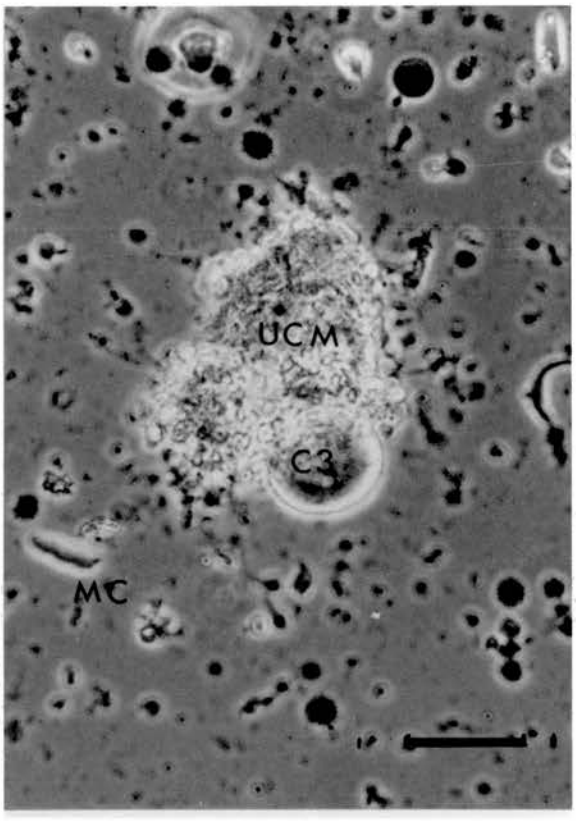
Scale bars=110um

C3 neurone

MC=Muscle cell

UCM=unidentified Cell Mass

a



b

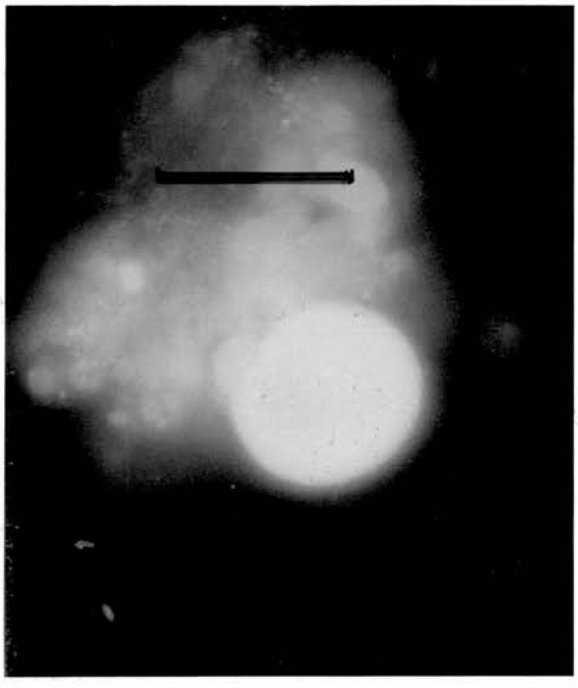


Figure 19

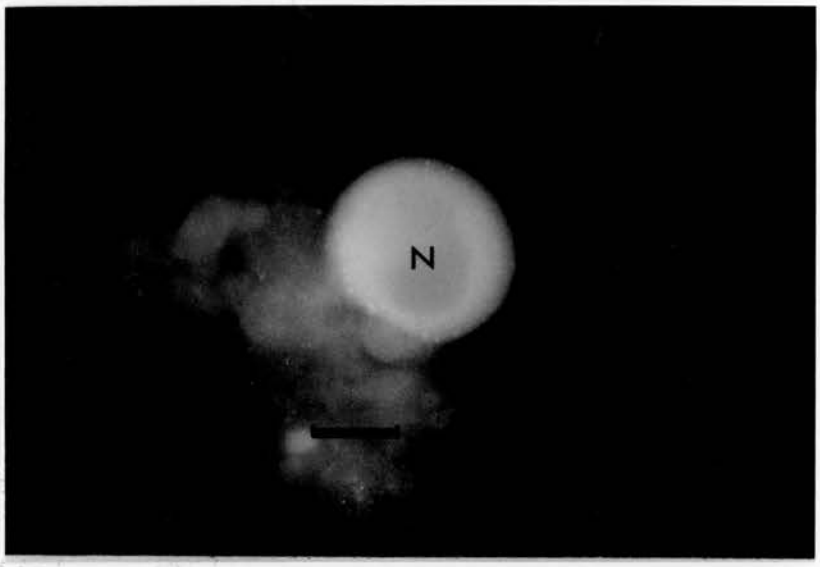
(a) C3 neurone showing discreet comparments of immunofluorescence, possibly vesicles or granules containing FMRFamide-like substances.(b)Focusing through the neurone shows first an overlying layer of the compartments and then the nucleus, devoid of fluorescence.

Scale bars=50um

G=Granules

N=Nucleus

a



b

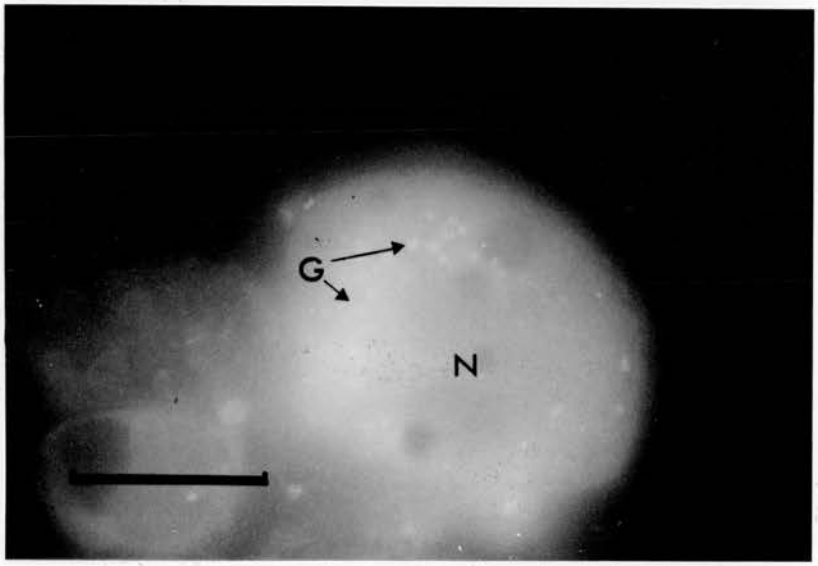


Figure 20

C3 neurone after 4 days in co-culture with tentacle muscle extract, which contains individual muscle cells, muscle fragments and small neurones associated with the tentacle. Neurites travelled under the muscle cells (TM). Phase contrast optics.

Scale bar=100um

C3 neurone

NT=Neuritic tree

TM=Tentacle muscle cells

TMNC=Tentacular muscle "neuronal" cells

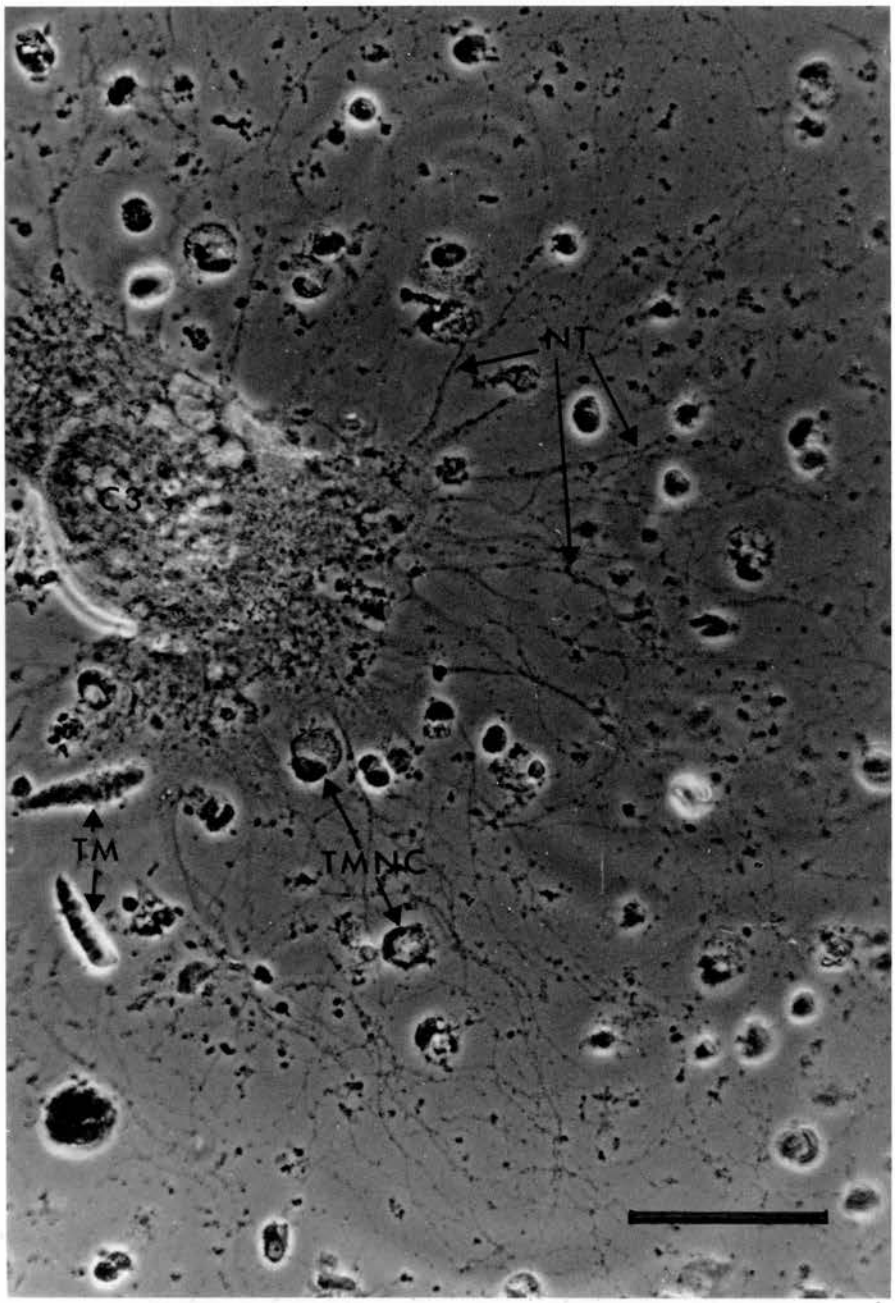

NT
C3
TM
TMNC

Figure 21a and b

The processes from the C3 neurone in figure 20 showing fluorescent immunoreactivity for FMRFamide-like substance.

Scale bar=10um

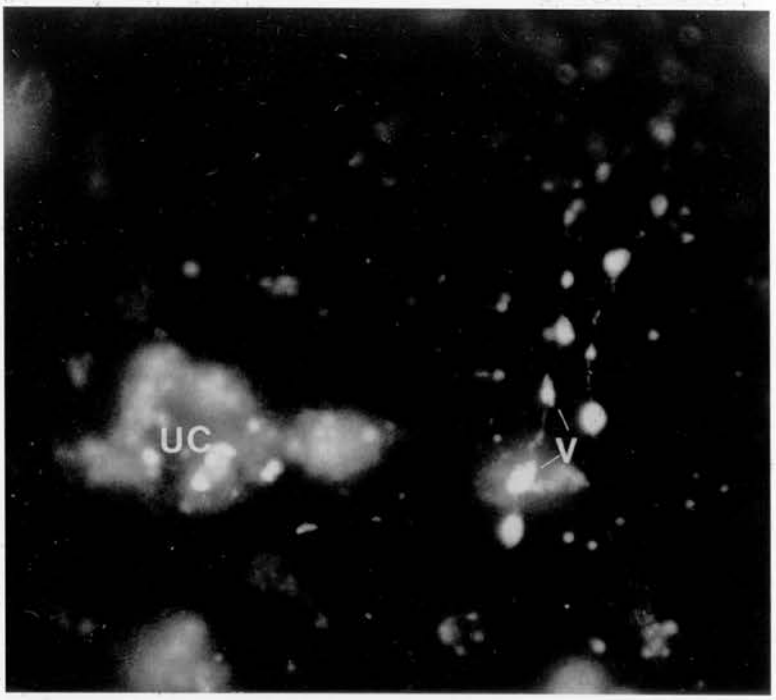
N=Neurites

T=Terminals

UC=Unidentified cells

V=Varicosities

a



b

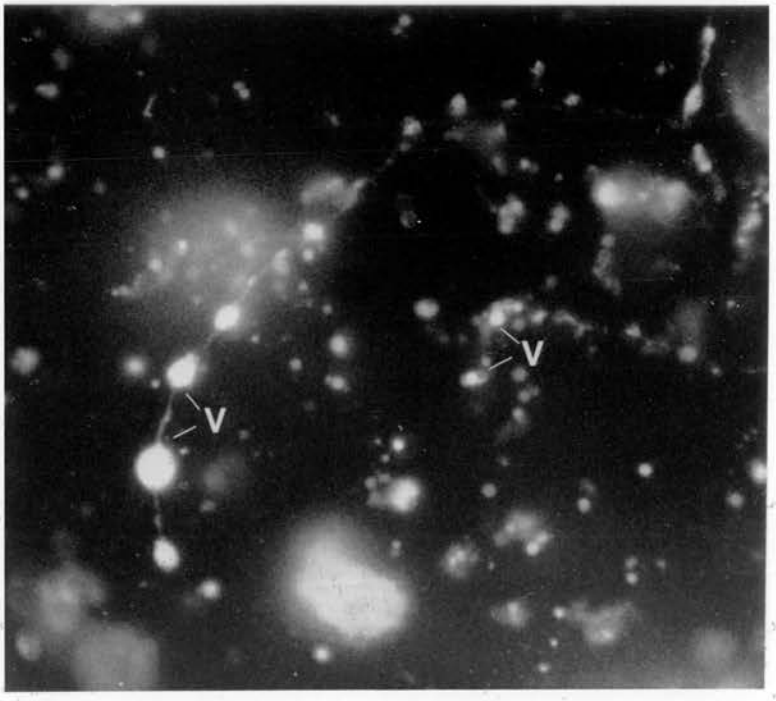


Figure 22

C3 neurone (4 days in culture) with the axon attached, before fixation for immunohistochemistry. This shows the area of development and the overlay shows the original cell body and axon. The length of axon between the lines showed dendritic remains when the neurone was dissected out. This area (DA) showed intense neuritic development . Phase contrast optics.

Scale bar=100um

A=Axon

C3 neurone

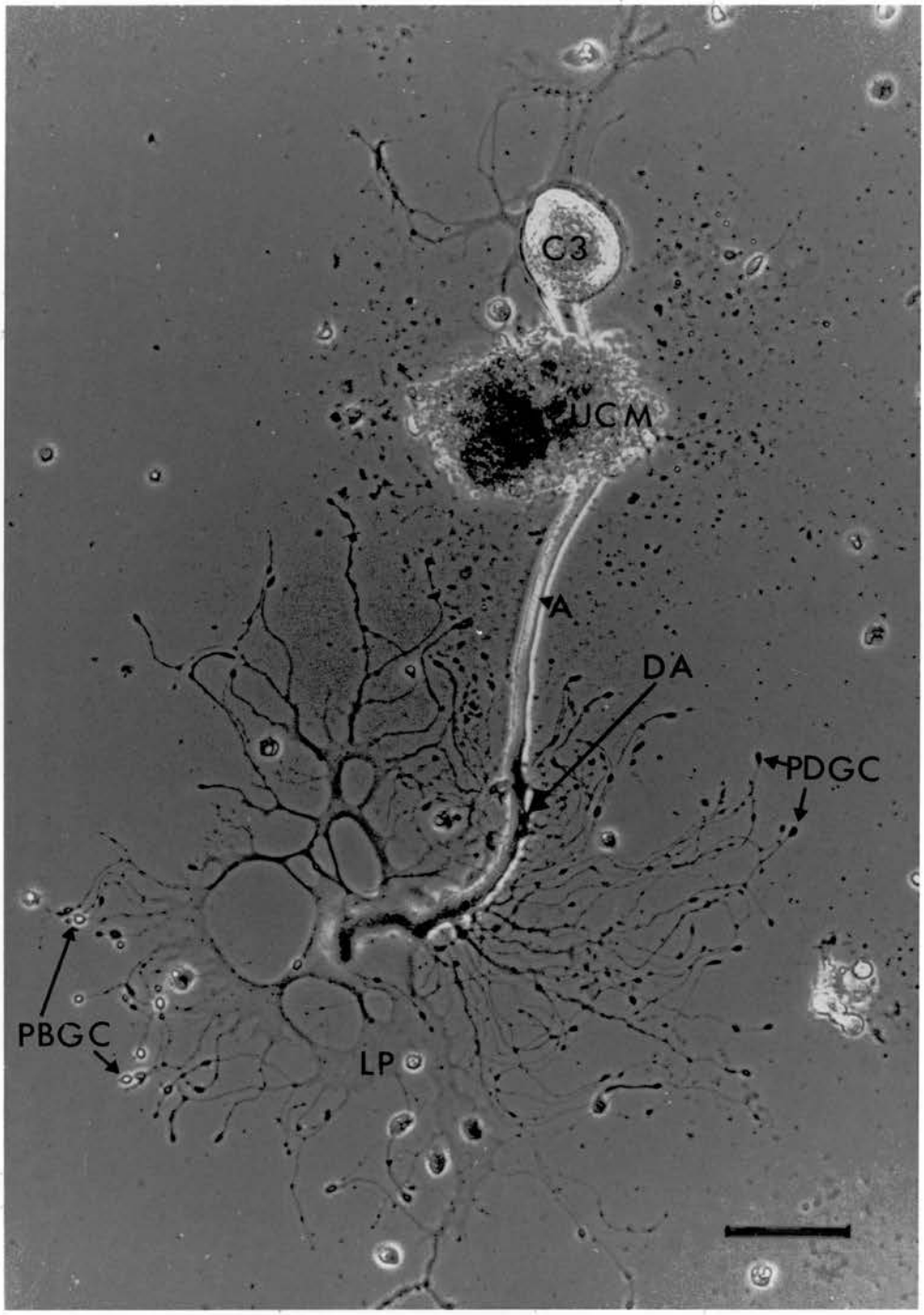
DA=Dendritic area

LP=Lamellipodium

PBGC=Phase bright growth cones

PDGC=Phase dark growth cones

UCM=Unidentified cell mass


C3
UCM
A
DA
PDGC
PBGC
LP

Figures 23a and b

(a and b)

Individual growth cones and varicosities from the C3 neurone in figure 23. (a) Phase contrast, (b) Showing staining for FMRFamide-like substances with fluorescence optics.

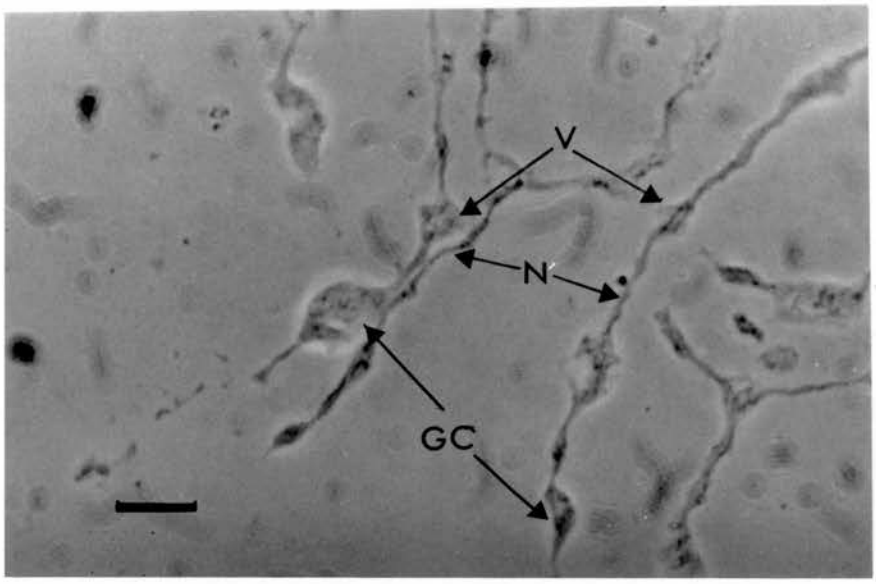
Scale bar=10um

GC=Growth cones

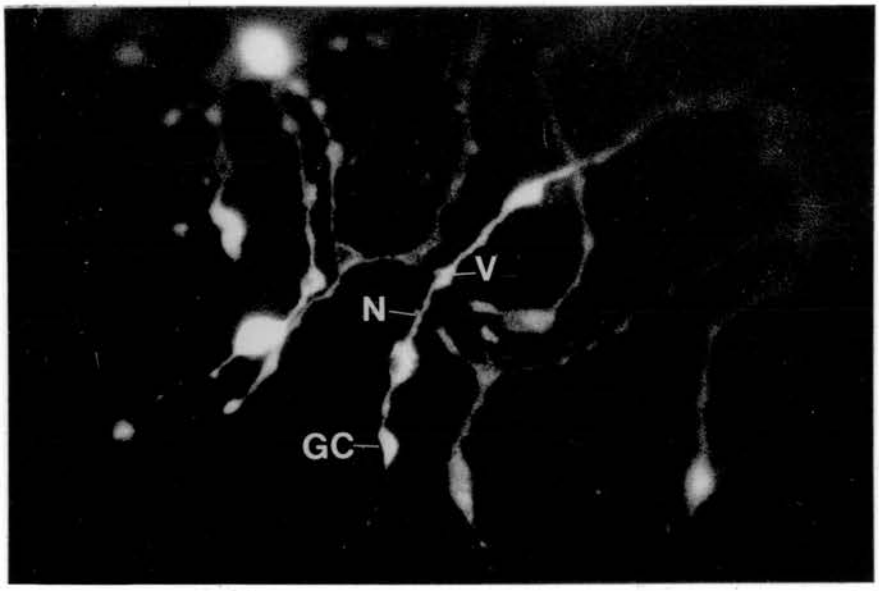
N=Neurites

V=Varicosities

a



b



3.10 **The C3 Neurone in Co-culture: Tentacle Muscle Cell and Tentacle** Neuronal Cell

Dissociation of the tentacle muscle using the method of Ishii and Takahashi (1982) yielded large numbers of muscle cells but when acetylcholine (10^{-7}-10^{-5}M) was applied topically by pipette no response could be illicited. The muscle cells (figure 24) could be made to contract by mechanical stimulation (touching with pipette end) or by osmotic shock (distilled water). Muscle cells were then dissociated using the method of Ishii, Takakuwa and Takahashi (1986). This was designed to remove the tryptic activity which had been shown by these authors to remove the acetylcholine receptors from the muscle surface. The yield of cells was much reduced and acetylcholine at the above concentrations had no effect. The acetylcholine was applied to muscle cells that had been isolated in culture for up to 36 hr.

C3 neurones were plated in association with isolated muscle cells (figure 20). However during the digestion of the tentacle muscle material other than intact muscle cells was found. Among the broken segments of the muscle cells were small round "fragments" which were at first considered to be rounded-up muscles endings. Many of these cells developed neuritic growth, with long, thin processes characteristic of neuronal cells (figures 25 and 24). In figure 16 these cells can be seen to make contact with processes from the C3 neurone. These cells, as far as I am aware, have not been shown before.

Figure 24

Extended tentacle muscle. Note the presence of "neuronal" cells extending neurites. Nomarski optics.

Scale bar=20um

Figure 25

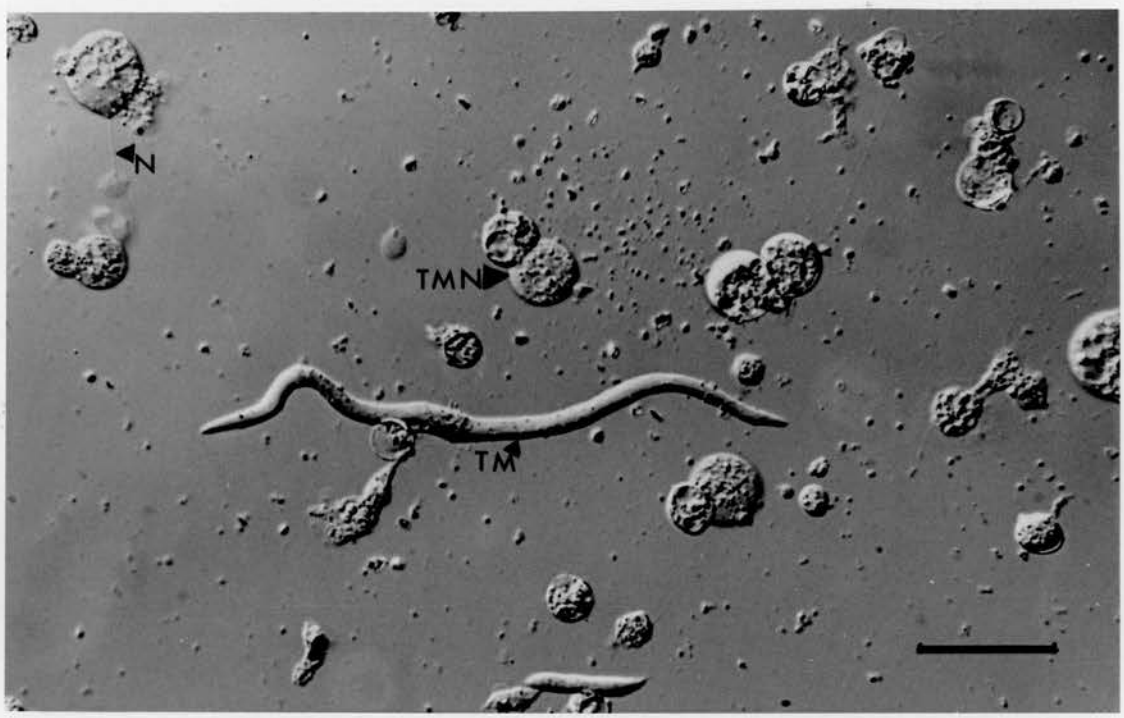
An extract of tentacle muscle cells(after 48hr) without the C3 neurone or other "neuronal" cells. A small tentacle "neuronal" cell producing neuritic growth. This cell is found in the dissociated tentacle muscle cultures. Nomarski optics.

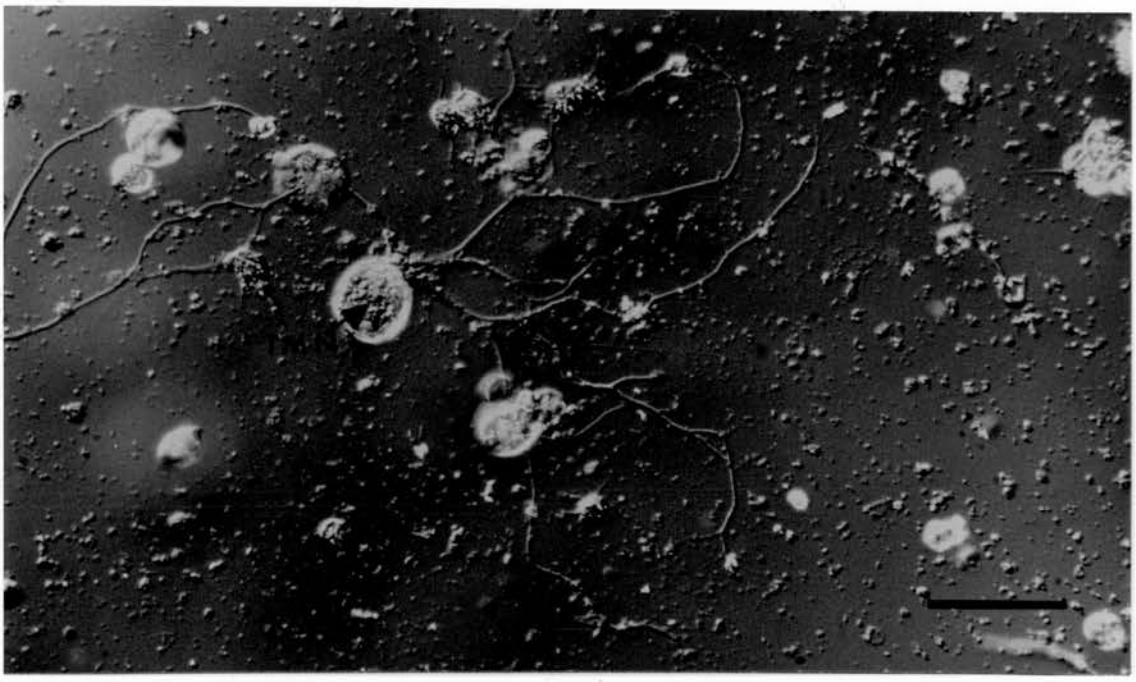
Scale bar=20um

N=Neurites

TM=Tentacle muscle cell

TMN=Tentacle muscle "neuronal" cell


N
TMN
TM



25

3.11 **The C1 Neurone: Morphology**

The C1 neurone exhibited some of the clearing actions shown by the C3 neurone. Between the neurites in figure 27a were areas free of debris but a line could be drawn where this ends and the intense neuritic crossings gave way to individual, exploratory processes. No large area of particle gathering could be seen in advance or around the growth cones from these individual neurites (c.f. the C3 neurone, figure 13).

3.11.1 **Growth Cones**

The growth cones were narrow with few short, filopodia. Although, interesting variations are shown in figure 28a, where a flat cone had developed one of its filopodia to form a neurite . Figure 28b had serial growth cones i.e. a growth cone had formed with filopodia, and from this cone a large neurite extended with a large cone. The filopodia in this figure showed beaded endings.

Figure 26

C1 neurone (6 days in culture) showing dense neuritic tree. Attached to the C1 neurone are many small cells. It is not known if these are neuronal or glial cells. Nomarski optics.

Scale bar=100um

C1 neurone

GC=Growth cones

UC=Unidentified cells

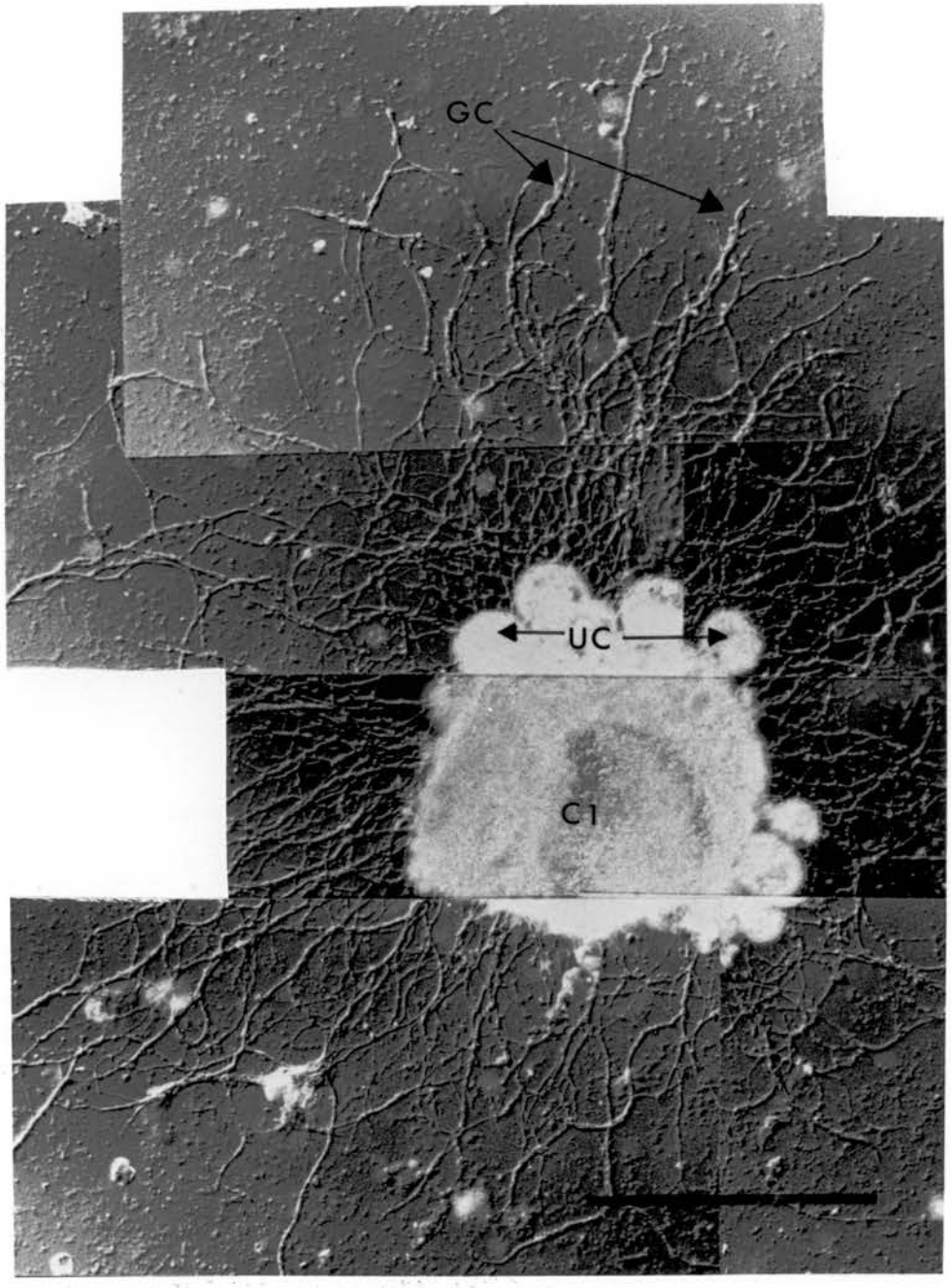

GC
UC
C1

Figure 27

Showing examples of growth cones from the C1 neurone in figure 26. (a) shows growth cones from the top right corner of figure 26. (b) Many of the cones on the neurone shown were narrow and with few filopodia. The curved line on the overlay showed the limit of the area cleared of background debris by the growth cones. (This contrast with the large areas cleared by the fine processes produced by the C3 neurone in tentacle muscle extract, figure 13). Nomarski optics.

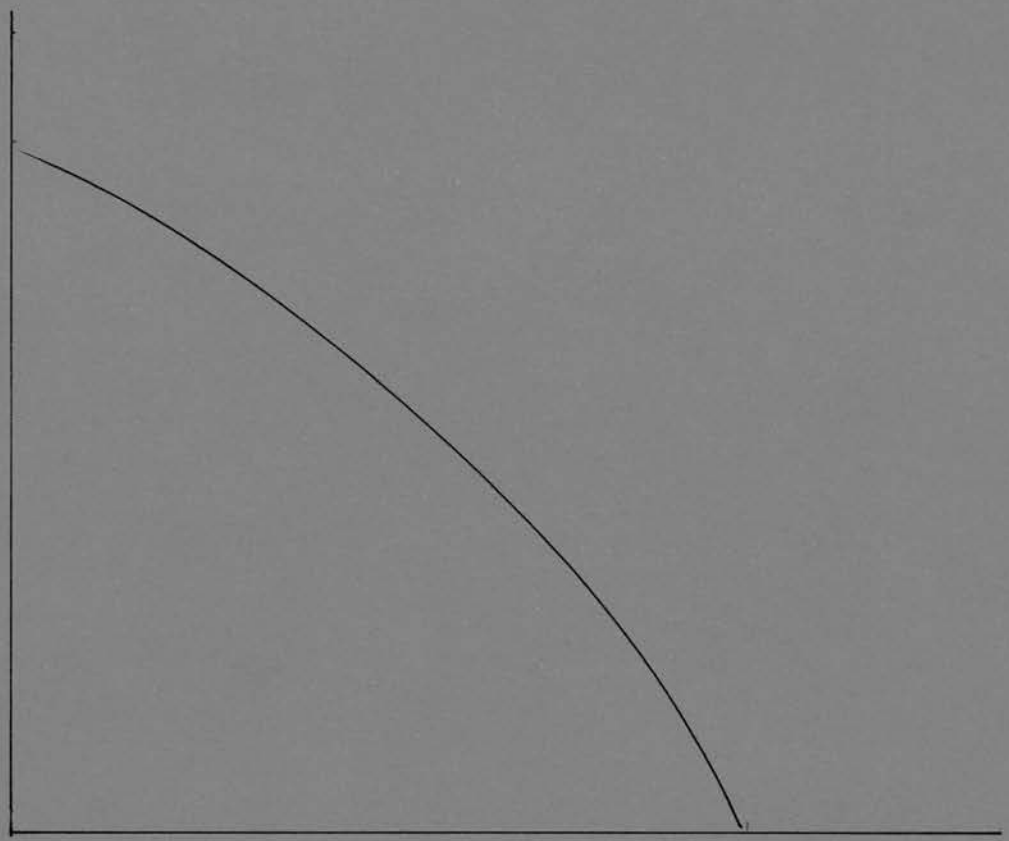
Scale bars=10um

BD=Background debris

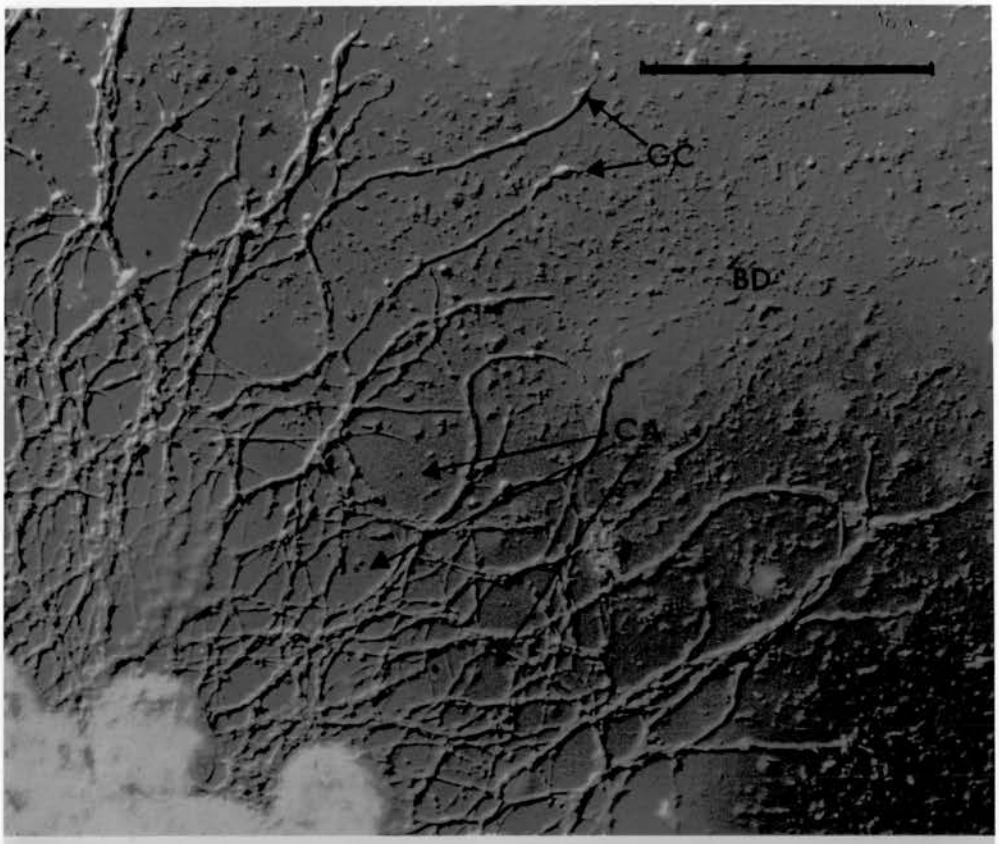
CA=Cleared area

GC=Growth cone

N=Neurite



a



b

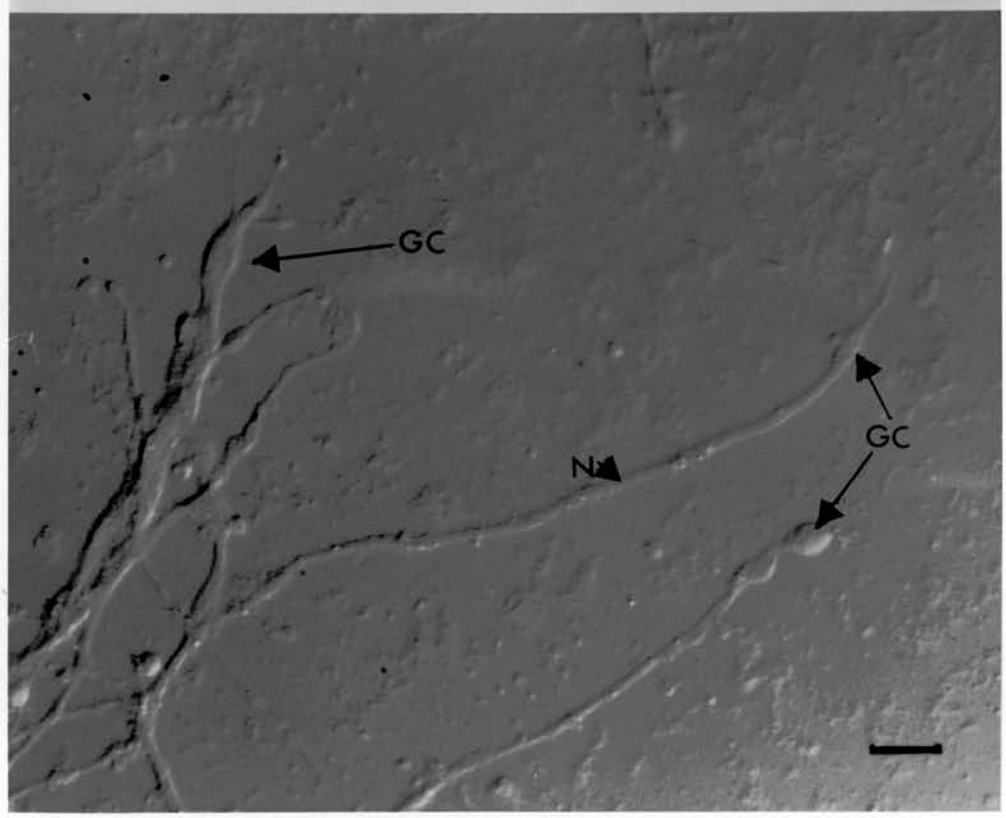


Figure 28

Growth cones from the C1 neurone. (a) is a growth cone where a filopodium has extended to form a new neurite.

(b) Multiple growth cones have formed on a single neurite. The filopodia had beaded endings.

Nomarski optics

Scale bar=10um

F=Filopodia

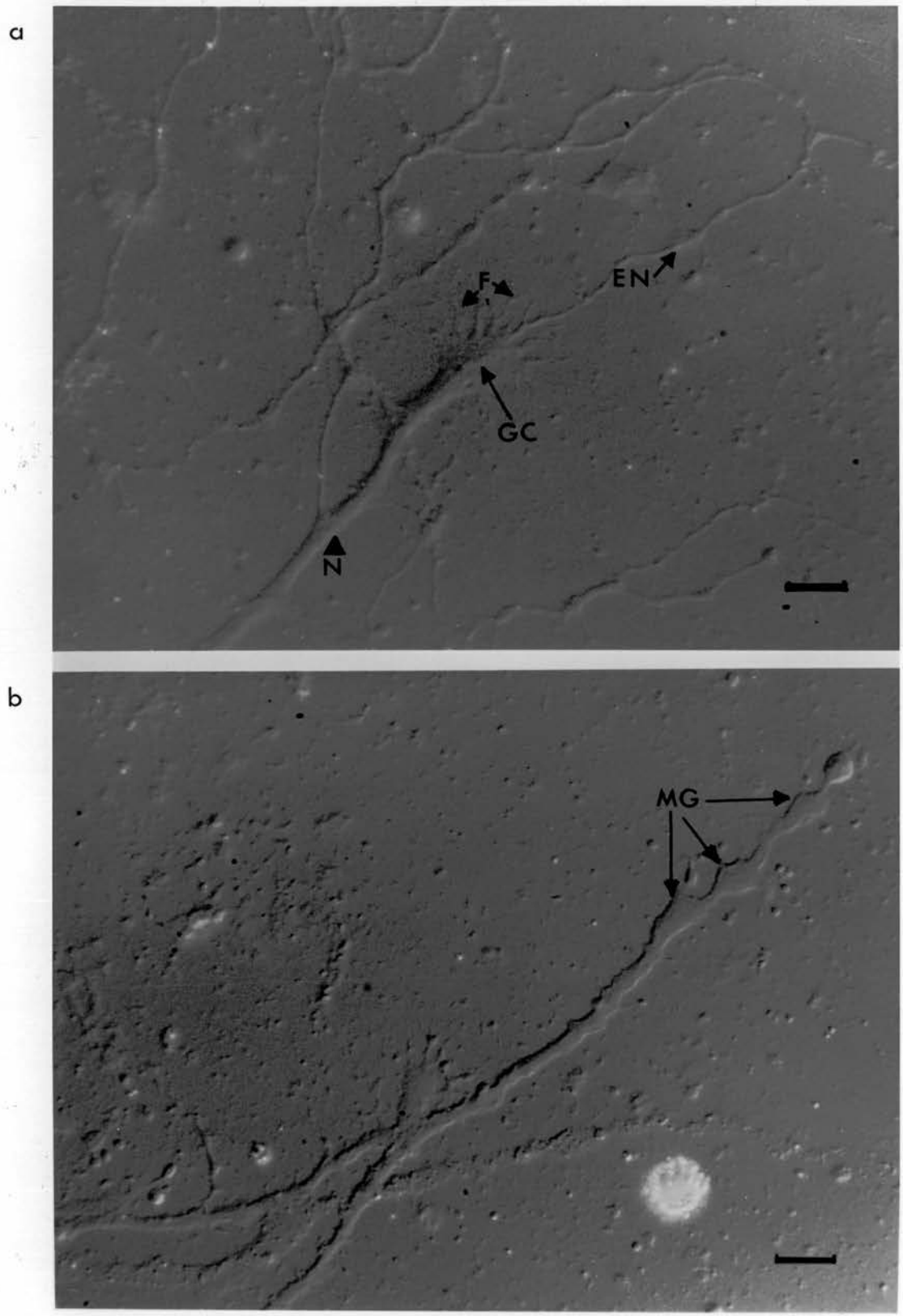
GC=Growth cone

N=Neurite

EN=Extended neurite

MG=Multiple growth cones

UC=Unidentified cells


a
F
EN
GC
N
b
MG

3.11.2 **Identification of Growth Cones and Processes from the C1** Neurone with the Dye, Lucifer Yellow

During the dissection of identified neurones it was often impossible to remove all the small cells attached to the remains of an axon or clinging to the soma without risking damage to the neurone. In culture these small cells might remain around the cell body (figure 30) or spread out around it. Microscopic examination gave a high degree of certainty of neuritic origins but the development of neurites and cones from these cells did raise some doubts. The C1 neurone (figure 30) showed this and was injected with the fluorescent dye, Lucifer Yellow (figure 29). The enlargments (figures 29a and b) show short processes growing out from along the length of the neurite. Processes of this kind could sometimes be seen close to the growth cones of many cells. However these processes were not seen spread so extensively along the neurites under normal light microscopy.

3.12 **Viability of Neurones and Neuritic Development**

The tables (1 and 2) of percentage attachments can be illustrated by figure 31 i.e. not all neurones that adhered to the substrate sprouted neurites. This shows C1 neurones in identical culture conditions however only one had formed a neuritic outgrowth. The other neurone, although it appeared the same under phase optics i.e. it was phase bright and was not opaque or granular and remained attached after several changes of medium, did not develop neurites.

Figure 29

(a)C1 neurone injected with Lucifer Yellow to show the spread of neuritic growth and the position of the growth cones.

Scale bar=200um

(b) and (c) are enlargments of the growth cones in bottom right hand corner of (a), note the distribution of short hair-like projections along the neurites. Fluoresence optics.

The brightness of the fluorescence causes distortion in the size of the processes making them look larger.

Scale bar=100um

The arrows show the position of growth cones for orientation with figure 30.

Figure 30

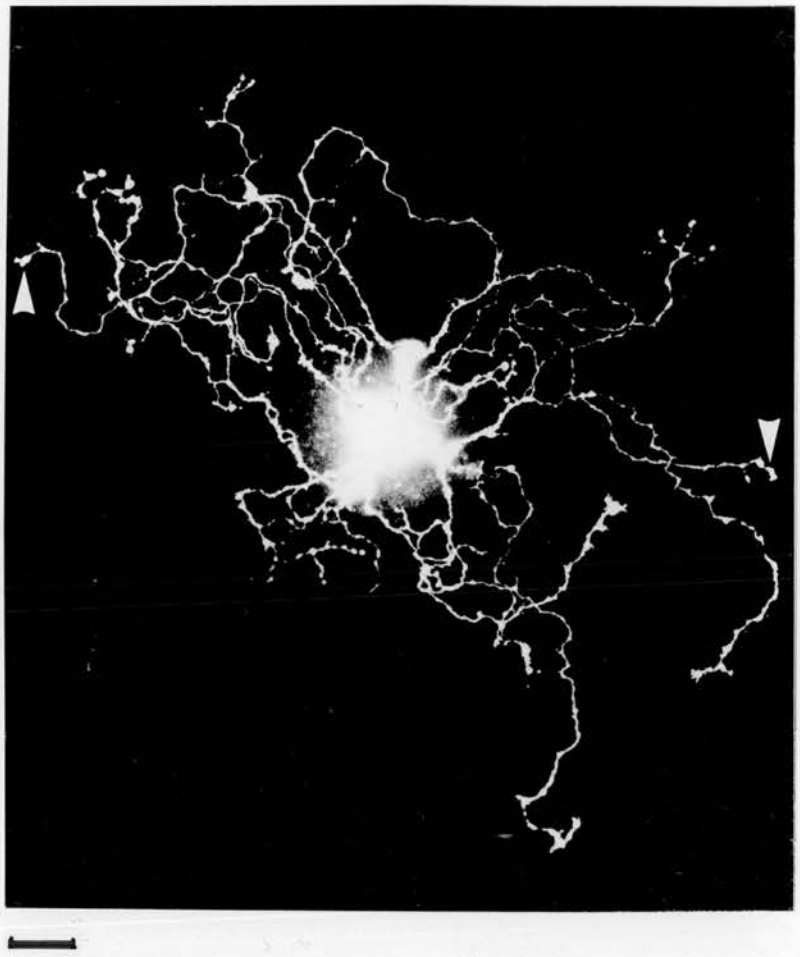
C1 neurone (6 days in culture) with extensive neuritic tree with groups of small cells from the cerebral ganglia. This figure is the mirror image of figure 29. The arrows on the left and right of the photograph show the position of the growth cones, also shown in figure 29a. Phase contrast optics.

N=Neurites

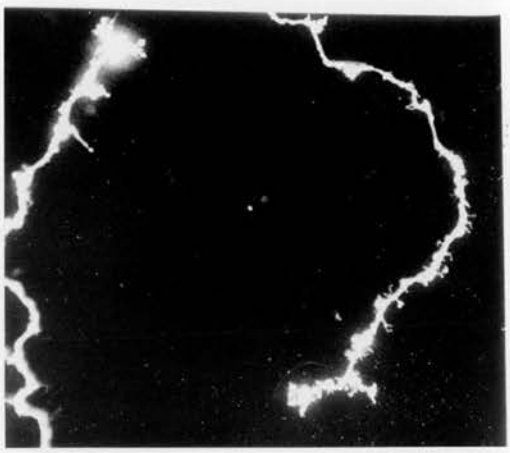
UC=Unidentified cells

Scale bars=200um

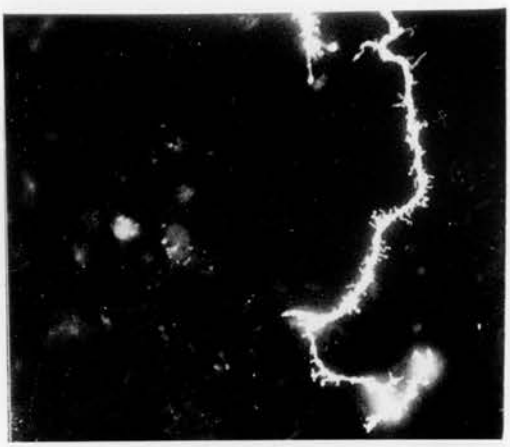
a



b



c



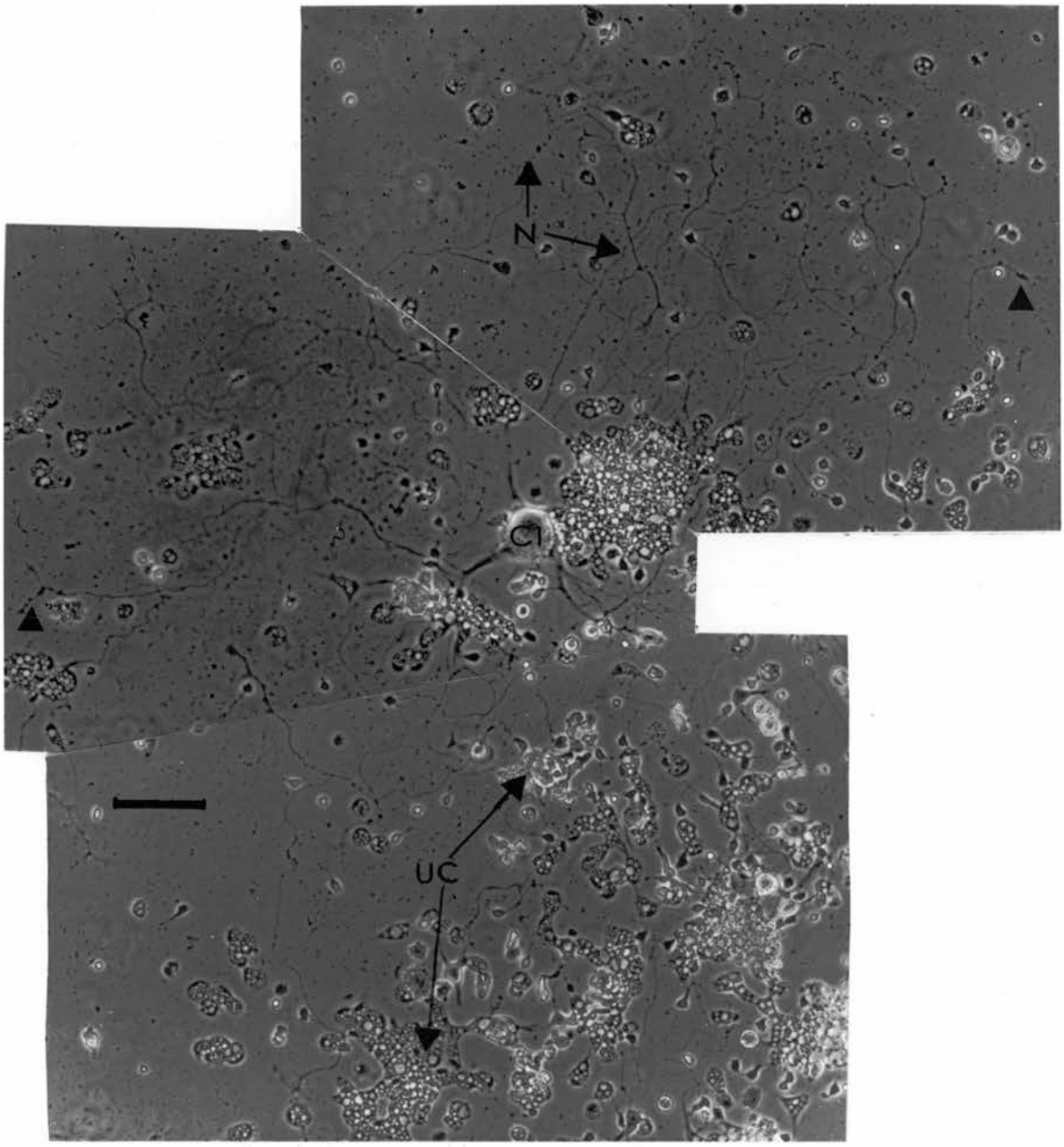
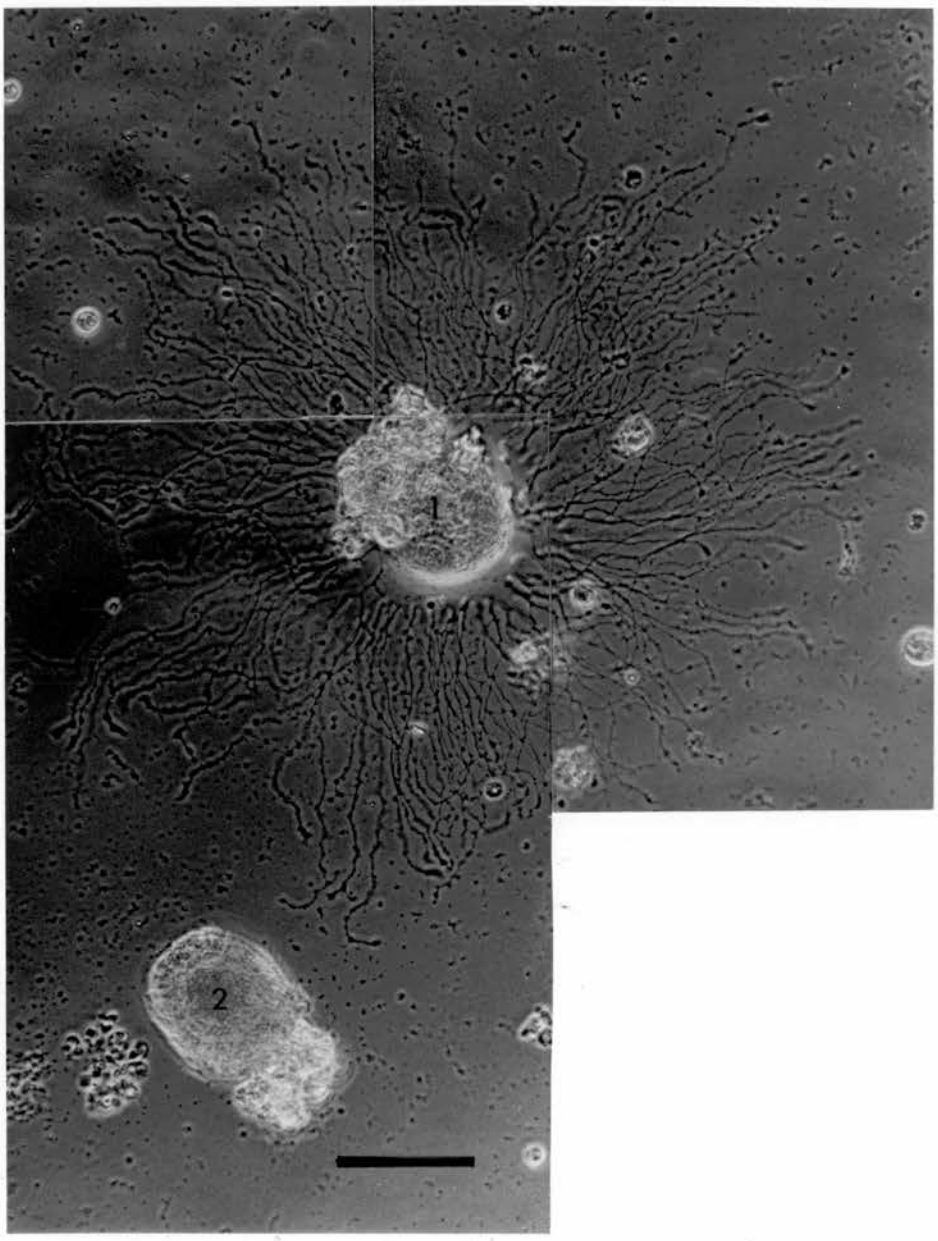

N
C1
UC

Figure 31

Two C1 neurones in the same culture conditions but while neurone (1) had extensive neuritic growth and many active growth cones neurone (2) produced no neurites. 5 days in culture. Phase contrast optics.

Scale bar=100um


1
2

3.13 **C1 Neurone with Both Axons Attached**

A C1 neurone was removed retaining both axons which were of different lengths. However this difference is the consequence of the dissection and is not related to their length *in situ* . A sequence of phtographs was taken to map the redevelopment. The pattern of development, from the first neuritic sprouting, is labelled 1,2,3 and 4 in figure 32. Despite the difference in length the longer axon started neuritic development but the shorter axon had the most prolific growth. Where the longer axon had folded there was neuritic development from the damaged area. In its first stage the folded area has the same phase bright, bulbous appearance of area 1. Although there was extensive arborisation from the existing axons the soma also formed a large neuritic tree.

Figure 32

A C1 neurone with both axons attached, showing the pattern of redevelopment. Before the first photograph was taken area 1, the end of the longer axon had collapsed to form a bulbous ending and neuritic sprouting had started(15hr). The following were taken under phase optics.

Scale bars=100um

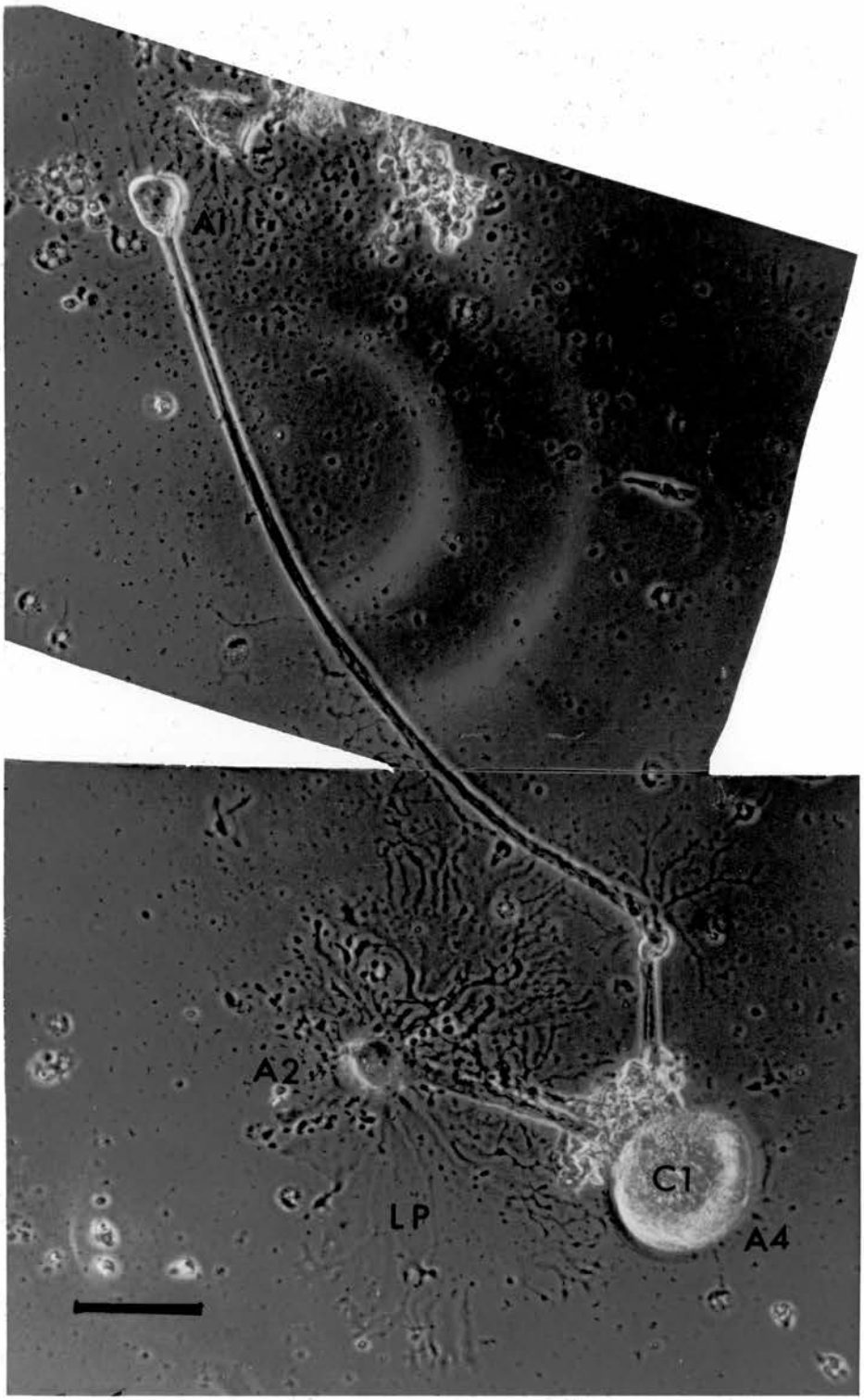
(I) 24hr after plating area 2 had formed a large lamellipodium and growth cones. At 30hr, when this photograph was taken, area 3 had developed neurites.

(II) 36hr and area 4(the cell body), had also sprouted neuritic extensions.

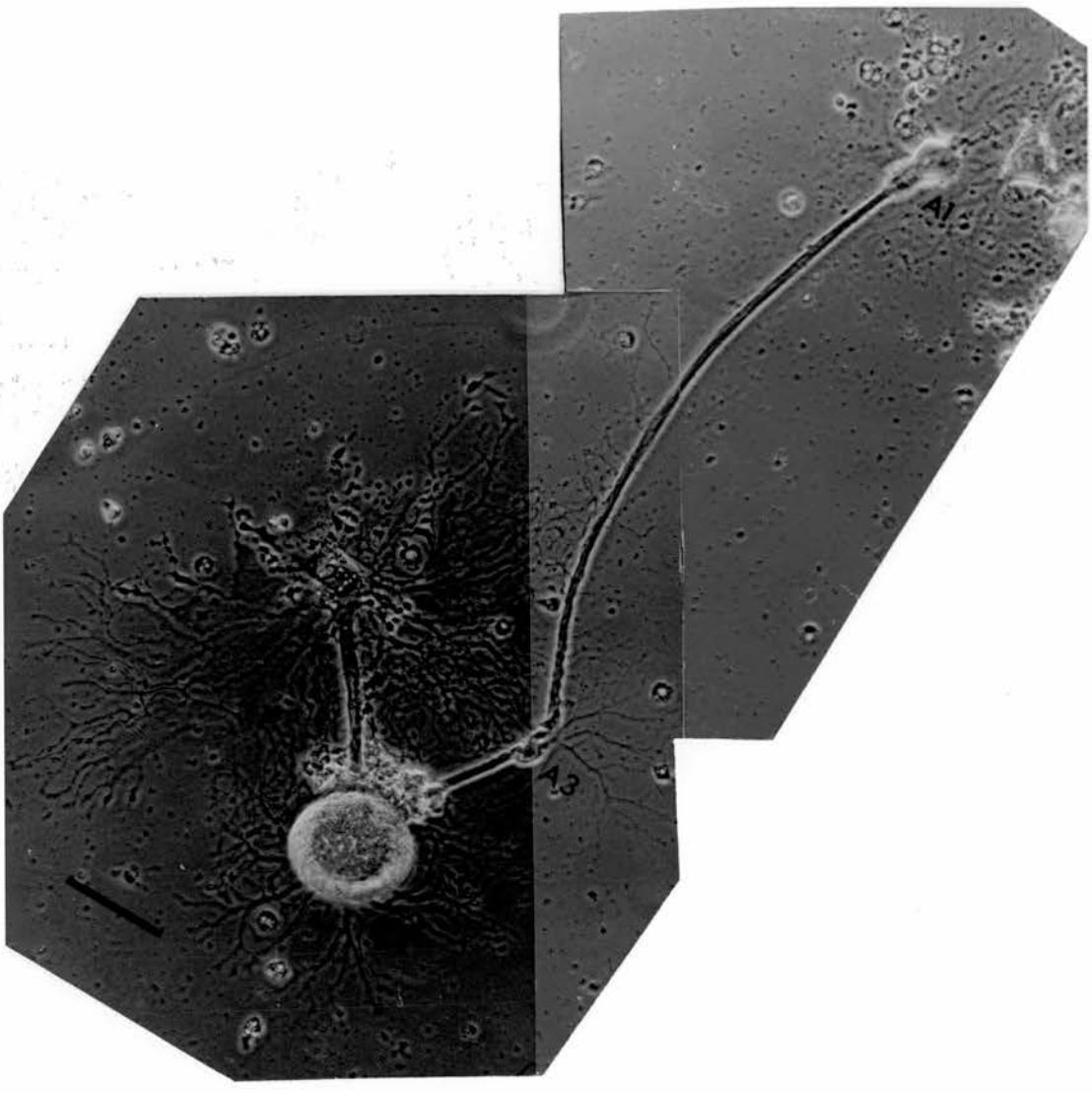
(III) 48hr, the area where axon twisted (area 3) had lost its definition. The lamellipodium around the C1 had disappeared.

(IV) 56hr , the axon in area 3 appears to have separated. Areas 2 and 4 show extensive arborisation.

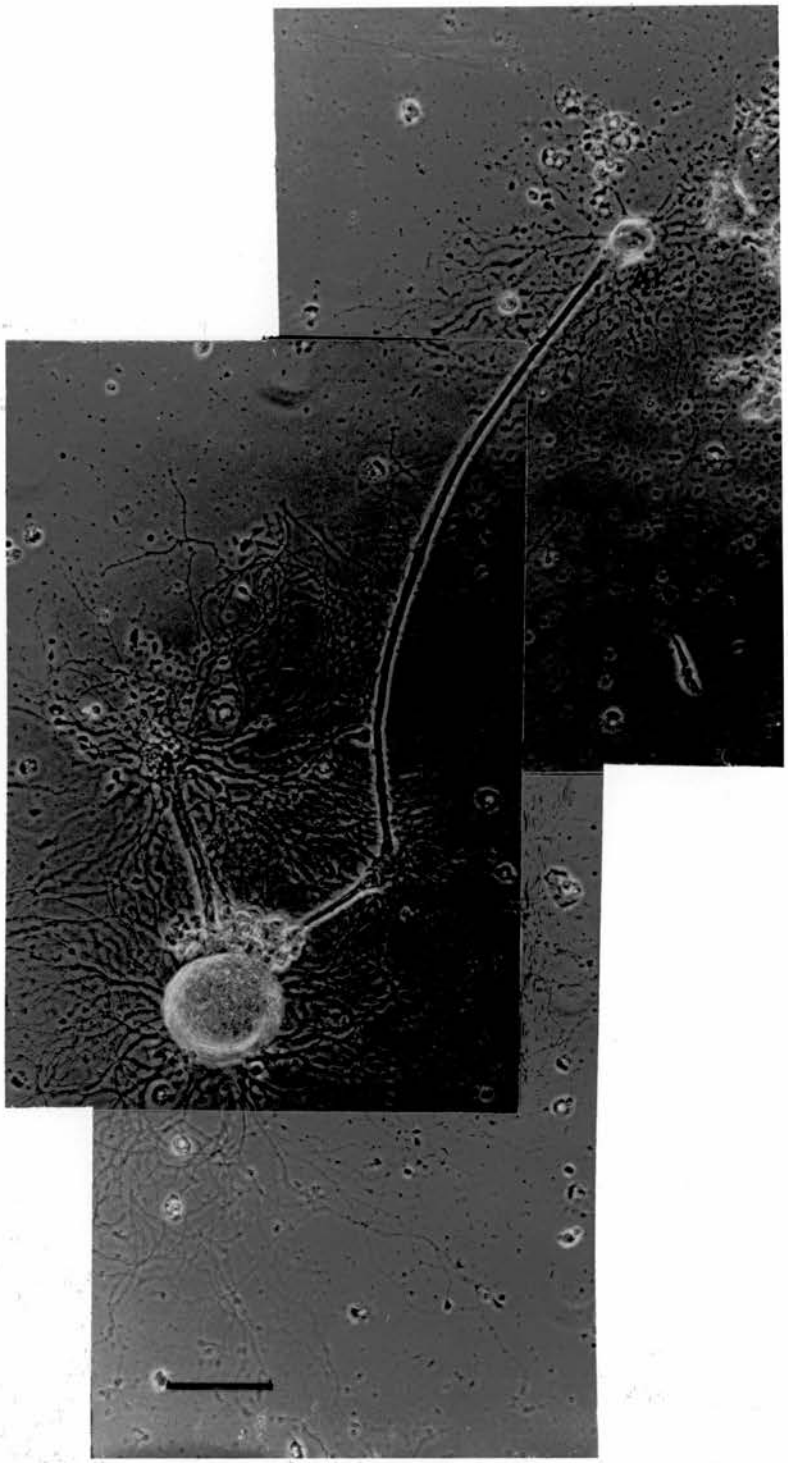
LP=Lamellipodium

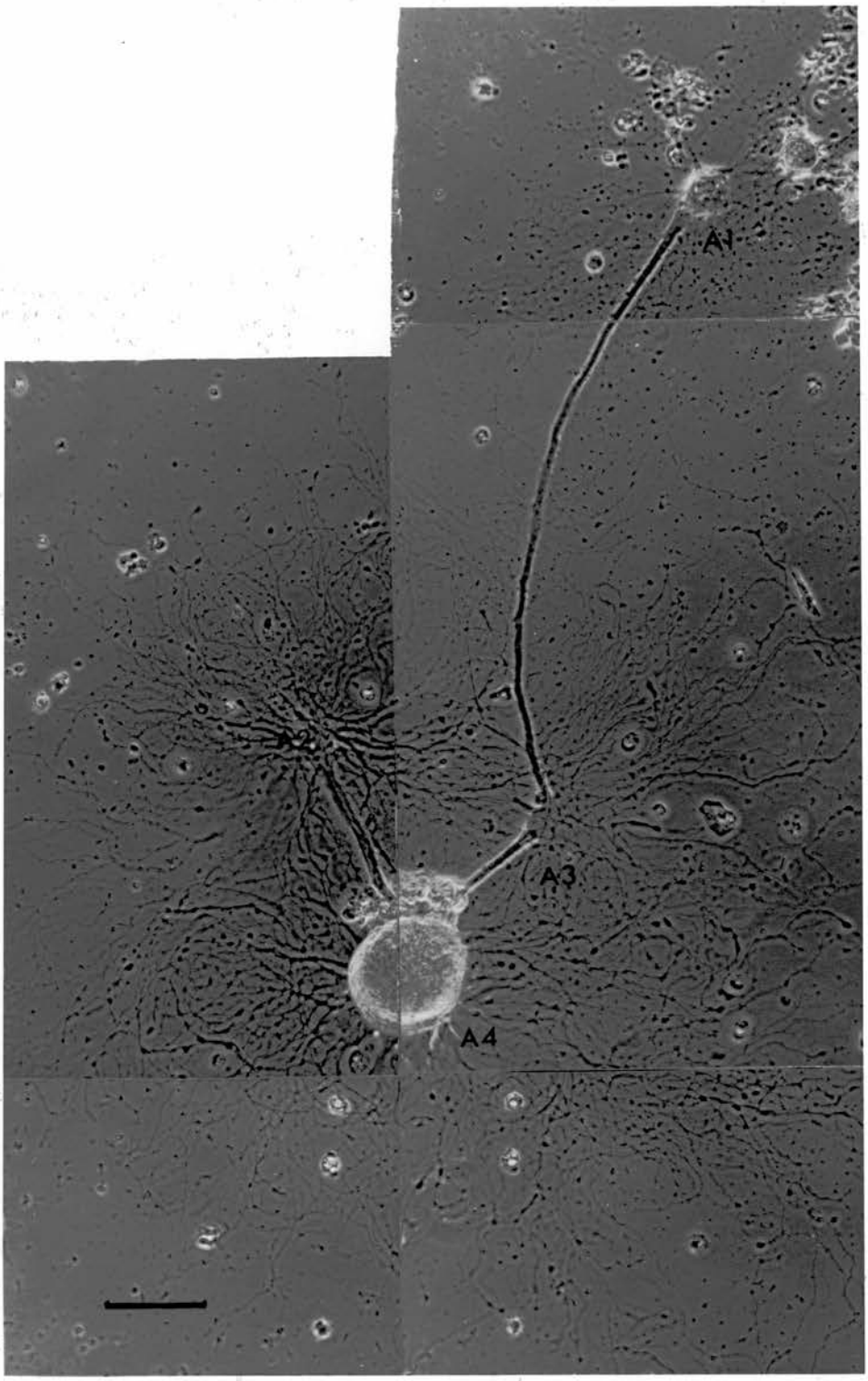


I



II





IV

3.14 **Influence of Fibroblast-like Cells on the Development of Neurites** and Growth Cones

The shape and extent of redeveloping processes were radically altered by adding neurones to cultures of fibroblast-like cells (f.c.) which had migrated from the ganglionic ring.

3.14.1 **Unidentified Neurones**

Plates which had circumoesophgageal rings added to them for 72hr went through several stages of colonisation by fibroblast-like cells. Within 18hr the area around the the ganglionic rings showed numerous individual, and clumps of small round cells (figure 33a). Over the following 24 hr these would flatten, put out a growth cone and migrated across the substrate. The rings were removed prior to the addition of neurones and fresh L-15 medium was added. The neurones quickly attached to the f.c. and formed broad, dense growth cones (figures 33b and c). The unidentified suboesophageal neurones extended neurites which were shorter than those developed without f.c.s (c.f. figures 34a and 8).

3.14.2 **C1 Neurone**

The C1 neurone when seeded onto a raft of fibroblast-like cells had thickened neurites and much enlarged growth cones. Adjusting for the difference in photographic scale figures 35 and 36 showed the effect of fibroblast-like cells on the size of the growth cones and neurites of the C1 neurone. Eight of the largest growth cones from the neurone in each figure were measured at the widest point and showed an average size of 8.5um (neurone without f.c.) and 19.6um(with f.c.). The width of the neurites were 1um (without f.c.) and 2.6um (with f.c.). The irregular

shape of the cones made accurate area measurement difficult and any difference in the depth of the cones could not be measured. In the absence of more detailed measurements the sizes of the processes should only be taken as orders of magnitude. The cones and neurites of neurones co-cultured with f.c.s.were approximately two times the width of those in cultures without f.c.s.

Figure 33

(a) Rafts formed from fibroblast-like cells which had migrated from the whole ganglionic ring over a period of three days. Neuronal cells from the suboesophageal ganglia, which were still rounded in appearance, had attached to the fibroblast-like cells 3hr after being seeded into the dish.

(b) and (c) after 15hr the neurites were short but broad.

Phase contrast optics.

Scale bar=50um

Figure 34

(a)After 24hr a neurone with a thickened neurite and large growth cone area. The growth cone showed exaggerated filopdial projections.

(b)Connecting neurones with large area of lamellipodium and a dense neuritic bridge.

Phase contrast optics.

Scale bar=50um

DNB=Dense neuritic bridge

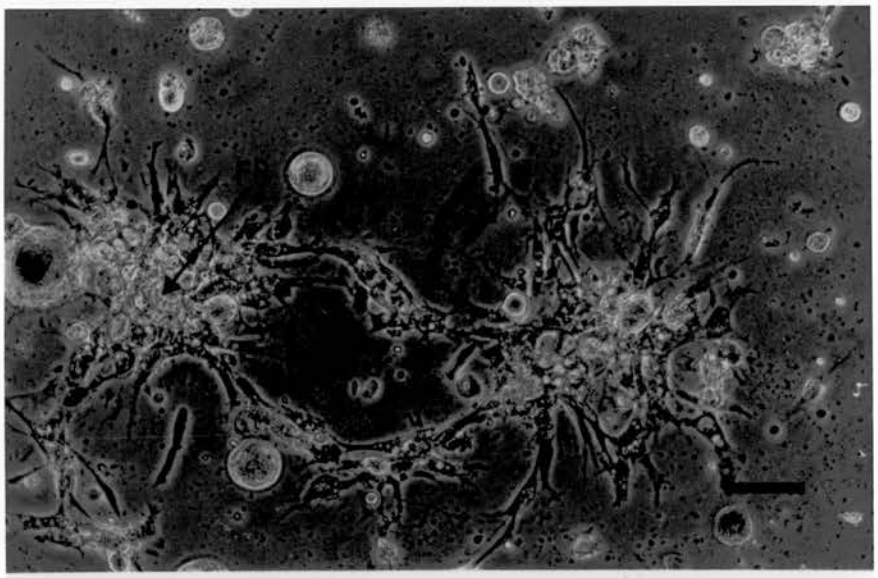
GC=Growth cone

FB=Fibroblast-like cell

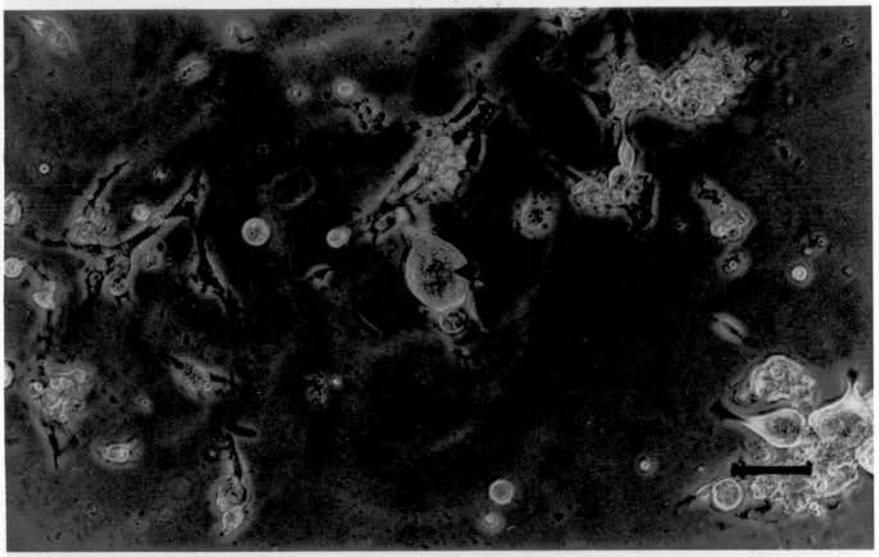
FR="Fibrobast" raft

SN=Suboesophageal neuronal cells

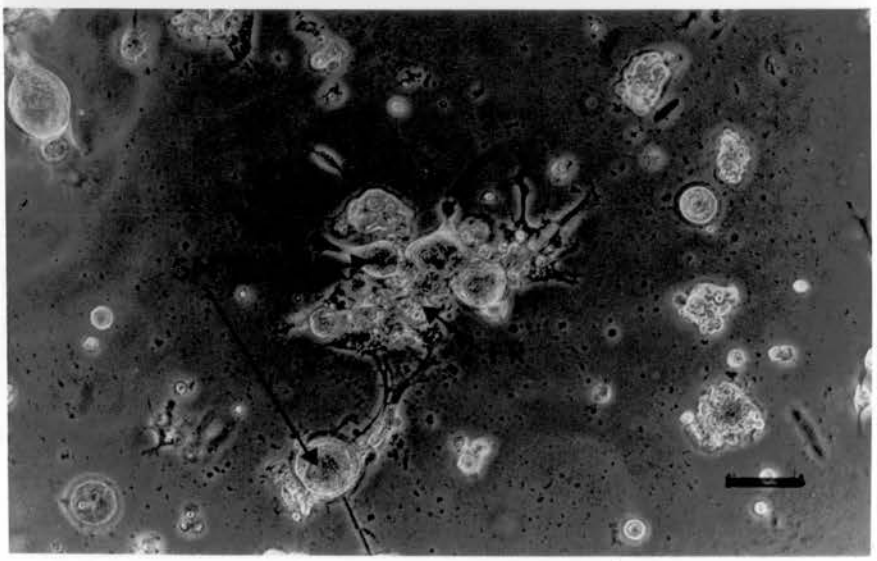
a



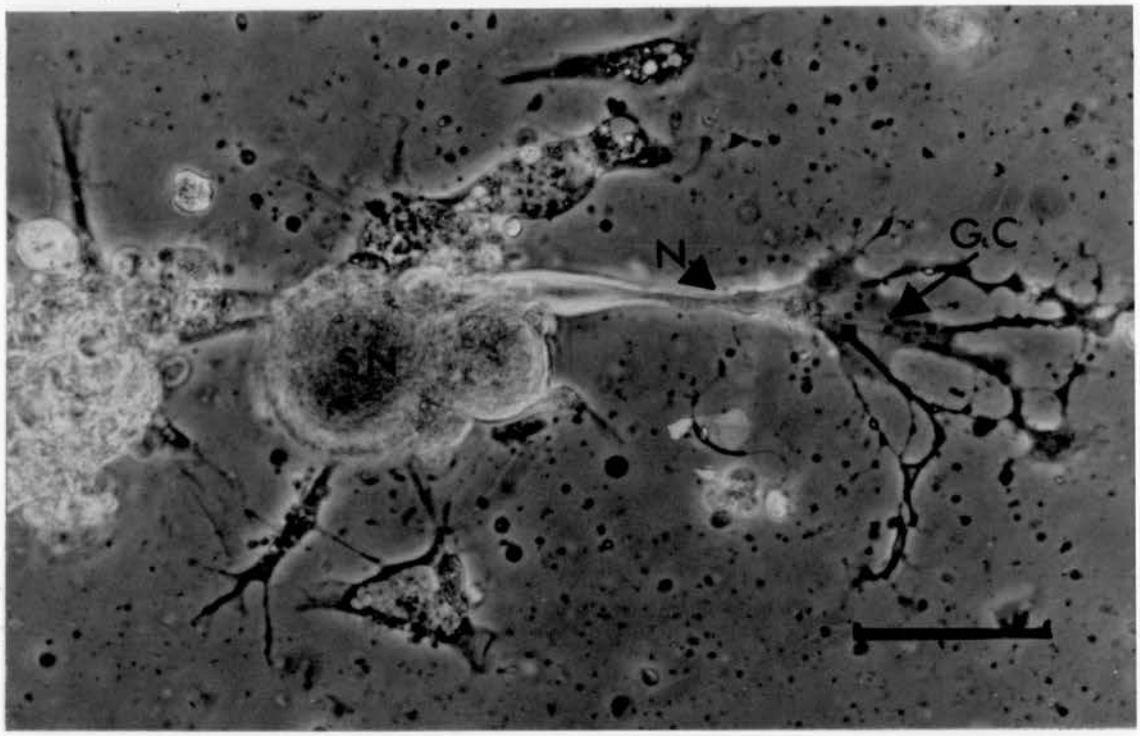
b



c



a



b

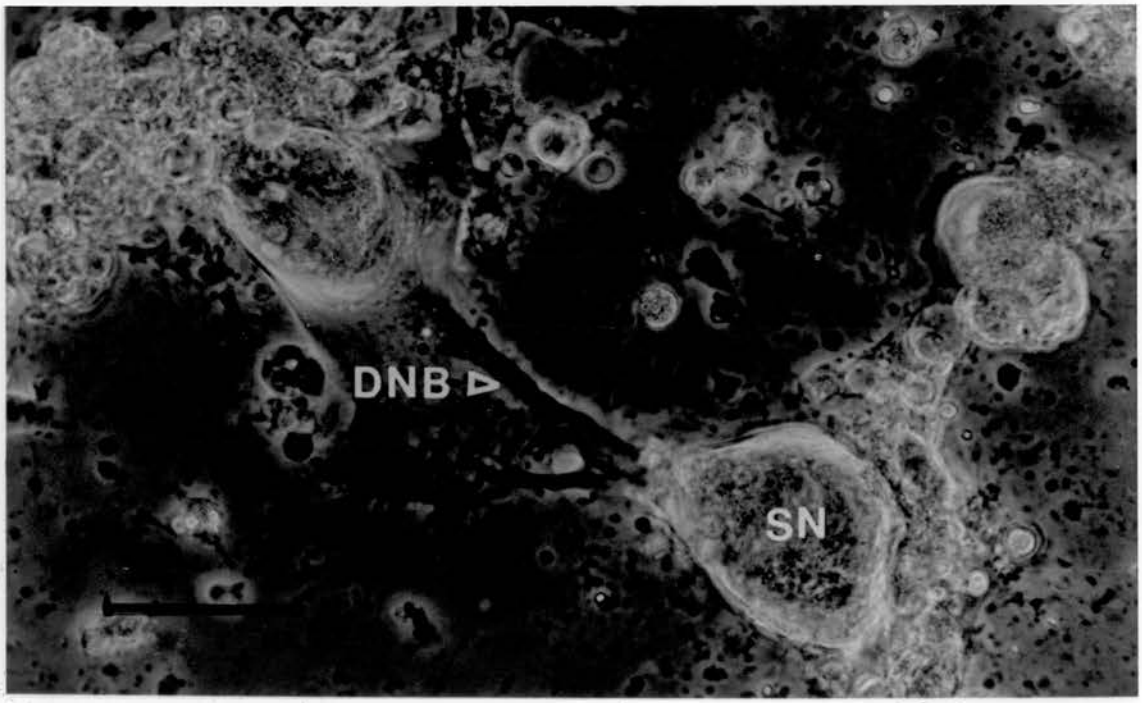


Figure 35

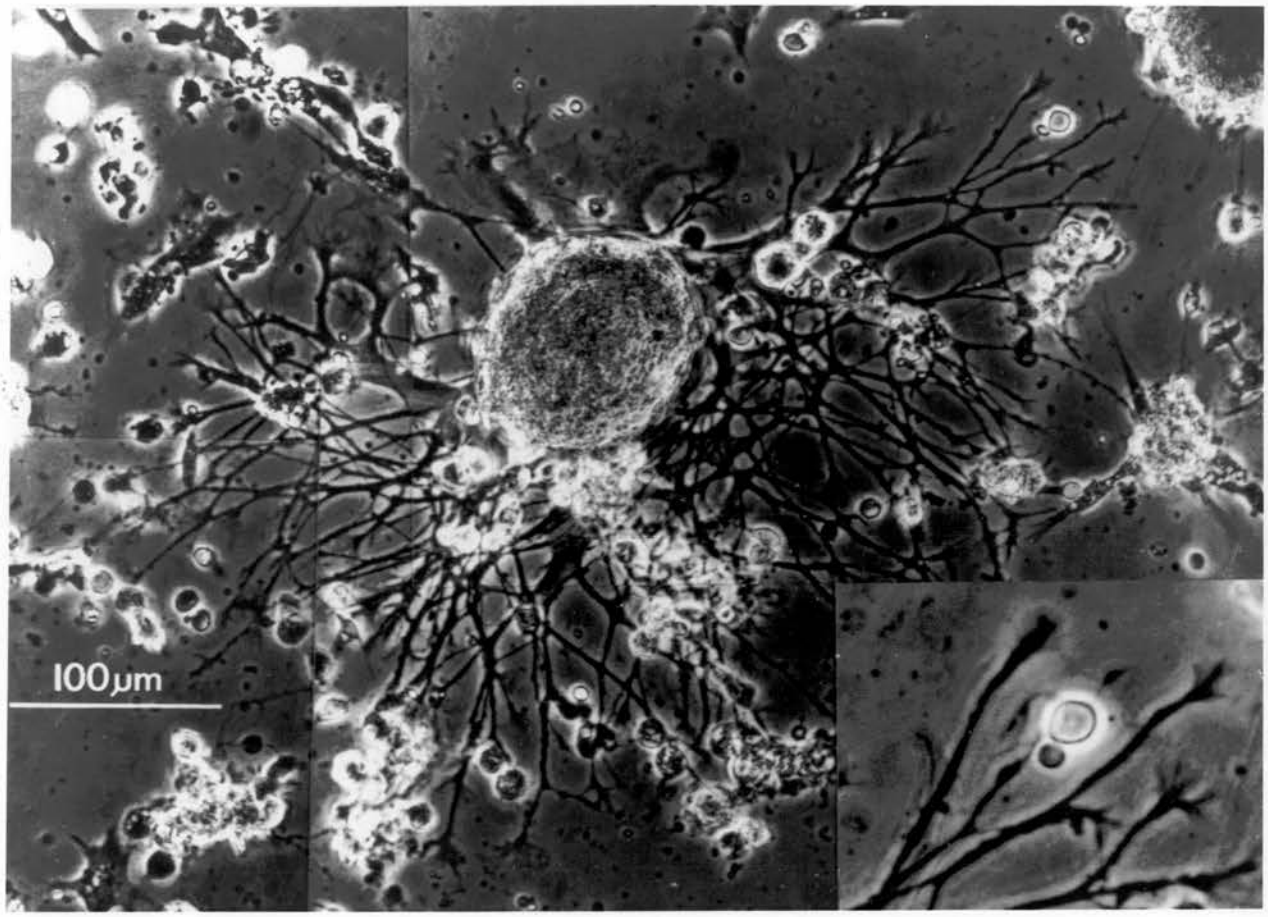
C1 neurone 3 days after being seeded onto fibroblast-like cells. The growth cones were enlarged and the neurites were broad and dense.

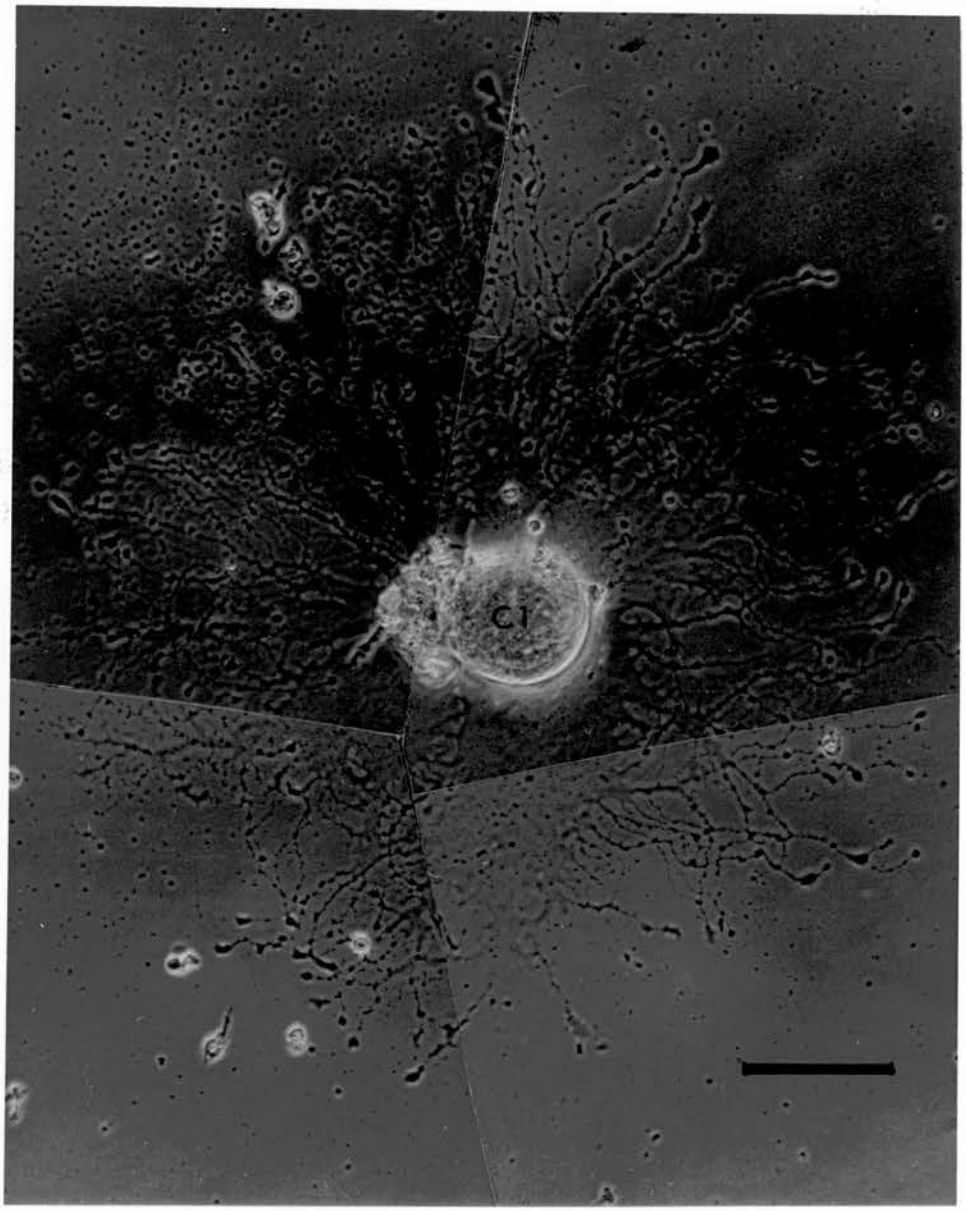
Scale bar=100um

Figure 36

C1 neurone (3 days in culture) serves as a comparator for figure 35 i.e. for the difference in size of growth cones and neurites of neurones on the substrate Con A. Both were photographed under phase contrast optics.

Scale bar=100um


100μm


C1

3.15 **The C1 Neurone on Plastic**

Neuronès of all sizes from Helix had a very low affinity for plastic surfaces and the success rate was inadequate for experimental purposes. However the C1 neurone in figure 37 illustrates the versatilty of neurone development and its "strategy" for dealing with low affinity surfaces. The C1 neurone when it is plated on Con A (a high affinity substrate) showed small growth cones and narrow neurites and on poly-l-lysine (a medium affinity substrate) large growth cones with narrow neurites. The C1 on plastic had developed short, very broad neurites with large cones. The lamellipodium, which had formed subsequent to these processes acted as an extra anchorage. The neurone developed over 4 days and the dish had been treated with conditioning factor. However it was not possible to know how much, if any, of the conditioning factor bound to the plastic surface.

Figure 37

C1 neurone attached to the surface of a plastic dish without substrate.

(a)18hr in culture dense, short neurites with large growth cones developed.

(b)56hr, a large lamellipodium grew out through the growth cones.

(c)72hr, the surrounding support, neurites and growth cones degenerated.

Phase contrast optics.

Scale bar=100um

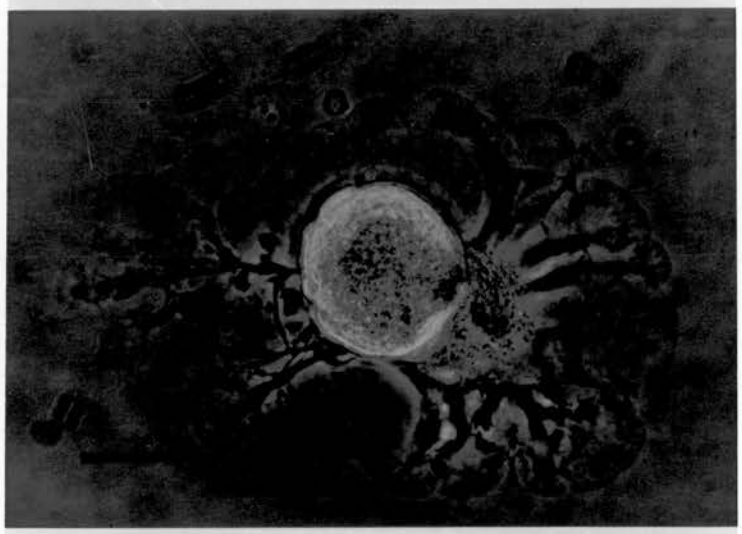
C1 Neurone

GC=growth cones

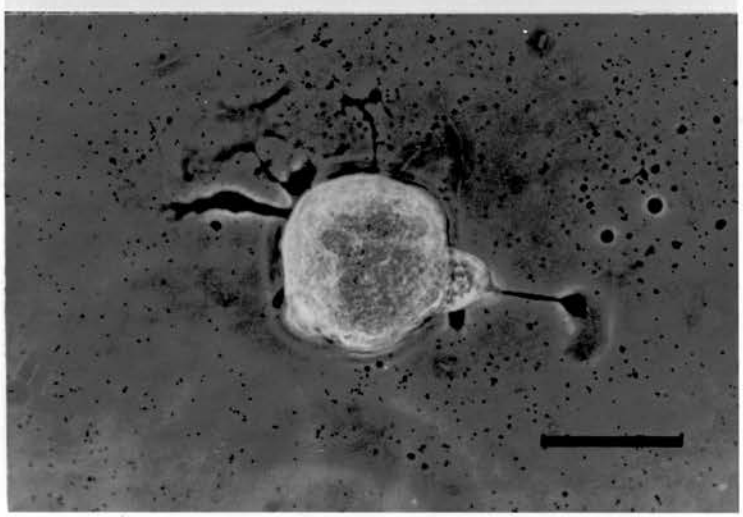
LP=Lamellipodium

a

b



c



3.16 **The C1 Neurone on Poly-L-Lysine (MW 300,000)**

The series of photographs (figure 38) followed the development of a C1 neurone from the first neuritic extrusion to cessation of growth and varicosity formation. The neurone was plated on poly-l-lysine and displayed important differences from C1 neurones on Con A. The neurites of these neurones went in an approximately straight line from the soma but on poly-l-lysine this is not the case. The neurites made many changes of direction, curving and describing circles. There were fewer neurites but the growth cones were larger (up to 50um across the widest part). The lamellipodium was thin and short filopodia were clearly visible through this membrane. In the mature stages of the neurone the growth cones became narrow and denser, taking on an elongated shape but maintaining very short filopodia along their length (figure 38, D7 b and c). This contrasted with the bulbous, smooth appearence of the growth cone on Con A. At the last stage (day 9) varcosities had formed along the length of all the neurites but the culture had become infected with bacteria.

Figure 38

C1 neurone on the substrate Poly-L-Lysine(MW 300,000). This series of photographs traced the develpoment of the neurone for 9 days. All of the following photographs were taken under phase optics. The growth cones shown as enlargments are indicated by arrows.

Scale bars=100um for photographs of C1 neurone and neuritic tree.

Scale bar=50um for photographs of individual growth cones and varicosities.

Day 1(24hr) after plating there was no lamellipodium and few neurites but the growth cones were large. What at first appeared to be a growth cone (MC) was a migratory cell (possibly glial).

Day 2(48hr) The C1 neurone as yet had few neurites. These were thin and and their direction was meandering. The growth cones (b, indicated by arrow in D2a) were up to 50um in width but with thin lamellipodia through which the filopodia could be seen.

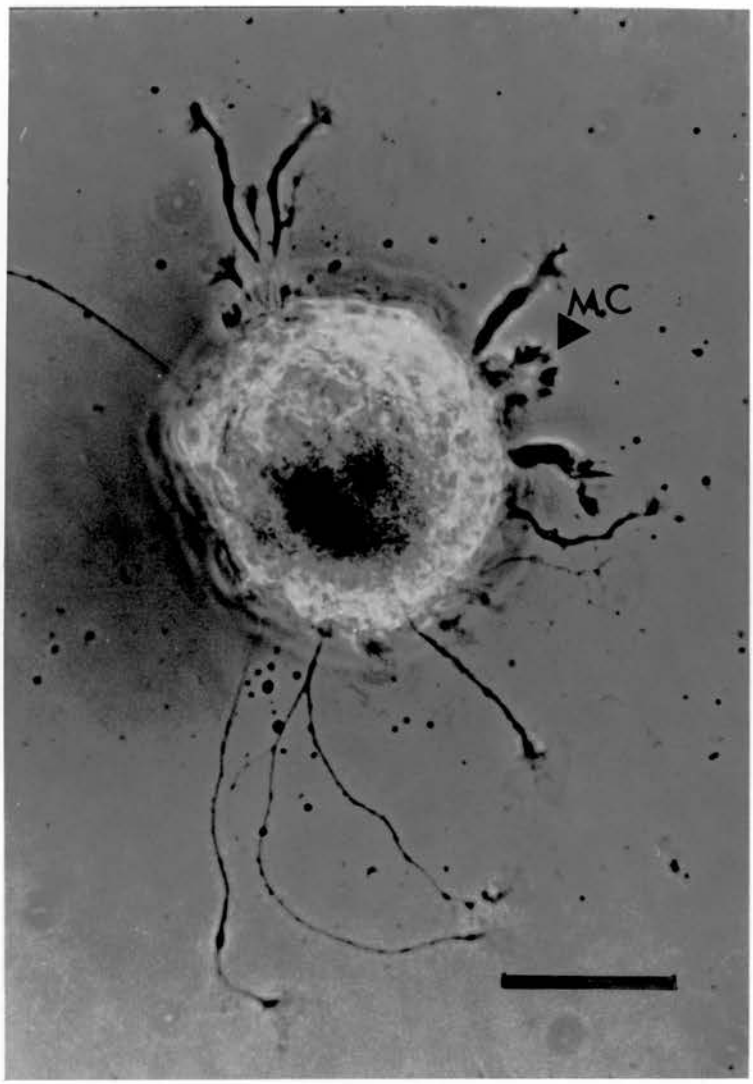
Day 4(96hr)Note the new position of the migratory cell. The growth cones were large and well defined.

Day 6(140hr) Some of the growth cones had lost the fan shape and become club-like, but with dense fiopodial growth along their edges.

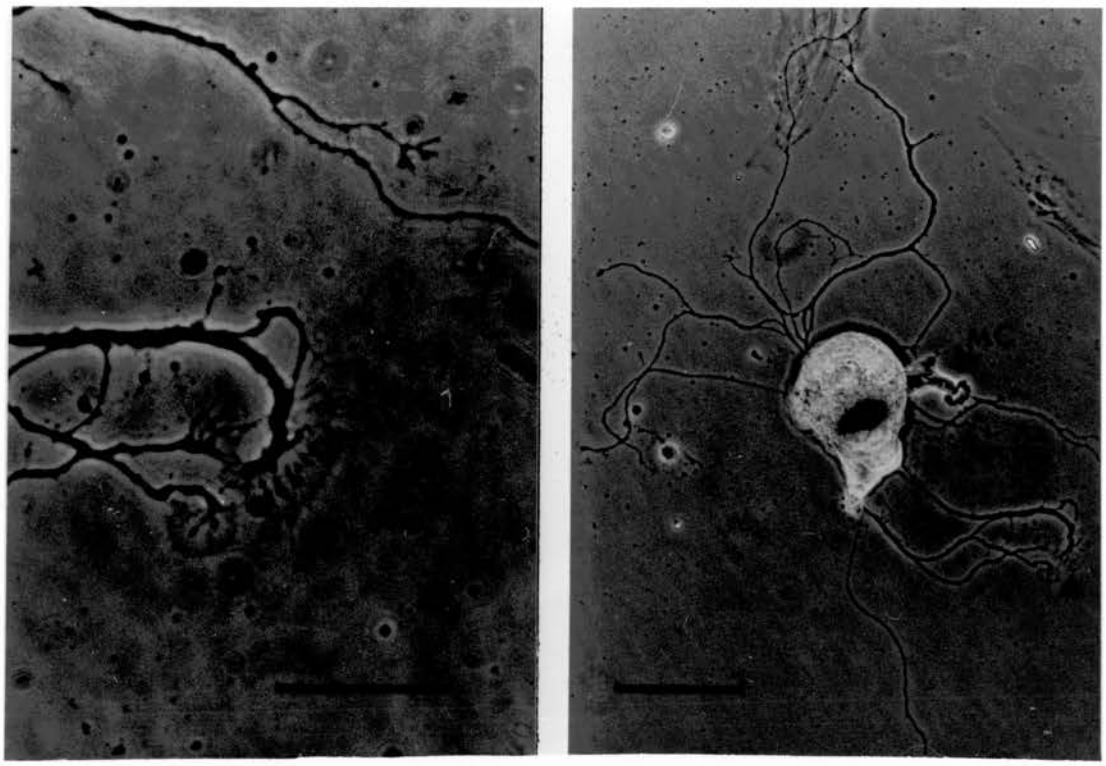
Day 7(160hr) All the growth cones had become elongated and club shaped.

Day 9(168hr) The culture had become infected with bacteria and the neurites developed varicosites protruding from along their whole length. Varicosities normally formed part of the neurite.

B=Bacteria. MC=Migratory cell. V=Varicosities.

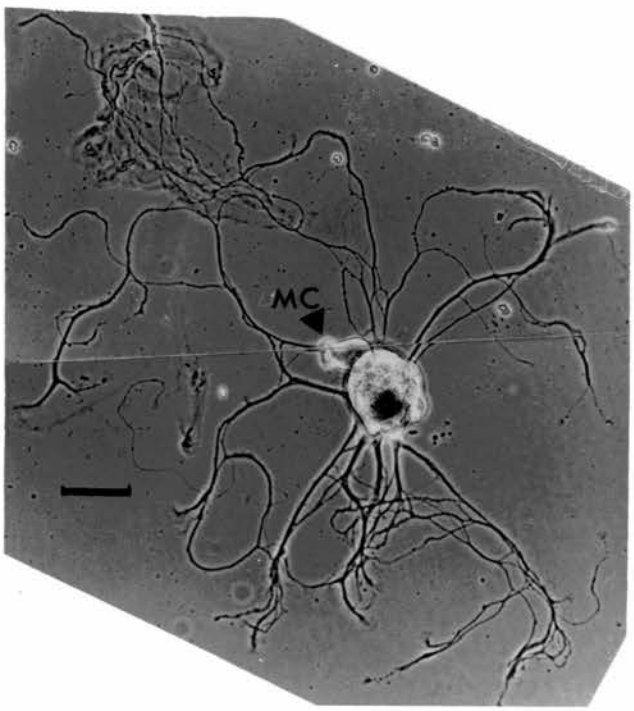


D1

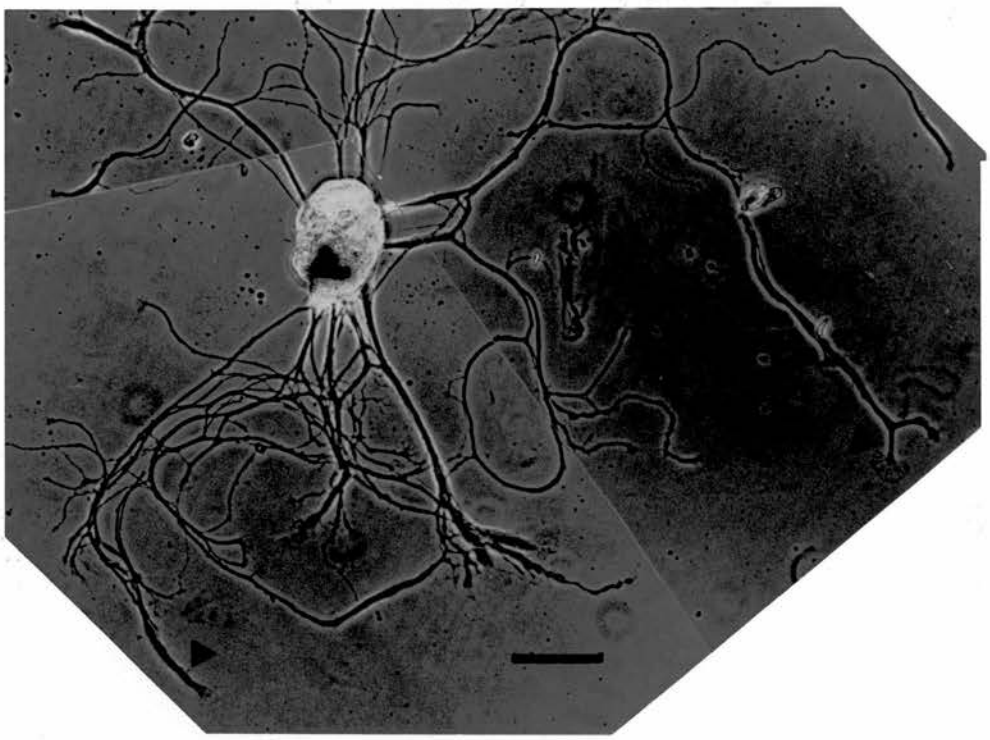


b

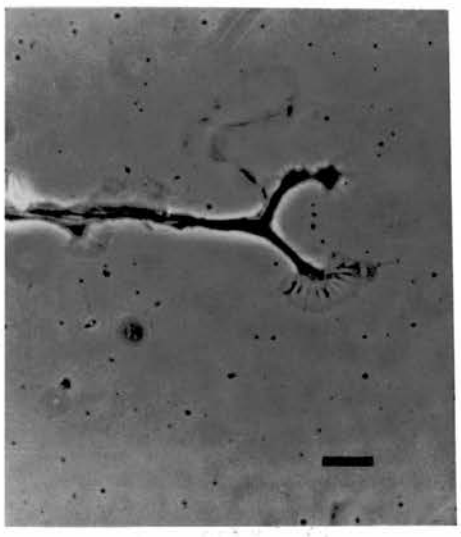
D2a



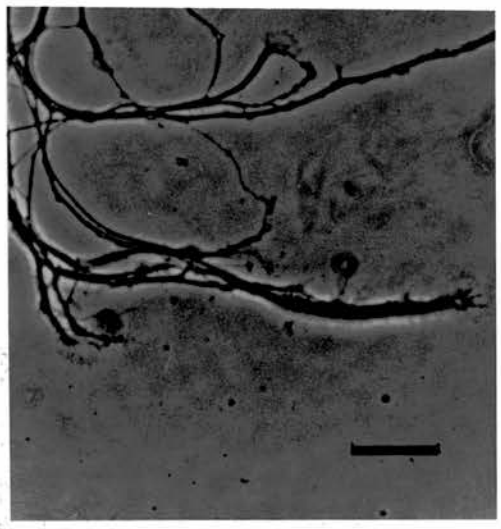
D4



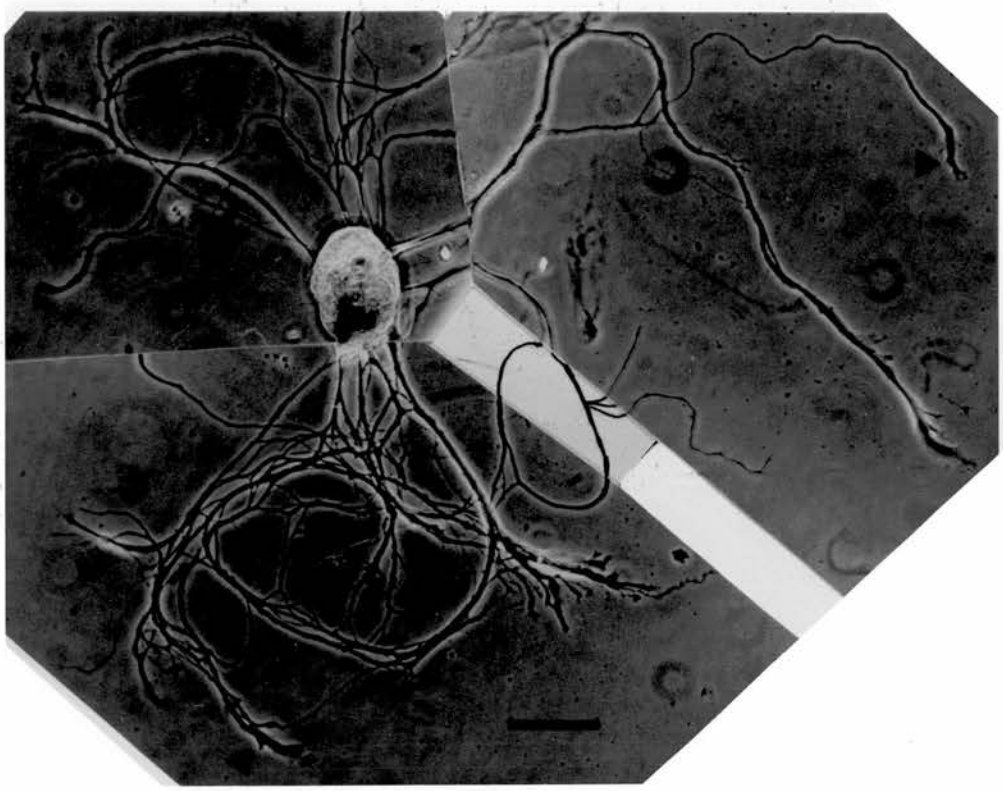
D6a



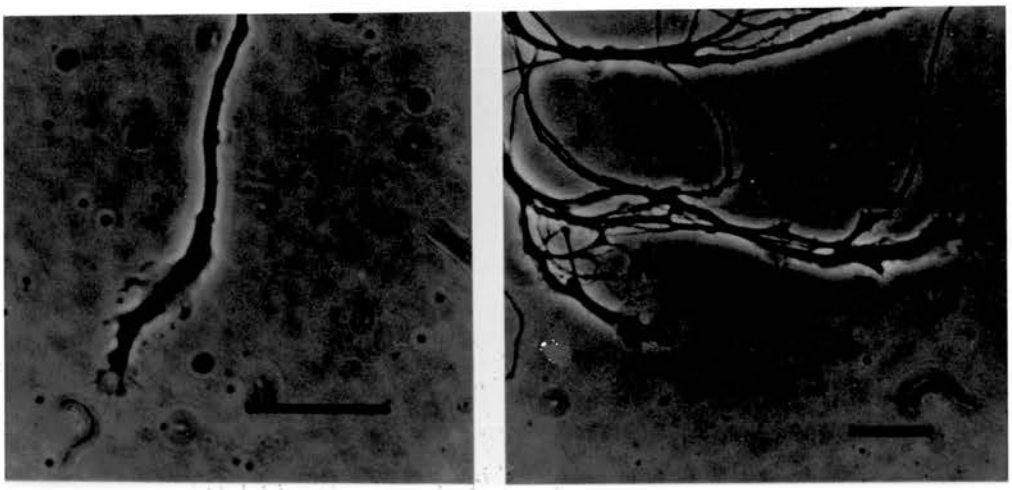
b



c

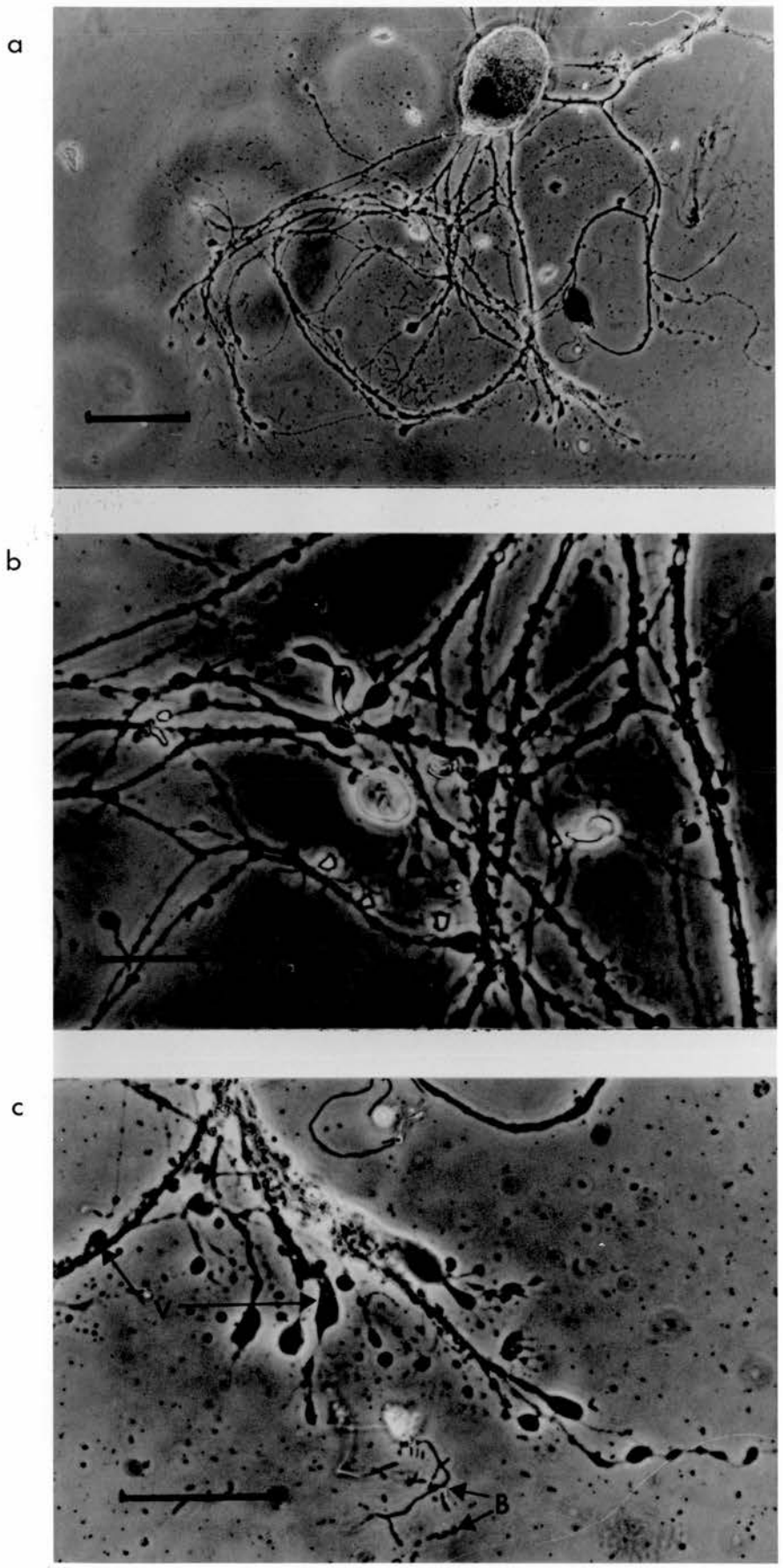


D7a



b

c


a
b
D
D
D
c
V
B

3.17 Connections of Neurones Developing on Poly-l-Lysine

Neurones that appeared to have connections were tested electrophysiologically. The examples shown were two unknown neurones from the cerebral ganglia and a C1 neurone and an unknown neurone from the cerebral ganglia (figure 39a and b). The C1 in figure 39b had attached to fibroblasts. The appearance of this neurone with many dense, straight neurites was markedly different from that of the C1 neurone on poly-l-lysine alone (Figure 38) but is comparable to the C1 neurone on fibroblasts on a substrate of Con A.

3.18 Buccal Neurones: Anterior, Median and Posterior

3.18.1 The Connections and Morphology. The redevelopment of these neurones followed the pattern of other large, identified Helix neurones. A lamellipodium or veil formed, followed by dense neuritic arborisation (figure 43). Figure 42 shows an middle neurone with the majority of the growth cones in a stable state i.e. the growth cones were large and round with no filopodia, and varicosites have developed along the neurites. This neurone showed no further development or elongation of the neurites. The normal appearance of the middle neurone is the same as that of the posterior and anterior neurones, however this photograph was included to demonstrate the anomalies in neuronal morphology caused by factors in cellular development. Reduced external calcium or blocked calcium channels in the membrane of growth cones can cause reduction or cessation of development of the growth cone and its lamellipodium (Goldberg 1988). This neurone went rapidly to this stage (30hr). Growth cones from this, and any other neurone, would usually only take on this appearance after 5-8 days, and with longer neuritic development. Figure 43 shows five anterior

neurones which were co-cultured. Three of the neurones developed in close proximity and two appeared to coalesce. The neurites formed a dense intertwining network, there was no demarcation between the areas covered by the neuritic growth from each neurone.

3.18.2 **Electrical Recordings from the Anterior Neurones**

Intracellular recordings were made in combination of pairs, 1 and 3,1 and 2, 2 and 3 (figure 43). Despite the close physical contact and overlapping of neurites electrical coupling could not be shown. Membrane potentials of -42mV,-50mV,-45mV and -54mV were recorded in cells 1,2,3,5 respectively.

Figure 39

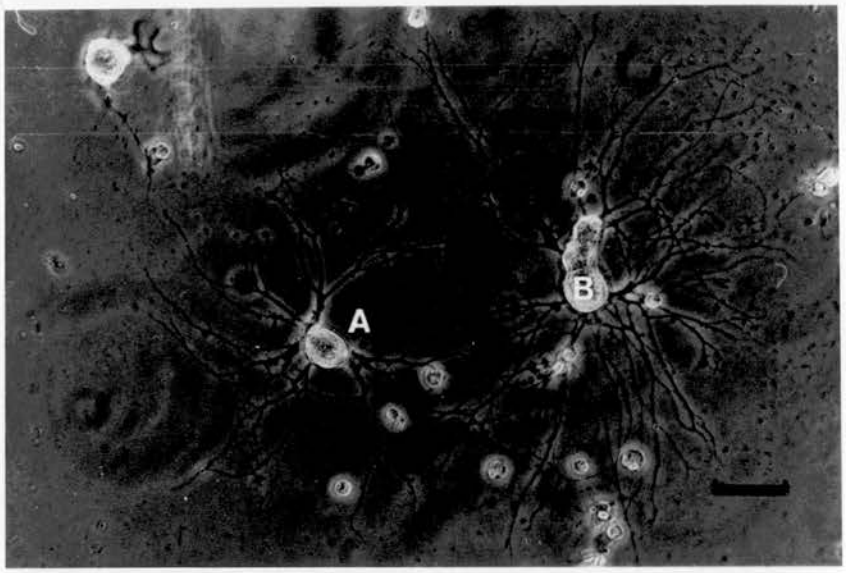
(a) Unidentified neurones from a cerebral ganglion developed in the same culture conditions as (b) and tested for connections. Phase contrast optics. (b) C1 neurone and an unknown neurone in co-culture on the substrate Poly-L-Lysine(MW 300,000). The C1 had attached to fibroblast-like cells. The unidentified neurone was enmeshed in the neurites of the C1 neurone. The smaller neurone had lamellipodial development. These neurones were tested for electrical and chemical connections.

Scale bar=100um

C1= identified neurone

UC=Unidentified cerebral neurone

a



b

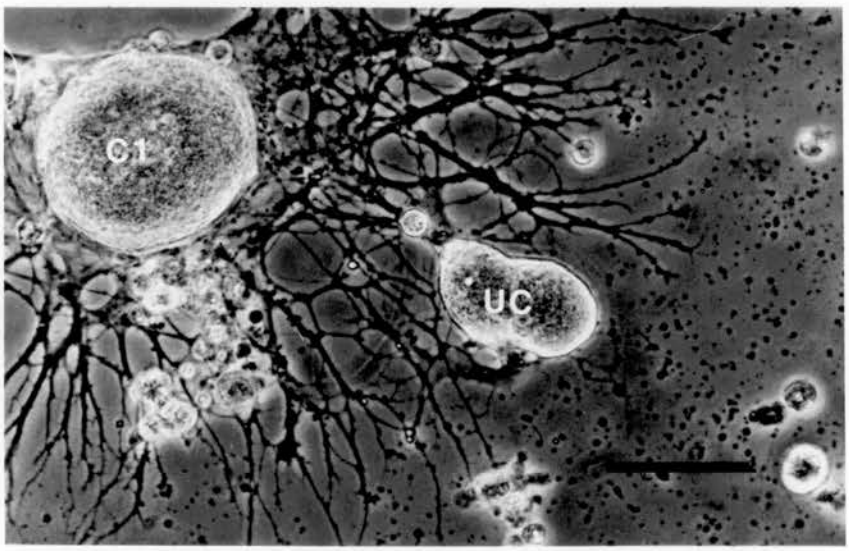


Figure 40

Recordings from the cells in figure 39

a) Electrical connection between the C1 neurone and an unidentified neurone (fig 39b). Each single spike from the C1 neurone caused a simultaneous spike in the unidentified neurone.

b) Evidence of possible chemical connections between the unidentified neurones in fig39a. Repeated firing in one neurone caused a single spike in the second neurone. There was a delay between these two events.

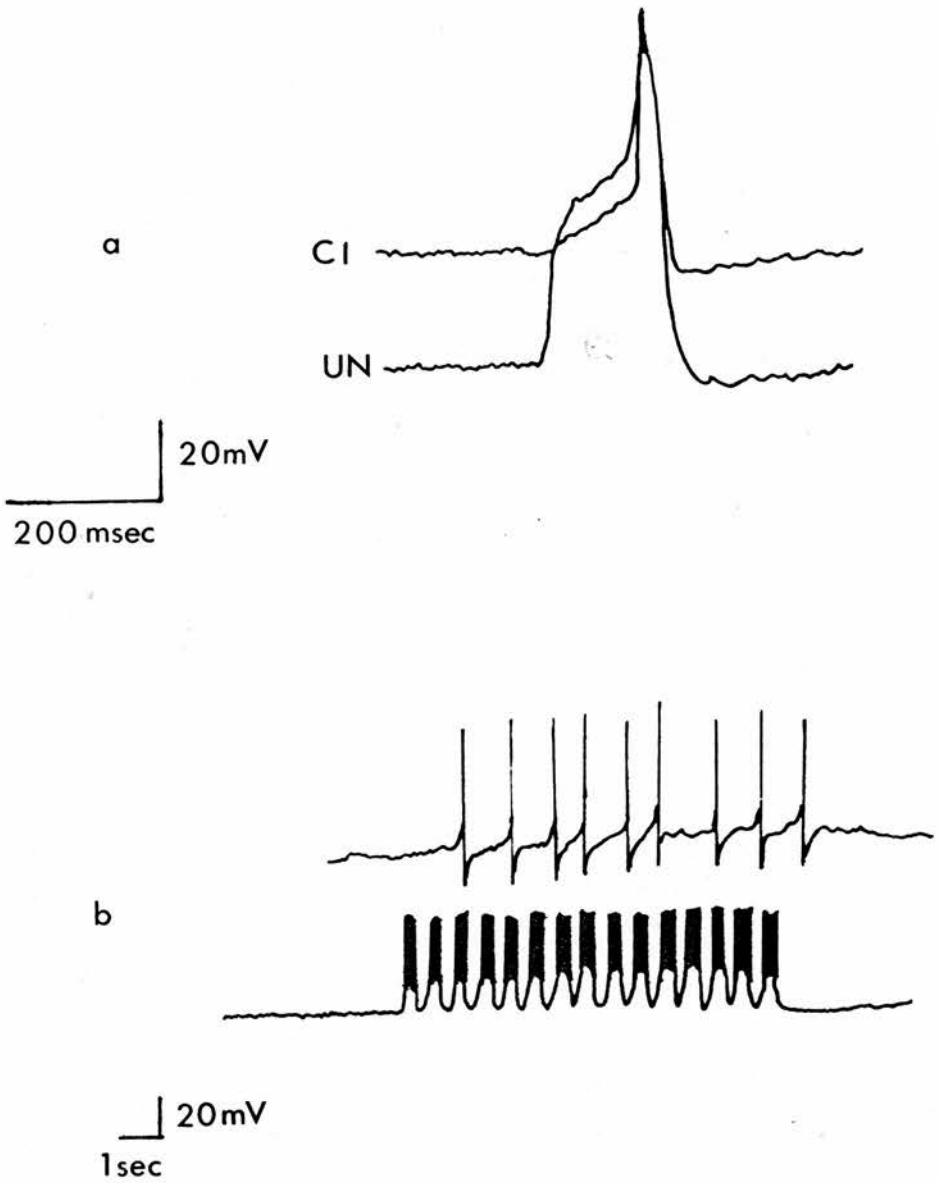

a
CI
UN
20mV
200 msec
b
20mV
1sec

Figure 41

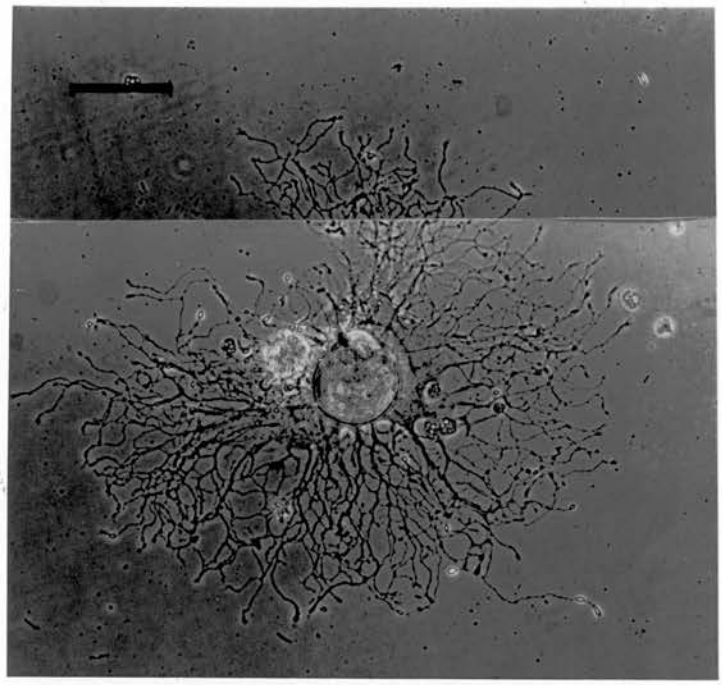
P neurone from the buccal ganglia. The nomenclature and the stages of development in these neurones follows that of the C1 and C3. A lamellipodium was formed from which the growth cones emerged. Phase contrast optics.

Scale bar= 120um

(a) Cell development after 20hr

(b) After 48hr

b



a

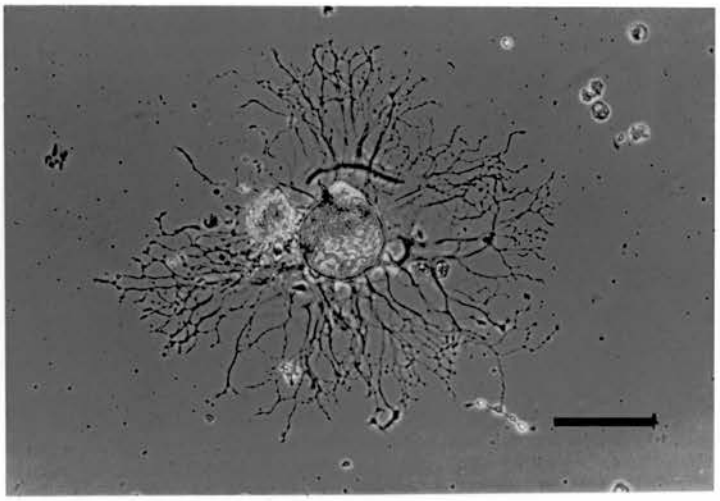


Figure 42

M neurone. This neurone exhibited thin neurites and in this figure the neurone has varicosities and large inactive growth cones. The neurites at the bottom of the figure were from an unidentified cell from the buccal ganglia. Phase contrast optics.

Scale bar=100um

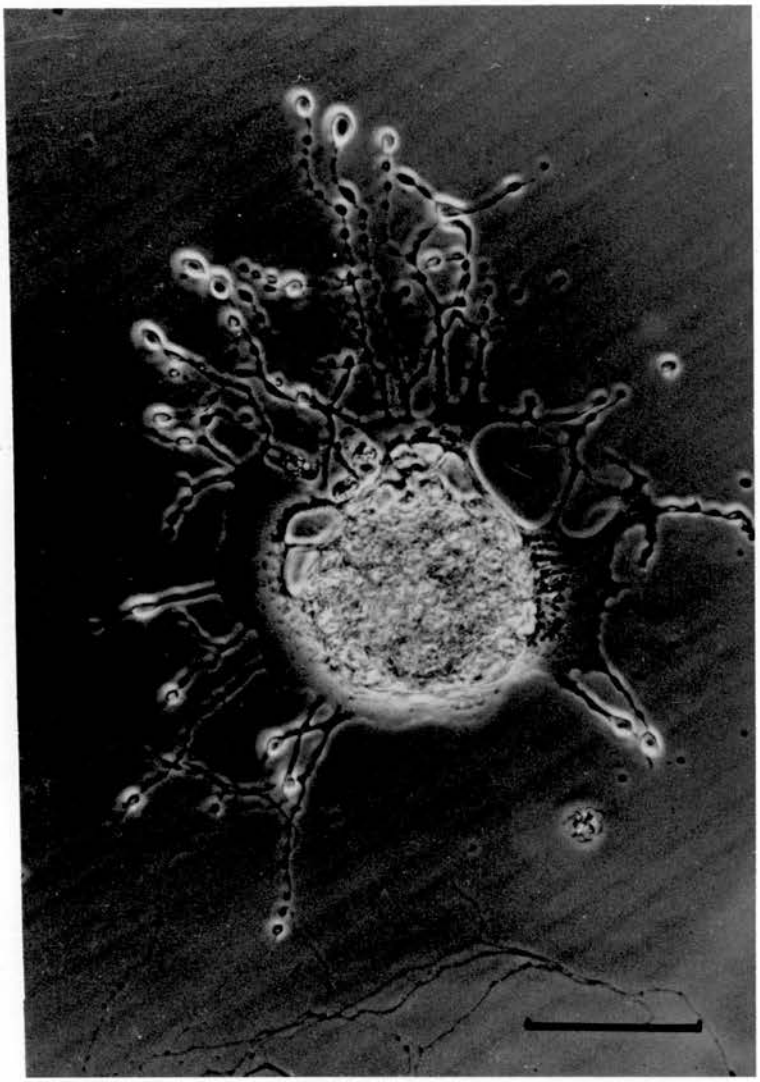
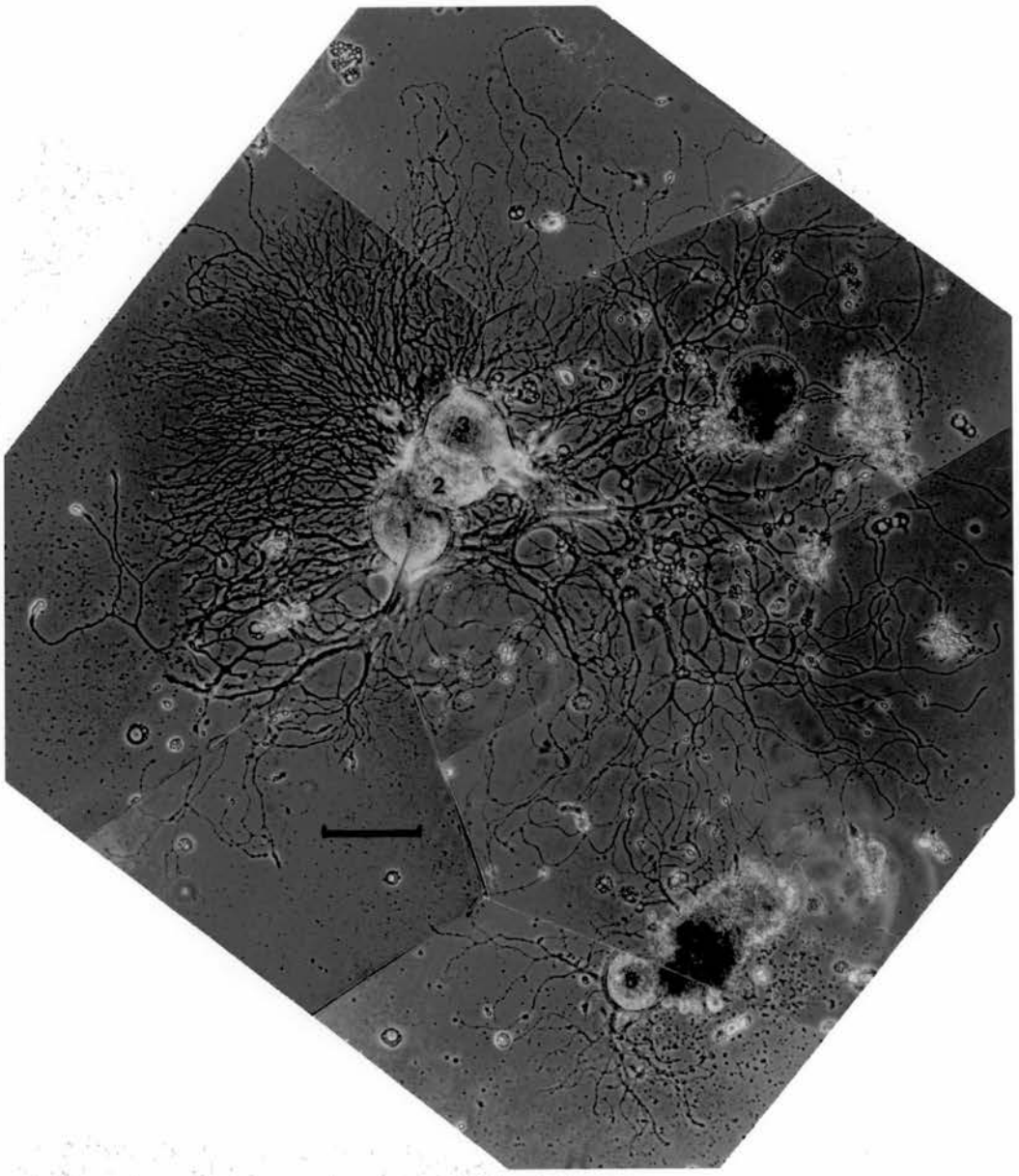


Figure 43

Anterior neurones. Five Anterior neurones in culture were numbered 1 to 5 for identification during a study of possible electrical or chemical connections.

Neurones 1,2 and 3 were in close contact, 2 and 3 appeared to have coalesced. Neurone 4 was firmly fixed but the neurites from the main group of neurones appeared to grow under it. The neurones had dense, overlapping neurites. The neurites from cell 5 were intermixed with those of 1 and 2. Phase contrast optics.

Scale bar=100um


1
2
3

3.19 **Varicosities**

All the identified neurones studied developed varicosities in their neurites. The whole neuritic network of some neurones would form varicosities (C1, figure 38(D9), C3, figure 21) and in others individual neurites or groups of neurites on a neurone would show swellings and stop growing while other neurites on that neurone would continue to extend (figures 10a and c).

3.20 **Growth Cones: Neurone Specific?**

The growth cones from C1 neurones (examples in figures 28a and b) and growth cones from the C3 neurone (examples figures 10b,11a) showed the shape and length of the growth cone and the length of the filopodia was specific to that type of neurone. The growth cones from the C1 neurone were narrow and club-like with short filopodia and few extrusions from the neurites. The growth cones from the C3 neurones , though also narrow, displayed long filopodia with extrusions from the neurite, giving it a spiky appearence. These comparisons were usually taken at the 3rd to 4th day, which was the middle of the development period. The unidentified cells did not usually form growth cones that were well defined or easily distinguishable from the neurite. Measurements were made of the growth cones from the end of the neurite along the longest axis of the growth cone and the two longest filopodia. Average values; for the C1 neurone from 20 growth cones was growth cone length 14 ± 3um and filopodia length 8 ± 2um ; for the C3 neurone from 20 growth cones was growth cone length 10 ± 1.6um and filopodia length 21 ± 3um.(Note: although no C3 were grown on poly-l-lysine for comparison the C1 on that substrate displayed short filopodia, figure 38, D2b.)

3.21 **The Influence of the Axon on the Neuritic Development of Isolated** Neurones

Two questions asked of the observed redevelopment of processes from identified neurones in vitro were: (1) did the neurone develop an axon from the neuritic network and (2) did the presence of an existing axon influence or preclude the formation of non-axonal neuritic growth from the cell body?

(1) Identified neurones, without axons,(C1, 5 cells; C3, 9 cells; buccals, 7 cells) were examined at four days. All of these neurones developed dense neuritic trees from the perimeter of the cell body. There was no preferential development of a single process among the neurites from these neurones which could be identified by apearance as an axon i.e. either longer or thicker than the other processes.

(2) Neurones with axons attached(C1, 4 cells;C3, 3 cells; no buccal cells were removed with intact axons) did not prefer to develop neurites from the axon alone, but also showed neuritic growth from the cell body. However from the C1 cell body the neuritic growth was profuse (figure 32, parts III and IV) but from the C3 (figures 13 and 22) only a few neurites had developed. Populations of neurones from the suboesophageal ganglia without axons, did in contrast, often develop specific polar neurites rather than a general neuritic tree (figures 8 c,d and e). (Neurones were examined at four days because at this stage the neuritic tree was well developed.)

DISCUSSION

DISCUSSION

The objective of this work, to maintain identified Helix neurones in culture, was achieved. The neurones could be isolated and were observed to develop processes. Throughout this study the word "isolated" forms part of a phrase to describe the neurones and this might be assumed to mean only their physical state, however this description can equally be applied to their functional condition. These neurones are not subjected to external seasons, nor normal physiological stimuli. Yet the apparently simple act of removing a neurone from its normal environment to the artificial conditions of the culture dish presents us with a library of responses. A single observed feature, such as a twist in the axon, has underlying it many complex changes in the organisation of the axon. The adherence of the cell to various and alien surfaces reveals not a naive cell but a "strategist".

4.1 Non-neuronal Cells

In the Helix cultures were non-neuronal cells which I labelled throughout this work as fibroblast-like. These cells had an important role to play when co-cultured with the identified neurones (see below). On the substrate Con A these cells were flattened but highly motile (figure 33). Motile cells could be seen on the surface of some neurones on a poly-l-lysine substrate (e.g. fig 38) but it had not been shown whether these motile cells and the fibroblast-like cells were the same type. Cells which have a similar morphology to these fibroblast-like Helix cells were identified as of microglial origin in the leech (Chiquet and Nicholls 1987) and as glial or fibroblast-like in Helisoma (Wong et al 1981). Chiquet and Nicholls showed that the behaviour of these cells was governed by the

substrate. On Con A they formed a stationary ring around the neurone but on poly-l-lysine they were motile. Although the movement of Helix cells was similar to those described by Chiquet and Nicholls their action was not. These authors found that the microglial cells inhibited the neuritic development of leech neurones whereas, in this study, Helix fibroblast-like cells produced development at an earlier stage than normal.

4.2 **Growth Cones: Shaped for a Purpose?**

In this study the growth cone shape and size showed variations from one neurone type to another and for the same neurone type on different substrates. If there is a correlation between a neurone and the shape of its growth cones and an answer is to be provided, even in part, the first questions that should be asked are: what is the purpose of the growth cone; what are the factors influencing its activity ; and what is its structure ? A clear purpose of the growth cone is to seek out the target cell and make appropriate connections with it, although that is not the end of the growth cone's existence. Embryonic leech axons will continue to develop after they have formed synapses, despite having a single target cell (McGlade-McCulloh and Muller 1989). The developmental structures, filopodia and lamellipodia can be found in the neuromuscular junctions of adult mice (Robbins and Polak 1988). These structures occupied the space between the Schwann cells and the muscle. It is known that the growth cones are not simply under the control of the neurone. The growth cone can respond independently, without affecting other cones common to the neurone(Haydon et al 1985). Release of a neurotransmitter from the growth cone can be detected when it is in contact with a receptor-rich membrane(Hume et al 1983), and that release is low prior to contact with the receptors(Xie and Poo 1986). Within the membrane of neuronal growth cones of Lymnea stagnalis

are potassium channels which open in response to stretching of the growth cone (Sigurdson and Morris 1989). The authors speculate that the potassium in modulating membrane activity might in turn cause calcium channels to open. The influx of calcium into growth cones, and neurites (Anglister, Farber, Shahar and Grinvald 1982), can cause neuritic growth.

Within the growth cone there are many dynamic changes taking place. Robinson and Karnovosky(1980) noted differences in the membranes of the cell and the filopodia in cultures of mice cells. The filopodial membrane has higher concentrations of intramembrane particles and lower levels of cholesterol than the cell (cholesterol is a membrane stabilising lipid). The cell attaches to the substrate along its length whereas the filopodium touches only at the tip. New formation of filopodial veil or lamellipodium has no microtubules, vesicles or organelles. Vesicles are present at sites of retracting veil, and identified when labelled membrane surfaces are sequestered within these vesicles (Tosney and Wessel 1983). Filament organisation is open in filopodia that are not attached and in active new veils, but it is in tight bundles in attached filopodia. Within the growth cones of embryonic rats, Dailey and Bridgman (1989) found that endoplasmic reticulum-like organelles were co-transported with microtubules into the peripheral domain and suggested that the calcium regulating role of the endoplasmic reticulum made it possible for the subsequent movement of other vesicles into these areas. Calcium is a key element for the assembly and disassembly of the proteins of the cytoskeleton (Forscher 1989).

Growth cones from Helix examined in culture were not negotiating a way through tissue, consequently their shape cannot easily be related to their purpose in vivo. However, although the type of substrate can alter the size of a growth cone, certain morphological characteristics of growth cones

hold true for identified Helix neurones. The C3 growth cones have numerous, long filopodia. The C1 growth cones when compared with the C3 on the same substrate had fewer, shorter filopodia and on other substrates had shorter filopodia. These differences raised the question of their functional significance to each neurone. This is as yet not known in Helix but in vivo the C1 and C3 have quite different routes to follow. The processes of the C1 stay within a neuronal environment from the cerebral ganglion through the cerebro-buccal connective to the neurones in the buccal ganglion and out to the pharangeal musculature (Pentreath and Cottrell 1974). The C3 passes out of the cerebral ganglion to the tentacle retractor muscle. Motor growth cone guidance to the appropriate muscle in zebra fish depended on preferential development on laminin rather than fibronectin. The growth cone would move into areas within filopodial reach. If the growth cone moved too far into an area with an inappropriate substrate it might be unable to retreat (Westfield 1987). It could be argued that there would be considerable advantage in longer filopodia to sample the surrounding area so that the correct choice would be made before commitment to one direction. The growth cones of the C3 neurone while making contacts with bundles of muscle cells could require more filopodia to give a larger area of contact with a motile target. Fischbach and Robbins(1969) found that the activity of different types of vertebrate muscles could alter the number of filopodia on a growth cone. There were more filopodia in slow twitch muscle terminals, where there was sustained stimulation and consequently a greater aggregate of impulses, than in fast twitch muscle terminals, which has fewer impulses.

The variation in forms of Helix growth cones from what is considered normal growth cone morphology in culture offer tantalising views of their

function *in vivo*. The C1 neurone (in figure 28 a and b) exhibits multiple growth cones and filopodium which extended many times their normal length. It is possible that these were abberations created in culture or that the growth cones were showing behaviour associated with the formation of synaptic connections on follower cells. A single neurite might make several serial connections on a neurone (compare this with, for example, synaptic connections on a ganglionic cell in the frog heart, Kuffler, Nicholls and Martin 1984).

The morphological changes shown by *Helix* growth cones e.g. larger cones had practical applications. The identified *Helix* neurones produce many growth cones but access to the membrane to identify, for example, types of ion selective channels, can be limited by the size of the growth cone. The application of patch clamping techniques, although already successful in examining a type of potassium channel in growth cones from isolated, identified *Helix* neurones (Green, Powell and Cottrell 1989) was improved by enlarging the size of the growth cones. In this study, co-culturing the neurones with fibroblast-like cells increases the size of the cones and the neurites. This increased the number available for study from each neurone (C1 neurone Figure 35). The C1 growth cones on poly-l-lysine (figure 38), were also enlarged, but lacked depth with thin lamellipodia and a near transparent central domain. The lack of depth in the growth cone meant that approaching its surface with a recording electrode increased the possibility of error i.e. not only making contact with the membrane but going through it to the dish surface. Growth cones from *Helix* neurones seeded onto fibroblast-like cells had a wide dense, phase dark centre indicating a greater depth. This also meant a larger area of accessible membrane, increasing the number of patches that could be

studied on each growth cone. This was important for a type of potassium channel where only a few channels could be shown on each growth cone. The surface membrane of neurites can contain ionic channels and enlarging the neurite could again give increased accesss to that membrane. Calcium channels have been shown in the surface membrane of neurites of neuroblastoma cells and can have a part to play in neuritic development of these cells(Anglister et al 1982).

4.3 **Growth Cones and Filopodia: Connections,Exploration and Other** Factors

In the introduction the electrical and chemical connections made by neurones are described. Before these connections could occur the growth cones made exploratory physical contact. Growth cones from mammalian central and peripheral neurones will retract and collapse when they meet unlike neurites(Kapfhammer and Raper 1987). The identifed neurones from Helix showed no obvious separation of neuritic pathways when several neurones were put together in culture. This was the case whether the neurones were of the same type e.g. the A neurones (fig 43, which showed closely interwoven neuritic processes) or the various cells of the suboesophageal ganglia.

The growth cones from Helix neurones will contact and navigate around small cells(figure 12). A growth cone was seen to change from the hand-like shape it assumes as it travels across the substrate. The growth cone retracted the filopodia on the side away from the unidentified cell. The filopodia of the C3 neurone can be twice the length of the growth cone but they were shortened and extended only as far as the cell surface. The growth cone was exhibiting expolaratory behaviour.

The C3 growth cones showed evidence of making connections with a small cell found in the dissociated tentacle muscle (figure 16). This cell exhibits long branching neurites which is a morphological characteristic of neuronal cells. The function of this cell has yet to be shown. Whether it is of neuronal origin could also be resolved by immunohistochemical staining e.g. with a monoclonal antibody A2B5, which is neurone specific (Eisenbarth, Walsh and Nerenberg 1979).

The processes of the C3 neurone, which was co-cultured with tentacle muscle extract, showed an unusual pattern of exploratory behaviour. Extensive areas of muscle cell fragments, small unidentified cells and particulate material were cleared from the surrounding substrate surface. The areas around growth cones are cleared by the filopodia but in these conditions the area extended many times beyond the normal reach of filopodia. Where neurites branch the growth cones will have areas of overlap. However it could be seen (figure 13) that several neurites extended straight out from the axon and the area in advance of these neurites was cleared. Processes smaller than filopodia were identified and labelled "sub-fiolopodia". The behaviour of the "sub-filopodia" suggested that they were most adhesive at the tip and were under tension. That is when the "sub-filopodia" made contact with the particulate material, which must have low adhesion, this was pulled from the substrate surface back to neurites or growth cones.

There was an unidentified factor from follower or target cells of C3 neurones that increased neuritic length and encouraged extended filopodial exploratory behaviour from C3 neurones in culture conditions. These changes in morphology were shown when C3 neurones were in co-culture with tentacle muscle extract (figures 13 and 16) but not when in culture alone

or with other neuronal cells. This factor was either released into solution from muscle or other cells within the muscle during the original dissociation of the tentacle muscle and subsequently attached to the substrate, or was from cells in the extract that had attached to the dish and continued to release it.

Barde, Edgar and Thoenen(1983) reviewed the action of neurotrophic factors on vertebrate neurones. They suggested that although there were factors in conditioned medium and tissue extracts which were able to maintain neurones in culture the factors producing neuritic growth were present only in conditioning medium. Conditioning medium is produced by cells releasing neurotropic factors into the culture medium over a period of time. The C3 neurones continued to extended their "superneurites" after medium changes but the rate of growth did not allow direct visual monitoring of changes in growth. If the growth rate slowed after medium changes this would have provided evidence for the factor being produced by cells in the culture dish rather than attached to the surface of the substrate.

The types of cells in the tentacle muscle are muscle cells and the rounded unidentified cells which did appear have connections with the C3 neurone. It was possible that these cells were releasing some form of attractant, however the neurites did continue to extend through large areas of muscle extract, by-passing many of these cells. This would suggest that the factor is generally growth enhancing rather than a cell specific signal.

4.4 Neuritic Initiation

The initiation of neuritic growth in identified Helix neurones took

longer than that reported for neurones in other invertebrates in culture. An extensive neuritic development could be seen in two to three hours in leech or Aplysia neurones while in Helix it can be twenty hours before neurites appear on identified neurones . The size of the neurones would seem to be significant. The diameter of the identified Helix neurones was 100-150um, which was two to three times that of neurones used from leech, Aplysia or Helisoma. By the end of the settling period of eighteen hours, smaller unidentified Helix neurones (up to 80 um) had also developed many neurites. The time taken to start neuritic development could be related to the time taken for the neurones to become fully attached to the substrate. Smaller neurones could attach more readily than large neurones (see also section on attachment below). The later development of neurites from identified neurones from Helix was not without exception. A few large C1 and C3 neurones had developed neurites during the settling period.

4.5 **Neurites: Budding Axons or Dendrites?**

The large identified Helix neurones showed no preference for the development of a new axon, despite the length of time in culture (up to nine days). The neurites continued to develop from all points of the cell circumference. It is possible that these neurones require a clearly identified or approriate target cell for the development of an axon. In comparison, unidentified neurones from the suboesophageal ganglia did show a defined bi-polar neuritic development in culture (Figure 8). The neurites developed from specific points leaving the rest of cell membrane clear. In cultures of hippocampal neurones of embryonic rats the axons and dendrites would develop without target cells(Dotti and Banker 1987) and when an axon was severed it would either regenerate or one of the dendrites would develop into an axon. The choice of dendrite which then became the axon

depended on on the length of the axonal stump. A stump 50um long ensured continued axonal function but if the stump was below 30um in length this allowed any dendrite 10um longer than competing dendrites to become the axon (Goslin and Banker 1989). Where dendrites were of equal length there was a delay of up to 18hr. Axons and dendrites do exhibit clear differences in proteins associated with membrane formation. The protein GAP-43, which is in greater abundance during regeneration, is found in the cell body, axon and its growth cones but not in the dendrites or their growth cones and MAP2 was found only in the cell body, dendrites and associated growth cones (Goslin, Schreyer, Skene and Banker 1988). The messenger RNA for MAP2 has been shown only in the dendrites(Garner, Tucker and Mattus 1988).

The characteristics described above are useful in deciding whether a process is an axon or dendrite. Would these differences be applicable for defining Helix neurites ? In the instances where the suboesophageal neurones had bi-polar neuritic growth this might be so. The C3 neurone in situ has dendrites emanating from the axon and this neurone isolated in culture did show dendritic remains on the axon (figure 17) and intense neuritic development from the dendritic area (figure 22). However the arrangement of axon and dendrites in the mature, identified neurones of the Helix is different to that of the mature hippocampal neurone. In the hippocampal neurone the axon and dendrites are separate processes but in the identified Helix neurone the dendrites come from the axon. It would be difficult to reconcile the mature internal structures of the processes of the mammalian neurone and the identified Helix neurone. The Helix axon would either have to contain two separate transport systems or the microtubule orientation would be the same in the dendrites and the axon. A neurone (the R2) in Aplysia formed dendrites in a ganglion distant from its

origin however these dendrites did not contain the organelles (e.g. ribosomes) exclusive to dendrites in vertebrates. Each dendrite with an associated glial cell was deeply invaginated into the axon and the organelles transported in the dendrite was the same as that in the axon (Ambron, Rayport and Babiarz 1988).

4.6 Lamellipodium

In the introduction I suggested that the lamellipodium or veil might offer an advantage in the study of organelle transport, but does it? The thin layer surrounding the neurone contained organelles. These organelles appeared in lines radiating out from the edge of the neurone. Where neurites formed the lines of organelles came together to form bundles which passed along the neurite to the growth cone.

As the veil was very flat and thin, this made it easier to see movement of organelles. Anterograde and retrograde movement of small synaptic vesicles and dense core vesicles in the C3 neurones dual transmitter system might be distinguished with greater accuracy and the differential rates of movement of the vesicles measured. In combination with the toxin, α-latrotoxin, which selectively depletes small synaptic vesicles (Matteali, Hainmann, Torri-Tarelli, Polak, Cecarelli and De Camilli 1988), the retrograde movement of vesicles containing FMRFamide-like substances could be further isolated. The combination of high resolution Nomarski optics and time lapse videophotography presently in use(Edmondson and Hallan 1987, Hollenbeck and Bray 1987, Aletta and Greene 1988) has made the study of living systems possible. The immunoreactivity for FMRFamide in the C3 neurone was present in the cell body (without neuritic growth) from the first day in culture. After

several days the presence of the FMRFamide-like substance was seen distributed throughout neurites and growth cones that had been developing. If there were stages in the C3's redevelopment when the transportation of organelles containing the FMRFamide-like substance was initiated, or increased when the growth cone connected to a follower cell, this could be established more clearly.

4.7 **Limitations of Photography**

The photographs in this work give a limited view of the life of the isolated Helix neurones. The rates of growth were difficult to monitor. Simple transfer from the incubator to the microscope stage meant changes of temperature and light required for photography. The time of onset of neuritic development was unknown for the first 18hr as the dishes containing the neurones could not be moved during this period because they detach from the substrate. The changes that might occur to neuritic network of the cells when neuronal or neuromuscular synapses are made would give useful insight into the development of the cell in culture. Do the cells make exploratory connections and retractions, do some cells inhibit the development of others? The use of the video systems above could resolve some of these questions.

4.8 **Organisation of Organelle Transport**

The advancing growth cone and the final synaptic connection requires the movement of packaged neurotransmitters, neuropeptides, cytoskeletal and membrane proteins and a system by which that can be effected. This transport has long been associated with microtubules and their links to vesicles (Allen 1975), mitochondria (Raine 1971), secretory granules (Supernant and Dentler 1982). Transport can be anterograde (from the soma

down the axon) or retrograde (back to the soma) and the link between microtubules and organelles are microtubules associated proteins(MAPs). Secretory granules attach to microtubules (MT) in the presence of cAMP but are released when exposed to magnesium-ATP (Supernant and Dentler 1982).

The final combination of filaments that make up the cytoskeleton is yet to be determined, but the present state of knowledge suggests a structure whereby microtubules and microfilaments are linked by MAP2 proteins; MTs linked by by a bridging protein, dynein; MAP1 is associated only with MTs, and microfilaments are connected by an actin binding protein, spectrin (Fulton 1984). Dealing with the complex variations of these proteins is beyond the needs of this work, however workers in this field have shown that named proteins are homologues acting in different parts of the cell transport mechanism.

In the general arrangement of organelle transport MAP1C is a cytoplasmic form of dynein. Dynein, a force generating protein which causes MTs to slide against each other, is involved in retrograde axonal transport. The equivalent protein in anterograde transport is kinesin (Vallee,Shpetner and Paschal, review 1989). The models Vallee et al propose are that organelles have the receptors of either dynein or kinesin, or that they contain both and only one is activated depending on the direction of movement. The bi-directional movement is of major importance in balancing the supply of materials to the growth cones. Growth cones can influence development depending on the surface or substrate and in neural cells the growth cone can be remote from the cell body. Unless the growth cone controls the production of proteins in cells it is reasonable to assume that the cell manufactures the proteins which are translocated to the active site and the excess is returned by the retrograde system.

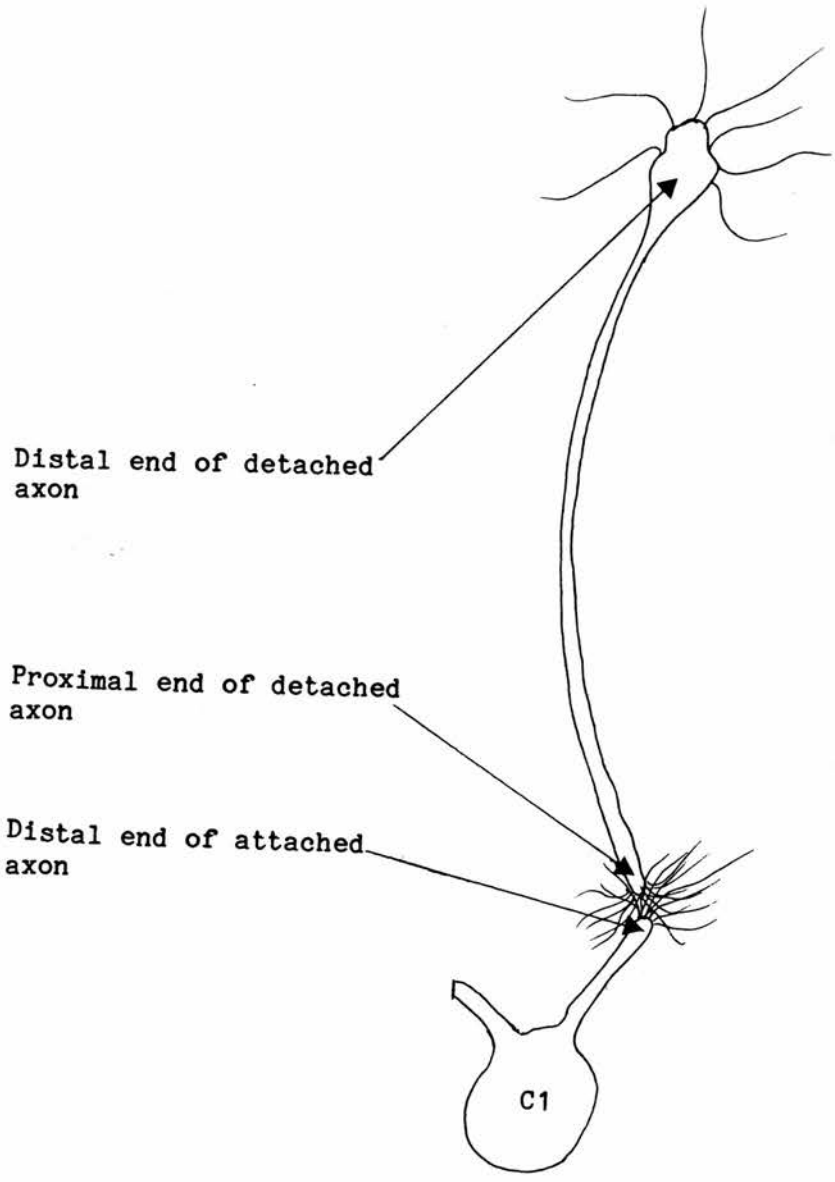
4.9 **Microtubule Orientation in Transport**

The polarity of microtubules are of major importance in the movement of organelles and in differentiating axons and dendrites. Microtubules have (+) and (-) ends which in axons are all arranged with the (+) end away from the soma but in dendrites are 57%(+) and 43%(-) (Black and Bass 1989). The significance of this for anterograde movement is the limitation of which organelles are transported down the axon or dendrite. Mitochondria and ribosomes, which are attracted to the + end of microtubules, are transported in axons and dendrites, while Golgi elements, which are attracted to the - end, are transported only in the dendrites.

4.10 **Microtubule Orientation in Developing Neurites**

Microtubules in mammalian neurites are found to reverse polarity when the neurites are severed. If a growth cone is severed from a cell with part of the neurite attached the growth cone will collapse and the neurite will form a bead at the cut end. A growth cone would redevelop from this cut end after about 20 min(Baas,White and Heidmann 1987). The microtubules at at the beaded end of the neurite which previously had a (-) orientation were now found to be reversed. Neurites, attached to growth cones, but which did not collapse maintained the normal polarity and no growth cone would form from that end. The authors suggested that the control of microtubule assembly occurs within the neurite.

The sequence of development of the C1(fig 32) leads to interesting questions about the organisation of microtubules in neuritic development. Both axons from the C1 had sprouted neurites. However the longer axon had twisted at one point along its length and this area had a phase bright, rounded appearance typical of collapsed processes. Subsequent development


Distal end of detached axon
Proximal end of detached axon
Distal end of attached axon
C1

showed that this section of the axon had lost its definition and structure, but the growth from the distal end of the long axon continued (see drawing opposite). It could not , at this level of microscopical examination, be certain if the sprouting from the twisted area originated in the proximal end of the detached axon but if it was then it could demonstrate polarity reversal described by Bass et al. The orientation of the microtubules in the axons of Helix neurones is unknown but the experiments of Bass et al did show that in neurites the formation of beaded endings was significant and indicated specific changes in microtubule polarity. If the growth was coming from the distal end of the axon still attached to the neurone i.e. below the break, then the continued development from the distal end of the separated axon shows organisation of growth was within the axon, for this continued its neuritic development for several days. To establish either of these proposals would require further experiments but both possibilities are equally exciting.

4.11 Attachment of Suboesophageal and Identified Neurones

The neurones chosen for study, C1 and C3, had a consistently lower rate of attachment to the substrate than the suboesophageal neurones. Neurones observed under the microscope immediately after dissection could be seen to move in reaction to vibrations in the laboratory. The identified neurones were amongst the largest in the Helix ganglia and it was at first considered that the large volume to substrate contact area would reduce attachment. However there were neurones of comparable size in the suboesophageal ganglia culture and these cells adhere without apparent difficulty. The initiation of neuritic growth in Helix depended on conditioning factors produced from Helix cells. This requirement raised the possibility that other factors were released from the large population of

cells in the suboesophageal ganglia cultures which could increase the attachment rate. This advantage was not available to the isolated neurones, a point also made by Bray (personal communication). However seeding the identified neurones into groups of cells or into miniwells did not improve the attachment rate. The miniwell reduced the volume of medium around the neurone by one thousand times and any growth promoting factors released by the neurone would be contained close to the isolated neurone.

The difference in the methods for isolating the identified neurones and suboesophageal neurones was also a source of variation for attachment rates. The manipulation of the individual, identified neurones required to remove them from the ganglion increased the number of contacts with dissecting instruments. The whole suboesophageal ganglion was taken out and transferred to a culture dish. The axons of the suboesophageal neurones could then be broken by being crushed against the bottom of the dish with a tungsten needle.

It was noted during this work that identified neurones removed retaining a length of axon had a higher attachment rate than those without an axon. This difference could also have contributed to the increased rate of attachment to the substrate of neurones from the suboesophageal ganglia. The isolation procedure for neurones from the suboesophageal ganglia left the majority of these neurones with a length of axon remaining attached. This contrasts with the procedure for identified neurones where the majority had no axon attached. Haydon (personal communication) found that a consistent and increased success rate of viable neurones, isolated from Helisoma, was achieved when these neurones were removed with a segment of the axon remaining attached.

The adherence and morphology of identified and unidentified Helix neurones were influenced by contact with fibroblast-like cells which were established in the culture dishes. The neurones in contact with the fibroblast-like cells adhered, flattened and developed neurites while those neurones, in the same dish but on the substrate alone, were still rounded in appearance (see figure 33). The neurones did not adhere to commercially produced fibronectin or collagen. One experiment was carried out using laminin as a substrate but again these neurones did not adhere. This does not neccessarily mean that the neurones have no receptors for these substrates. The Helix neurones are exposed to trypsin and this enzyme is known to remove fibronectin-binding receptors from mycobacteria (Ratcliff, McGarr, Abou-zeid, Rook, Stanford, Aslanzedeh and Brown 1988).

The explanations for the contrast, for both adherance and development, between neurones in the same culture conditions, where some were on the substrate alone and the others were attached to the fibroblast-like cells might be:

(1) The trypsin treatment had removed the attachment receptors from the neurones and either (a) the fibroblast-like cells facilitated the production of new attachment receptors on the neurone or (b) the fibroblast-like cells readily attach to any attachment receptors that are produced by the neurones, whereas on the substrate alone the neurone might need to produce many of these receptors before there was effective attachment.

(2) The neurones and the fibroblast-like cells had cell attachment receptors that did not involve fibronectin, laminin or collagen.

4.12 Significance of Varicosities Seen in Neurites

Varicosities could be seen in the neurites of the identified and unidentified neurones of Helix. In healthy C1 and C3 neurones, the varicosities were seen in developing neurites, with active growth cones(fig 10a) and also in sessile neurites. The unusual formation of the varicosities on the neurites of a Helix C1 neurone (fig 38, D9) in the presence of bacteria might have been part of a degenerative process. Varicosities are normally continuous with the neurite but in figure 38 the varicosites were protruding, as distinctive structures, from the neurites.

C3 neurones and their neurites which were to be examined for the presence of a FMRFamide-like substance had to be fixed and stained. Varicosities can be artifacts produced when fixation and staining techniques are used. Beading formed in dendrites which were being stained with Horse Radish Peroxidase and electronmicroghraphs showed these beadings contained swollen mitochondria (Brown and Fyffe 1984). Ross,Arechiga and Nicholls (1988) found varicosities developed in regenerating neurites of leech Retzius cells were the result of exposure to ultraviolet light after the cells had been filled with the fluorescent dye, Lucifer Yellow. The C3 neurone and its processes were examined under phase contrast optics prior to fixation and staining and the varicosities could be seen.

Although, as shown above, the varicosities are not produced by fixation and staining they might still be physiological artifacts. The cellular mechanism for the production of the peptide in the C3 neurone, for example, could be active but in culture there is no target cell. The signals for any large release of transmitters are not available so that there is a build up of peptide containing vesicles in the processes. However Kobeirski and Beltz(1987) found FMRFamide-like peptides in the ventral nerve cord and neurosecretory organs of the lobster, Homerus

americanus, and distributed through fine fibres and terminal varicosities which had no immediate targets cells. Neurites from interneurones in locust contain many varicosities wich are sites of electrical imput and output (Watson and Burrows 1985).

Varicosities along an axon serve a function in the transport of axoplasmic material. Edmonds and Koenig (1987) using videomicroscopy showed swellings in the regenerating axons of goldfish retinal ganglion cells. The mitochondria and small organelles were separately identified. In the movement of bulk membrane proteins the varicosities, which carried a mixture of these proteins, were considered as the transport mechanism. Hollenbeck and Bray (1987), by immunofluorescence staining, identified tubulin and neurofilament proteins being transported in varicosities along chick embryo neuronal cells. Rat brain cell in culture displayed processes with varicosities rich in kinesin, a protein responsible for axonal transport (Pfister, Wagner, Stenoien, Brady and Bloom 1989).

4.13 Electrical Activity of Helix Neurones in Culture

Some electrophysiological experiments were made on the Helix neurones in culture. The neurones were those identified in intact ganglion maintained in Leibovitz-15 medium, and also identified and unidentified isolated neurones maintained in culture.

These experiments were made to:

(1) Test if the culture conditions were appropriate for maintaining the neurones.

(2) Establish whether identified neurones with known responses to 5-HT would respond in culture conditions.

(3) To examine if neurones had made synaptic connections.

Resting membrane potential and impulse activity were recorded from isolated Helix neurones in culture conditions. Identified neurones in the intact ganglion, maintained over a period of one to sixteen days, gave repeated spiking activity (figure 6) when stimulated. The C1 neurones had shown their normal response and fired when depolarised (figure 39). 5-HT was applied to the buccal neurones and the C1 neurones in intact whole ganglia (long term maintenance) and to buccal cells isolated in culture. In each case the neurone responded by an increase in rate of firing as was seen with these neurones in situ. The action of 5-HT on the C1 neurone is to reduce the activity of K^+ channels (Barnes 1987) and this is mediated possibly by the second messenger protein kinase C (Hill-Venning and Cottrell 1988). In situ each of the buccal cells had synaptic connections with the C1 (Cottrell 1980).

4.14 **Synaptic Connections in Culture**

The Helix neurones did make electrical connections (figure 40) in culture. When an electrical connection was shown between neurones it was tested to establish that the response was not an artefact produced by the recording conditions. One of the intracellular electrodes was removed from one of the pair of neurones, but left close to the cell surface. The impaled neurone was stimulated and the response which had been recorded in the second neurone was absent. There was no conclusive evidence for chemical synaptic connections but not many experiments were made to test this point. Nevertheless in one or two cases where the response of the follower cell was delayed the connection may have been mediated by the release of a chemical transmitter. More experiments need to be done to

confirm these results.

A group of anterior neurones from the buccal ganglion of Helix (figure 55) presented apparently ideal conditions for synaptic connectivity i.e.many intertwinning neurites and very close contact between the cell bodies but did not produce any conclusive evidence for connections. Work in Helisoma has shown that no direct cell-cell connections are possible after the initiation of neuritic extension(Haydon, personal communication) and that electrical synapses can only form between mutually growing neurites. Individual neurites from the A neurones could be seen extending before and after the recordings were made, it might be that the main growth had stopped and connections from small processes at such distances from the neurone were too weak to record. The age of the animals could have an effect. In this work the buccal neurones were from adult Helix. Neurones from adult Aplysia in culture had greatly reduced synaptic connections compared with juvenile neurones. The presence of juvenile neurones in adult cultures, whether they were target or not, improved the formation of synapses between the adult neurones (Schacher and Flaster 1987). Were the A neurones from Helix approriate cells to form connections ? In the intact buccal ganglia of Helix Cottrell and Macon (1974) could not find electrical or chemical connections between the A,M or P neurones. Arechiga et al (1986) in the leech and Camardo et al (1983) in Aplysia found that the synaptic connections between neurones were not random but specific.

The percentage of connections formed between neurones in culture can vary considerably depending on the time in culture and the type of connections formed i.e, electrical or chemical. Arechiga et al (1986) found in leech neurones while electrical connections formed easily, chemical synapses was present in only a few of coupled neurones out of

hundreds tested. Where Helisoma neurones were in cell-cell contact Haydon (1988) showed over half of the pairs had formed electrical synapses but no chemical synapses were present in the first two days. On the third day all coupled neurones had made chemical synapses. In cultures of molluscan neurones, where none of the neurones were identified, Arshavaskii et al (1987) found only one fifth of neurones tested had made synapses.

No connections could be established between C3 neurones and the tentacle muscle cells even after five days in culture. The Ach receptors were removed from the muscle cells by the initial dissociation procedure (Ishii et al 1982) and a subsequent refinement of the technique to remove tryptic activity (Ishii et al 1986) did not produce the conditions for connections.

A possible explanation could be the use of Con A as a substrate. The cholinergic sites of the neuromuscular junction are blocked by Con A(Alman and Appel 1976). In receptor extracts from the normal and denervated leg muscle of the rat Con A, at concentrations of 10^{-9}M blocked 50% of the receptors and at 10^{-7}M caused complete block. These workers showed that the binding of α-bungarotoxin (a known Ach receptor blocker) to the Ach receptor could be prevented by prior exposure to Con A. In this work on Helix neurones the Con A is not free in solution but the area of the muscle cells which was attached to, or was close to the substrate might have had its available receptor sites filled at the level of the growth cones approach. The extreme adhesiveness of Con A, which is an advantage when attaching the large neurones, could have inhibited vertical exploratory behaviour of the growth cone. Sites available on the side and upper surfaces of the muscle cell might be beyond the reach or pulling power of the filopodia.

The effect of Con A on muscle has been described above and its effects on other types of connections described in the introduction. To examine synaptic connections in culture the substrate and the neuritic development are clearly important. To eliminate the influence of Con A other substrates could be used. To remove the need for, and difficulties of, substrates or neuritic connections there is another approach. Haydon(1988) co-cultured Helisoma neurones, that make synaptic connections in situ, in conditions that inhibited cell-substrate adherence and neuritic growth. Neurones placed together for three days formed soma-soma chemical synapses. Alternatively these neurones were kept seperately for three days and when put together formed chemical synapses within a few seconds.

4.15 **The Membrane Potential**

Neurones from Helix had lower resting membrane potentials in culture conditions than those in situ. Studies in this laboratory showed that neurones in culture had membrane potentials with a mean of -47 ±12mV compared to neurones in the intact ganglion with a mean value of -59 ± 8mV (Greene, Powell and Cottrell 1989). The membrane potential had become depolarised or less negative.

Neurones which are unhealthy can have low membrane potentials. However the neurones used appeared normal. The soma was well defined and phase bright. The neurites had been developing, with active growth cones. Neurones that were monitored after recordings were made continued to extend neurites. The damage caused by impaling the neurone with the recording electrode can affect the membrane potential. This is usually a short lived effect caused by leakage of ions in and out of the cell where the membrane is damaged. The neurone recovers as the membrane seals around the

electrode.

The digestive enzymes used during dissection can effect the membranes of cells. In this work the neurones covered by a thin layer of connective tissue had been exposed to Pronase. Subsequently the connective tissue was removed and the neurones treated directly with trypsin. Hofenmann and Miller(1967), looking at the effect of digestive enzymes on connective tissue and any changes this might cause in the electrical properties of Aplysia neurones, used concentrations of Pronase twice that used in this work. They found it did not change the responses.In isolated Helix neurones (but not in culture) exposed to 0.1% trypsin for a short period of time(4 min) the inward current of sodium was not affected. Increasing the time of exposure to 10 min reduced both the inward currents of calcium and sodium (Lee,Akaike and Brown 1977). In the present study neurones were exposed directly to trypsin for much longer periods (60-90 min).

The incorporation of phospholipids into the membrane of epithelial cells was reduced when exposed to trypsin (Kirkpatrick, Melzner and Goeller 1985). Phospholipids have an important part to play in controlling the permeability of the membrane (Hall and Baker 1977).

Segel(1987) showed that trypsin could raise levels of cAMP in rat thymocytes. The second messenger cAMP activates a specific protein, protein kinase C, within the cell. This protein interacting with phospholipids in the cell membrane influences the structure of ion channels in the membrane (Kaczmarek 1987). When a cell fires the membrane is depolarised and it is the activation of potassium channels to allow the efflux of potassium from the cell which returns the cell membrane to resting potential. Certain types of potassium channels are closed by the

action of cAMP and raised levels of cAMP within the cell could lead to prolonged depolarisation of the cell membrane (Smith 1989). The effect that trysin might have had on Helix neurones should, however, have been short lived as trypsin inhibitor was used to inactivate it.

There is another external factor which might have a long term effect on the ionic mechanisms of the membrane. This effect could come from cells removed with the neurones during dissection (e.g. figure 33). The migratory behaviour of these cells, which is not a characteristic of adult neurones in culture, suggested they were glial cells (see example in figure 45 and section on non-neuronal cell above). Glial cells are closely associated with neurones and other cells and one of their functions is to control extracellular levels of potassium. This is done by taking up potassium released from the neurones and slowly re-releasing it. The membrane potential is governed mainly by the potassium concentration (Kuffler,Nicholls and Martin 1984). An increase in external potassium can cause the membrane to become depolarised. There is a wide range in the change in potassium concentrations required to alter the membrane potential. Increasing the external level of potassium threefold caused a large depolarisation of 20mV in neuroblastoma cells within an hour (Anglister et al 1982). However this reponse was reversed when the potassium level was returned to normal. In leech a five times increase in external potassium concentration causes a depolarisation of 5mV (Nicholls and Kuffler 1964).

The concentration of potassium in the medium for maintaining Helix neurones was never altered from 5 mM. However glial cells, for example from the leech ganglion, can accumulate a potassium concentration of 110 mM (Nicholls and Kuffler 1965). Isolation in culture might create a novel

relationship between the neurone and glial cells. The continuing attachment of glial cells to neurones in culture could cause localised concentration and action of potassium unrelated to the levels of potassium in the medium.

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