

# **A radical reassessment of the cellular structure of the mammalian nervous system**

by Harold Hillman

(Unity Laboratory of Applied Neurobiology, 76, Epsom Road,  
Guildford, Surrey, GU1 2BX, U.K. E-mail:

[harold.hillman@btinternet.com](mailto:harold.hillman@btinternet.com) (Telephone: +44(0)1483 568332).

The current consensus is summarised. Much of it is shown to be wrong, because it is based on histological and electron microscopical procedures, the effects of which have been recognised by histologists, but largely ignored in interpretation of micrographs. A revised structure based largely on observations of unfixed neurons and neuroglia by phase contrast microscopy is proposed.

## **Current consensus about the cellular structure**

The brain has a cortex, the 'grey matter' on the outside, and the 'white matter' deep to it. In the spinal cord, the white matter is on the outside and the grey matter is deep to it. In the living and fresh tissue, the grey matter is pink due to the presence of blood in very small blood vessels. The white matter is intensely white due to the predominance of axons and fibres. The apparent greyness of the grey matter in pathology specimens is due to the long term preservation which makes the blood appear blue or black and the cortex grey.

The brain and spinal cord consist of neurons surrounded by neuroglial *cells*, with very many nerves coursing through it in all directions. Many fibres are in bundles or tracts. Each neuron has a cell body, out of which arise main dendrites. Like the stem of a plant, many dendrites arborise from the axon, with smaller branches arising from the larger dendrites. These fine dendrites join pre-synaptic fibres attached to synapses adhering like limpets to the surface membranes of other neurons. A main, larger fibre, an axon, arises from the cell body and travels a long distance until it divides, to be attached to synapses on other cell bodies, or neuromuscular junctions on muscle fibres.

The neuroglial cells fill the space between the neurons. The neuroglia is believed to consist of three different types of *cells*, namely astrocytes, oligodendrocytes and microglia. The astrocytes have long processes and several synapses on neurons; the oligodendrocytes have large nuclei and rather few processes; the microglia have no processes and appear in sections as lone nuclei.

I now present a critique of the above consensus followed by a revised view of the cellular structure of the nervous system. However, it is first necessary to review the procedures which were used to arrive at the currently accepted views.

### **Histology, histochemistry and electron microscopy**

All these procedures involve the following steps: killing the animal or obtaining a biopsy or a post mortem specimen; excising the organ, biopsy or specimen; cutting a small piece; fixing it; dehydrating it; embedding it; cutting sections; rehydrating them; staining them; mounting them permanently; examining them under the microscope. The electron microscopist coats the tissue with salts of heavy metals, because, --unlike living tissues -- these salts are not affected significantly by low pressure, electron bombardment, high temperature and X –irradiation. Living tissue could not survive such assault. The electron microscopist examines mainly this metal salt deposit (Weakley, 1972).

Histology developed in the 19th century as a branch of pathology. The pathologists, who were generally physicians, compared the appearances of diseased organs from patients who had been diagnosed clinically, with the appearances of the same organs of healthy human beings, in order to diagnose illness. They knew very well that nuclei were not violet and that cytoplasm was not pink. Their original purpose was diagnosis of disease, but histology was gradually employed to elucidate the structures, physiology and biochemistry of normal tissues (Hillman, 2000).

These three procedures use reagents and manoeuvres quite empirically. The concentrations of fixatives, dehydrating agents, embedding agents, stains, differentiating agents and mounting agents, as well as the time to which the tissue is exposed to them, are decided largely empirically. Relatively few experiments have been carried out to examine these effects of

variables involved (Mann, 1902; Baker, 1958; Kushida, 1962; Stoward, 1973; Bancroft and Stevens, 1977; Chughtai, Hillman and Jarman, 1987; 1988).

Histology, histochemistry and electron microscopy, suffer two fundamental difficulties. Firstly, the reagents cause considerable shrinkage, and also exchange with the constituents of the tissues. The volume of the tissues and therefore the concentrations of all the solutes change during the procedure. Thus the chemistry of the stained tissue is very unlikely to be the same as it is in the parent tissue. Secondly, but equally seriously, a thin *section* is cut from the block of the tissue, whose volume has already changed from the original. The thickness of the tissue is measured by the advance of the microtome, but this is not accurate. The block is compressed when it is cut, and the section expands when the pressure is removed from it. When a section cuts through an organelle, one has no way of knowing from which direction the blade has come. We cut three boiled eggs vertically, diagonally and horizontally in the same relative thickness to a whole egg, as is used when sections are made of nervous tissue, (Hillman, 1986, p. 51). This showed that from a single section, one could *not* tell the following: the dimensions of the cell or organelle; its shape; its incidence in a cell; its absorption of light; whether it is present; its position; whether it had moved; its rate of movement; whether it is a natural structure in the cell or an artefact of preparation; of what chemicals it is composed; its metabolism; the concentration of any constituents in life. It is optimistic to think that one could elucidate such information by carrying out measurements on histological slides, if one knew the thickness of the sections and the measured dimensions of the organelle, as they appear in the sections, and the changes of dimensions which occur during preparation.

The following other effects occur in tissue during preparation:

- (i) dehydration causes osmotic effects which cause movement of water and solutes, and changes in compartmental volumes;
- (ii) considerable heat is generated at the cutting edge of the microtome knife, whether it cuts continuously or vibrates;
- (iii) as indicated above, cutting compresses embedded specimens;
- (iv) freezing causes shrinkage, high-pressure, dehydration and sometimes cracks. It may form sharp crystals which may cut tissue, and it sometimes denatures proteins;

- (v) pressure on a section is increased when the cover slip is applied.

### **Electron microscopy**

Electron microscopy has its own particular problems:

(a) as indicated above, the electron microscopist looks at the heavy metal deposited on the tissue. Any structure which is soluble in any of the agents used, or has no affinity for the heavy metal salts, or is broken down by them, will not be seen;

(b) the salts used are powerful and toxic, and denature proteins, including enzymes; they inhibit metabolism;

(c) an enormous amount of heat is dissipated in the specimen when it is bombarded by electrons. The section may be burnt-up;

(d) living tissue cannot be viewed in the electron microscope;

(e) freezing is sometimes used in electron microscopy to avoid chemical fixation, although sometimes the fixatives are used later on in the procedures.

If one looks by light microscopy at a living tissue or unfixed unstained cells, one is looking at *tissue*. If one looks at a histological section one is looking at tissue, *minus* constituents which dissolve in the reagents used *plus* the artificial reagents, which are used to stain it. If one examines an electron micrograph, one is looking at a heavy metal deposit *plus* some of the reagents used; little tissue is present. It is useful to compare a living tissue with a histological or electron microscopic section

#### ***Living Cell***

#### ***Tissue studied***

respires

does not

intracellular movements

none

membrane potential

not polarised

structures exist in 3 dimensions

many structures only seen in 2 dimensions

changes induced are reversible

changes are not reversible

electrically excitable	not after histology, histochemistry or electron microscopy
intermediate metabolism	no metabolism
slightly variable in volume	swells
resists infection	becomes infected
can heal	cannot heal
does not denature	denatures
cytoplasm and nucleoplasm are fluid	both precipitate

Table 1. These are very significant differences.

### **Analysis of histology, histochemistry and electron microscopy**

Optimally, one would like to study the cell biology of the nervous system in the living intact animal (Table 1). Attempts have been made using reflected light, and confocal microscopy, to examine brains and spinal cords in situ, but this has not yet been done successfully, except with sciatic nerves. The probable reasons are that the tissues are too translucent and the membranes do not reflect much light.

However, the next best approach is to separate the tissue using as little energy as possible, and examine it in an environment as closely as possible mimicking its natural extracellular fluid.

The following minimum steps occur in histology;

- (a) the animal is restrained, and then killed, preferably with minimum pain;
- (b) the tissue undergoes agonal changes;
- (c) the nervous tissue is excised;
- (d) the part of the tissue is separated;
- (e) the cells or tissues are characterised optically;
- (f) they are photographed.

These steps may be examined individually. Killing an animal changes its biochemistry grossly. For example, its blood carbon dioxide, phosphate, lactate, and potassium ion

concentrations, rise, while its oxygen, sodium ion, adenosine triphosphate, phosphocreatine, concentrations go down. These changes affect much of the tissue metabolism. It is hoped and normally assumed that they will reverse during incubation. There is no realistic way of testing this, since the volume and chemistry of the tissue changes during incubation. In this circumstance, it is worth asking whether cell biologists should use tissues in vitro at all. Perhaps, they should confine their experiments to working on intact animals and human beings, tissue cultures, unicellular organisms and plants.

Teasing tissue breaks connections and also separates compartments which were formerly adjacent. However, it is extremely unlikely that separating parts of cells, which were previously adherent to each other, would not affect the permeability of their membranes. Nevertheless, the quantity of energy injected into tissues by homogenisation and centrifugation is likely to be orders of magnitude greater than is used in teasing or micromanipulation.

Microscopy of cells subjects them to a great deal of light energy, much of which is dissipated in the structures in focus. This may break down volatile compounds, and also cause or accelerate intracellular chemical reactions. Photography is an important aid to microscopy, but, nowadays, electronics is often used to enhance micrographs, to highlight features, and to eliminate imperfections believed to be artefacts, etc. Electronic circuits 'improve' micrographs, but it would be useful if microscopists and bio-ethicists would discuss this practice to clarify its morality.

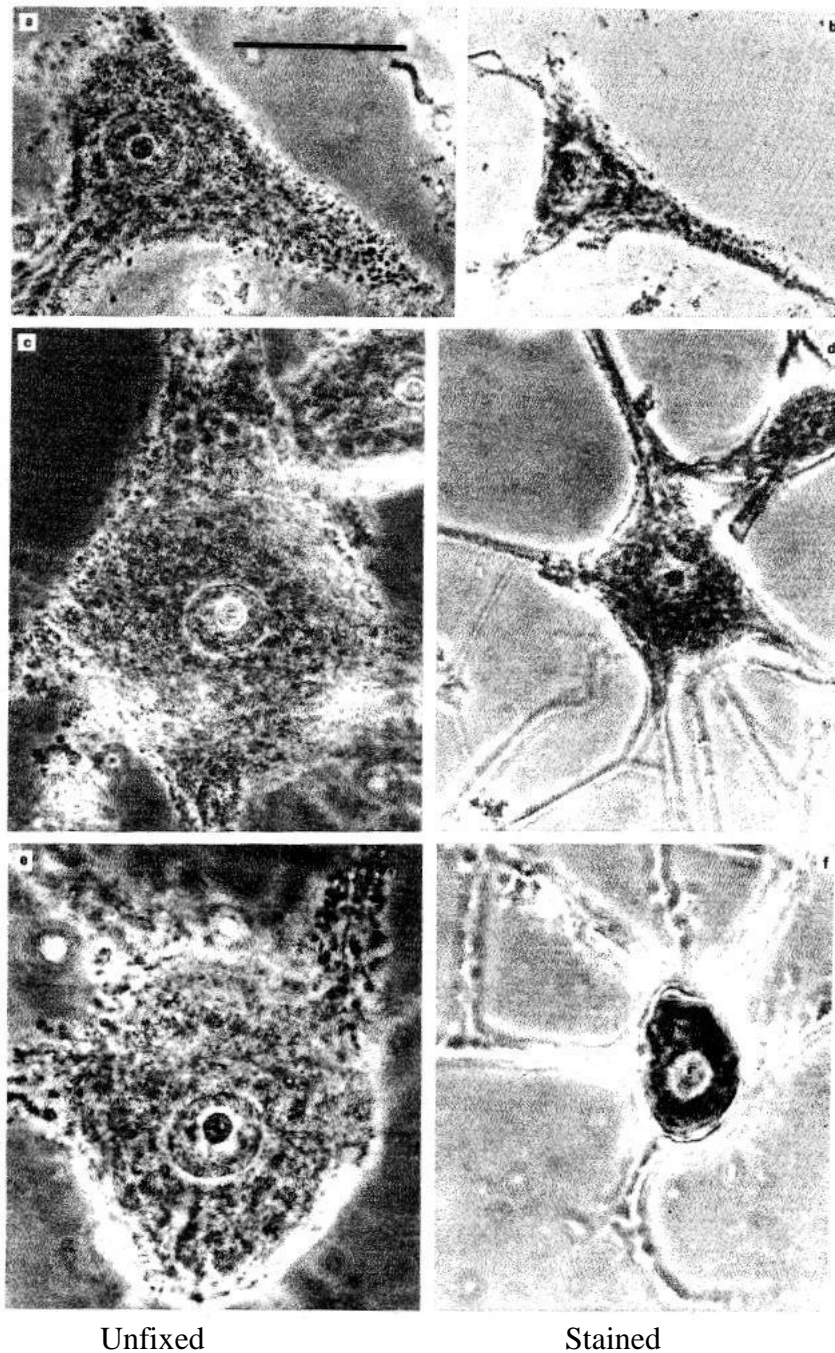


Figure 1. Rabbit medullary neuron cell bodies isolated by hand, *a*, before, *b*, after haematoxylin and eosin staining; *c*, before, and, and *d*, after, Palmgren's staining; *e*, before, *f*, after osmium tetroxide staining. The bar is 50  $\mu$ m. This figure is from Chughtai, Hillman and Jarman (1987).

### Effects of staining

We examined directly what staining procedures did to the apparent structure of cells. We dissected out rabbit medullary neuron cell bodies using the procedure of Hydén (1959), and we

took advantage of his finding that isolated cell bodies attach firmly to clean microscope slides. We placed these cells in parallel-walled chambers with channels, through which the agents could be introduced, and drawn across the tissue, using filter paper from the other side. We viewed the single cell bodies by phase contrast microscopy until they were stained, when we switched over to bright field. We could see each reagent arriving as we introduced it from alternate sides, and we photographed the cell body at each stage. We studied haematoxylin and eosin, the commonest procedure used in histology: Palmgren's, widely used in neurology, and osmium tetroxide, extensively used in electron microscopy. All the three staining systems caused the areas of the cells to considerably: haematoxylin and eosin *to* 20% of their original areas, Palmgren's *to* 19%, and osmium tetroxide *to* 14% (Chughtai, Hillman and Jarman, 1986) (Figure 1 here). Peripheral nerves with visible axoplasm shrink so much that they looked like non-myelinated fibres (Chughtai, Hillman and Jarman, 1987) The shrinkage of the cytoplasm was greater than that of nuclei, probably because the former contained more water.

One must be a little cautious in interpreting these results. It is likely that a neuron cell body in life is near spherical. When it adheres to a microscope slide, it appears to collapse like a fried egg. Changes from the latter may not be the same as those from the original shape. This procedure for watching the effects of reagents on cells could possibly be used on other cells, such as hepatocytes and kidney tubule cells, which keep their shapes better.

### **The likely structure of the neuron in the living intact mammal**

The cell body of a large neuron in life is probably up to 120-150  $\mu\text{m}$  in maximum diameter. Its lower limit is not known, as little attention has been given to small cell bodies in the cortex; they cannot be dissected out. Neurons in the tissue culture pulsate slowly all the time, but it is not known if they do in intact animals. They each possess one long nerve fibre which is designated the 'axon'. It may be millimeters to metres long, and its distal end breaks up into fibres which end near, but not on, other nerves; they join neuromuscular junctions. The cell body is more spherical and convex, rather than the concavity in which it is usually represented, which is caused by the shrinkage during staining. The cell is covered by a single membrane, which is not 'trilaminar' or 'unit'. Protein molecules, glycolipids and glycoproteins do not protrude from its surface. Nor does it have receptors, antigens, transmembrane molecules,



transport enzymes, carriers, ion channels, within or near it. All of these macromolecules are large enough to be seen by the electron microscope, but are not seen at all. The evidence for them comes from experiments using subcellular fraction, which is a procedure which ignores the second law of thermodynamics (Hillman, 1972). Nor has any explanation been offered by electron microscopists, pharmacologists, cell biologists or biochemists for the invisibility of these macromolecules (Hillman, 2008, Ch.20). So I think the conclusion must be that *either* they are not present in or near the cell membranes, *or* that the electron microscope fails to detect them. I believe the former to be the case.

The chemistry of the membranes of the neuron has been assumed to be composed of lipid and protein since the classic experiments of Gorter and Grendel (1925), and the lipid bi-layer hypothesis of Davson and Danielli (1936). This is a very interesting hypothesis originally worked out for red cell membranes and since assumed to be true for all cell membranes.

The orientation of the molecules within the cell membranes is pure speculation; there is no way of proving it, however many elegant diagrams are drawn of it. The finding that one face appears hydrophobic and the other hydrophilic is not enough evidence upon which to base current models.

Meanwhile, one only knows for certain, that the cell membrane appears to consist of one layer of unknown thickness or chemistry. The diameter of 7-10 nm given in textbooks is measured from electron micrographs, and completely ignores any shrinkage of the membranes (of which the two dark lines and one light line are believed to be composed), during the preparation for electron microscopy.

The membranes of cells including neurons are believed to be perforated by tubular ion channels. These channels are believed to open for particular ions, at particular voltages, particular delays and other parameters. Despite the fact that 30-40 channels have been reported in the literature (Conley, 1996), only *one*, the acetylcholine sodium channel, has ever been claimed to have been detected by electron microscopy (Unwin and Zampighi, 1980; Toyoshima and Unwin, 1988). Even the latter represents a construction based on electron microscopy, not a direct image. The same is true of the model by Kistler et al. (1982). Neither Unwin, nor any of the other channellers have explained why they so rarely see these allegedly ubiquitous structures.

Thousands of receptors are also believed to be present on, or in, the cell membranes (Burgen and Barnard, 1992; Lauffenberger and Linderman, 1993; Kennakin and Angus, 2000). Many of these receptors have been isolated and sequenced, and their size is such that they are within the resolution of the electron microscope. There are also believed to be many transmembrane molecules. One may say quite categorically that neither the membrane receptors, nor the transmembrane molecules, nor the transporters are seen by electron microscopy. One possibility is that during the preparation for electron microscopy the macromolecules have moved to different locations in the cell, but where? If one were to entertain this possibility, it would immediately throw doubt on any localisation of a macromolecule or structure by electron microscopy. There seems to me to be much simpler hypotheses, including: (i) the ligands are not reacting with the receptors; (ii) the macromolecules are only formed as a consequence of the procedures for their isolation; (iii) receptors do not exist. My hypothesis is that the receptor property represents the molecules on the cell membrane, the cytoplasm, the mitochondria and the nucleus which react with the ligands under the conditions of the experiment. I beg pharmacologists and biochemists to offer another explanation for the failure of their favourite macromolecules to show up face to face with the microscopists. Under the electron microscope, the cell membranes of neurons appear to be as smooth as an angel's cheeks.

The cytoplasm of the neuron is continuous with that of the axon and dendrites, since one cannot see any barriers between them. The cytoplasm contains mitochondria, which can be seen in living cells, but the following other structures are seen only in two dimensions on electron micrographs, and are artefacts: the endoplasmic reticulum; the cytoskeleton; contractile systems; mitochondrial cristae. Lysosomes, peroxisomes and phagosomes are not seen in unfixed cells. The Golgi apparatus, which was detected when neurons of barn owls were stained with silver stains (1898), is an artefact (Hillman and Sartory, 1980, p,63-66). (Table 1). The two dimensionality of these structures on electron microscopy does not seem to have been commented on in print, but privately electron microscopists have asserted that the micrographs used to illustrate these structures have been chosen from the clearest images available and these are obviously those which cut the plane of section normally. In response, I have repeated my plea in private conversations, in correspondence, in lectures and in publications, for anyone to provide me either with published references, or with individual micrographs, showing the full range of

orientations of each of these structures in *single* micrographs. So far, (2011), there has been no response.

Observations on living cells in tissue cultures in unicellular organisms, in axons, and in plants, show the following intracellular movements: Brownian movement; streaming; movements due to convection; diffusion; phagocytosis; pinocytosis; diapedesis; meiosis and mitosis. Furthermore when particles of diameters, 0.5-5  $\mu\text{m}$ , are injected into the cytoplasm, they appear to move freely, and not be deviated by transparent or invisible cytoplasmic bodies. The movements above are visible by low power microscopy of x200 to x400, and they would not be able to occur in the presence of all the cytoplasmic furniture mentioned in the last paragraph. Measurements by electron microscopy indicate that these structures are beyond the resolution of the light microscope. However, there are several methods of light microscopy, some of them claiming to achieve magnifications beyond the Abbe limit. These include differential interference contrast; confocal; tandem scanning; quantum dot microscopy; and lensless. These methods should enable microscopists to demonstrate the reality in life of some or all, of the structures, which I assert to be artefacts (Table 2).

### **Artifacts of the nervous system**

<i>Artifact</i>	<i>Why it is an artifact</i>
Trilaminar 'unit membrane'	Impossible solid geometry
Ion channels	Extremely rarely seen
Membrane receptors	Not seen by electron microscopy
Transmembrane molecules	Seen only in diagrams
Membrane carriers	Not seen by electron microscopy
Endoplasmic reticulum	Could not allow intracellular movements
Golgi body	Rarely seen in unfixed cells
Cristae of mitochondria	Impossible solid geometry
Molecular motors	Not seen even by electron microscopy
Myelin lamellae	Impossible solid geometry
Nuclear pores	Impossible solid geometry
Synapses	Impossible geometry

Synaptic vesicles

Too uniform in diameter

Table 2. With the exception of the Golgi apparatus, these artifacts were found by electron microscopy. Structures seen in unfixed cells, which are not seen in stained sections of central nervous system, are fine granular material, nucleolonema, nucleolar membranes.

If the above structures are, indeed, artefacts, how may they arise?

(i) The unit membrane *appearance* of the cell, nuclear and external mitochondrial, membranes - as opposed to the membranes *themselves* - is easily explained. A line is a geometrical abstraction, because it has position but no thickness. Any real membrane has two surfaces. Stains used in electron microscopy deposit on both surfaces, so that if one cuts any real stained membrane and magnifies it sufficiently, it must appear as two lines. The uniformity of distance apart or of the two 'laminae' arises because the electron bombardment explodes the tissue under the state, leaving a groove (Hillman, 2008, p.58);

(ii) many of the reagents used in staining for electron microscopy are very near their solubility products. So many and heterogeneous are the agents used and the constituents of the tissue, that it is extremely likely that during the lowering of pressure in the electron microscope or during the electron bombardment, some of the chemicals in the region will precipitate, and deposit. There would be considerable difficulty in distinguishing the latter from cell structures;

(iii) most of the mass of cells is cytoplasm and nucleoplasm, containing proteins, carbohydrates, fats and salts. When they are dehydrated, those constituents which were not dissolved *or* suspended in water, precipitate or crystallise. The latter would be part of the apparent cytoplasm and nuclei of stained tissue;

(iv) freezing techniques are sometimes used instead of chemical fixation. Early cryobiologists examined frozen solutions of glycerine, amino-acids and salts. These appeared in beautiful crystalline patterns which looked remarkably similar to the cytoskeleton (Luyet, 1960; Meryman, 1966; Mackenzie and Luyet, 1967; Smith, 1961). It is a useful exercise to examine the shapes and properties of these crystalline forms;

(v) the apparent two-dimensional nature of the 'unit membranes', the endoplasmic reticulum, and the mitochondrial cristae, has not formally been noticed, criticised or explained:

(vi) one suggestion about how intracellular movements could occur in the presence of a cytoskeleton is that the mitochondria, lysosomes or peroxisomes, could secrete lytic enzymes which dissolve the cytoskeleton, and then they re-form in real time. While it is just conceivable that these biological particles might contain such enzymes, it is hardly credible to suggest that ground glass, carbon black, or iron filings would do so;

(vii) many biologists believe that intracellular movements are caused by ‘molecular motors’, attached to the intracellular structures. The motors are believed to be macromolecules (Vale and Fletterick, 1997;Vallee, 1998). However, the motors are simply not seen by electron microscopy.

Intracellular movements can occur from the energy imparted by the ambient temperature and from the illuminating light source (Hillman, 2008, p 292-294). For over 100 years, movements have been studied in mixtures of salts, water and oil , called colpoids, (Butschli, 1893; Herrera, 1900; Bastian, 1930, Herrera, 1928; Fox and Dose, 1977). Brownian movements, diffusion, streaming and convection movements may be seen in colpoids. Modern cell biologists should regard it as a duty to exhaust the properties of simple systems, before generating biological theories to explain physico-chemical properties. William of Ockham would have not expected this.

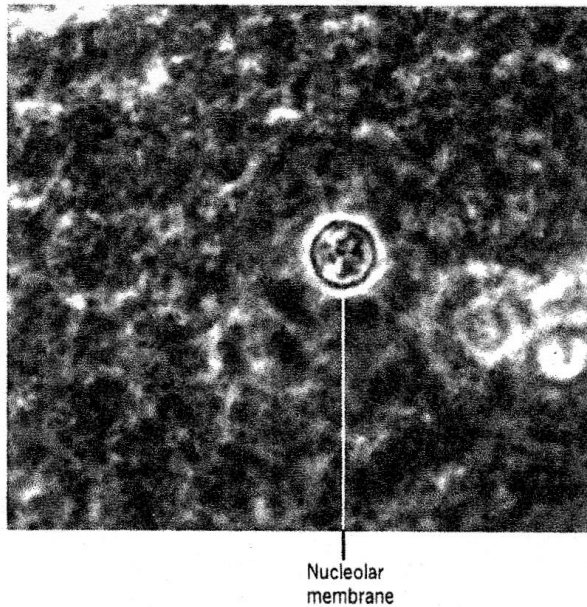


Figure 2. Nucleolar membrane and nucleolonema in isolated rabbit lateral vestibular neuron (phase contrast, x 1200), from Hillman and Jarman (1991).

### **A nucleolar membrane**

Hydén (1959) took out cells bodies in 0.25 M sucrose solution, but, Hinton, Burge and Hartman (1969) showed that sucrose inhibits a number of enzymes, so we decided to dissect out our cells in Krebs-Ringer bicarbonate buffered saline, or in normal saline. When we did this, we noticed that the cell bodies became much more translucent. The nucleolus became much clearer, and the nucleolonema could be seen. This is a strand within the nucleolus first seen by light microscopy in 1951 by Estable and Sotelo. It has almost disappeared from the literature, since the electron microscope was introduced, although it is occasionally seen as a blob. In our unfixed cell bodies, the nucleolonema could be seen moving around the nucleolus slowly, and occasional pieces broke off and escaped into the nucleus (Sartory, Fasham and Hillman, 1971). These movements ceased when 10% formalin was applied to the cells. As the nucleolus could now be seen clearly, we detected around it a nucleolar membrane in neurons from all the rabbit, rat, guinea pig, frog and human cells we examined (Hussain, Hillman and Sartory, 1974; Hillman and Jarman, 1991, pps 23, 24, 53, 54) (Figure 2). We have also shown it as a demonstration at several meetings of the Physiological Society. At one place, J.R. Baker saw it, and agreed with our observation and our interpretation. When the cells are taken out in 0.25 M sucrose, or are

fixed and dehydrated, the nucleolus precipitates, so that neither the nucleolonema nor the nucleolar membrane are seen clearly.

There seems to be considerable resistance to publishing micrographs showing the nucleolar membrane, I believe, mainly from electron microscopists. 'Nature' would not do so, although I offered to send its Editor slides of cells showing it. Two chief editors of different editions of 'Gray's Anatomy', and the Wellcome Museum, initially agreed to exhibit micrographs, then subsequently changed their minds without giving any reason.

I have often been asked, "What is the chemistry and the function, of the nucleolar membrane?" It seems very unlikely that the chemistry will ever be known, because the nucleoli are too small to be harvested in such quantities for microchemical analysis, although there is some scope for analysing them, for example, using optical methods, such as tweezers, and micro-spectrography. Since I have shown that the procedure of subcellular fractionation ignores the second law of thermodynamics (Hillman, 1972; 2009), I do not believe that it can contribute to our understanding of the chemistry of nucleolar membranes, even if they could be collected in bulk.

## **Synapses**

In a living neuron, the cell body is believed to have dendrites issuing from it. These are believed to join fine pre-synaptic fibres, which end as synapses on other neuron cell bodies. Other than on diagrams, (see, for example, De Robertis, 1959), I can find no *micrographs* showing the dendrites continuous with the pre-synaptic fibres joining the synapses decorating the surfaces of neurons. Occasionally, one sees short fibres looking like cilia attached to cell bodies. We showed that the light microscope *does* have the resolution to show up unmyelinated fibres in white matter (Hillman, 1986, pp.116-117). So why can these crucial dendritic-presynaptic connections not be seen? Of course, those in a plane at right angles to the section will be cut off, but those in the plane of the section should be seen radiating like a halo from a saint's head. A synapse which does not have a dendritic connection is like an old fashioned telephone, which is not connected to the telephone exchange. At several hundred lectures in Britain, Europe, Israel, the United States, Thailand and Australia, I have invited members of the audiences to either send me references or micrographs showing these missing links, but so far, none have been sent to

me. Perhaps you can do better? When I challenged a Professor Lieberman at University College, London, who asserted vehemently that he knew of many such micrographs, he told me that he did not intend to do my literature searches for me. We have given other evidence that the anatomical synapses are artefacts of staining. The first illustrations of Held (1897) and Auerbach (1898), showed knobs on the nerve and muscle cell bodies and dendrites, after they were stained with silver stains, but they also showed knobs of similar appearance away from either of these two structures (Figure 3). Such knobs are seen in nervous tissue stained for light microscopy with silver and osmium salts, and they have been ignored. If they are synapses, they must have been displaced during histological preparation. If this explanation is accepted, how can one accept any localisation following a histological procedure? If such black granules are not synapses when they appear away from cell bodies and dendrites, how does one know that the granules in the latter sites, which look similar, are indeed synapses? The salts of silver, osmium, lead and gold are deposits.

It was believed that the 'ultrastructure' of the synapses seen by light microscopy was confirmed by electron microscopy. Unfortunately, authors did not notice that the light instrument showed them to have diameters of 2-8  $\mu\text{m}$ , while the electron microscopists saw 0.5-1  $\mu\text{m}$ . They could not be looking at the same objects. Furthermore, while the light microscopists saw 10's to 1000's, the electron microscopists saw 1000s to tens of thousands (Hillman, 1985). Of course the electron microscopists would see more because their instruments have higher resolution, but one may ask why they never see the larger ones detected by light microscopists.



When isolated neuron cell bodies are examined by transmitted light, they appear rather mottled. We showed that the mottling was *not* due to synapses, but due to mitochondria. The former would be fairly evenly distributed on the outside the cell body, but the latter would appear in much lower concentration over the nucleus, since the nucleus does not contain mitochondria (Hillman, 1986, p.148).

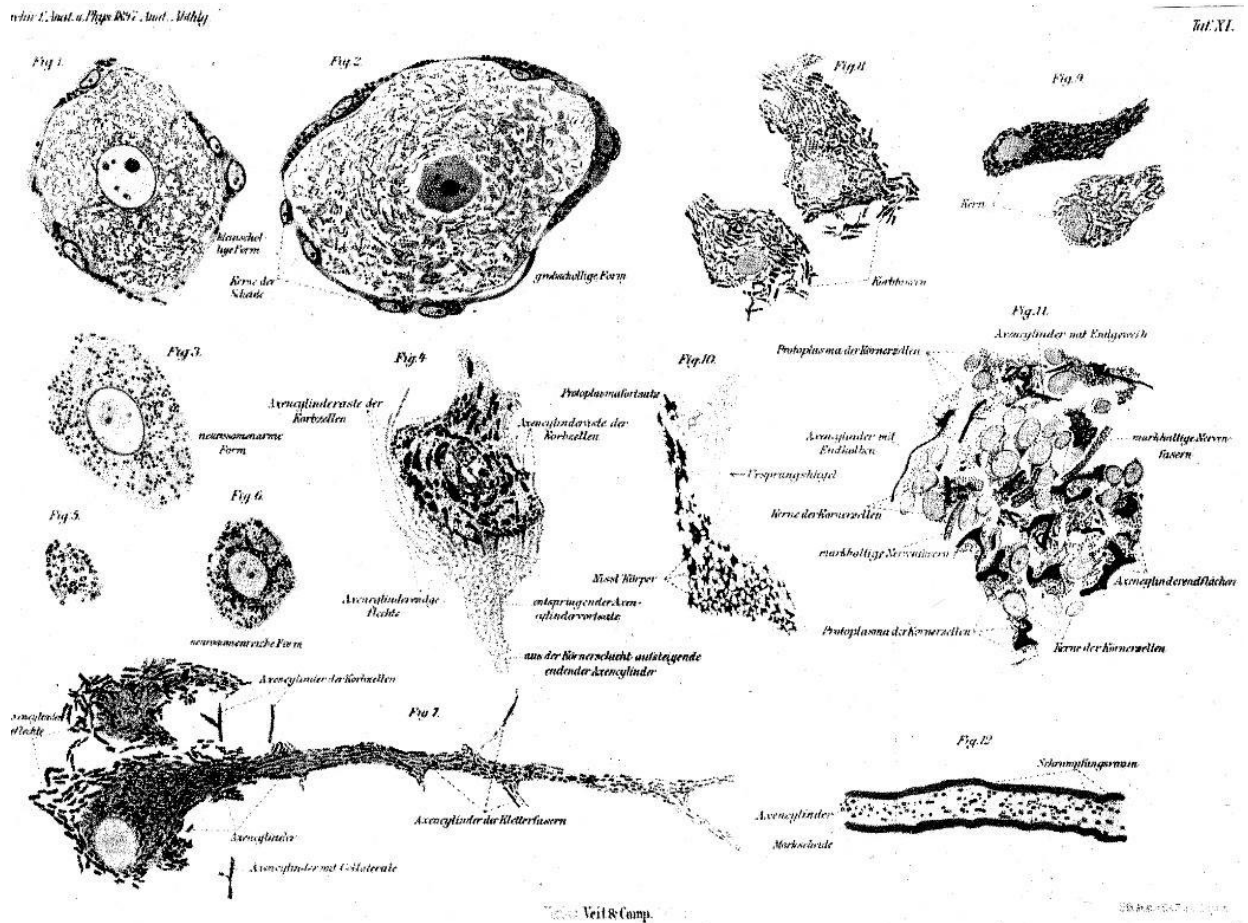


Figure 3. Drawings of neurons from rabbit trapezoid nucleus showing silver stained end-feet, which are considered one of the first demonstrations by light microscopy of synapses. Note particularly figures 1, 2, 3, 7, 8. These illustrations are from Held (1897, his plate XI)

Synaptic knobs seen by electron microscopy appear to have pre-synaptic and post-synaptic thickenings (Palay, 1956; Pappas and Waxman, 1972; Peters, Palay and Webster, 1998). Unfortunately, solid geometry would dictate that if these thickenings were circular or oval, they should be seen in sections normal or face-on in approximately equal frequencies, and obliquely much more often. They are not seen under the electron microscope face-on or obliquely, at all, so

one must conclude that the heavy metal causing the thickness, is two-dimensional and is deposited after the sections have been cut (Hillman, 1986; pp 135-156). That is to say, the synaptic thickenings are artifacts.

Synaptosomes are believed to be a fraction of the central nervous enriched in synapses (Rodriguez de Lores-Arnais, Alberici and de Robertis, 1967; Whittaker, 1972; Pappas and Purpura, 1972). If, indeed, they were spherical bodies, sections of them should result in circles of different diameters. Also, the 'stalks' from where they have been pulled off the presynaptic fibres should be evident sometimes. I can find neither of these expectations from solid geometry either in the literature or under the electron microscope. Can anybody?

In denying the existence of *anatomical* synapses (but not neuromuscular junctions), I am not saying that there is no electrical signal where the nerves approximate to each other, but I am asserting that the microscopic location of the tip of a micro pipette is not accurate enough to say for certain precisely where it is.

If synapses and synaptic clefts do not exist in life, the chemical hypothesis of transmission (Katz, 1969) is thrown into doubt. This is beyond the scope the present discussion, but I have examined it in extenso (Hillman, 1991a,b). My main reasons for rejecting it are that it contains too many unprovable assumptions, and that most of the evidence for it is collected from experiments on neuromuscular junctions, which are nowadays assumed to operate by the same mechanisms as synapses, but are very different (Hillman, 2008, p.246).

## Myelinated and unmyelinated fibres

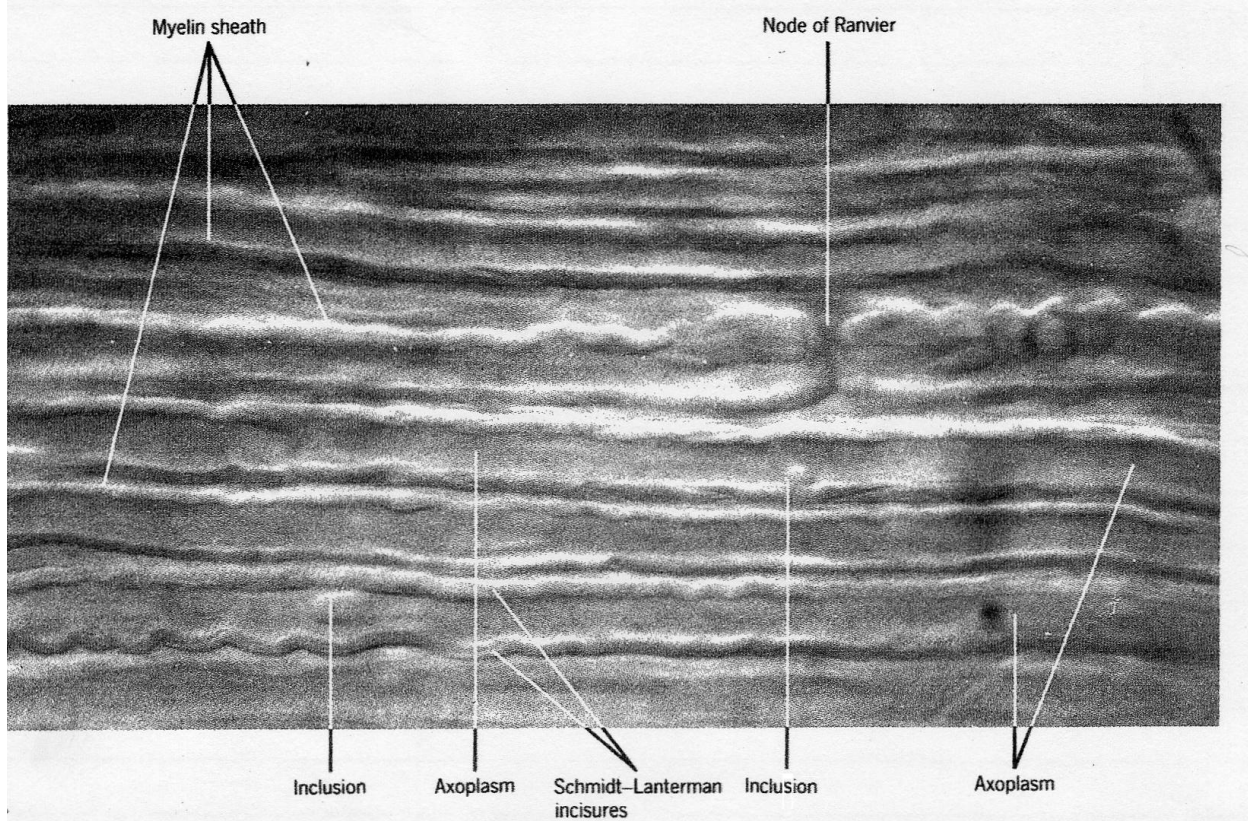


Figure 4. Sciatic nerve of a living mouse *in situ* (incident light, dark ground, x 1000). (By kind permission of Professor Susan Standring). Note the presence of narrow Schmidt-Lanterman incisures and intra-axonal inclusions (Wilson and Hall, 1970).

Myelinated fibres are easily recognised (Ochs, 1982; Landon, 1986). On light microscopy, a single sciatic nerve fibre appears as a tube about 15-20  $\mu\text{m}$  in diameter, the axon, containing a fluid axoplasm (Figure 4). This tube is surrounded by another tube, the myelin sheath, which, at intervals, is narrowed by nodes of Ranvier. It has faults, called Schmidt-Lanterman clefts and a network, the Golgi-Rezzonico apparatus; no one knows what the latter two structures do, but it has been suggested that they enable the fibres to elongate and shorten without breaking. The possibility that they are artefacts resulting from teasing can not be dismissed. High power magnification of living myelinated fibres reveals Brownian movement of particles, both in the axons and in the myelin sheath. Occasionally, spherical inclusions appear in the axoplasm.

On electron microscopy, the myelin sheath on longitudinal section appears to consist of sheets of lamellae, and on transverse section, these appear as circular rings. A spherical or oval nucleus is seen by the side of myelinated axons and it has been called the Schwann cell nucleus. Geren (1954) put forward the idea that during early development, the Schwann cell membrane wrapped itself around the axon several times, and this scroll of membranes formed the lamellae of the myelin sheath. This has been generally accepted. Unfortunately, this model is wrong for the following reasons: (a) under high power phase contrast microscopy, the sheath is seen to have a low refractive index and Brownian movement of particles can be seen within it. If it were full of Schwann cell membranes, it would have a high refractive index and Brownian would not be possible; (b) if the lamellae were cut in the long axis at the maximum diameter at the sheath and axon, they would appear to be equally spaced apart. If they were cut longitudinally they in any other parallel plane, the lamellae should appear splayed. That is, if the lamellae *do* appear equally distant apart, the axon should be clearly visible in its maximum diameter. If the axon is not visible, the lamellae should appear clearly splayed. Neither of these mandatory requirements of solid geometry is obeyed; (c) the Schwann cell membrane is simply not seen on its way towards joining the myelin lamellae; (d) by electron microscopy, oblique views of sections of the myelin sheath should show the individual lamellae at a farther distance apart in the long axis of the section that they do in the short axis. Oblique views of lamellae are simply not seen (Hillman, 1991b). Here, also, is an attempt to defy three dimensional geometry; (e) the myelin lamellae were previously supported by evidence from low angle diffraction studies (Schmitt, Bear and Clark, 1933; Schmidt, 1936), but these were carried out on partially dehydrated nerves; (f) the myelin sheath has a lower impedance and resistance at the nodes of Ranvier, than at the antinodes. It is said to act as an insulator, but this insulation does not seem to be necessary for non-myelinated fibres; (g) the higher impedance and resistance of the anti-node was regarded as evidence for the idea that excitability passes from one node of Ranvier the next without decrement. This was called 'saltatory conduction' (Tasaki, 1939; Stämpfli, 1956). In this model, the node of Ranvier acts as a site of relay of the action potential. However, I think that Kerchhoff's Law would dictate that the nodes would short-circuit the electric signal.

The probable explanation for the myelin lamellae is that the cytoplasm in the myelin sheath is fluid in life, and is dehydrated during histology or electron microscopy. The two

dimensionality of the lamellae means that they were formed after the sections were cut, and they deposited in lines of precipitate on longitudinal section, and in circles of precipitate on transverse section.

If one looks at a myelinated axon, one sees occasional spherical inclusions, sometimes apparently adherent to the axonal membrane, (Figure 4) giving the possibility that they budded off from it. Unidentifiable small amorphous particles are seen flowing along it, and it shows the usual range of intracellular movements. In the unfixed cells, there is no evidence of endoplasmic reticulum, cytoskeleton or lysosomes. Nor, I believe, have electron microscopists reported 'molecular motors' plying their way along the axoplasm.

### **Demyelinating diseases**

Several diseases, such as multiple sclerosis, acute disseminated encephalomyelitis, lead poisoning and optic neuritis, are classified as 'demyelinating diseases', because, in these conditions, their myelin sheaths appear unstained in sections. If one examines the lesions closely, one sees a wider area than the myelin sheaths in which other adjacent tissue does not appear stained. But there is a much larger area around the nerve which does not appear to be stained either. Rather than the lesion being confined to the myelin sheath, the larger unstained area looks as if, in the unstained region, a substance has been excreted by the lesion which has prevented access to the stains of the whole region. The nature of such a possible substance should be investigated in an unfixed lesion and the normal looking tissue adjacent to it. I am not aware of any such studies carried out in fresh, unfixed, untreated lesions.

### **Non-myelinated and Remak fibres**

These are fine, and thus difficult to study, so they have received little attention from neurobiologists.

### **The neuroglia**

When Virchow in 1846 examined brain he noticed a granular ground substance between nerve cells. He named it 'neuroglia,' which means 'nerve glue' in Greek. During the 19th century, mainly before the complex procedures of histology were developed, fine granular material was described by such authors as Leeuwenhoek, Hodgkin, Lister and Valentin, but it

seemed to have rather disappeared in recent years. An account of the history of neuroglia is being submitted for publication (Hillman and Jarman, 2010), but I will give a brief summary here.

Virchow regarded the neuroglia as a connective tissue, containing granules. In the late 19th and early 20th century, the view of its nature gradually changed. When Ramon y Cajal (1906; 1930) and Del Rio Hortega (1932) described astrocytes, oligodendrocytes and microglia, the idea gradually evolved that neuroglia was the name of a tissue which was composed of these three kinds of cells. This if one were able to stain brain or spinal cord simultaneously with stains specific for neurons, astrocytes, oligodendrocytes and microglia, virtually the whole tissue would be stained. We showed this not to be so in the following experiments:

(a) we stained several 6  $\mu\text{m}$  thick sections with the 'specific' stains recommended by the textbooks for each alleged different kind of cell, and then marked the cells in one section, which could be identified in the next. It turned out that between 40% and 50% of cells in each section could be identified in the next (Hillman, 1986, p.102-105),

(b) we also stained sections with stains recommended for each kind of neuroglial cells, scanned the sections with a densitometer, and then added up the areas stained by all the procedures, as if they had been carried out simultaneously. In total, more than half of the areas of the sections did not stain.

(c) We also took isolated rabbit medullary neurons and the neuroglial clumps adjacent to them and stained them each with Mallorys, or with Weil and Davenports, or with Marsland, Glees and Erikson, or with Gallyas's procedures, all recommended for neuroglia, and the neurons showed up just as well (Hillman and Deutsch, 1978).

We concluded from these experiments that neuroglia did not stain in histological sections, but if separated by dissection, neuroglial clumps could do so.

In 1959, Hydén described a simple technique for isolating neurons and adjacent pieces of neuroglia, which he called 'neuroglial clumps'. He regarded the latter as samples of neuroglia, whose properties could be compared to the neuron cell bodies (Hydén and Pigon, 1960). The respiration of the two were compared, so was their succinic dehydrogenase and cytochrome oxidase activities, their auto fluorescence and other properties. The following is a list of most of

the research workers who dissected out and published papers on neuroglial clumps: H. Hydén; A. Pigon; A. Bäckman; I. Augustsson ; A. Hamberger; H. Hillman, P. Sartory, K. Deutsch, T.S. Hussain, J. Allen, I. Chughtai, D. Jarman, L. Hertz and others.

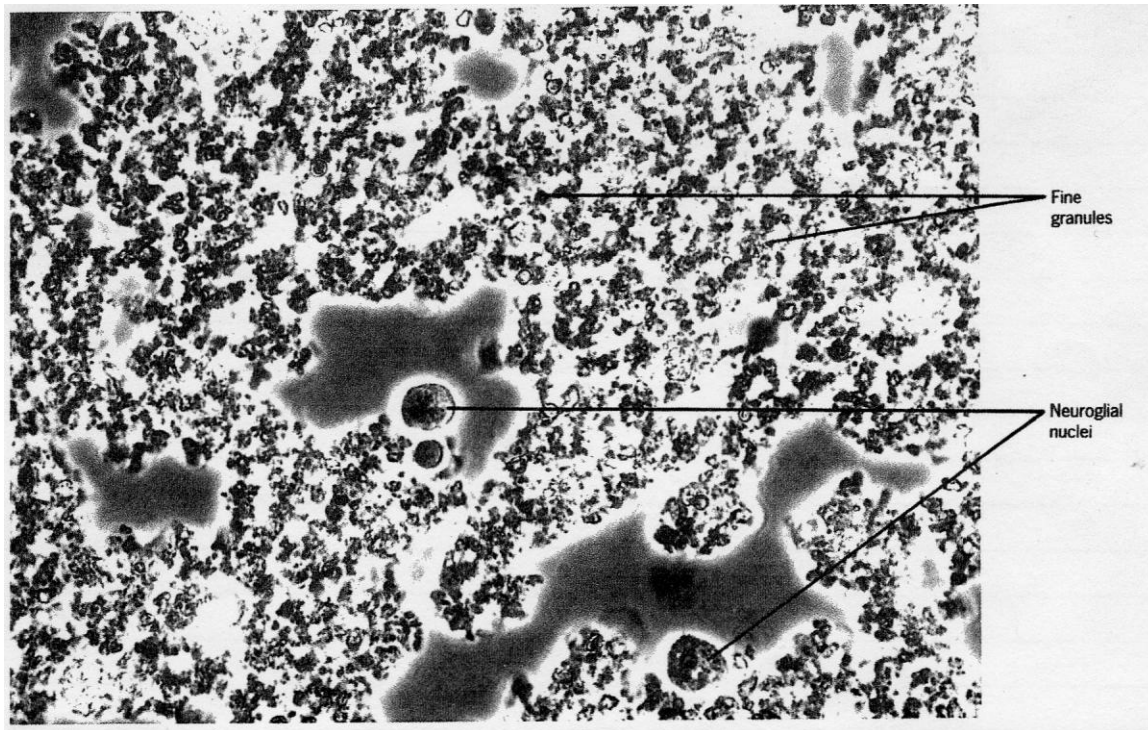


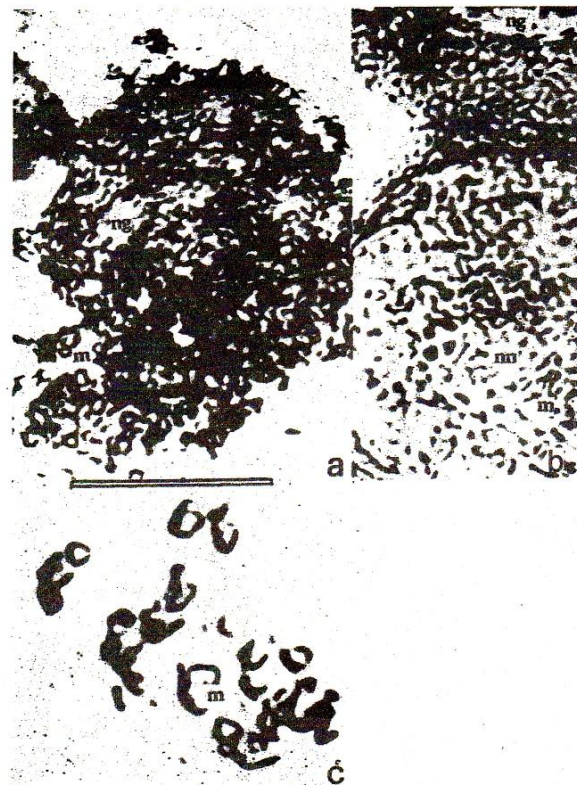
Figure 5. Partially teased human caudate nucleus from a 70-year old male to show neuroglial nuclei and fine granular material. Phase contrast, x 700.

In the unfixed state, the neuroglial clumps appear in 0.25 M sucrose, in Krebs-Ringer solution, in '199' medium or in normal saline, as intensely white, because they are made of a very large number of granules, mostly appearing 0.5-2  $\mu\text{m}$  in diameter (Figure 6). They are very friable and adhere together like pieces of rice pudding. Occasionally, neuron cell bodies can be found in the neuroglial material, and unattached nuclei (Figure 5) can be seen apparently moving around the neuroglia. When teased apart, it can be seen that vast numbers of fine fibres course through the neuroglia. Probably, most of these fibres are dendrites, and non myelinated fibres, with some myelinated fibres visible (Hillman and Jarman, 1991).

We took pieces of brain and spinal grey matter, 2-3 mm in diameter, and sandwiched them in similar-sized pieces of liver or kidney. We then stained the sandwiches with four stains recommended for *neuroglia*. In each case, they showed the membranes of the liver cells and

kidney tubules. However, in the nervous tissue we could see no continuous membrane between those of the neurons and those of the naked nuclei. The next step was to examine the neurological clumps directly. Hydén and his school generally believed that the neurological clumps consisted of astrocytes or oligodendrocytes, although - as far as I could find - they did not test them with the 'specific' classical stains or with antibodies.

We decided to look at the clumps directly. We took out individual clumps adjacent to cell bodies, and dissected out any neurons which might be present in them. Then we teased each of them and stained them directly with Janus-green neutral red, which shows up mitochondria, with which we also stained individual neuron cell bodies. Both the neuron cell bodies and the clumps



were seen to be full of aggregates of mitochondria (Figure 6).

Figure 6. Fine granular material stained with Janus green-neutral red, viewed by phase contrast microscopy. *m* is mitochondria. The bar is 20  $\mu\text{m}$  for *a* and *b* and 50  $\mu\text{m}$  for *c*. *a* is an isolated neuroglial clump; *b* has a neuroglial clump in the upper part of this picture, and a neuron cell body in the lower, and *c* shows the clump separated to show that the fine granular material



consists of mitochondria. This illustration comes from Hillman, Deutsch, Allen and Sartory, (1977) by kind permission of the Quekett Microscopical Club.

When one looks at histological sections of nervous tissue stained by any standard procedure, one sees neuron cell bodies with long axons and many dendrites. One also sees the main volume normally regarded as neuroglia within this, these are many neuroglial nuclei, or naked nuclei - as I will call them. The latter are believed to be the nuclei of astrocytes, oligodendrocytes or microglia, and each is believed to be invested with thin layers of cytoplasm. Around these thin layers, one would expect to see cell membranes of these neuroglial cells, but they are *not seen at all by light or electron microscopy*. I have asserted this at lectures and in publications, and the explanation given for it is that the cell membrane will only be seen by electron microscopy, if it is cut normal to the plane of section. This ignores the fact that the cell membranes, the nuclear membranes and the mitochondrial membranes are all seen clearly by electron microscopy, when they could not, of course, all be normal to the plane of section of the time.

### **Identification of cells in brain and spinal cord**

(i) the classical textbook of the central nervous system by Peters, Palay and Webster (1998) shows diagrams -not micrographs- of typical neurons, astrocytes and oligodendrocytes. It is rather surprising that they have not managed to show *light micrographs* of these cells. Indeed the latter are extremely rarely found in the literature, as they are rather shy to appear under the microscope. Perhaps it has not been appreciated that if one took any neuron, astrocytes or oligodendrocyte in a section, and made three-dimensional models of it, one would see many of the latter images overlapping. For example, if one sectioned the apex of a triangular neuron, where there are few or no dendrites, the cell would look like an oligodendrocyte;

(ii) early histologists often wrote that they could not distinguish for certain between neurons and astrocytes, and oligodendrocytes and microglia;

(iii) pathologists, biochemists and tissue culturalists often describe cells as 'glia' or 'neuroglia', indicating that they cannot specify to which particular type of glial cell they are referring;

(iv) clinicians and pathologists often use clinical criteria, such as the age of the patient, the site of the lesion, or the rate of its spread, to characterise the type of cell from which a tumour originates;

(v) in an extensive literature survey, recorded in many tables (Hillman, 1986, pps. 52-73), I examined, in respect of the neurons and the three different kinds of neuroglial cells, the following parameters as described *in textbooks*: their dimensions and shapes by light microscopy; their dimensions and shapes by electron microscopy; their appearances in tissue culture; the staining procedures believed to be characteristic of them; the markers believed to show up each.

I came to the conclusion that neurobiologists had not appreciated that the characteristics of the cells so overlapped that one could not distinguish on any objective grounds between the particular kinds of neuroglial ‘cells’. These conclusions were summarised in my book ‘The Cellular Structure of the Mammalian Nervous System’ (1986). This required a complete reassessment, as follows: in the central nervous system, there are only two kinds of cells, in addition to the ependymal cells and the blood vessels. There are neurons, which comprise all cells with processes, including those currently named neurons, astrocytes and oligodendrocytes. There is an all-embracing parenchyma or ground substance of neuroglia, which consists of fine granular material interwoven with dendrites, fibres, myelinated and non myelinated axons in a fluid. The fine granular material consists largely of free mitochondria. Naked nuclei float freely in the fine granular material. The brain and spinal cord derive their cohesion from the axons, dendrites, fibres, tissue viscosity, ependymal cells and meninges, and together neuroglial acts as a bulky viscous fluid, which can transmit impact as a result of concussion. Any surgeon or pathologist taking a biopsy is aware of the fluid nature of the central nervous system.

The naked nuclei (Figure 5) are named by neuropathologists as: neuroglial nuclei, oligodendrocytes, reactive astrocytes, or microglia. They are enveloped by fine granular material, and are probably mobile in life. However, the neuroglia is a syncytium, with individual naked nuclei surrounded by mitochondria in their cytoplasm, but this cytoplasm does *not* have its own cell membrane. This can be seen in any light or electron micrograph of brain or spinal cord. I cannot find a single histologist, who agrees with this assertion, who denies it, or who is prepared to explain it.

## **Critique of the current consensus on cell structure of the nervous system**

(a) the specificity of the staining systems for neurons, astrocytes, oligodendrocytes and microglia, is not generally discussed. We showed that seven different classical neuroglial stains showed up such undoubted neurons as cerebellar Purkinje cells, medullary cranial nerve nuclei and ventral horn cells;

(b) the staining systems, which the histologists consider to be specific, were originally tested on tissue sections, but do not appear in the literature to have been tested on the *same* normal cells in tissue culture, which would establish that the histologists and tissue culturalists were looking at identical cells. It is possible that they were found *not* to show up the same cells, or that the results of such experiments were not published. I have examined the literature intensively on this subject;

(c) it is difficult to know how to compare the histology of brain with that of the tissue in culture, because jobbing tissue culturalists know that when tissues are placed in the culture media, they change their morphology over a period of days. They often subsequently ‘de-differentiate’;

(d) when one attempts to compare the biochemistry of the alleged different kinds of neuroglia in culture, there is a serious problem. The cells are cultured in completely different media, so that is not at all surprising that they should be expected to exhibit different biochemical properties. Obviously the biochemistry of cells in culture is totally dependent on the chemistry of the culture media;

(e) the immunologists have failed to compare the antigens present in cells in the brain and spinal cords cut freshly, with those present in the cells in tissue cultures of what are believed to be the same cells. The implication of the use of antibodies to seek out the location of antigens in the body is that there are different antigens in different adjacent neurons and neuroglial cells in the same brains and spinal cords. One may ask, ‘Why does the body not produce antibodies to each of the antigens present in each different kind of cells?’ If it did, the central nervous system would be swirling with ‘specific’ antibodies binding to its own antigens. This would create a cellular Armageddon;

(f) it is proper to ask why it is so difficult to find micrographs of astrocytes, oligodendrocytes and neurons, in the same field. It is very rare to find micrographs -as opposed to diagrams- in which neuron cell bodies are *not* stained, when neuroglial cells are. Again, this is an invitation to anyone who knows publications showing this, or has any, to contact me.

## The likely cellular structure of the living mammalian nervous system

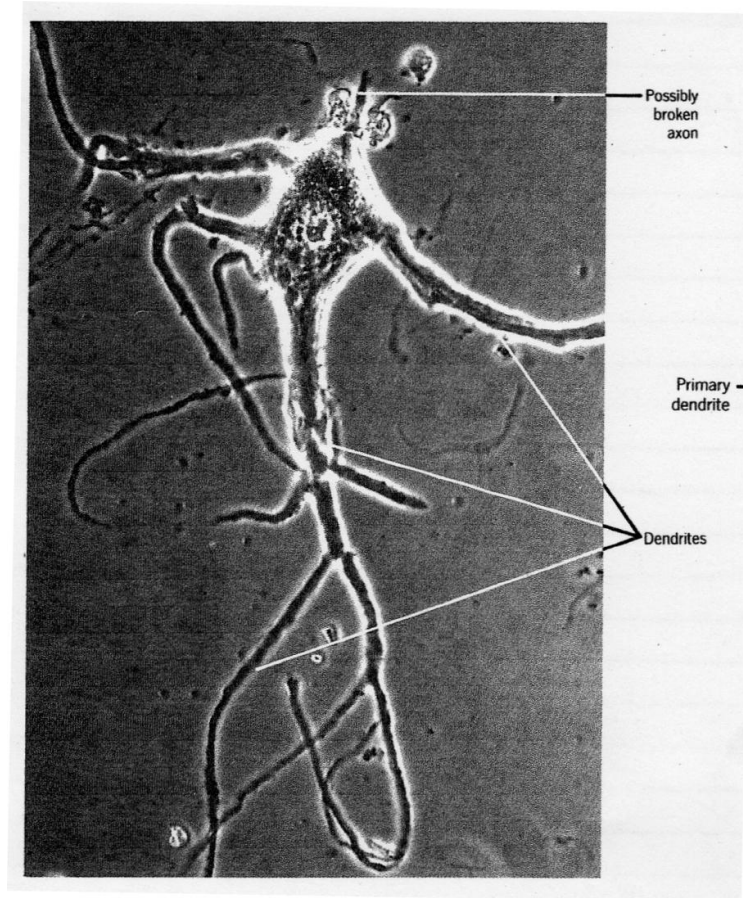


Figure 7. Isolated human hypoglossal neuron from a 62-year-old female, to show the dendritic tree (phase contrast, x 375).

A neuron is a bloated looking cell shaped like a depth charge with up to about 12 fattish dendrites arising from it (Figure 7). The concave body usually illustrated is that of a cell shrunk by histological or electron microscopic preparation.

The cell body is enclosed by a single-layered membrane, whose thickness is unknown and possibly unknowable. The figures of 7-10 nm are measured from electron micrographs. The chemistry of the cell membranes is currently only speculation. It was worked out for red blood cells, and assumed to be applicable to all cell membranes, and some of the measurements were made using subcellular fractionation, which ignores the second law of thermodynamics. The orientation of the lipid and protein molecules of which it is believed to consist is largely based on

the finding that one face of the membrane is hydrophilic and one face is hydrophobic. The cell membrane does not possess protruding structural molecules, glycoproteins or glycolipids; these are not seen in intact tissue by electron microscopy, but may be found in subcellular fractions. The cell membrane is not punctured by ion channels, which open and close when ions pass through. Only one such channel has ever been identified, although the physiologists claim that there are at least 30 different kinds (Conley, 1996). Although ion channels, as described, do not exist, the cell membranes are porous and contain passages which permit ions to cross, as do dialysis tubing and collodion membranes. The currents of pAs seen in patch clamp experiments arise from the high impedance pipettes, the chemicals used in preparation and the electronic components of the circuits (Hillman, 2008, pp 114-121).

There are no macromolecular membrane receptors for transmitters, hormones, antibodies, G-proteins, vitamins, drugs, toxins or death. The apparent receptors found arise when ligands - not the substances themselves- react with any constituent of the tissues or the reagents used in the experiments. The macromolecules are simply not seen by electron microscopy. The action potential is generated probably between the cytoplasm or axoplasm and the extracellular fluid. It is very unlikely to be generated *in* the cell membrane, which has a high impedance and resistance.

It is very unlikely, also that the cell membrane of the neuron contains ATPases or other 'transport' enzymes within its wall. Many of the enzymes have been characterised and sequenced and calculations also indicate that they should be visible by the electron microscope. The time is due for an explanation of these anomalies.

The dendrites are not attached to the pre-synaptic fibres joining synapses ending on neuronal cell bodies. The apparent frosty glass appearance of cell bodies by transmission light microscopy is due to mitochondria (Hillman, Deutsch, Allen and Sartory, 1997). If the dendrites of one cell are not joined to pre-synaptic fibres, excitability must spread from one neuron to another by a different mechanism than chemical transmission. An alternative hypothesis has been put forward (Hillman, 1991c).

In life, the cytoplasm of the neuron is a slightly cloudy liquid containing mitochondria and several small unidentifiable particles moving around it. There is no endoplasmic reticulum,

cytoskeleton, Golgi body, lysosomes, peroxisomes, molecular motors, contractile systems or any other identifiable particles or structures (Table 2). The mitochondria are tubes about 1  $\mu\text{m}$  wide and several  $\mu\text{m}$  long. They are seen in all orientations from circular, to oblique, to worm shaped. They are full of a liquid called mitochondrioplasm, which dries out to give the appearance of cristae; the cristae always appear to be normal to the plane of the section, which makes it certain that they are two-dimensional artefacts. So are their inner membranes. The subcellular fraction in which mitochondria are believed to dominate is believed to be the site of oxidative phosphorylation, and mitochondria are also believed to contain DNA, but in both cases, it is not known how much the procedure to separate the mitochondrial fraction is responsible for the apparent localisations.

The nuclear membrane of neurons is not trilaminar, nor is it perforated by nuclear pores. It contains nucleoplasm, whose chemistry in life is not known precisely, but it probably contains DNA. The evidence for this comes from nuclear transplants not subcellular fractionation. Nuclear transplantation is a low energy manipulation, and permits the nucleus to develop in the cell to which it has been transplanted. The nuclear pores are heat-induced cracks.

The nucleolus in neuron cell bodies is surrounded by a nucleolar membrane, and a nucleolonema gyrates within it, occasionally releasing pieces into the nucleoplasm. The clear part of the nucleolus is called the 'pars amopha'.

The neuroglia consists of a fine granular material enveloping the neurons in the living brain and spinal cord. The fine granular appearance is due to the presence to a large number of mitochondria, and some unidentified and unidentifiable small granules immersed in a fluid. It does *not* consist of cells. There are no astrocytes or oligodendrocytes. Any cell with a long process is a neuron. The neuroglia is a syncytium containing many naked nuclei, which appear to be able to move. There is cytoplasm around the naked nuclei, but each naked nucleus does not have its own cell membrane, either by light or electron microscopy. Naked nuclei, are usually called neuroglial nuclei or reactive astrocytes. They can be easily teased out of unfixed brain or spinal cord (Figure 5). Their lack of cell membrane does not seem to have been noticed in the literature. The generalised cell structure of grey matter of brain and spinal cord is shown below.

### **Recent developments**

During the last two years, Mr. David Copestake and I have been looking at histological sections of brains and spinal cords of mice, rats, human beings, guinea pigs and rabbits, stained by Davenport's (1934) modification of Ramon y Cajal's (1903) procedure. We examined directly only the tissue, named 'neuroglia' by Virchow in 1846. We made the following experiments: (i) we followed the courses of the dendrites for hundreds of microns; (ii) we looked for the presence of knobs, granules, spheres, ovals, or deposits, indicating synapses, on the surfaces of neuron cell bodies. We saw none; (iii) we saw no continuities between dendrites and any fibres ending on neuron cell bodies, usually called 'presynaptic' fibres; (iv) we observed a 1000 sites at which dendrites from different neurons apparently crossed, and then measured the vertical distances between the sets of two fibres. The fibres touched in only 11 out of the 1000 crossings. In the other 989 pairs, the fibres were between 0.5 and 3.0 micrometers apart; a very few dendrites appeared to have swellings on them, but when we focussed on the swellings, they turned out to be only bends of the dendrites.

So far, we have been unable to obtain publication of these results. The main reasons given were that "synapses were not central to the interests of neurobiologists", or that we had not used electron microscopy. We pointed out that anatomical synapses were described by light microscopy over 50 years before they were seen by electron microscopy. Referees have usually neither sent our manuscript to referees, nor criticised our histology or microscopy.

We urge colleagues to repeat these experiments.

Mr. David Jarman and I were examining the history of neuroglia. We noticed that since Leeuwenhoek described fine granular material in 1684, it was seen several times in unfixed brain in the 19<sup>th</sup> century. In 1827, Hodgkin and Lister described it briefly, when they examined the brains using achromatic objectives, but they did not publish a definitive account of these observations. Hyden and Pigon (1960) using hand dissection, separated neuron cell bodies from the tissue surrounding them, which they called 'neuroglial clumps'. Mainly in the 1970s and 1980s, at least 12 authors separated the clumps, photographed them, measured their oxygen uptake and enzyme activities, and published micrographs of them. Among these authors were Hyden, Hamberger, Hertz, Epstein, O'Connor, Deutsch, Jarman and myself.



We decided that we would look at fresh clumps directly, without any disruption or preparation under phase contrast microscopy. It soon became obvious that they consisted almost entirely of mitochondria, similar to the appearance of mitochondria in neurons. We published this (Hillman and Deutsch, 1978; Hillman, Deutsch, Allen and Sartory (1977). However, in recent years, neuroglial clumps have disappeared from the literature. We concluded that mitochondria and naked nuclei were the main constituents of the neuroglia, and occupied the majority of the volume of the brain and the spinal cord.

While examining the literature, I came across an extraordinary anomaly in the electron microscopy of myelin lamellae. In every single publication, one saw an oblique view of a complete myelin sheath, as judged by the long axis being significantly more than the short axis. However, the myelin sheath itself appears to be of uniform thickness all the way round even in these obviously oblique sections. This observation appears to be true in every electron micrograph in the literature. The only conclusion that one can come to about this is that the image at which one is looking is two-dimensional, that is to say, that it arose after the section was cut.

Furthermore, in unfixed myelin sheaths, viewed by phase contrast microscopy, the refractive index of the sheath appears higher than that of the axons. If the myelin sheath were composed of scrolls of Schwann cell membranes, as indicated by the Geren model (1954), one would expect the refractive index of the myelin sheath to be higher.

High power light microscopy allows one to see Brownian movement of particles in the myelin sheaths (Singer and Bryant, 1969). This would not be possible, if the myelin sheath were solid with lamellae.

### **Consequences of proposed new cellular structure**

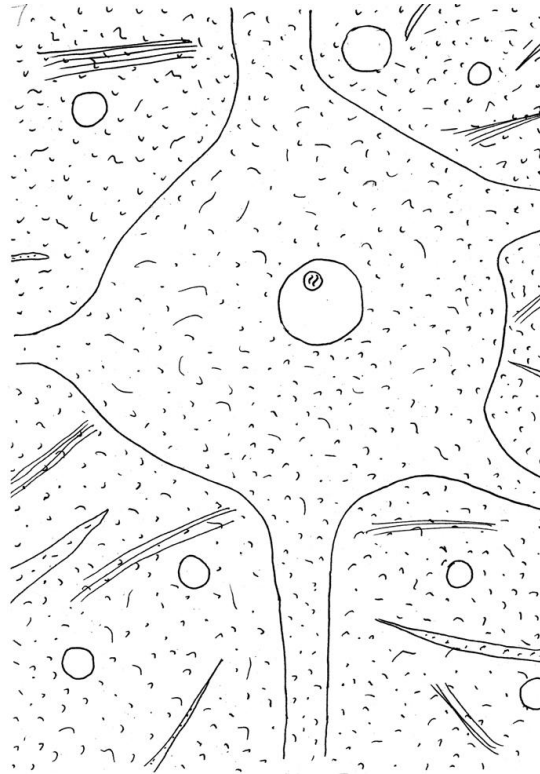


Figure 8. Diagram of the proposed cellular structure of the central nervous system.

In Figure 8, please note that there are few neurons; the neuroglia (the fine granular material) enveloping the neurons consists mainly of mitochondria; there are no astrocytes, oligodendrocytes or synapses; naked nuclei are distributed within the neuroglia, and can probably move within it; dendrites and axons also traverse the neuroglia; there is a nucleolar membrane. In life, the fine granular material is much denser than is represented in this diagram. Detailed evidence for this proposed structure may be found in Hillman, (1986), Hillman and Jarman, (1991) and Hillman,(2008).

This clarifies the following problems:

- (i) the apparent dearth of light micrographs showing astrocytes and oligodendrocytes in healthy nervous tissue;
- (ii) the lack of membranes around the neuroglial 'cells';

(iii) the difficulty of distinguishing, unequivocally between neurons and astrocytes, between astrocytes and oligodendrocytes, and between oligodendrocytes and microglia;

(iv) the uncertainty of criteria for distinguishing the different kinds of neuroglial cells unequivocally by objective criteria;

(v) the finding that astrocytes and oligodendrocytes are of similar developmental origin;

(vi) the lack of specificity of the stains for the different kinds of neuroglial cells;

(vii) the finding that gliosis occurs around a lesion within a few hours, which it could not, if the neuroglia were solid with three kinds of cells;

(viii) the fact that the central nervous system behaves like a liquid, when subjected to sudden pressure. There is often a lesion opposite a concussion to the brain, and another one opposite it, called 'contre-coup'.

(ix) tumours of neuroglial or glial cells are found in the brain. They are rich in nuclei, which we believe to be free in the syncytium of the neuroglia, which may explain their rapid spread and malignancy.

## References

- Auerbach, L. (1898). Nervendingung in Centralorganen. *Neurol. Zentralbl*, **17**, 445-454.
- Baker, J.R. (1958). *Principles of Biological Technique*. Methuen, London.
- Bancroft, J.D. and Stevens, A. (Editors) (1982). *Theory and Practice of Histological Techniques*, 4<sup>th</sup> edition, Churchill-Livingstone, New York.
- Bastian, H.C. (1913). *The Nature and Origin of Living Matter*. Fisher Unwin, London.
- Burgen, A. and Barnard, E.A. (1992). *Receptor Subunits and Complexes*. Cambridge University Press, Cambridge.
- Bütschli, O. (1884). *Investigations on Microscopic Foams and on Protoplasm*, translated by Minchin, E.A. Adam and Charles, London.
- Chughtai, I., Hillman, H. and Jarman, D (1987). The effect of haematoxylin and eosin, Palmgren's and osmic acid procedures on the dimensions and appearances of isolated rabbit medullary neurons. *Microscopy*, **35**, 625-629.
- Chughtai, I., Hillman, H. and Jarman, D (1988). The effect of staining with haematoxylin and eosin, Golgi's stain and osmic acid on the appearance of single sciatic nerve and corpus callosal fibres of guinea pig. *Microscopy*, **36**, 76-83.
- Conley, E.C. (1996). *Ion Channels*, Vols **1-4**. Academic Press, London.
- Davenport, H.A. (1933). Block staining of nervous tissue with silver. Pericellular end bulbs or boutons. *Stain Technol.*, **8**, 143-147.
- Davson, H. and Danielli, J.F. (1936). *The Permeability of Natural Membranes*. Cambridge University Press, Cambridge.
- Del Rio Hortega, P. (1932). Microglia, in *Cytology and Cellular Pathology of the Nervous System*. Vol. **2**, edited by Penfield, W. Hoeber, New York, pp. 482-534.
- De Robertis, E. (1959). Submicroscopic morphology of the synapse. *Int. Rev. Cytol.*, **8**, 61-96.
- Estable, C. and Sotelo, R. (1951). Una nueva estructura celular; el nucleolonema. *Publ. Inst. Invest. Sci. Biol.*, **1**, 105-123.
- Fox, S.D. and Dose, K. (1977). *Molecular Evolution. The Origin of Life*, 2<sup>nd</sup> edition. Dekker, New York.
- Geren, B. (1954). The formation from the Schwann cell surface of myelin in chicken embryos. *Exper. Cell. Res.*, **7**, 558-562.
- Golgi, C. (1898). Sur la structure des cellules nerveuses des ganglions spinaux. *Arch. Ital. Biol.*, **30**, 278-286.
- Gorter, E. and Grendel, F. (1925). On biomolecular layers of lipoids on the chromocytes of blood. *J. exp. Med.*, **41**, 439-441.
- Held, H. (1897). Bertrage zur Struktur der Nervenzellern und ihrer Fortsatze. *Arch. Anat. Physiol. Anat. Abt.*, **21**, 204-294.

- Herrera, A. (1900). The origin of the individual. On the initiation of protoplasm. *Mex. Soc. Alzate Mem.*, **15**, 23-30.
- Herrera, A.L. (1928). Plasmogeny, in, *Colloid Chemistry*, Vol 2, edited by Alexander, J. Chemical Catalogue Company. New York, pages 81-91.
- Hillman, H. (1972). *Certainty and Uncertainty in Biochemical Techniques*. Surrey University Press, Henley on Thames.
- Hillman, H. Deutsch, K., Allen, J. and Sartory, P.K. (1977). The histology of neuroglial clumps in rabbit brain. *Microscopy*, **33**, 175-180.
- Hillman, H. and Deutsch, K. (1978). Staining of isolated rabbit neurons and neuroglial clumps. *Experientia*, **35**, 771-772.
- Hillman, H. and Sartory, P.K. (1980). *The Living Cell*, Packard Publishing, Colchester.
- Hillman, H. (1985). The anatomical synapses by light and electron microscopy. *Medical Hypotheses*, **17**, 1-32.
- Hillman, H. (1986). *The Cellular Structure of the Mammalian Nervous System*. M.T.P. Press, Lancaster.
- Hillman, H.(1990). An electrical hypothesis for transmission in the central nervous systemsystem. *Medical Hypotheses* ,**34**, 220-224.
- Hillman, H. (1991a). A re-examination of the vesicle hypothesis of transmission in relation to its applicability to the mammalian central nervous system. *Physiol. Chem. Phys. Med. NMR.*, **23**, 177-198.
- Hillman, H. (1991b). Some microscopic considerations about cell structure. Light versus electron microscopy. *Microscopy*, **36**, 557-574.
- Hillman, H. (1996). A new look at the cellullar structure of the brain, in, *Perspectives in Neuroscience Research*, edited by Tan, C.K., Ling, E.A. and Tan, C.B.C. Singapore Neuroscience Association, Singapore, pp. 17-30.
- Hillman, H. (2000). Limitations of clinical and biological histology. *Medical Hypotheses*, **54**, 553-564.
- Hillman, H. (2008). *Evidence-based Cell Biology*. Shaker Publishing, Maastricht.
- Hillman,H. (2009). Procedures used in modern cell biology ignore natural laws. *J. Biol. Phys. and Chem*, **9**, 130-135.
- Hillman, H. and Jarman, D. (1991). *Atlas of the Cellular Structure of the Human Nervous System*. Academic Press, London.
- Hinton, R.H., Burge, M.L.B. and Hartman, G.C. (1969). Sucrose interference in the assay of enzyme and proteins. *Analyt. Biochem.*, **29**, 248-256.
- Hodgkin, T. And Lister, J.J. (1827). Notice on some microscopic observations of blood and animal tissues. *Phil. Mag.*, **2**, 130-138.
- Hussain, T.S., Hillman, H. and Sartory, P.K. (1974). A nucleolar membrane in neurons. *Microscopy*, **32**, 348-352.

- Hydén, H. (1959). Quantitative assay of compounds in isolated fresh nerve cells and glial cells from control and stimulated animals. *Nature*, **184**, 348-352.
- Hydén, H. and Pigon, A. (1960). A cytophysiological study of the functional relationship between oligodendroglial cells and cells of Deiters' nucleus. *J. Neurochem.*, **6**, 57-72.
- Katz, B. (1969). *The Release of Neural Transmitter Substances*. Sherrington Lecture, Liverpool University Press, Liverpool.
- Kennakin, T. and Angus, J.A. (editors). (2000). *The Pharmacology Function, Biochemical Recombinant Receptor Systems. Handbook of Experimental Pharmacology*, **148**, Springer, Berlin.
- Kistler, J., Stroud, R.M., Klymkowsky, M.W., Lalancette, R.A. and Fairclough, A.M. (1982). Structure and function of an acetylcholine receptor. *Biophys. J.*, **37**, 371-383.
- Kushida, H. (1962). A study of cellular swelling and shrinkage during fixation dehydrating and embedding in various standard media. *J. Electron Microsc. (Japan)*, **11**, 135-141.
- Landon, D. (Editor) (1976). *The Peripheral Nerve*. Chapman and Hall, London.
- Lauffenberger, D.A. and Linderman, H. (1993). *Receptors, Models for Binding, Trafficking and Signalling*. Oxford University Press, New York.
- Leeuwenhoek, A. van, (1684). Opera Omnia; Seu Arcana Naturae (1772) De structura cerebri diversorium animalium 1, 29-41, in *The Human Brain and Spinal Cord*, edited by Clarke ,E and O'Malley, C.D. (1968). University of Berkeley Press, California. P. 419.
- Luyet, B. (1960). On various transitions occurring in aqueous solutions at low temperatures. *Ann. N.Y. Acad. Sci.*, **85**, 540-569.
- Mackenzie, A.P. and Luyet, B.J. (1967). Electron microscope study of recrystallization in rapidly frozen gelatine gels. *Biodynamica*, **10**, 95-122.
- Mann, G. (1902). *Physiological Histology, Methods and Theory*. Clarendon Press, Oxford.
- Meryman, H.T. (editor) (1966). *Cryobiology*. Academic Press, New York.
- Ochs, S. (1982). *Axoplasmic Transport and its Relation to Other Nerve Functions*. Wiley, New York.
- Palay, S.L. (1956). Synapses in the central nervous system. *J. Biophys. Biochem. Cytol. Suppl.*, **2**, 193-203.
- Pappas, C.D. and Purpura, D.M. (editors) (1972). *The Structure and Function of Synapses*. Raven Press, New York.
- Pappas, G.D. and Waxman, s.G. (1972). Synaptic fine structure: morphological correlates of chemical and electronic transmission. In, Pappas and Purpura (1972), pp. 1-44.
- Peters, A. Palay, S.L. and Webster, H. (1998). *The Fine Structure of the Nervous System*. Oxford University Press, New York.
- Ramon y Cajal, S. (1903). Uno sencillo metodo de coloracion selectivo de reticulo protoplasmic y sus efectos en los diversos organos nerviosos. *Trab. Lab. Invest. Biol. Univers. Madrid*, **2**, 129-221

- Ramon y Cajal, S. (1906). The structure and connection of neurons. *Nobel Lectures in Physiology and Medicine*. Nobel Foundation, Elsevier, Amsterdam, pp. 220-253.
- Ramon y Cajal, S. (1913). Uno nuevo proceder para la impregnacion de la neuroglia. *Rev. Clin. De Madrid*. **101**, 401-405.
- Rodrigues de Lores-Arnaiz, G. Alberici, M. and de Robertis, E. (1967). Ultrastructure and enzymic studies of cholinergic and non-cholinergic synaptic membranes isolated from brain cortex. *J. Neurochem.*, **14**, 215-225.
- Sartory, P.K., Fasham, J. and Hillman, H. (1971). Microscopical observations of the nucleoli in unfixed rabbit Deiters' neurons. *Microscopy*, **32**, 93-100.
- Schmidt, W.J. (1936). Doppelbrechung und Feinbau der Markscheide der Nervenfasern. *Zeitschr. f. Zellforsch.*, **23**, 657-676.
- Schmitt, F.O., Bear, R.S. and Clark, G.L. (1935). Xray diffraction studies on nerve. *Radiology*, **25**, 131-151.
- Singer, M. and Bryant, S.V. (1969). Movement in myelin Schwann sheath of the vertebrate axon. *Nature*, **221**, 1148-1150.
- Smith, A.U. (1961). *Biological Effects of Freezing and Supercooling*. Arnold, London.
- Stämpfli, R. (1952). Bau und Funktion der isolierte markhaltiger Nervenfasern. *Ergebn. Physiol.*, **47**, 70-165.
- Stoward, P.J. (1973). *Fixation in Histochemistry*. Chapman and Hall, London.
- Tasaki, I. (1939). The electrosaltatory transmission of the nerve impulse and the effect of narcosis upon the nerve fibre. *Amer. J. Physiol.*, **127**, 211-227.
- Toyoshima, C. and Unwin, P.N.T. (1988). Ion channels of acetylcholine receptor reconstructed from images of post-synaptic membranes. *Nature*, **336**, 247-250.
- Unwin, P.N.T. and Zampighi, G. (1980). Structure of the communication between communicating cells. *Nature*, **283**, 545-549.
- Vale, R.D. and Fletterick, R. (1997). The design plan of kinesin motors. *Ann. Rev. Cell Dev. Biol.*, **13**, 745-777.
- Vallée, R.B. (editor). *Molecular Motors and Cytoskeleton Part B. Methods in Enzymology*, **298**, Academic Press, San Diego.
- Virchow, R. (1846). Uber das granuliertte Aussehen der Wandungen des Gehirnsventrikel. *Allg. Zeitschr. f. Psychiatr.*, Berlin, **3**, 242, 250.
- Weakley, B. (1972). *A Beginners Handbook in Biological Electron Microscopy*. Churchill-Livingston, London, plate 2.
- Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J.A. (1964). The separation of synaptic vesicles from disrupted nerve end particles ('synaptosomes'). *Biochem. J.*, **90**, 293-303.
- Williams, P.L. and Hall, S.M. (1970). In vivo observations on mature, myelinated nerve fibres of mouse. *J. Anat.*, **107**, 31-38.

## **Postscript**

I invite dialogue with anyone who would dispute the findings or interpretations quoted here, including my own and those of my colleagues. Obviously expensive studies of artefacts of research procedures are a waste of personnel and resources. In my opinion neurobiologists have a duty to address the difficult questions, and to produce micrographs, which show those images, which are alleged not to be present. If the images cannot be found, it is their duty to consider the consequences.

(Last revised 28<sup>th</sup> June, 2011).