The synaptology of the L4 neuron in the first visual ganglion of the fly, musca domestica

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THE SYNAPTOLOGY OF THE L4 NEURON IN THE FIRST VISUAL GANGLION OF THE FLY, MUSCA DOMESTICA

A thesis submitted in candidature for the degree of M.Sc. of the University of Durham

Paul Debbage 1975

ABSTRACT

Results of optomotor experiments on Musca and Drosophila led to the formulation of a conceptual model which allows the prediction of the insect's response to defined moving stimuli. A basic requirement of the model is that there should exist in the ganglion chain, between the compound eye and the motor output, cross-connections between the channels of information flow in the ganglia. Further optomotor experiments provided evidence about the directions within the ganglion of the cross-connections.

The first visual ganglion of Musca is built of sub-units called cartridges, each of which is composed of six retinular cell axons synapsing onto five different neurons: some of which are second order neurons, some of higher order. One of these five neurons, "L4", has two long collaterals at the base of the lamina, which run from the cartridge to the two neighbouring cartridges in the hexagonal array. This configuration is compatible with that suggested by the optomotor experiments for the cross-connections postulated by the conceptual model. Thus it was of interest to examine the synaptic connections made by the terminations or "endfeet" of these collaterals. This study reports the results of examination, by electron microscopy, of serial ultra-thin sections of the first visual ganglion containing L4 collaterals.

The results show that the three collaterals which terminate in each cartridge (two long collaterals from neighbouring cartridges plus one short one from the L4 within the

cartridge) do so in intimate contact with each other and with the two second-order neurons L1 and L2. The three collaterals are presynaptic to each other, each to the other two. Each of them is also presynaptic to the L2 neuron in that cartridge, and it seems that each synaptic contact between two members of the three collaterals is also simultaneously a contact with L2. No contacts are made to the L1 fibre, in contrast to the systematic pattern of contacts to the L2 fibre. In addition to this basic scheme of connections the collaterals are also presynaptic to some of the retinular cell axons, usually R3 and R5, apparently in cartridges from the more central parts of the ganglion, or there may be a gradient of connectivity across the ganglion.

Since no studies, apart from the anatomical ones, exist on this network, the conclusions drawn about the significance of these results must be tentative. The results are not incompatible with the idea that these L4 collaterals could be the cross-connections postulated in the conceptual model. One function possibly mediated by the network could be lateral inhibition, which has been shown to occur in Musca.

INTRODUCTION

Insect nervous systems have been a popular object of study recently as is demonstrated by the following list: the beetles Chlorophanus, Lixus, Zophobas, the bug Oncopeltus, the bee Apis, the grasshopper Locusta, the praying Mantis, the dronefly Eristalis, the blowfly Calliphora, the housefly Musca, and the fruitfly Drosophila, have all been the objects of optomotor investigations alone[13,14,17,35]. Electrophysiological and optical studies have been performed on a wide range of insects, sometimes in conjunction with each other and optomotor studies.

This popularity reflects not only the advantages of small size and ease of culture found with insects, but several other features attractive to those investigating nervous systems. Firstly, the number of neurons comprising the insect nervous system is small in comparison with that comprising the most vertebrate systems. The eye of Man contains about 10^8 receptors, that of an insect only 10^3 to 10^4. Man can discriminate 4×10^5 points in his optical surroundings, an insect only 10^2 or 10^3. Man has about 10^8 neurons in his optical system, an insect about 10^5[25]. The parsimony of neurons found in insects is further illustrated by the fact that muscle innervation in insects is commonly with 2 axons, sometimes only with one, sometimes with four, but this is to be contrasted with the innervation of muscles by hundreds of axons in vertebrates.

The behaviour of insects is also attractive to

investigators. It includes various simple stereotyped reactions which can be easily and predictably evoked, and are thus available for precise quantitative measurements[14,16,17].

The optic systems of insects offer these same advantages to the experimenter in an enhanced form: the optic lobes and retinas are frequently large and conspicuous, yet their construction is simple, being to a large degree achieved by repetition of small subunits[2,3,5,24]. Such periodic construction simplifies the anatomical study, for if the structure of one subunit is described, then the total structure is known in its essentials. The optic systems are not only of obvious significance to the insect, but they also detect and process stimuli mediated by a medium which is well-described physically: light. This means that stimuli applied during experiments can be accurately described, a quality of great value in building models to account for the behaviour observed.

The flies Musca and Drosophila have been studied with a variety of techniques. Optical experiments have demonstrated the properties of the photoreceptor dioptric apparatus[7,8,9,23,24,28]. The study of optomotor responses has revealed aspects of the primary photoreceptive processes, and also of the information processing performed by the nervous system[8,13,14,16,17,38,39]. The retina and lamina of these flies have become well-known structures, due to use of reduced silver techniques and of the electron microscope[2,3,5,24,42,45-48]. The other ganglia of the optic lobe have also been partly described. Electrophysiological techniques have given insight into the functioning of individual cells and of cell systems in the retina and optic ganglia[21]. Genetic experiments are

being performed on Drosophila[20], with the aim of understanding aspects of nervous function through the use of neurally defective mutants. Some of these lines of research will be described in some detail here, insofar as they provide a close background and a motivation for the study presented here. These are:

1. the histological studies
2. the optical experiments
3. the earlier optomotor experiments

Anatomical studies

The anatomy of the Musca visual system will be briefly outlined here. Drosophila has been shown to be similar. The anatomy has been the subject of studies with the light microscope, using the Golgi[43], Bodian[3,4,5,42], Golgi-Cox, Holmes-Blest[3,4,5] etc., methods of staining. These all deposit reduced silver, mercury or another metal, into the neurones of the optic ganglia in a non-selective fashion, so that individual neurons can be distinguished from among the dense masses of neurons which comprise the ganglia. The anatomy has been subject also to a thorough but far less extensive study using the electron microscope[2,45-48]. In this case the fixation has been after Karnovsky[22,23], using formaldehyde and glutaraldehyde, and a post-fixation with osmic acid[36]. This method has been supplemented in different studies by mixed Golgi-Karnovsky fixations[43], and in yet other studies by degeneration and auto-radiography techniques. The following structures have been described in the chain of visual ganglia which are shown schematically in Fig. 8.

Retina

The Musca compound eye has about 3,200 facets arranged in a hexagonal array. This array is not regular but is stretched at right angles to the animal's long axis. Nevertheless it is easy to identify the three axes of this array, and they have been labelled (x,y,z Fig. 6) [3,4]. These three axes can be followed through the chain of optic ganglia (Fig. 7) and spatial relationships within each ganglion can thus be related to the relationships present on the outer surface of the eye, (fig. 6b).

The facets of the cornea are part of the dioptric apparatus. Their curvature is sufficient to explain the image-forming properties of the eye, and no recourse need be had to explanation in terms of cylindrical lenses[24]. Beneath the cornea lie the crystalline cones, one to each facet. These cones are gelatinous. The light rays traverse these cones and are brought to a focus below them in the retina. Separated from the crystalline cone by four Semper cells there is the photoreceptive system, the "retinula". The dioptric system plus the retinula constitute the "ommatidium", one of which lies beneath each facet of the eye. The retinula consists of eight retinula cells, of which two, numbers 7, 8 are arranged in tandem. These cells each contain a microvillar rhabdomere, the seven rhabdomeres present at any given depth of the retinula being arranged as an unfused rhabdome. The constant asymmetry of this rhabdome allows each retinula cell to be identified with a number. The retinula is separated from its neighbours by pigment cells, so that the ommatidia are optically isolated[2]. Fig. 9 is an

electronmicrograph of the retinula.

The rhabdomeres are supposed to be the light-sensitive elements of the retina. Their tips lie in the focal plane of the dioptric system[8]. Further, the microvilli of the rhabdomeres are oriented in a specific way (see Fig. 9) explaining the dichroic absorption described above. Extraction of photopigments from the rhabdomeres is unsuccessful, but were the pigment molecules arranged along the membranes of the microvilli, one would expect the dichroism that is observed. The microvilli of rhabdomeres 7 and 8 are arranged orthogonally to each other. The rhabdomeres show waveguide properties, (Franceschini et Kirschfeld, 1970)[9,10], and the pseudopupils appearing inside the eye are images of the rhabdomere endings (or are virtual images of real images of those endings) or can only be explained on the assumption that the rhabdomeres are the light-carrying elements[9].

The retinula cells convert the light stimuli they receive into signals (graded potentials) and these are fed into the next unit of information processing, the first optic ganglion or "lamina ganglionaris". The retina cells send axons down into this ganglion, eight axons from each ommatidium.

The Retina-Lamina Projection

The functional units of the retina are called ommatidia, and those of the lamina ganglioaris are the "neurommatidia", or "cartridges". Eight axons project from each ommatidium into the lamina, and each cartridge receives eight retinula axons. However, the eight axons projecting from one ommatidium are not the same eight as those arriving at the corresponding cartridge. The axons from one ommatidium diverge (see Fig. 8)

and project to seven different cartridges, and since there are exactly as many cartridges as ommatidia, it follows that each cartridge receives axons from seven different ommatidia. From its corresponding ommatidium each cartridge receives the retinula axons number 7 and 8. If the map of those rhabdomeres of seven ommatidia which look at the same point in the environment (Fig. 11) be superimposed on the map representing the projection of seven axons from seven different ommatidia onto one cartridge (Fig. 10), then it is seen that the information received by the eye from one point in the environment is brought together at one cartridge in the lamina ganglionaris. The value of this for the animal has been discussed above[3,24].

Lamina ganglionaris

This will be called simply the lamina. It is composed of subunits called "cartridges", which are arranged in a hexagonal array. The three axes of this array have been traced through from the retina, so that the x-axis of the retina corresponds to the x-axis of the lamina, etc. (see Fig.12) [3]. The cartridge is composed of the eight retinula cell axons which project down from the retina. Six of these are arranged in a circle and the two others lie beside each other outside the circle. These two are the axons of retinula cells 7 and 8. (Retinula cells and their numbers will be denoted as R1, R2, etc., in the following). These two axons make no synaptic connections at all in the lamina, but simply project through it into the next optic ganglion, the medulla[2]. R1-R6 do make synaptic connections in the lamina. They enclose the axons of two large monopolar neurons, whose cell

somas lie at the distal end of the lamina. Three other monopolar cells also send axons through the lamina, and these lie outside the ring of endings of R1-R6. The spatial relationships of all these axons remain sufficiently constant that in a transverse section of the lamina they can be identified with some certainty[2,3,5], (Fig. 13, Fig. 12). As in the retina, the lamina in the northern part of the eye is a mirror image of that in the southern part, and the right eye is a mirror image of the left. Thus from a section it is possible to identify the part of the lamina from which it came (Fig. 12).

The five monopolar cells have been named L1-L5. Their profiles are shown in Fig. 14. L1 and L2 branch in a regular fashion, sending processes between neighbouring R-cell endings these processes themselves sometimes being branched. It is frequently impossible to identify such a process in a section as originating from L1 or L2. The remaining monopolars have less regular branching patterns, the significance of which remain unclear. L4 is of particular interest in that two of its branches are long collaterals (Fig.15) reaching from the cartridge containing the "mother fibre" to two neighbouring cartridges in the -x and +y directions of the hexagonal array (Fig. 16) [42].

The cartridge contains two other well-described components. These are the "alpha" and the "beta" cells. The alpha cell is intrinsic to the lamina, its cell soma lying just beneath the ganglion. The branching pattern of this cell remains unknown, but spreads over several cartridges. The alpha cell contributes processes to a number of different

cartridges. These processes lie between and just outside the R1-R6 cells. They are closely apposed to those of the beta cell. This cell arises in the medulla and sends up a single fibre to the lamina, where each of these fibres then branches into a crown of six fibres, all of which contribute to the components of one cartridge. These lie beside the processes of the alpha cell.

The various cell types are readily recognised in electron microscope sections by the following characteristics. (Picture 1a of the serial section shows these clearly and may be compared with Fig.12). R-cells contain a clear central cytoplasm, usually surrounded by a ring of mitochondria. The periphery of the cell is filled with numerous small vesicles, all of the same size, and whose contents are not seen on electron micrographs. Among these vesicles are seen "capitate projections", which are glial projections into the R-cell, capped at the end with a thickened membrane which appears to consist of the inner unit membranes of the R-cell and the glial cell, together with a fused membrane derived from the outer membranes of R-cell and glial cell. Black pigment granules occur in these cells. Also pre-synaptic specialisations, Figs. 17 and 18. All the R-cells are identical in electron micrographs, and none branch.

L1 - L5 contain clear cytoplasm with few mitochondria, and a few neurofilaments. The only specialised structures found in these cells in the lamina are post-synaptic specialisations and, very rarely, a presynaptic specialisation. The two long L4 collaterals, where they come to lie inside the ring of R-cells, between L1 and L2, contain vesicles which

are apparently the same size as those in the R-cells, and very occasionally presynaptic and post-synaptic specialisations (Figs. 17 and 18).

The alpha cell also contains similar vesicles and pre- and post-synaptic specialisations. The beta cell contains clear cytoplasm and large invaginations with a complicated membrane structure. These "gnarls" contain membranes contributed by the alpha cell and also by the epithelial cells. The resulting combination of three membrane system is very electron dense after normal fixation, so that the exact relationships are unclear.

The epithelial cells are of two types, one type occurring more distally in the lamina, the other proximally. The distal type usually contains dark cytoplasm after fixation involving phosphate ions. The presence of many mitochondria enhance the dark effect, and the result is that the cartridges stand out very clearly in electron micrographs, against a dark "background" of glial cells. The proximal type of glial cell contains numerous neurofilaments and electron-dense granules, in clear cytoplasm.

The synaptology of the lamina has been semi-quantitatively studied[2]. The main classes of synapses, a synapse being defined as the characteristic pre-synaptic specialisation opposite a characteristic post-synaptic specialisation, are listed below. The preponderance of synapses presynaptic in the R-cells and postsynaptic onto L1-L5 is evidence for the synaptic character of these morphological structures, since the information flow in the lamina can reasonably be expected to be from the R-cells to the L-cells.

Medulla (See Fig. 8)

In contrast to the retina and lamina this ganglion is relatively unknown. It is readily seen, using the light microscope, to be a layered structure, but use of the Golgi technique reveals the presence of a bewildering variety of different cell types, with very differing branching patterns. Use of the electron microscope shows that here too, a periodic system of subunits is present, but these subunits have yet to be described, so that their basic construction is unknown. It is known that the chiasma between the lamina and medulla shows a crossing-over, so that the +direction of the z-axis becomes the -direction,[5]. (See Fig. 7).

It is also known that the endings of the L1-L5 neurons in the medulla occur at different depths and have different branching patterns. R7 and R8 terminate at different depths in the medulla, but it has been shown that both terminations are in the "parent" cartridge. Therefore it is in the medulla that all the information originating in one ommatidium is brought together in one processing unit.

Lobula and Lobula Plate

Only four orders of insects have a lobula plate structure: the Diptera, Lepidoptera, Orthoptera and the Heteroptera. In the lobula there is also a periodic structure, but this is as yet undescribed. This ganglion will not be described further here.

The Optical Studies

There exist three principal types of compound eye[9,24, 28,25]. These are the apposition eye, which is the commonest

type, the optical superposition eye, found for example in moths active by night, and the neural superposition eye, found in Hemiptera, Orthoptera, and Diptera.

Apposition eyes (Ref. 24,28)

In these eyes each ommatidium acts as a functional unit, and is optically isolated from its neighbours. (Exner's definition). In the case of Limulus (the horseshoe crab) there are several sense cells ("retinular cells") and an eccentric cell. Only the axon of the eccentric cell can be stimulated to produce impulses by light stimuli, so that each ommatidium has precisely one output, and corresponds precisely with the definition. However, most species with apposition eyes have several retinular cells in each ommatidium, and the rhabdomeres of these cells are close together, and form a "fused rhabdome". This rhabdome acts as a light pipe, due to its having a higher refractive index than the surrounding media, so that light waves are guided along it and do not escape. The light is absorbed by the rhabdome pigments, and causes impulses in the axons of the retinular cells. Although the rhabdome is regarded as an optical unit for this reason, the individual retinular cells might be different from each other with respect to their colour sensitivity or their sensitivity to polarized light. Since each ommatidium contains one rhabdome acting as an optical unit, then each ommatidium is perceiving light from one point of the optical environment only. Thus, even though each of the retinular cell axons are sending signals to the optical ganglia, all the signals from one ommatidium are related to one point only of the visual surrounding, and

therefore this type of eye too is regarded as an apposition eye. (Examples: bees, locusts, ants, etc.)

Optical Superposition eyes (Ref. 24,27,29)

In this type of compound eye there is no isolation of an ommatidium from its neighbours. The essential, and anatomically easily recognisable, difference between this type and the apposition eye is that a superposition eye must have a thick layer of transparent tissue between the dioptric apparatus and the sensitive layer of the retina. Then it is possible that the light rays leaving the tips of the crystalline cones can converge in such a way that one retinal element can be illuminated by the rays leaving the tips of several cones. In the case of the Firefly Lampyris the dioptrics produce erect images, which is unique among the insects. In this case no sacrifice of spatial resolution is made in order to achieve the high sensitivity of an optical superposition eye. However, in all other cases, where the dioptrics produce inverted images, there is a pronounced pigment migration. During light-adaptation the pigment granules are found in a proximal position, beneath the dioptric system, thereby blocking the oblique ray paths necessary for optical superposition to occur. The result is that the eye functions as an apposition eye, with each facet of the cornea illuminating only one retinal element, allowing of a high spatial resolution. In the dark-adapted state the pigment granules are found to be very distally placed, so that they do not block the oblique ray paths, and thus several corneal facets illuminate each retinal element. The result is a higher light-gathering power, but a marked decrease in spatial resolution. It can be shown from geometrical

optical arguments that the light-gathering power of optical superposition eyes, in terms of the number of quanta gathered per receptor from an extended optical surround, is about 1000 times greater than that of an apposition eye. Optical superposition eyes are found in Lampyris, night-flying Lepidoptera, Neuroptera and Coleoptera. Evidence for an optical superposition eye is found if (1) a large space separates dioptics and retina, (2) limited illumination of eye causes extensive eye glow, and (3) optomotor responses can be induced by projecting a moving pattern onto one ommatidium.

Neural Superposition eyes (Ref. 9,24,25)

This type of eye contains ommatidia that are optically isolated from each other. However, here too there is a device for increasing the light-gathering power of the eye. The ommatidia contains "unfused" rhabdomes, in which the rhabdomeres of the retinular cells are not close together but widely separated. It can be shown that the angles between the optical axes of adjacent rhabdomeres are the same as the angles between the optical axes of adjacent ommatidia. The rhabdomes lie inside the ommatidia in such a way that a point source of light may be aligned to illuminate seven different rhabdomeres of seven different ommatidia. It can further be demonstrated optically (the phenomenon of the "reduced corneal pseudopupil") that seven rhabdomes, each from a different ommatidium, are "looking" at the same point in the environment. The axons from these retinular cells then diverge in such a way that the axons from one ommatidium do not all travel to the same element in the first optic ganglion, but to seven

different elements. This pattern of divergence brings together axons from seven different ommatidia, the result being that the rhabdomeres which are lookingat one point in the environment send axons to the same element in the first optic ganglion[3,24]. Here the signals induced in the axons by optical changes at that point are superimposed. This increases the effective aperture of the eye by a factor of seven in comparison to the apposition eye[27]. Since it is not light-rays that are superimposed, as in the optical superposition eye, but light-induced signals, this type of eye has been named "neural superposition". (Schematically shown in Fig. 5) [3,24].

If a given compound eye can be shown to exhibit a "reduced corneal pupil"[8,9] showing that the rhabdomeres of different ommatidia are looking at one point in the environment, and if the retinal elements map onto the elements of the first optic cartridge in such a way that the resolved points of the optical surround are represented as elements in that ganglion, then the eye may be considered to be of the neural superposition type. This type of eye is found in Musca and Drosophila. Unfused rhabdomes are also found in, e.g. Gerris.

In Musca, Drosophila, and Calliphora, it can be shown that vision is dichromatic[7,24]. Two receptor systems are present, and they differ in absolute light sensitivity, sensitivity to the plane of polarised light, contrast sensititivity, and spectral sensitivity[7]. The first system has an extinction spectrum with a maximum at about 515nm (the green receptor) and is associated with the rhabdomeres Nos.1-6 in a given rhabdome. The angular light-sensitivity of this

system is 3.5 degrees, and it has a greater light-sensitivity than the other system. The second system has an extinction spectrum with a maximum at about 470nm (the blue receptor), and is associated with the rhabdomeres Nos. 7 and 8 in a given rhabdome. The angular resolution of this system is 1.7 degrees and the light-sensitivity is lower than that of the first system[24]. A material shows "dichroic absorption" if the amount of polarised light absorbed by it depends on the angle of incidence of the polarised light[24]. The abosrption happening in the two systems described here is in each case dichroic. Maximum absorption occurs in the different rhabdomeres of a rhabdome at different angles of incidence.

The Optomotor Experiments

The flies Musca and Drosophila exhibit a common insect reflex, the tendency to follow the angular motion of objects within their visual field. This is called an "optomotor response", such responses being defined as the behavioural response to movement relative to the insect in its visual surroundings[8,38,39].

It is part of the insect's navigational control system: should the animal turn involuntarily from its straight course, then the apparent movement of the optical surroundings elicits the optomotor response, which compensates for the deviation, returning the animal to its previous course[14,17].

Such behaviour is the result of a "feedback loop" established between the animal and its environment, which means that incoming information is used by the animal as a reference for the control some variable, upon which in turn

depends the quality of the information being monitored. The loop may be represented as in Diagram 1. It may be analysed by (1) injecting a disturbance into the input and then tracing the response through the loop, a "closed loop" analysis which leaves the loop intact and the input under the control of the animal; or (2) by breaking the loop, injecting a disturbance into the input and measuring the response at the point of breakage, the "open loop" analysis.

In the optomotor experiments with flies the disturbance injected into the loop is movement in the fly's visual environment, the fly perceiving only the movement (see Fig. 2a), which is usually periodic, being characterised by its angular velocity, contrast, spatial frequency, and spectral characteristics. It is completely characterised as a stimulus if the angle it subtends to the fly's long axis is known.

The control system is contained in the fly's nervous system, and so cannot be dissected without greatly perturbing it. However, the variables it controls can be measured exactly: the force of flight thrust or torque, or the directional choices made in walking. Both open-loop and closed-loop analyses have been performed on the optomotor responses of Musca and Drosophila. Only the open-loop experiments and their results are directly relevant here, and only they will be discussed.

Open-loop experiments

The method is as shown in Fig. 2a. The animal is held rigidly in the axis of a hollow cylinder, the inside of which is lined with a contrast pattern. Rotation of the

cylinder causes the fly to attempt to turn in the direction of the movement. The fly and the movement form a feedback loop, which is broken because the fly is unable to effect its turning movement. The rate of rotation of the cylinder remains under the control of the investigator. The simplest experiment possible with this arrangement is to allow the fly to manifest its response by choosing left or right turns as it walks along a Y-maze. The strength of the fly's response is then measured by: $R = W-A/W+A$, where W represents the number of choices made in the direction of the moving pattern, and A represents the number of choices made in the opposite direction. It can be shown that R is then a linear measure of response strength if it is not greater than 0.7. This type of experiment has been performed on the beetle Chlorophanus and on the flies Musca and Drosophila[13,8,38,39]. Later developments in technique allowed the turning response to be continuously measured in flying flies. In such experiments the fly is attached to a coil turning in a magnetic field produced by a further coil. The turning movement made by the fly disturbs the equilibrium in the magnetic field of the coils, thereby inducing a small voltage in the outer coil. This voltage is amplified, and fed to a meter from which the torque of the fly's movement can be read. The arrangement is open-loop when part of the amplified voltage is returned to the coils as a compensation current, producing a torque in the coils opposing the torque of the fly, so that the fly is restricted in its turn to only very small angular displacements, about 1/100 of a degree.[39]

However, the results obtainable with the simpler

experiments on Chlorophanus provided the data from which the conclusions relevant here were drawn.

(a) A sequence of two light stimuli impinging on adjacent facets of the compound eye is the elementary event that evokes an optomotor response.

(b) Two stimuli impinging on the same facet do not evoke an optomotor response. (It follows that the optical resolution of the compound eye in these experiments is determined by the angle between the axes of neighbouring ommatidia).

(c) The stimulus received by one ommatidium can interact only with the stimulus received by the neighbouring ommatidium, and by those once removed. No interaction for movement perception exists between ommatidia separated by more than one unstimulated ommatidium.

(d) The visual fields of adjacent ommatidia do not overlap.

(e) A maximum response is elicited by stimuli impinging successively under a time difference of about 1/4 second on neighbouring ommatidia. Smaller and larger time differences elecit smaller responses. Responses still occur with time differences of up to ten seconds.

(f) If + denotes a stimulus change from dark to light, and - from light to dark, then the light stimuli S_{AB}^{++} applied to a pair of horizontally neighbouring ommatidia A and B elicits a turning response $+R_{AB}^{++}$, where the + sign indicates that the direction of the turn follows the direction of the stimulus succession, A to B.

(g) S_{AB}^{--} elicits a response $+R_{AB}^{--}$, and $+R_{AB}^{--} = +R_{AB}^{++}$.

(h) S_{AB}^{+-} or S_{AB}^{-+} both give responses opposite to the direction of the stimulus successions. $-R_{AB}^{+-} = -R_{AB}^{-+}$, where the - sign indicates that the direction of turn is opposite to that of the stimulus succession. Thus, the relation between stimulus input and response output of the feedback loop follows the rule of algebraic sign multiplication (Fig. 3).

Fig. 3

	S_A^+	S_A^-
S_B^+	+R	-R
S_B^-	-R	+R

(i) The strength of response depends not only on the speed of stimulus succession (e), but also on the changes of light intensity representing the stimuli. If ommatidium A receives a stimulus intensity x, and B receives y, then R is proportional to (xy), that is, to the product of the 2 stimuli.

(j) A succession of stimuli to ommatidia ABCD of a horizontal row produce a response as in (f), (g), (h) above, e.g. R_{ABCD}^{++++}. This response is equal to the sum of all the partial responses evoked, so that $R_{ABCD}^{++++} = R_{AB}^{++} + R_{BC}^{++} + R_{CD}^{++} + R_{AC}^{++} + R_{AD}^{++} + R_{BD}^{++}$ and so on.

These results show that in the Chlorophanus nervous system there must be physiological processes working in accordance with the mathematical operation of multiplication, and linking sensory input with motor output[8].

Later work with Musca and Drosophila provided evidence

that similar processes exist in those species also[8,13,14,16,17,25,38,39]. On the basis of these conclusions a minimum mathematical model was designed that accounts for the functional properties of the physiological system (Fig. 4)[8] allowing the prediction of response strengths to previously untested pattern stimuli. The model takes no account of the anatomical structures of the system, since these were not considered during the optomotor experiments: it supplies the simplest conceptual scheme which quantitatively describes the processes occurring inside the intact animals that were observed[14]. It will be seen that it makes some anatomical predictions, however.

The model has two detector inputs (ommatidia) A and B, and one motor output. A and B feed into two channels, which at one point are cross connected. Before the output is reached, the information in one channel is subtracted from that in the other, and the difference is the signal which directs the output response.

The nature of the signals passing along the two channels is described as follows. The two detectors transform the input stimulus into two time-functions, which are signals varying over time in a manner correlated with the variations of the stimuli. These two time-functions undergo filtering, which transforms them into related time-functions. The filters in the model are linear, since experimental data from Chlorophanus shows there to be linear filtering during the optomotor response. Linear filters have the property that their outputs may be superposed, which implies that their outputs for any given input can be expressed as the sum of

elementary output signals triggered by a sequence of narrow input pulses, (into which the arbitrary input is decomposed). So the response of the filter to a single impulse is a time-function.

For an arbitrary input L(t), the output is the superposition of the elementary responses which gives the responses, L*(t). If the stimulus input be described by a function varying around an unchanging mean value, so that

$$L_A = C + G(t) \qquad \text{and}$$

$$L_B = C + G(t-\Delta t),$$

then it follows that the transformations effected on these functions by the linear filters of the model can be followed exactly through the model as they occur, resulting in an exact quantitative description of the output expected from the model for any arbitrary input stimulus.

Each filter transforms the time-functions into new time-functions, and the symmetry of the left-to-right and the right-to-left response indicates that the filters in each channel are identical, i.e. $F_A = F_B$, $G_A = G_B$, etc.

At the cross connection each channel receives an input from the other channel. This is multiplied with the information already present in the channel, so that after this each channel carries information originating from both detectors. (This does not imply that each channel is carrying the same information, because each detector generally receives a different input at a given time, and also because the filters F and H have modified the time-functions L_A and L_B in different ways).

Each channel then performs a time-averaging on the time-function it is carrying, and then the contents of one

channel are subtracted from those of the other. The result governs the response output.

The model can be tested for its predictive power. This has been done[39] and the responses agree with the curves derived from the model. Furthermore, the model predicts that the optomotor response of the beetle should be invariant to phase shifts in the Fourier components of the visual surroundings of the insect, and this also has been demonstrated to be true[16,38,39].

There is evidence that the model applies both to Musca and Drosophila[13,14,17].

Use of the model led to increased understanding of the optical resolution of the compound eye. The fundamental properties of a compound eye are (1) the divergence angle $\Delta\phi$ between the axes of adjacent ommatidia, and (2) the spatial light-sensitivity range of an ommatidium, expressed by the "opening angle" $\Delta\rho$. The values of both these parameters may depend on the method applied for their measurement. It is thus desirable to investigate them with varied methods, and therefore optical measurements, histological measurements, and optometer behavioural measurements have all been made[38,39]. The optomotor measurements proceeded by predicting the expected quantitative response of the model on the parameters $\Delta\phi$ and $\Delta\rho$ This was done by deriving the equations which express the response in terms of $\Delta\phi$ and $\Delta\rho$. The two parameters were then measured from Drosophila and Musca, the results being: $\overline{\Delta\phi}$ (average) = 4.6 degrees, for Drosophila, and 2.0 degrees for Musca. These both coincide with the measurements made histologically of the interommatidial angle, in horizontal

sections. $\Delta\rho = 3.5$ degrees for Drosophila, and 1.7 degrees for Musca. It can be shown that the two parameters $\Delta\phi$ and $\Delta\rho$ are so related that they form an optimal pair[38,39].

That is, increased resolution would involve having a greater density of smaller ommatidia, which would then also have greater contrast transfer: but ultimately the light flux entering each ommatidium would be so small that the signal, with its excellent resolution and contrast transfer, would be swamped by quantum noise. Therefore the product $\Delta\phi.\Delta\rho$ should exceed a certain limit so that the signal/noise ratio is favourable. Given this limit, then the ratio of $\Delta\phi$ to $\Delta\rho$ should have an optimum value at which the resolution is not too coarse to exploit the available contrast transfer, but also the contrast transfer is not so great that the limits of resolution are exceeded. It can be shown that, in this sense, $\Delta\phi$ and $\Delta\rho$ form an optimal pair[38,39].

It then follows that the optical resolution of the compound eye is determined by the angular density of the ommatidia, and not by the much higher angular density of the receptor cells in the retina. This is true regardless of whether the compound eye is of the apposition, optical superposition or neural superposition type. This result is important for it defines the number of information channels present in the eye.

It is to be expected from the above results that there exist in the nervous system of Musca cross-connections between functional channels, and that these cross-connections are essential for the optomotor reflex: for any movement detection, in fact. It is on these cross-connections that the study presented here concentrates. It was possible in

further optomotor experiments to discover further properties of these cross-connections, and these results will be stated here. The experiments were done mainly with Drosophila and later verified for Musca[13,14,17]. These later experiments provided the following conclusions:

1. Both eyes of the fly are equal in their motion-detecting abilities, and both are sensitive to motion in any direction.

2. The motion-detecting subunits possess certain orientations on the eye surface, and they discriminate between pattern motions that are pro- and regressive to these orientations.

3. Pro- and regressive stimuli elicit opposite responses in the flight system.

4. The subunit orientations are expected to group in at least two different directions that share a common line of symmetry with the internal eye structure. The minimum model requires the axis of symmetry to be the animal's long axis[13,14].

Many more conclusions were drawn from these experiments, but are not directly relevant here. Of particular interest are some statements about the minimum number of neural connections necessary to explain the observed behavioural responses. These conclusions are to be found in references 13,14 & 17.

The Statement of Intent

The research described briefly above provides a number of conclusions which made it interesting to examine the neuron L4 in the lamina more closely. These conclusions are gathered and stated in order below:

(a) The optomotor response in Musca involves physiological processes in the nervous system, working in accordance with the mathematical operation of multiplication. (p.21-22)

(b) The minimum mathematical model accounting quantitatively for the observed behaviour (optomotor response) is one involving first-order correlation. The model has a principal anatomical requirement: that the nervous system of the animal should contain fibres linking neighbouring channels of information flow; these fibres are expected to mediate the cross-connections necessary for the multiplication process to occur. (p.20-21)

(c) The motion-detecting subunits possess certain orientation on the eye surface, and these orientations are expected to group in at least two different directions that share a common line of symmetry with the internal eye structure. The minimum model satisfying this condition requires that the axis of symmetry by the animal's long axis, the z-axis of the hexagonal array of ommatidia. (p.24)

(d) Musca possesses a "neural superposition" type of eye. Thus the information originating in one point of the optical environment is brought together as signals entering the cartridges of the lamina. These, and not the ommatidia, are to be considered the channels of information flow carrying signals derived from the visual environment, for the resolved visual environment is mapped point-for-point onto the cartridges, and not onto the ommatidia. (p.14)

(e) In the lamina there is found a cell, L4, which sends collateral branches to the two cartridges neighbouring its

parent cartridge, in the +y and -x directions of the hexagonal array formed by the cartridges[42]. This means that these collaterals are symmetrical with respect to the z-axis of the array, as well as providing the links between information channels required by the mathematical model of the optomotor response. They are therefore the first structures which could come under consideration as being these links. The other neurons of the lamina either do not have collaterals passing between cartridges, or in those which do, then the branching pattern has not been shown to satisfy the conditions required of the linking fibres.

These conclusions provided a motivation for studying the synaptic connections made by L4 in the lamina. Possibly the pattern found would indicate that this neuron mediates the multiplication process of the mathematical model, in which case this model could be tested and extended by further study of this neuron. Such a finding would, to a large extent, help in explaining the purpose and functioning of the remaining cells of the lamina.

Choice of technique.

Use of the light microscope was prohibited by the small size of the neurons in the lamina: the whole cartridge is of the order of 20μ across[2]. Further, supposing that the synapses of L4 could be rendered visible in the light microscope, there remained the fact that the three collaterals of three different L4 cells which converge onto each cartridge, are twined intimately together. It would be impossible to distinguish the cell containing a given synapse, and this was to be the whole point of the study. It was, therefore, necessary

to have recourse to the electron microscope. This could be done in different ways. One way was to assume that the L4 cells of a given area of the lamina all have identical synaptologies, and then to proceed with the Golgi-electron-microscopy technique. This was being done by other workers in the institute, as part of a programme to study the whole lamina by this technique. Their results appeared before the results of this study[43].

This study proceeded the second possible way. Since the cartridge is the information-processing unit of the lamina, it is of some interest to study the synaptologies of the three collaterals of three different L4 cells which converge onto one cartridge and end there (Fig. 15). From this one could perhaps deduce the interactions mediated by these collaterals inside one cartridge. The results of this study largely confirm the results achieved with the Golgi-EM procedure[6,43].

Serial sectioning for the electron microscope is a slow technique, but it allows of a quantitative description of the object thus observed. Since synapses inside the L4 collateral endings were obviously rare, whereas other classes of synapses were very common, e.g. the R1-R6/L1,L2 synapses,[2] the quantitative measurement could be valuable in later attempts to explain the different frequency of occurrence of different classes of synapses. This aspect would assume greater significance if the L4 neuron did in fact turn out to be the one mediating the linkages required by the optomotor response model. These considerations, together with the fact

that serial sectioning was almost the only way of making the required observation, led to its use in this study.

METHODS

The conditions imposed upon an object being observed in an electron microscope column are well known. A vacuum of about 10^{-4} Torr is necessary to allow electrons to pass from cathode to observation screen, and these electrons are focussed by the condensor lens to a near crossover or cross-over point at the specimen, so that high fluxes of electrons are available to render the image visible to the observer. Since electrons traverse matter poorly, and also because the electron microscope objective lens has a very great depth of focus, it is necessary that the specimen be sliced very thin to enable its observation. (Thick sections are completely in focus so that the image is very difficult to interpret, the possibility of excluding information from it by use of a very shallow focal plane, as in the light microscope, being absent). Finally the sections will be imaged faintly unless they are contrasted with heavy metal deposits in some way, because the image is formed by use of elastically scattered electrons: this scattering depends on the atomic numbers of the atoms encountered in the specimen, and since in biological specimens the atomic numbers of the main constituents are very similar the images will have an inherent lack of contrast[34,49].

Some of these factors are especially significant during any attempt to observe a serially sectioned object

in the electron microscope, and the methods chosen for preparation of the specimens used here reflect this fact. Of the factors mentioned above the ones that can cause difficulties are:

(1) necessity of a minimum flux of electrons through the specimen to provide a sufficiently bright image at the observation screen,

(2) the requirement that the sections be thin,

(3) the use of a contrasting procedure.

To reduce these difficulties as far as possible various steps of the preparative procedure were modified, and these modifications will be mentioned below.

Fixation:

Strains of flies bred in the MPI.Bio.Kyb. were used in the experiments. These flies were therefore freely available. The animals were stunned by a two-minute exposure to cold, at about -5°C. Other methods, such as stunning with ether vapour or CO_2 were tried, but were less convenient than use of a cold shock; there was also a suspicion with the use of ether that cell membranes appeared smudged when seen on the final pictures. In most cases no dissection was carried out before fixation. The head was sliced vertically in half with a sharp razor, and the eye required dropped into the fixing fluid. To avoid later uncertainty about the location of the tissue being examined, only the right eye was fixed in these experiments. Thus, any ambiguity could be resolved directly from the micrographs. (p.7) The fixative finally used was Phosphate-buffered formaldehyde glutaraldehyde mixture, after Karnovsky[41,22,23]. A series of fixations using this fixative at varying pH, temperature, osmolality, and molarity of glutaraldehyde, gave the following

solution (see below) as the best result. In an attempt to block-stain the tissues, to avoid the need to stain ultrathin sections on grids, specimens were stained after the Karnovsky mixture with aqueous phosphotungstic acid or ethanolic tungstic acid[1], with uranyl acetate, with potassium permanganate[30,37], and with osmic acid[36]. No better method was found than osmic acid followed by uranyl acetate and phosphotungstic acid in ethanol. This is a standard procedure, and none of the others matched its quality of fixation although permanganate did give better contrast, without on-the-grid staining[37]. Different periods of post-fixation in osmic acid showed that 90 minutes was optimal. The fixation finally used was as follows:

Fixing procedure

Glutaraldehyde/paraformaldehyde, pH = 7.20, $2\frac{1}{2}$ hrs, 4°C.
Phosphate buffer, pH = 7.20, three 5-minute washes.
Veronal-buffered osmic acid, pH = 7.20, for 90 minutes.
Veronal-buffer, pH = 7.20, three 5-minute washes.
30% ethanol, for 10 minutes.
50% ethanol, for 10 minutes.
70% ethanol + 2% Uranyl acetate + 0.1% Phosphotungstic acid for 12 hours.
100% ethanol, three washes at 10 minutes each.
Embedding.

Formulae for fixative reagents:

Paraformaldehyde/glutaraldehyde:

0.9 gm NaOH

8.0 gm paraformaldehyde

200 ml distilled water. Heat this mixture to 60°C,

not less, and wait till the paraformaldehyde dissolves. Cool under tapwater, then add:

3.75 gm $NaH_2PO_4.H_2O$

4 ml glutaraldehyde, 25%. Stir, check pH - should be between 7.1 to 7.4. Use fresh, because the glutaraldehyde oxidises and the pH falls to about 6.0, which is unacceptable.

Phosphate buffer (pH = 7.20)

(I) 9.08 gm/litre KH_2PO_4, prepare one litre.

(II) 11.875 gm/litre $Na_2H\ PO_4.2H_2O$, one litre.

Take 72.6 cc of solution (II), make up to 100 cc with solution (I).

Veronal buffer

Veronal (sodium barbiturate) 14.7 gm + $Na.OOC.CH_3.3H_2O$, 9.7 gm, + 55 ml N/1 HCl, make up to 1000 ml with distilled water. pH = 7.25.

Veronal-buffered osmic acid

5 ml of 2% aqueous OsO_4 solution, + 5 ml veronal buffer, giving a 1% buffered solution of osmic acid. pH = 7.25.

The two fixative solutions were prepared in a fume-cupboard.

Embedding

The fixed brains were embedded in Araldite. This is also a standard method[53]. Araldite is an epoxy resin, and these have the disadvantage that they significantly decrease the contrast available in the tissue when it is viewed in the electron microscope. This makes grid staining essential, and thereby increases the difficulties associated with serial sectioning. However, the other characteristics of epoxy resin are so favourable in comparison to those of, for example, methacrylates, that use of Araldite is to be preferred[53,31,33].

Araldite embedments are stable under high electron fluxes, and penetration of the tissues is excellent. Also the cutting properties of these embedments are very good.

Dehydration in alcohol, 10 minute soakings in 30%, 50%, 70%, 90% and three times 100% ethanol successively, was followed by two 20-minute soakings in propylene oxide since this is a better solvent for Araldite than ethanol. Also, any traces of propylene oxide remaining in the tissues when the resin is cured are chemically bonded into the plastic, so that the tissue is not disturbed by gassing.

The Araldite was mixed as below, stored till needed at -10°C, and after embedding, cured for 12 hours at 40°C, then 12 hours at 50°C, then 24 hours at 60°C. 6 hours cooling was allowed for cross-linking of the polymer to occur.

Araldite

(Ciba/Geigy Araldite)

100 ml Component A
+ 100 ml Component B
+ 5 ml Component D

Mix, stir thoroughly with glass rod for at least 30 minutes.

Then add

1 ml Component C — Stir thoroughly for at least another 30 minutes; Store at 0°C.

Preparation of grids

Copper grids of 3 mm diameter and containing a circular hole of 1 mm diameter were used. Use of grids with bars or nets was not possible because of the requirement that every section on the grid be visible. Grids with bars were used once or twice, with the experience that on every occasion the ribbon of sections aligned itself with the bars and so could

not be observed. Stout grids were preferred. It is possible to obtain thin ones which tend to bend very easily, and the risk of breaking the film thereby is too great.

The grids were coated with films of Formvar. This was dissolved in dichloro-ethane. It was found that solutions made with chloroform dried too quickly and produced uneven films: in the electron beam these films are less stable. The solution of Formvar was made by dissolving 0.4 gm of Formvar in 100 cc of dichloroethane. This was stirred with a magnet-stirrer for at least five hours before use. It was found that too short stirring times did not allow the Formvar to dissolve perfectly, and again unstable films were the result. Stirring was done at room temperature, but if storage of a Formvar solution was necessary this was done at 4°C. Formvar solutions were not stored longer than overnight.

Formvar films were prepared by dipping a microscope slide into the solution for half their length, and allowing excess solution to drain off one corner. After a period of 10-15 seconds the dichloroethane had evaporated and the film was then stripped off the slide. During evaporation of the solvent great care was taken not to breath on the slide because droplets of water vapour falling onto it produce holes in the final film, and dust particles introduced into the film at this stage are a source of dirt on the final photographs of the specimen. Stripping of the film away from the slide was done immediately because on those occasions where it was allowed to remain on the slide for half an hour or more, stripping became extremely difficult. In all cases the slides used had been cleaned by wiping them carefully with tissue paper. More vigorous cleaning methods, such as rinsing

in alkali solution, made stripping of the film an impossibility. The film was stripped off the slide by immersing the slide at an angle of about 45 degrees into water, one end first, then with a smooth movement immersing the whole slide at the same angle, so that the water surface moved continuously and at regular speed up the slide. The film then usually floated off the slide and remained on the water surface. The process was facilitated by breathing lightly onto the slide before immersion but after the evaporation of the solvent. If the film failed to detach from the slide the slide was dropped and allowed to sink to the bottom of the water. Many such slides can be prepared in a short time and so their preparation represents no bottleneck in procedure. The nature of the water used for this procedure is of critical importance. If the surface is dirty then the final pictures will be marred with black dirt particles. Since the dirt allowed to contaminate the film on a grid is additive, that at one stage accumulating with that of the previous stage, and is impossible to remove, the final picture can be heavily polluted unless strict cleanliness is observed throughout the preparative procedures. Therefore, the water used at this stage was always freshly distilled water or de-ionised water prepared commercially for medical use: but then only from a freshly opened bottle. It was poured into a large trough and immediately covered with a lid. The stripping process was carried out underneath the tilted lid.

The film floating on the water surface was made clearly visible by illuminating it brightly from above. Grids were then taken up singly with tweezers and dropped matt side down

onto the film. Onto each film were dropped only a few grids. The corners of the film tend to be thicker, also the edges. Grids were spaced about 5 mm from each other, so that when they were removed from the film, no mechanical stresses were applied to the films of neighbouring grids. The film with its grids were then removed from the water by laying the edge of a strip of thick plastic film on the water surface near the Formvar film, and pushing the thick film forward into the water at an angle of 45 degrees. The formvar film was thus caught on the lower surface of the thick film, and submerged with it. When the thick film was removed from the water the Formvar film adhered firmly to it, with the grids sandwiched between them. The combination was blotted carefully and laid on a filter paper in a Petri-dish. The dish was placed in a desiccator and the grids were ready for use after 24 hours. They could be stored for two or three weeks if necessary. For longer periods it was found preferable to prepare fresh grids. This procedure produced stable grids which were reliably clean, although occasional dirty grids did appear.

Sectioning

The L4 collaterals to be examined lie in the proximal part of the lamina. Therefore, the whole retina and the distal lamina must be trimmed away. The rough trimming, to expose the tissue by clearing away excess Araldite, is easily done using a razor-blade and a binocular dissection microscope. Usually most of the compound eye was exposed in this way, so that a choice of the exact location for

sectioning could be made. The Araldite block was then inserted into the holder of the ultramicrotome and semi-thin sections cut off normal to the surface of the eye at that point chosen for study. These semi-thin sections, 2 microns thick, were removed from the glass knife with a camel-hair brush, and placed on a drop of water on a microscope slide. This slide was warmed to 60°C for a few minutes so the sections dried down onto it, and then the following stain applied to the sections at 60°C for about one minute:

1% azur 2 in distilled water + an equal volume of

1% methylene blue in 1% borax solution. (Mallory's Azur 2-methylene blue stain)

This stain will be referred to as the "methylene blue stain". It stains Araldite-embedded tissues. When these were fixed as described above, the cytoplasm of most cells stain deep blue, and some neurons have cytoplasm staining only little. The result is that the grosser features of the tissue can be easily examined in the light microscope with a x25 objective, without phase contrast. After staining the slide can be rinsed with tapwater and allowed to dry, and then a permanent preparation made using Permount to mount the coverslip.

Using these semi-thin sections it was easy to check that the correct part of the eye was being sectioned by examining the retina. Later these sections could give information about roughly how deep in the lamina the sectioning had reached. Since the eye and lamina are convex domes, then a transverse section showing a small area of chiasma in the middle could be taken to guarantee that a number of cartridges would be visible on the final ultrathin

section, cut across at varying levels (Fig. 19). The choice of cartridge to be followed could then be made at the electron microscope.

At this point the block was removed from the ultramicrotome, and a final trimming done under the dissecting microscope. This was again done with razor-blade. The aim was to produce a sectio ning-face with the smallest possible surface area and the correct trapezoidal shape. The depth of the trapezoid was usually limited to three or four cartridge rows, at the most to about 100 microns deep: this allowed the placing of ribbons 10-20 sections long onto a single grid. Such trimming was very delicate, but gave the advantage that cutting artefacts such as chatter, badly expanded sections, and uneven thicknesses of neighbouring sections, were easily avoided. Also, the glass knife was no trouble during sectioning: no scratching occurred, mainly because the ribbon of sections could be allowed to grow quite long without disturbance.

The microtome used for ultrathin sectioning was usually a Porter-Blum MT2-B, but sometimes an LKB 300 was used. The time chosen to perform serial sectioning was usually an evening during a weekend, to avoid problems due to vibrations caused by traffic or walking people.

Various modifications to normal cutting technique were used. These were:

(1) <u>The knife</u>

Glass knives were used. More than 200 sections could be obtained without pause and without scratching from a normal glass knife, providing the sectioning-face was small. The standard piece of Tesa-band was curved around the knife to

form a trough, and the trough was sealed with nail-varnish. The difference was that a looped injection-needle was taped onto the knife in such a way that its point rested inside the trough, and below the level of the cutting edge. This needle was sealed into position with a piece of Tese-band and nail-varnish.

During use of the knife the needle was attached to a 1-metre long piece of plastic tubing, which at its far end was attached to a syringe mounted on a board and provided with a fine screw system attached to the plunger. The syringe, the tubing and the knife were all filled with distilled water before sectioning began, and during sectioning the level of the water surface could be easily controlled by moving the screw knob slightly. This arrangement allowed the problem of evaporation from the trough during serial sectioning to be obviated without disturbing the water surface in any way. Also, if a ribbon of sections proved difficult to remove from the cutting edge, a gentle squeeze on the tubing allowed a current of water to be applied to them from beneath, a procedure which frequently dislodged them. The whole arrangement has been described before[51].

(2) Removal of sections from the trough

Fishing for the sections with a grid causes great disturbance to the glass knife, and can result in a section being skipped or a thick section being cut on the next cycle. The LKB microtome could be halted, cooled with its fan, and restarted after the sections had been caught on the grid. This was not possible with the Porter-Blum, and even with the

LKB the result was frequently that the grid brushed against the knife cutting-edge, and caused scratches on subsequent sections. The following technique allowed ribbons of sections to be removed from the trough with minimal disturbance to cutting.

A glass rod was heated with a bunsen burner, and stretched to provide a very thin diameter length of glass. This length was heated gently and folded into a loop. The resulting glass-loop of about 3 mm diameter could be easily cleaned in acid and kept in easy reach of the ultramicrotome. Wire loops have also been used for this purpose.

When a sufficiently long ribbon of sections had been cut the glass loop could be immersed in the trough water and brought up beneath the ribbon. The ribbon then remained floating on the drop of water in the loop, when it was pulled away. It was easy to perform this sequence quickly and with no disturbance of the trough water. With care the loop could be used to lift up a ribbon of sections still adhering to the cutting edge of the knife, avoiding the need for any other movements near the microtome.

(3) Capture of sections on grids

The aim of catching a whole ribbon of sections on a grid without any sections lying over the metal of the grid is difficult to achieve if the ribbon is nearly as long as the grid is wide, and without ample time and space. These latter factors were provided in the following way. Small slabs of steel were bored with a countersink drill to produce a cavity in one surface, about a centimeter in diameter. The slabs were then sprayed with black paint.

A small pool of water was put into the cavity of several slabs before sectioning began. A fluorescent light was used to illuminate the surface of the pool of water in one slab in such a way that sections floating on the pool were made clearly visible in the same manner as occurs at the ultramicrotome itself. The pool of water could be observed with a binocular dissecting microscope. It was easy to release the ribbon of sections picked up on a glass loop onto the pool of water on the black slab and pass the whole slab over to an assistant, or indeed simply to leave it until sectioning was finished. Then the ribbon of sections could be accurately aligned at leisure with the hole of a grid, with plenty of working space and the possibility of discarding a defective grid if this were necessary. Sections were captured on grids by lowering the grid into the water perpendicularly until the water surface covered half the hole, and then edging the grid toward a section. Tilting the grid slightly under the section and pulling it up from the water would then cause the sections to adhere to the Formvar film.

Sections

Silver sections were cut. These had a thickness of between 600-900 Angstroms. During a long serial section some slightly thicker and slightly thinner sections were always produced, for unknown reasons. Lack of vibration or air movement, no manual contact with the microtome, and even heating etc. in the room, did not suffice to remove this problem. If a serial section contained an unacceptable number of gold sections (1200 Å), or, on even less fortunate

occasions blue ones (1500 Å), it was discarded. The series used contained mainly silver sections together with a few grey ones and pale gold ones.

Contrasting of Sections

Grid-contrasting using Reynold's lead citrate was used[40]. The two-step contrasting procedure involving uranyl acetate and then lead citrate, was not used. This implied a minor loss of contrast on the sections, but this could easily be recouped at the microscope or during the photographic procedures as will be described. The reason why the two step procedure was avoided was the following. It can be shown (see Appendix, p.53 that if a collection of grids is to be subjected to a number of procedures each of which involves a possibility of destroying the grid, then the number of grids lost after all the procedures are completed will increase in a fashion related to the binomial series, so that each added procedure involves an addition to the number of grids lost that depends on an added term in the binomial series. Although the probability of destroying a grid by puncturing the Formvar film is an unknown quantity for a given procedure, it is certain that the more procedures that are used, the greater the probability is that a set of grids containing a serial section will not be intact at the end of the last process. Therefore, the simplest and fewest procedures were used on the grids. In particular, to avoid handling the grids often with forceps, the following method was used: a gridbox was drilled out so that each cavity for a grid became a hole open on both sides of the box. The lid was drilled appropriately also, so that with the lid

on the box it was possible to immerse the whole box into a solution and thereby expose each grid to the solution. The grids were stored in such a box immediately after capture of the sections, and the staining with Reynold's solution was performed by immersing the box in the solution for two minutes, then moving it gently up and down a few times in the 0.02N sodium hydroxide, then many times in fresh distilled water. Then the whole box was blotted off and dried. It was not found necessary to dilute the Reynold's solution.

Observation in the Electron Microscope

The choice of magnification to use is of paramount importance when a serial section is to be observed. If too high a magnification is used then the intensity of the electron beam that must be passed through a section in order to produce an image on the fluorescent screen sufficiently bright for focussing, will be high. This intensity may be produced by using the condensor lens to provide a crossover beam, but the effects of this on the section are significant and damaging. The high flux of electrons easily evaporates the lead deposit used to give contrast to the tissue, and this lead will then condense again onto the section and provide background contamination. The lead may also melt and coalesce into granules *in situ*, and these granules are sufficiently large to prevent the resolution of unit membranes. Thirdly, the electron beam vapourizes the Formvar film of the grid, and also the Araldite of the section. If a focussed beam is used to illuminate one small area of the section, this vapourization can produce mechanical tension sufficient to rupture

the film and destroy the preparation. Since the grids used for serial sections are not provided with net or bars, there is no efficient heat conduction away from the site of illumination, where electrons are interacting with matter to produce heat: the resulting build-up of heat at that point also tends to rupture the film.

For these reasons it is essential to choose a magnification which is not higher than the minimum required to resolve the smallest details which should be studied. A smaller magnification than this will not allow the synapses, for example, to be visualised. The choice of magnification was calculated as follows.

The smallest object that should be resolved on the final print is the unit membrane. This has a width of about 200 Angstr. The smallest object comfortably resolved by the human eye is about 0.5 mm in diameter, held a few inches from the eye. Thus, the total magnification required can be calculated:

Given that 10,000 Angstroms = 1 micron, and

1,000 microns = 1 millimetre,

the required total magnification will be:

500 microns/200 x 10^{-4} microns = 2.5 x 10^{4}.

This is then a magnification of about 25,000 times.

Since part of this magnification can be achieved during the preparation of the final print, only the grain size of the negative need be considered. If a magnification of five times is applied to the negative to produce the final enlargement, the grain size of the negative will not appear on the print, so that an initial magnification of about 5,000 times will be

required at the electron microscope.

In practice the second step magnification of the Zeiss EM 9S was used: 4,700 x. This was followed by a 3x magnification for the final prints, giving a total magnification of 14,100 x on the print. The beam intensity thus required to focus the sections was sufficiently low that few preparations were destroyed in this manner. Focussing of the image was usually performed by finding a small hole in the film near the object to be photographed, and then strongly overfocussing. The diffraction ring thus produced was very bright, and by gradually underfocussing the objective this ring could be observed as it decreased in diameter. The point at which it vanished by becoming the same diameter as the hole could be observed easily by focussing through it and observing the dark edge that then began to appear round the hole. Two or three such through-focussings always sufficed to note the point of exact focus, and then the grid was translated the short distance to the object, and the photograph taken. In this way focussing required a minimal intensity of the beam: much too small an intensity to enable observation of the object being photographed. Such observation was never indulged in because the object could be studied at leisure later on the final print, and because of the danger of rupturing the film. During photography of a serial section the small striplike sections described above (p.37) were valuable. Once the required object was located on the first section, then the direction of translation required to move to the next section was always the same, as was the

distance to be traversed. Thus, the whole strip of sections on one grid could be worked through very quickly and with certainty that the correct object was on each photograph. This had an especial value if the Formvar film ruptured during the observation: the film then slowly contracts toward the edge of the grid, so that if the pictures can be taken quickly enough, the remainder of the sections can be photographed before the film is doubled over them or otherwise obscures them. It will be seen that loss of three or four sections already is a great hindrance in analysing the cartridges of the lamina. (p. 61)

Contrast

This was enhanced by use of contrast diaphragm just below the object, so that only electrons passing through the tissues were allowed to reach the photographic material. The electrons coming at large off-axis angles diminish the available contrast appreciably, and this diaphragm closed them out. For this purpose too, the intermediate lens diaphragm was inerted into the beam. This diaphragm lies nearer the photographic plate, and serves the same end.

Exposure

The Zeiss EM 9S is equipped with an automatic system to determine the correct exposure. At the beginning of the work this system was calibrated with test plates and the developer to be used. Thereafter a simple calibration before each series of exposures was sufficient. The intensity of the beam was reduced before exposures so that an exposure time of about one second resulted.

The microscope was checked for correct alignment before

each series of exposures were made. In particular, astigmatism could be closely watched at the focussing stage.

Photography

(1) Developing

Plastic plates, Agfa type Scientia were used in the Zeiss EM 9S. The instrument holds a few more than 60 of them, which is helpful when many sections have to be photographed routinely. Loading and unloading of plates was performed in total darkness. Two developers were tried with these plates, Kodak D19 and Neutol. It was found that Neutol gave better contrast and this was used routinely. This developer also gave excellent results with respect to fogging of the plates, which was absent, and to grain size. Graininess on the final prints was not a problem. This stage of the photography was one at which contrast could be increased, compensating for the small loss of contrast due to omission of the uranyl acetate grid stain. Developing was performed in total darkness.

(2) Printing

Developed plates were stored in individual envelopes. These were numbered appropriately with the plates; the Zeiss EM 9S numbers each plate.

The final print was required to be enlarged sufficiently that inspection with the naked eye would reveal the small details, and use of a magnifying glass would clarify any doubts concerning these details. The plates of the Zeiss EM 9S were 6 x 6 cm, and a magnification enlarging this to fit onto 18 x 24 cm paper, 3 x , satisfied this requirement. It was, therefore, not possible to turn the paper beneath the enlarger

so that the orientation of each picture was the same. This was a minor hindrance during serial reconstruction, but to alleviate it would have been very wasteful of paper.

The enlarging process provided another point at which contrast of the final picture could be increased. Subject to the requirement that six steps of grey tone be visible on the final print, a choice of paper hardness was possible, "harder" paper giving greater contrast than "softer". It turned out that normal grade paper gave sufficient contrast.

Serial Reconstruction

Since the pictures comprising the series were frequently interspersed with pictures representing the work of others, and sometimes more than one cartridge had been traced through a series of sections and two sets of pictures (of different cartridges) were intermingled, the plate numbers did not provide an ordering for the series. Also, a ribbon of sections on a grid is not marked for first and last section, and sometimes these ribbons were photographed in reverse. Therefore, the first part of serial reconstruction was to order the pictures representing one series and number them as members of that series. This numbering was done on the prints themselves. It was found that careful notation at the electron microscope was essential, otherwise the assignment of a given plate to a definite grid was difficult. If this notation had been done and was available, then the task of ordering pictures was easy after some practice has been acquired at recognising similar pictures.

Once the prints were ordered the various elements of the cartridge could be unequivocally identified on the first

print, and marked on that print with coloured pencil. The tracing through of the identified elements was then easy, except where a section was missing: at such places the elements of diameter large in comparison to section thickness could be easily traced, but smaller branches whose diameters were small in comparison to section thickness could often not be followed through. Branches of L1 and L2 have a diameter of about 0.5μ or less, when these were cut lengthwise they could not be identified if a missed section intervened. The identification of each element was marked onto each print in coloured pencil. After this any element could be located at any point of the serial section by specifying its name and the number of the picture in the series.

Two courses could now be followed. The first would be the reconstruction of the three-dimensional shape of the cells, which for the purposes of this work was not really needed. A number of methods for proceeding with such a reconstruction have been described, such as drawing the cell outlines onto glass plates and stacking the plates, or tracing groups of pictures onto pieces of tracing-paper and then tracing groups of these groups onto other pieces of tracing-paper, so that eventually one piece contains a contour map of the whole object, and others. Special problems of aligning neighbouring pictures exist in such work, and a number of computer programmes exist allowing most of this work to be routinely completed by computer.

In this work the second procedure was used: this is to simply tabulate the supposed synaptic connections and use the table to produce a diagrammatic presentation of the "wiring-

diagram". Although a definitive statement about the properties of the resulting diagram is not possible from the anatomical results alone, and given the difficulties encountered in distinguishing whether a certain object on the picture is a synapse or not, it is nevertheless to be expected that such a diagram will disclose any symmetries or asymmetries present in the "wiring", and provide some clues about the functions mediated by the connections thus demonstrated. (Compare ref. 2).

This tabulation involves the following problems:
(1) First, it is necessary to define the characteristics of what is to be considered a synapse. Since the simplest explanation of the lamina function is that the receptor cell axons are presynaptic to the monopolar cells, then the connections most frequently observed between these cell types may be labelled as synapses. This consideration also defines the pre- and post-synaptic sides of the synapse. Since it has been shown that the information flow in the lamina is indeed from the retinular cell axons to the monopolar cells, then the object to be called a synapse in this study will be the same as that called a synapse by Boschek in 1968[2]. This procedure gives no guarantee that all the types of synaptic connection will be tabulated: it does, however, tabulate the connections most certainly identified as synaptic. It turned out that this was the only type of synapse in the L4 collateral network.
(2) Once the "synapse" is defined, another problem arises: if the serial section is incomplete because in some places one section or two sections are missing, then it is not certain that the final tabulation will contain all the synapses

that were present in the original material. The probability of producing a serial section of the required length with no missing sections is very small. Light microscopy shows that collaterals of L4 have end-feet which extend from the proximal edge of the lamina to about one-third of its length distally[42]. The lamina is of the order of 60-80 microns deep, so that the L4 end-feet are about 20-25 microns long. Since 10,000 Angstroms = 1 micron, and the ultrathin sections cut for the electron microscope are between 600 and 900 microns thick, it follows that the number of sections required to cut the total depth of one L4 end-foot is

25 x 10,000/600 = approximately 400

or 25 x 10,000/900 = approximately 250

This would be the number of sections required to section the total depth of an L4 end-foot in the deepest part of the lamina, using the sections normally produced. In practice the part of the lamina chosen was quite high in the northern hemisphere of the eye, and sections of about 800 Angstroms thickness were used. This reduced the number of sections required to about 150 or less.

However, the chances of cutting and observing 150 sections at the electron microscope without losing any are very small.

The dimensions of the synaptic structure observed are about 1,000 Angstroms long. It has been noted that these structures occur in corresponding positions of three serial sections, and infrequently in only two sections. Thus, if one place of the serial section more than two sections are missing, then there is no guarantee that the tabulated results will mention all the synapses originally present in

the tissue.

(3) The third problem is also to do with the synaptic structures. These normally are found, in the L4 end-feet, to be positioned where the presynaptic cell contacts two post-synaptic cells at one point (see Figs. 17 and 18): that is, the synapses are positioned in the corners of the cells, where membranes meet each other in close proximity. The result of cutting a membrane obliquely is a blur on the final picture, and since in these corners the membranes curve sharply, there will always be blurs at these positions because a cut perpendicular to one part of membrane will be oblique to other membranes. In the resulting confusion the synapse may be difficult to discern. For this reason a structure was only tabulated as a synapse if it was visible on three consecutive sections. This requirement diminished the chances of mistakenly discerning a non-existent synapse in such a corner, or of not seeing one that was there.

One final problem existed in the tabulation procedure. The part of the cartridge under consideration contains the end-feet of three different L4 cells: one from the cell which is a component of that cartridge, one from a cell which is a component of the neighbouring cartridge in the -y direction, and one from a cell which is a component of the neighbouring cartridge in the +x direction (Fig. 15). These three end-feet are in intimate contact with each other and with L1 and L2 and some of the R-cell axons. Each end-foot makes contact with each other end-foot and with L1 and L2, and with some of the R-cell axons. This involves much branching and bypassing of neighbouring end-feet, as well as of any branches arising

from L1 or L2. Frequently the membranes lie along the plane of sectioning, so that large blurred areas intervene where such bypassings occur. It can sometimes be difficult to assign a profile on one picture to a known profile on the preceding picture under these circumstances. However, the tabulation requires, ideally, that all the profiles on each picture be assigned the correct identity: otherwise a synapse, for example, between the end-foot of the -y component and L2, may be assigned to the +x component. This problem could not always be solved, and in these cases any such synapses were not assigned to a definite component. They were assigned, with a question mark, to those components which were the possible "parents".

METHODS

Appendix

Consider a family of processes $P_1..P_h$ to be applied to a collection N of n objects, in order $P_{1,2,....h}$. If to each process there corresponds a destruction rate expressed as probability p_1 of destruction occurring during process P_1, then after each process a definite proportion of N will be already destroyed.

Consider p1 ≠ p2 ≠ p3 ≠ p4, etc.

After P_1, p1.n objects will have been destroyed.

" P_2, p2.(n-p1n) = p2n-p2p1n, objects destroyed.

" P_3, p3.(n-p1n p2(n-p1n)) " "

Number left after process:-

$P_0 = n$

$P_1 = n-p1n$

$P_2 = n-p1n-p2n+p2p1n$

$P_3 = n-p1n-p2n-p3n+p2p1n+p1p3n+p3p2n-p3p2p1n$

This is binomial (Count terms of different order)

If p1 = p2 = p3, etc. then:-

Number left after process:-

$P_0 = n$

$P_1 = n-pn$

$P_2 = n-2pn + p^2n$

$P_3 = n-3pn + 3p^2n - p^3n$ (Binomial)

RESULTS

The structure representing a synaptic connection is shown in Figs. 17,18. These T-ribbon structures are apparently limited in occurrence to a few insect orders, but they occur as the major type of synapse in the lamina of Musca, and are frequent in the other visual ganglia too. The T-ribbon is situated against the membrane of the presynaptic cell. It appears as a T-ribbon in cross-section, but in fact its three-dimensional structure is considerably more complicated[2]. It consists of a flat membranous sheet, which forms the upper arm of the T in cross-section, and is situated at a distance from the membrane of the cell. Between the sheet and the membrane there is a perpendicular structure of two crossed bars, which is in contact with the sheet, but ends a very short distance from the cell membrane. This presynaptic structure is common to all the cell types of the lamina, being found as the connection made where branches from L1, L2 or L3 meet the R1-6 cells. See picture 20a), R4. In this case the T-ribbon is present in the R1-6 cell. It is found again in the alpha cells, always in apposition to one of the gnarls, and also where alpha is presynaptic to L4. It is the type of presynaptic structure found in the L4 collateral network.

The post-synaptic structures vary in shape, although no known correlation exists between the type of post-synaptic structure and the type of function mediated by the synapse. In L2 the post-synaptic structure is a short bar. In L4 it is a rectangular fuzzy structure. Fig. 18 shows both these

types simultaneously, and in Fig. 17 both the post-synaptic elements are L4 collateral endfeet.

In the serial sections the post-synaptic structures are poorly seen because of the low magnification employed. Also, the synaptic site is often sectioned obliquely, so that the sheet part of the presynaptic element is missing, and the perpendicular crossed bars are sectioned in close proximity to the cell membranes of both pre- and post-synaptic cells, together with the post-synaptic structure. In such a case it may be difficult to decide that the resulting dark smudge represents a synaptic site, and it may be impossible to decide which cell is post-synaptic, or whether two or three are. (See synapse y→x+L2, picture 44a).

Several series of sections through the proximal portion of the lamina were examined. Most of them were prepared from male flies four days old, and from the northern hemisphere of the right eye.

The results from these series constitute a survey which provides an indication of the types of connection found among the L4 collateral endfeet in the northern hemisphere of the lamina. Tables 1-6 present these results. In these tables, the horizontal rows of entries record the neurons that were observed as being post-synaptic to L4 collateral endfeet. The vertical columns record which type of collateral endfoot was observed to be presynaptic. Thus, an entry in the "x" column and "y" row records an observation than an L4 collateral endfoot running along the x-axis to a certain cartridge was observed to be presynaptic to another L4 collateral endfoot running along the y-axis to the same

cartridge. (The x and y axes, and the projection of the L4 collaterals along them to meet in a given cartridge, are presented in Figs. 12, 13, 15 and 16).

The numbers constituting the entries in one of the Tables 1-6 record the number of times that that type of synaptic connection was observed in the series of which the table is the record. Thus, in Table 2 the entry in the column "x" and row "y" is "1": this records the observation that the L4 x-endfoot was presynaptic to the L4 y-endfoot, in the same cartridge, only once in this series of 23 sections. The diagonal entries in the Tables 1-6 would imply an L4 collateral being synaptically connected to itself, and therefore are void. The columns denoted "R" record observations of retinular cell axons being presynaptic to L4 collaterals, this was only rarely observed. The rows L1 and L2 record occasions when the L4 collateral endfeet were observed to be presynaptic to the monopolar fibres L1 and L2.

Table 1 records a preliminary survey made on sections from the northern hemisphere of the left eye of an old male fly. The sections were not serial, and the entries in the table record the occurrences of the different types of connection observed in the material which were summed to provide the entries.

Table 2 records the observation, in a series 23 sections long, of one cartridge from the same fly as Table 1, and the same area of the eye: northern hemisphere, left eye.

Table 3 records the observation, in a series 50 sections long, of one cartridge from the northern hemisphere of the right eye of a 4-day-old male fly.

Table 4 records the observation, in a series 30 sections long, of one cartridge from the northern hemisphere of the right eye of a 4-day-old male fly.

Table 5 records the observation, in a series 60 sections long, of one cartridge from the northern hemisphere of the right eye of a 4-day-old male fly.(Tables 3-5 are derived from 3 different flies).

Table 6 is a composite table: its entries are the sums of the entries in the Tables 1-5.

Tables 2-5 are all consistent with one another, and the Table 6 therefore provides a general survey of the preliminary results. It shows that the three L4 collateral endfeet clustering inside each cartridge are presynaptic to each other, with approximately the same frequency. Each of the three endfeet are presynaptic to L2, with similar frequencies, and none of them were seen to be presynaptic to L1. The synaptic relationships between the three L4 collateral endfeet and the retinular cell axons are not obvious from Table 6. Each endfoot was observed to be presynaptic to retinular cell axons at least once, but to which one of the six was not recorded. The retinular cell axons were not observed to be presynaptic to the L4 collateral endfeet.

Table 1 is not derived from serial observations, but it is consistent with Table 6.

None of the results in Tables 1-6 are derived from observations of a series of sections containing the complete cluster of L4 endfeet within one cartridge. The longest series recorded, in Table 5, contained 60 sections, which contains only part of the cluster of endfeet in one cartridge (see

p.50: about 150 sections would be required to contain a complete cluster).

The numbers of synapses recorded in Tables 1-5 are also low. It was thus impossible to exclude the possibility that the synaptic connections made by the L4 collateral endfeet in one cartridge could be different, perhaps in a systematic way, to those in another cartridge. None of the Tables 1-5 contain a full complement of the possible connections, although Table 6 does.

For this reason an attempt was made to cut a long series of sections, of sufficient length to contain a complete cluster of L4 collateral endfeet in one cartridge. A series of sections was prepared from the lamina underlying the corneal facets about 15 to 20 rows south of the northern perimeter of the right eye of a 4-day-old male fly. The preparation was thus from an area near the edge of the eye, where the lamina is about three times thinner than at the centre of the eye. A shorter series of sections suffices to contain the whole length of the L4 collateral endfeet from this area of the lamina.

From this series were photographed two cartridges, which are presented here as two sets of photographic prints. The cartridges are labelled "a" and "b", and cartridge "a" was the neighbour of "b" along the x-axis of the hexagonal array of lamina cartridges. The results obtained from "a" and "b" are presented separately below. The two sets of photographic prints are presented in a separate binding, the number of each print being written beside the EM plate-number (e.g. 91a: cartridge "a", section 91).

Results from a) and b)

139 sections in series were prepared as has been described under "Methods" above. Table 7 contains the notes made during sectioning, and shows which grid of the gridbox contained given sections, and the nature of those sections, whether thick, thin, etc. These grids were treated as described, with the Reynold's lead citrate solution, to provide contrast in the EM. Two neighbouring cartridges, a) and b), were chosen on the first section, and were photographed on the different sections comprising the series at a magnification of 4,700 times using the Zeiss EM 9S. Table 8 contains the notes made during this photography, and shows the date of work, which grid was photographed, the setting on the Zeiss EM 9S, and which plate number corresponds to which section on the grid. Later, the serial numbers of each picture were added to the table, so that quick identification of a picture was possible during analysis of the series. (Table 8 also contains entries made by other workers using the EM). The two series a) and b) are presented as two series of photographic prints, to which Table 9 provides a key, giving the plate number from the EM, the grid number in order, and the serial numbers of the prints, and also the orientation of the series with respect to the lamina. Using Tables 7-9 it was possible to reconstruct the two series in correct order as photographic prints, because the three tables contain all the information required to correlate any of the prints to the original sections viewed in the EM. A description of each series individually follows.

Series b

Cartridge b) was chosen because it was the neighbour of cartridge a), and could therefore be easily located and photographed. Also, any asymmetry between neighbouring cartridges would be seen. However, since the lamina is a curved plane, cartridge b) is sectioned at a level considerably more distal in the lamina than is cartridge a). (See Fig. 19). The result is that series b) does show features not found in series a), but the whole depth of the L4 collateral endfeet in cartridge b) are not recorded in series b).

Series b) shows the stem fibre of L4 giving rise to the "0" branch of the collateral network, picture 70b: that branch which enters the cartridge to make synaptic connections with the other collaterals entering the cartridge from the -x and +y axes of the array. The fibre thus identified as L4 is post-synaptic to the alpha cell at more distal levels of the series. (Pictures 29-30b, 15-16b, and 3-5b). This class of connection is not evident in the Series a). Series b) yields little information on the connectivity of the L4 collateral network. The only synapse seen in the network is on pictures 82-83b, where the y-collateral is presynaptic to L2 and the 0-collateral simultaneously, as a "dyad". The synaptic connections evident in Series b) are presented in Table 10. Series b) shows two other features which make it valuable for comparison with Series a). The first is that the position of the L4 fibre in series b) is normal with respect to the other components of the cartridge, being close to the L3 fibre throughout its length. In Series a) the position of the L4 fibre is unusual, the fibre being displaced

to a position beyond the R6 ending, see for example picture 55a. The second is the long extension that the x-collateral sends around L2 in the pictures 81-87b. This is equivalent to a similar extension of the x-collateral found in Series a), pictures 74-60a. This extension gives rise to a branch of the x-collateral which is quite important in Series a), because the branch makes synaptic connections. Unfortunately the Formvar film on the grid (B5) ruptured during photography at the EM; the photography was continued but the pictures were blurred by movement as the Formvar film retracted during the exposures (Cf. p.45). The extension of the x-collateral occurs on the pictures which are blurred in Series a), but the equivalent extension in Series b) occurs on pictures which are clear, because the cartridge b) is displaced on the series due to the curvature of the lamina. In Series b) the extension also forms a branch of the collateral, lying beside L2, R6 and R1, that is, in the same position as the branch in Series a) at pictures 57-58a. It is quite apparent from pictures 69-71a that the x-collateral in Series a) is branching, but the extra evidence from Series b) makes the conclusion firmer. In neither Series a) nor Series b) does the y-collateral branch in this way, so the branching of the x-collateral is unexpected. (A simple explanation for it could be that since three different L4 collaterals are clustered together in a limited space between L1 and L2 (see Fig. 15), and if each must make physical contact with the other two and with L1 and L2, then there must be branching in order to allow every combination of types of contact to occur. Such branching is easily accommodated if one collateral

branches around the outer side of L2 and re-enters the cluster a second time, from the opposite side. This would not disturb the close packing of the cluster.

Series a)

This Series is complete in the sense that it contains the total endfeet of the L4 collateral network; cartridge a) was chosen for study because the first section of the Series, picture 1a, shows the most distal traces of the collateral endfeet. All the synaptic connections within the collateral network of cartridge a) can be traced, and it is the analysis of these connections that provides the immediately verifiable results of this study. The other serial sections that were studied produced results in accordance with the ones that will here be derived from Series a). (See Tables 1-6 and p.57).

In both Series a) and Series b) the cells of the lamina have been identified with ink on the prints of the Series, in different colours. These identifications are as follows: the neurons L1, L2, L3 and the receptor axons R1-6 are identified in blue. Small branches of L1 and L2 are important because they sometimes receive synaptic connections from the collateral network, as in pictures 36a-40a, where a branch of L2 in picture 37a is post-synaptic to the y-collateral on pictures 38-40a. (The O-collateral is simultaneously post-synaptic to the y-collateral on these pictures). In such cases the L1 or L2 branches may be marked merely with a small blue "1" or "2", as in pictures 39-40a, because space does not permit the "1" to appear.

The x-collateral is identified in black ink, the y-collateral in green ink. The L4 cell native to the

cartridge is identified in red ink, its different parts being labelled appropriately: thus, the stem fibre is labelled "L4" and appears on all pictures, and the O-collateral is labelled "O", and the x- and y-collaterals of the native L4 neuron are labelled in red as "x" and "y". Pictures 84-87a show the points where the O-collateral and the x-collateral arise from the stem fibre, and the markings have been made accordingly. The x-collateral thus marked can be followed through Series a) as far as picture 37a. The point where the y-collateral of this L4 arises is not shown, but the collateral itself is marked, pictures 87-91a.

Neurons not relevant to this study have not been marked, so that L5, and beta are marked, but R7 and R8 are not marked. One case of a suspected synapse, L4 presynaptic to beta, led to the labelling of the beta profiles in green ink from the site of the supposed synapse, as far as they could be followed unambiguously.

The synaptic connections noted in Series a) are presented in Table 11. The synaptic connections have not been marked on the pictures because most of them are clearly visible without it, but also because there is too little space to mark them without at the same time obscuring either the synapse or its surroundings in some way. In cases where the existence of a synapse is in doubt, because it is poor in contrast or sectioned obliquely, or where there is doubt as to which of three profiles in close proximity to the presynaptic profile are really post-synaptic, any obscurance of the evidence would be very unhelpful.

In Table 11 the columns denote the types of synapse recorded, and are labelled "y--x", etc. The latter indicates

a synapse in which the y-collateral is presynaptic and the x-one post-synaptic. The synapses are nearly always, if not always, "dyads", see below. The rows of Table 11 indicate the pictures of the Series. An ▨ entry in a row indicates a synaptic connection to be seen on that picture, and involving the profiles noted at the head of the column. Doubtful synaptic connections have been denoted with ◢ in Table 11, and where the doubt is severe, as "?".

Table 11 represents the data gathered from Series a) by inspection of the pictures. It is summed in Table 12, the numbers of occurrences of the various types of connections being the contents of the Table.

If the doubtful entries be disregarded, the results presented in Table 12 are equivalent to the following propositions:

1) The three L4 collaterals which cluster in cartridge a) are each presynaptic and post-synaptic to the other two at least once, and not more than three times.

2) Each of these three L4 collaterals is presynaptic to L2 at least once, and not more than three times.

3) None of these three L4 collaterals is presynaptic to L1.

4) The three L4 collaterals are post-synaptic to each other but to no other cell. (Propositions 1) and 4) are illustrated in Fig. 15).

Identical conclusions were drawn from the evidence of earlier serial sections (see Tables 1-6). One feature of earlier serial sections is missing from Series a); synaptic connections from the L4 collaterals to the R1-6 cells were evident in earlier series, but are completely absent from

Series a), except possibly in picture 13a, which is a doubtful connection.

The four propositions given above can be simplified if it is observed that the synaptic connections in Series a) are all "dyads". In a dyad one profile contains a presynaptic structure, and two profiles, both apposed to the first, both contain a post-synaptic structure. It is much more difficult to observe a dyad than it is to demonstrate an ordinary synapse: it may be that more than two profiles are apposed to the presynaptic structure but not all of them are post-synaptic, a quality which is demonstrable only if the post-synaptic structures are easily recognisable. Difficulties also occur if the section lies in a plane transverse to the axis of the synaptic connection, because the picture will then show a blur due to membranes sectioned obliquely and obscuring the presynaptic and post-synaptic structures. This difficulty was mentioned above regarding the recognition of simple synaptic sites: it is compounded if the synapse is a dyad. In spite of these difficulties the evidence in Series a) seems to justify the construction of Table 13. In this Table, the LHS of the entry gives the serial number of the pictures in Series a) (on which the synapse is to be found), and the RHS of the entry is a description of the synaptic connection visible on these pictures. For reference, each entry has been numbered, 1-10, so each synapse recorded has a number. From Table 13 the following result can be framed:

5) Each of the three L4 collaterals clustering within the cartridge a) is presynaptic to both of the other two collaterals, and to L2 simultaneously with each of them.

This proposition involves synapses 1, 2, 3, 4, 5, 6, probably 9, and 10 in Table 11. Synapse 7) is a doubtful case: its existence as a synapse can be seriously questioned. Synapse 8) would indicate some predominance in the connection from the "0" branch of the L4 cell native to cartridge a). This synapse would also be a dyad.

Since not all the synapses mentioned in Table 13 are very distinct, the clarity with which they can be seen is marked beside the entries in the Table. It will be observed that if synapses 3) and 10), which are the least distinct, be omitted from consideration, much of the force of proposition 5) remains. The connections made are still dyads with one member always being L2. What is lost is that the ring of connections is no longer closed: the 0--x connection is missing. The 0--x connection has been observed in other serial sections, (Tables 3, 4, 5) - in fact, all the connections listed in Table 13 are known from other serial sections (cf. Table 6). The fact that is newly observed in Series a) is that all the observed synapses are dyads and that L2 is always a member of the dyad. (The L2 connections had not been considered earlier, and the fact that many synapses in the lamina, including the L4 ones, were dyads had not then been noticed). If no synapses have remained unobserved in pictures 61-68, then proposition 5) summarises the scheme of connections found in cartridge a). The evidence from all earlier series is compatible with this proposition and support it although none of them was sufficiently complete to provide it (Tables 1-6, Table 10).

Therefore this proposition will be taken as being the main statement of this Thesis. The Discussion will be concerned with its relevance to the results of other workers which were stated in the Introduction.

DISCUSSION

Limitations of Technique

There are two limitations involved in the use of the technique of serial-sectioning for the electron microscope, in an investigation into neural connectivities. The first is that there is no simple correlation between the structures observed and their physiological significances. The preparation of the specimen for electron microscopy subjects the tissue to various polar and non-polar solvents, to abnormally low and high temperatures, and to exposure to heavy metal salts of different kinds. The final tissue, embedded in plastic and observed in a vacuum, shows no physiological properties at all, and resembles its original only in its structure. The correlation of structure and function becomes very difficult, yet this correlation is of great importance for the understanding of the types of events occurring at synaptic sites.

Some correlations have been made between structure and function in the lamina of the housefly. These involved the marking of neurons by dyes during electrophysiological recordings, and then identifying the elements so marked. Procion Yellow was used for this purpose by Zetler and

Järvilehto[21,52], to study the properties of some of the retinular cells. However, L4 has yet to be studied in this manner, and will provide difficulties to such an approach because of the small cross-section of its axon. Also, the normal results of recording in the lamina are that the neurons show responses of the graded potential type, which are not easy to interpret.

Another approach that was attempted was autoradiography. If the labelled neurotransmitter had been concentrated within one or two neuron types, the assumption could have reasonably been made that these neurons were using the transmitter. The transmitter used was GABA, but the results were not specific: the labelled GABA was found to become selectively concentrated in the glial cells of the ganglion. The hope had been that L4 would be indicated as using GABA as the neurotransmitter, but this was not shown. Therefore, no physiological information is available which might indicate the type of function mediated by the synaptic contacts made by the L4 neuron.

The second limitation arises from the delicacy of the serial-sectioning technique itself. It is not suitable for describing a large population of objects, so that variations within a population cannot practicably be described by means of it. In this study this drawback was minimised by preparing all the series from flies of the same age and sex, and sampling the lamina underlying the same area of the compound eye in each case. This removed from the results as far as possible, variations which were due to differences in different parts of the eye, between the sexes, or age.

The work also rested on the assumption that since the cartridges under observation were functional then they should exhibit characteristics due to this, and that different cartridges should display a certain set of features in common due to their common function. This assumption was strengthened by always sampling from the same part of the ganglion, where a difference in roles of different cartridges is least likely. Since a common set of features was indeed found in different cartridges from different animals, as expressed in proposition 5) on page 65, it appears that these features do represent a characteristic part of all the cartridges that could be examined from animals of the age and sex used (Tables 10, 1-6, 12).

However, apart from the set of features noted in every cartridge that was sampled, there were other features that were not constant from cartridge to cartridge. These were the synaptic connections involving the retinular cell axons. Some partial series showed synaptic sites in which the L4 collaterals were presynaptic to R3 and R5, and in one doubtful case, to R2. The series presented here (a) is entire, yet it shows no synaptic connections to any of the retinular cell axons from L4. (The blurred photographs 61 to 68 occur at, and proximal to, the level of termination of the retinular cell axons). This discrepancy between different cartridges could be explained by assuming that the cartridges sampled did not all lie in precisely the same area of the ganglion, and that whereas some lay further to the southern part of the northern hemisphere of the eye, the cartridge a) presented here lay further to the north of the eye, where

the cartridges are smaller and the cell processes less elaborate, than nearer the equator. Such a scatter is very probable because it is unlikely that each series of sections could be taken from exactly corresponding rows of the eyes of different animals. Strausfeld[43] has suggested that the L4 neuron displays a gradient of connectivity across the lamina; if some cartridges that were sampled contained L4 cells that made connections to retinular cell axons, whereas others contained L4 cells that did not, this would then be explicable by supposing that the samples were taken from different points along this gradient. Cartridge a) would represent the extreme case possible in the gradient (no connections) as well as proving that this extreme is realised. However, the existence of such a gradient would be less easy to prove than to suppose. The minimum proof possible would be the examination of a serial section prepared from the equatorial region of the lamina. This would be a large undertaking, because a series of about 400 sections would be required: 25 microns at 15 sections each. The description of the properties of such a gradient of connectivity would require that collections of series be made at three or four points along the supposed gradient. Since the collection of serial sections described here demonstrates that an interesting group of common features exists among cartridges from one area of the eye, and since it is likely that this group of features would also be an important part of cartridges showing more complex L4 connectivities, this discussion will be limited to this group of features, for which evidence is available. The

larger problem would become involved with shortcomings inherent to the serial-sectioning method itself.

Interpretation of the results

A lamina-type ganglion has been observed in all visual systems involving compound eyes. This is true of apposition eyes, and of optical and neural superposition eyes, and it is also true of species throughout the Arthropods. It seems, then, that the lamina performs some function essential to the compound type of eye. This function has not yet been explicitly described.

The laminas that have been examined all show common features of construction: the retinula axons project into the lamina and make synaptic connections with second order neurons there, which in turn project into the medulla. The neurons are arranged more or less clearly into cartridge-type units, each of which involves a group of the retinular axons synapsing onto a group of different types of second-order neurons. In addition there are tangential fibres entering the lamina from the medulla, and these may be more or less prominent; in the Housefly they are not prominent at all, in some other groups (e.g. the Prawn Pandalus - personal communication, D. Nässl) they are the most prominent fibres in the lamina. However, it is a common feature of laminas that there is more than one type of second order neuron. These differ in their branching-pattern, in their patterns of synaptic connectivity, in their variability throughout the lamina, and in the types and levels of terminations they make in the medulla. It is a reasonable assumption that

this may be so because the functions of the lamina require a variety of cell types, or perhaps because the medulla requires a variety of different inputs. The common tasks of the lamina may be divided in different ways among the second-order neurons of lamina in different species, yet the examination of the lamina of one species in detail may still give an insight into those functions, and will at least provide a comparison with other species. The lamina of the Housefly has been exhaustively studied by different authors, and is very thoroughly described (refs. 3,4,5,21, 42,44,45,46,47). However, the L4 neuron had been less well described, partly because the clustering of three long collaterals from three different L4 cells, within the small volume at the proximal end of the cartridge (Fig. 15), produced problems of identifying the profiles belonging to different collaterals. This made it difficult to describe the synaptic connectivity of each collateral separately.

This inadequate description of the L4 cell was an important lacuna in the description of the Housefly lamina, for the following reason: the ability to discriminate moving objects is common to animals possessing compound eyes. Not only is the ability of intrinsic interest, but there also exists an explanation of it in precise quantitative terms, and this explanation has been demonstrated to be valid in the case of Musca (See page22). Since the model postulated is one that can be realised in electronic and mechanical terms, it is of interest to examine how the same principle is realised in a biological mechanism. Predictions from the model were used to suggest experiments to determine the type

of neural connections necessary to embody the model in a biological system, and from these experiments was derived a description of the minimum neural connections needed to do so. The L4 cell is the most distal neuron in the Musca visual system which satisfies these conditions, which were: "the subunit orientations are expected to group in at least two different directions that share a common line of symmetry with the internal eye structure. The minimum model requires the axis of symmetry to be the animal's long axis". (See page 24). The L4 neuron satisfies these conditions because the two long collaterals arising from it at the proximal edge of the lamina, run from one cartridge to those cartridges neighbouring it in the Y and -x directions of the hexagonal array. When transposed back to the corresponding facets of the cornea, the axis of symmetry of the long collaterals is the z-axis: the animal's long axis. Since the L4 neuron satisfies the conditions required for it to be a movement detecting neuron, and since it is the only neuron in the lamina known to do so, the synaptic connectivities of the long collaterals are of considerable importance. These long collaterals represent links between neighbouring "channels" of information flow in the lamina, and although the alpha cells are also such links, it is only the L4 cells that have been exactly described from the point of view of which "channels" they join. Also, only the L4 cells satisfy result 4) on page 24. The following discussion will therefore analyse the possible significance of the L4 neuron with respect to movement detection. The question underlying the analysis will be which directions of information flow are

possible between cartridges by way of L4.

Possible significance to the fly of the L4 collateral network

The first consideration is the information flow along the collaterals of L4. The direction of this flow is unknown, but in the case of the two long collaterals there are three distinct possibilities: a) If the signals can only travel outwards along these collaterals, from the axon in the parent cartridge to the endfeet within the cartridges neighbouring on the -x and y axes, then the net flow will exert a directional influence on cartridges in the +z direction: each cartridge will be influenced via two collaterals arriving from two other cartridges, one along the x-axis and one along the y-axis. The result will, in toto, be a directional property of the lamina along the z-axis, and symmetrical to it (Fig. 22). This case would satisfy the conditions for L4 to be a motion-detecting neuron, or involved in one stage of motion-detection. b) If the signals can only travel inwards along the two long collaterals, then the net result would again be a directional property of the lamina along the z-axis (in the -z direction), and acting on the axon which the L4 cell projects into the medulla. In view of the fact that the endfeet of these collaterals are presynaptic, this direction of information flow seems unlikely; however, the same reasoning would also rule out a) above, because the endfeet of the collaterals are also postsynaptic. c) If information can flow both backwards and forwards along the two long collaterals, then it is possible to produce the construction equivalent to the simplest scheme of connections within a hexagonal array, published earlier (Fig. 16,20,21)[6].

This construction, however, assumes that the results of information flow outwards along the collaterals is similar or identical to those of flow in the other direction. Should this not be so, then the removal of arrows from the diagram to produce the simplest scheme of connections is not valid (i.e. Fig. 21 is not valid). Since spike-conduction is not demonstrated as occurring in these collaterals, there seems to be no reason to exclude this possibility.

These considerations leave the role of the short O-collateral vague. Since the synaptic contacts made by this collateral are similar to those made by the other two, it would appear that the cluster of endfeet within a cartridge is a unit of importance within the cartridge, and that the synaptic contacts made by the endfeet are important within the cartridge where they are made, rather than as contacts to external cartridges. If the cluster of endfeet is the functional unit, then this would probably favour a) above, in which the information is regarded as flowing towards the clusters.

There is a further point in favour of case a) above, which is that all the collateral endfeet are presynaptic to a cell within the cartridge containing the cluster. (L2). This makes it more plausible to believe that information is flowing towards the cluster from the neighbouring cartridges (Fig. 22), and then influencing L2. If cases b) or c) were true, then it could be expected that the three collateral endfeet were postsynaptic to some cell within the cartridge containing the cluster, and that information flowing inwards along the collaterals towards their parent axons would be

originating from the cartridge containing the cluster. Such synapses have not been noted. Thus, as far as the morphological evidence alone is concerned, it seems that case a) above is most likely to be true, and thus no evidence is provided against the idea that L4 is involved in motion-detection.

In this discussion of the collaterals as connections between cartridges, it has been assumed that the synaptic contacts made by each collateral are similar to those made by the other two. The introduction of any more complex assumption leads to very complicated patterns of connection, and is unwarranted by the evidence. (The synapses are all morphologically similar).

The second consideration is the asymmetry of the L4 collateral network with respect to L1 and L2. In no case yet examined by serial sectioning has L1 been observed postsynaptic to any of the L4 collaterals. This result is in contradiction to the published findings of other authors[43]. The absence of a synaptic connection is difficult to prove, since failure to observe one does not necessarily imply its absence. However, the frequent occurrence of synaptic connections in which the L4 endfeet are presynaptic and L2 is postsynaptic, and the orderly pattern of connections thus produced, has been observed. It was a feature of several serial sections, including the ones presented here. In fact, the orderly pattern of these connections with L2 is so striking since it appears that L2 is postsynaptic to the L4 collaterals at every synaptic contact that they make. (Proposition 5, page 65). If a similar set of connections to L1 does exist, it is difficult to explain why serial sectioning has not

revealed it when one complete series suffices to demonstrate a similar set of connections to L2. One possible explanation is that the use of the Golgi-EM method does not permit unambiguous identification of the L1 and L2 profiles. This would easily lead to an impression, possibly erroneous, that both were postsynaptic, when in fact only L2 was.

The presence or absence of such a set of connections with L1 is worth establishing because it helps to answer the question: why does each cartridge contain five neurons of different types in addition to the receptor cell axons? Also, why are two of these different types, L1 and L2, so similar to each other? The fact that one of them, L2, is influenced in a very orderly way by a network of collaterals at the base of the lamina, whereas the other, L1, is not, is of some importance, especially since the network of collaterals seems to have no other obvious output, in some cartridges at least.

The third consideration is the functional significance of the synapses of the collateral network. The structures recognised as synapses are described above, page 54. It is these structures that mediate information flow from the retinular cell axons to the second order neurons L1, L2, and L3. It would thus seem possible to regard them as excitatory synapses, yet if this were true in the L4 collateral network, the net would stimulate itself to fire continuously, a meaningless condition. It may be that the postsynaptic structure is of importance in this connection, for it is different in L2 and L4, see Fig. 18. Whether the L4 collateral network synapses are inhibitory in nature

remains to be seen. In that case the net would have the property of lateral inhibition. Such inhibition has been demonstrated in Musca[27], and is a common phenomenon in both vertebrate and invertebrate eyes.

There is one clear difference between the synapses in the L4 collateral network and those connecting the retinular cell axons with L1-L3: the latter are far more numerous. Each retinular cell axon may be synaptically connected with L2 about 20 times, whereas the x-collateral of a given L4 cell is not connected to the y-collateral within the same cluster more than two or three times. The reason for this quantitative difference is unexplained.

Whatever the function of the network, it is noteworthy that L2 appears to be influenced by every signal passing from any L4 collateral within the cluster to any other. This input is the most proximal one to L2 that occurs in the lamina, and the functioning of the L4 collateral network may have considerable significance in determining the input of L2 into the medulla.

Finally, the character of the individual L4 neuron is of interest. It is not simply a second-order neuron transmitting information from the retina to another location; it receives no direct inputs from the retinular cell axons. Its inputs are in the distal part of the lamina, from the alpha cells, themselves fibres intrinsic to the lamina, and spreading over fields of several cartridges. They receive inputs from the retinular cell axons, and are also involved in the "gnarls" which protrude into the beta cells and are possibly synaptic. These alpha cells then are presynaptic

to the distal branches of the L4 cells (See page 60).

The L4 cell also makes long collateral branches in the upper layers of the medulla[43], which run from the column of the medulla corresponding to the cartridge in which the L4 cell is situated, to the columns corresponding to the cartridges of the lamina invested by the L4 lamina collaterals. Thus the same three channels are linked once in the lamina and once in the medulla by the same L4 cell. It may be that the two sets of collaterals together form a functional unit, and that the understanding of the lamina collateral network must wait upon knowledge of the medullary collateral network.

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FIGURES 1, 2,

AND 4-22.

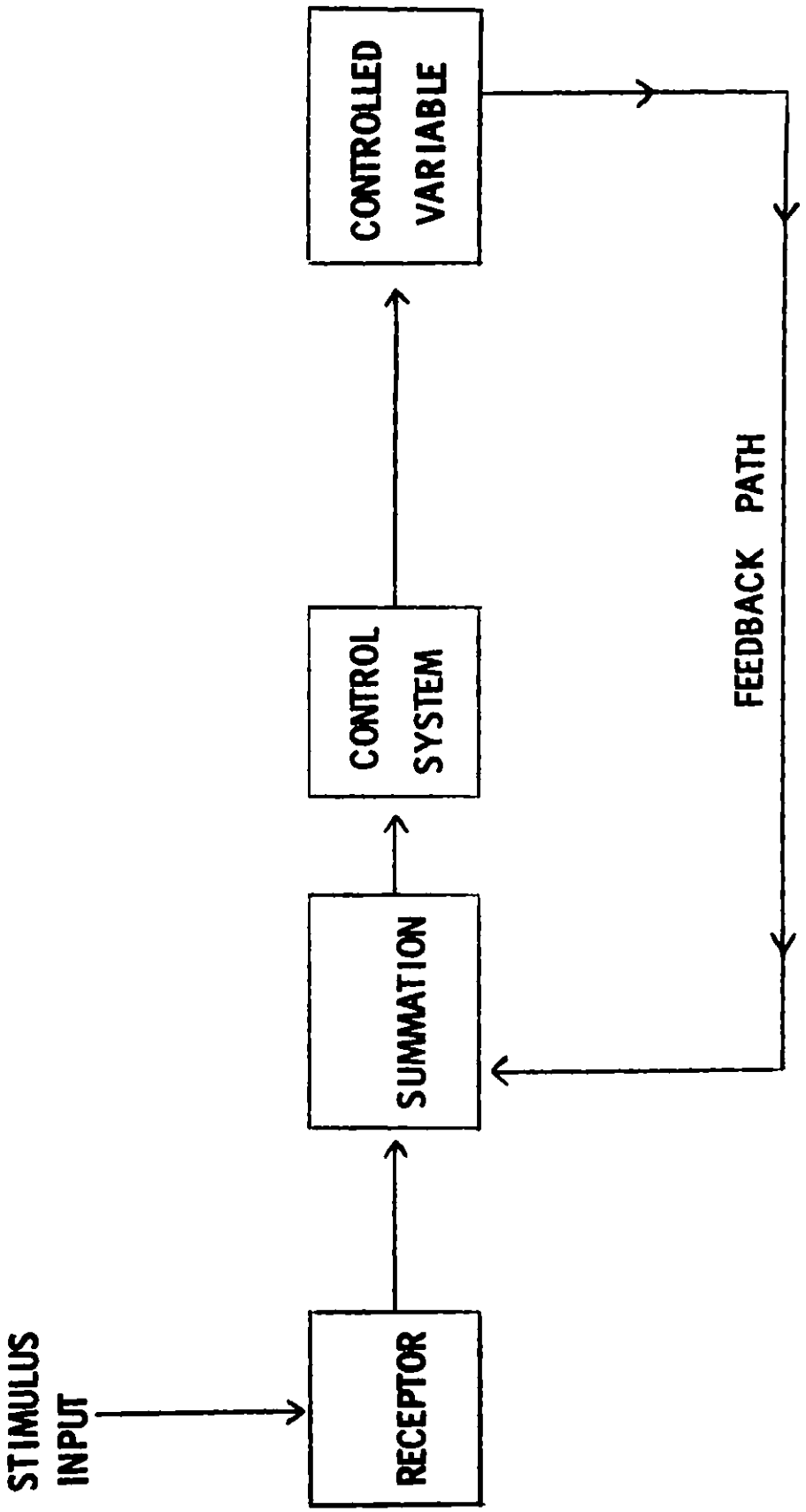


FIG. 1. Diagram of control loop; in text p.16 .

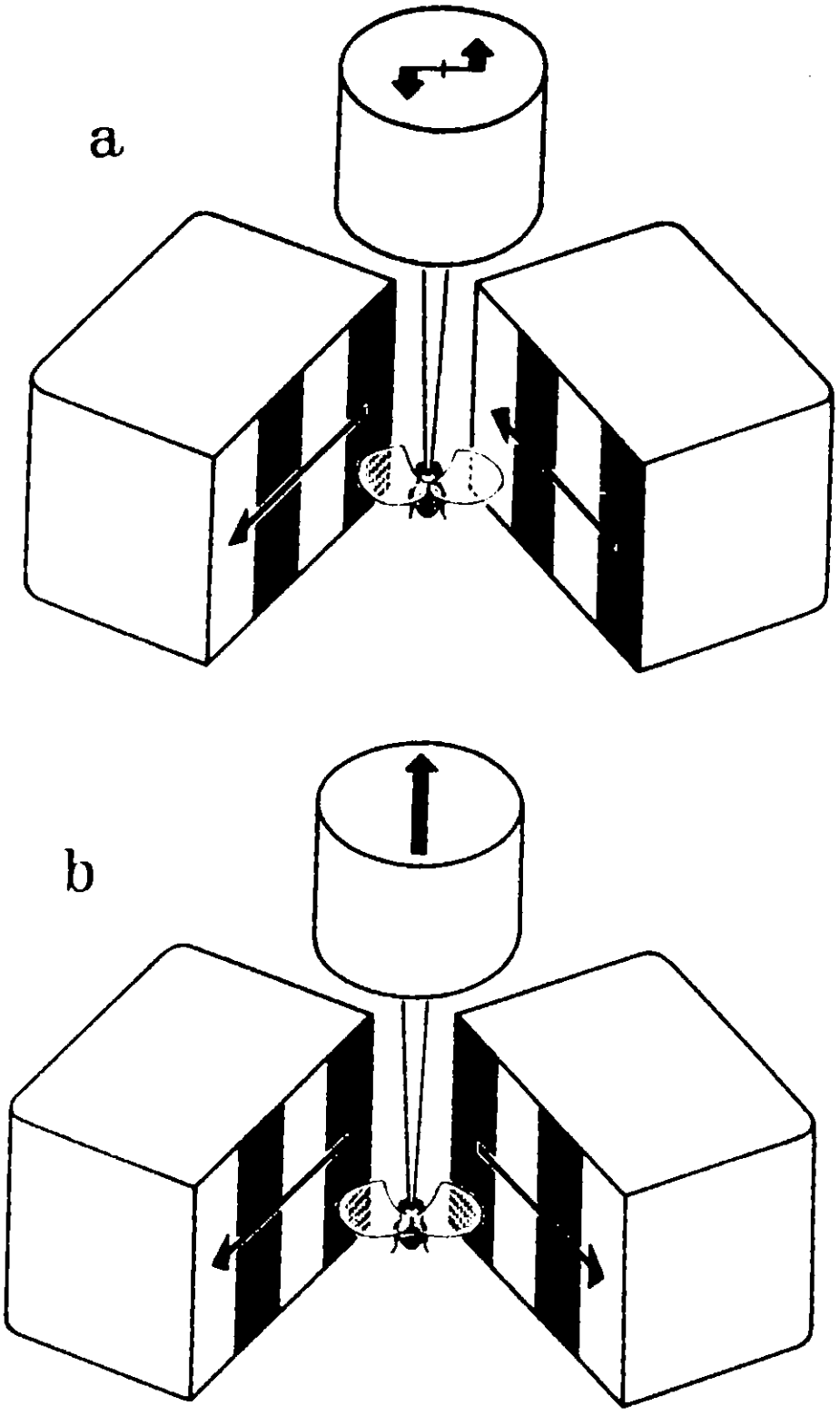


FIG. 2. Experimental arrangement for optomotor experiment: the fly is suspended rigidly and the moving stimulus presented to cover its whole visual field. Measurement of the fly's response to the stimulus is shown on the meter. (From ref. 15).

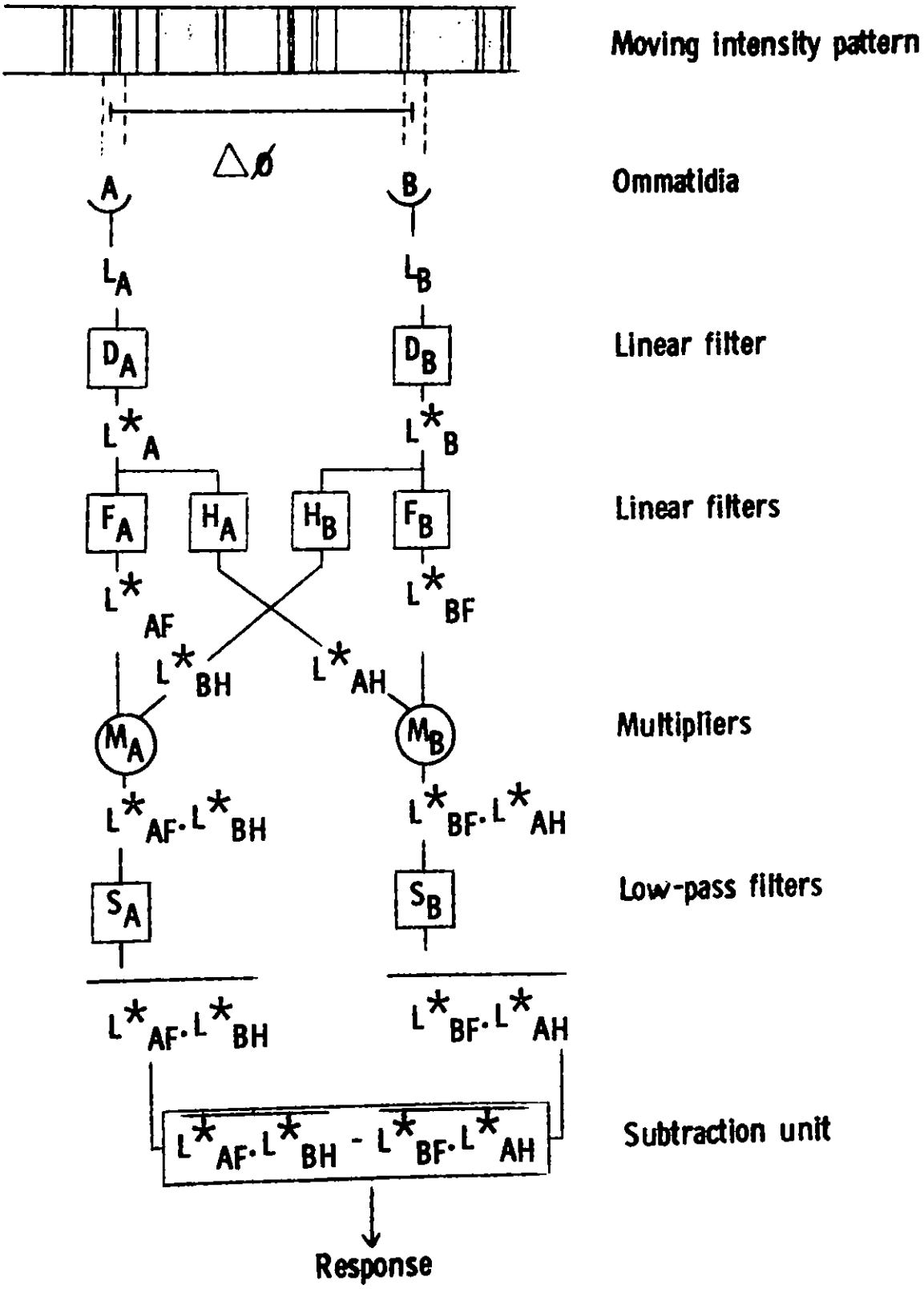


FIG. 4. Diagram showing conceptual model for the optomotor response. A and B are receptors, D,F,H,X are filters, M is the multiplier process, S is a long time-constant filter. The inputs L(A) and L(B) are transformed by the filters and multipliers into L*(A), etc.

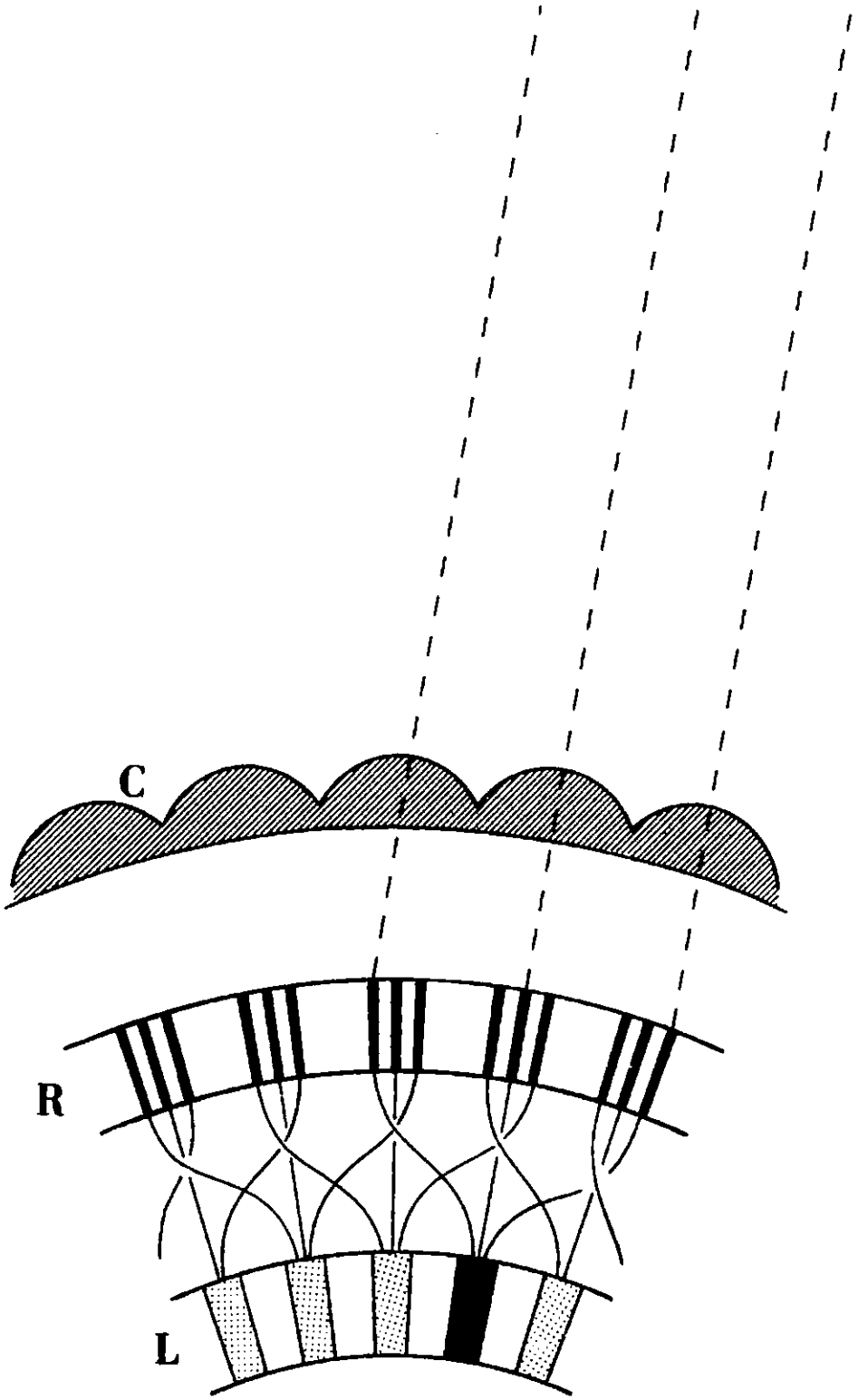


FIG. 5. Diagrammatic representation of the "neural superposition eye": light rays from one point in the environment are shown passing through three corneal facets, being absorbed by different rhabdomeres beneath the facets, and the signals induced in the receptor cells being brought together at one [illegible] in the lamina by means of the crossing over which is

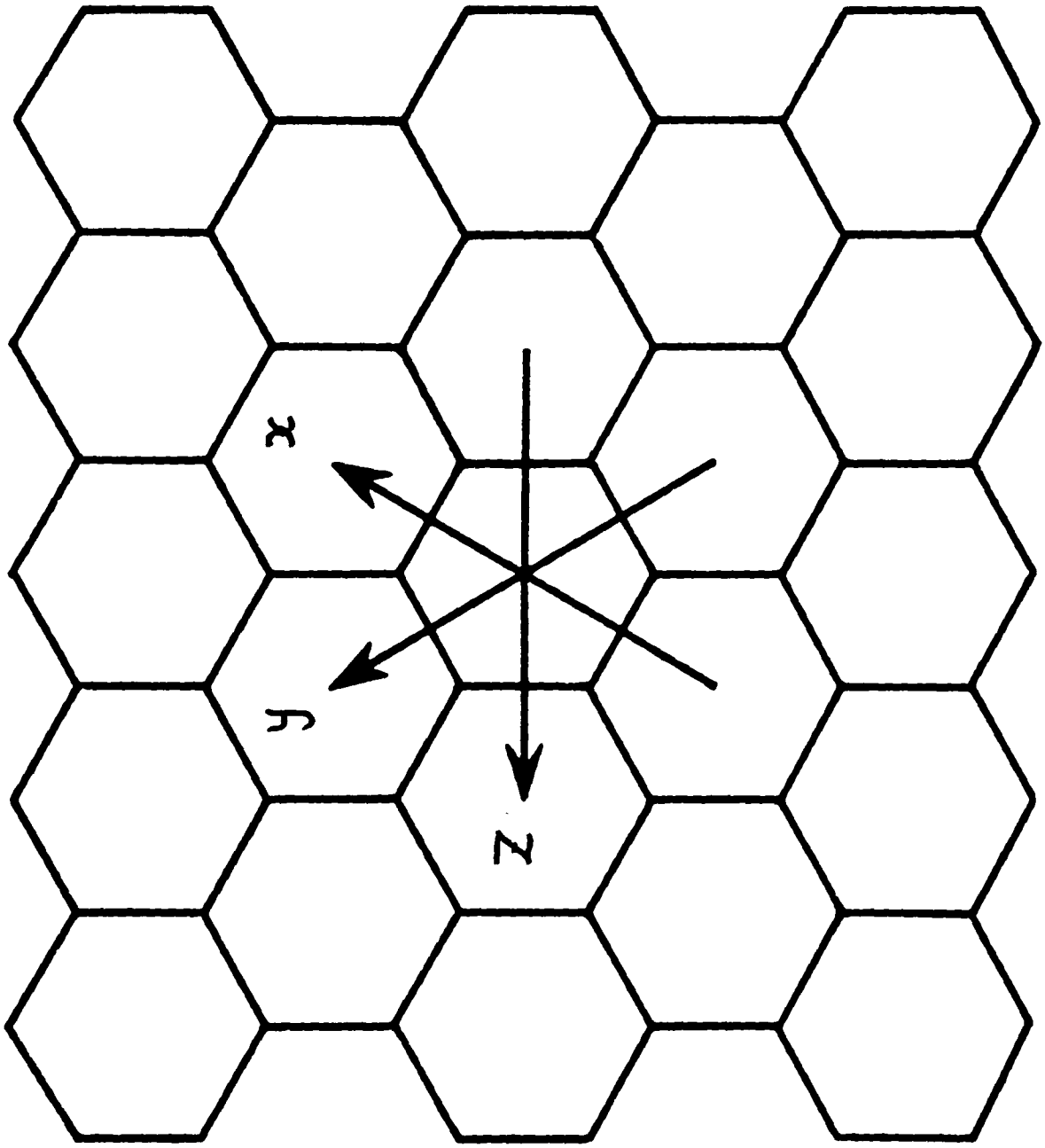


FIG. 6a. Definition of the three axes, x, y, and z, of a hexagonal array.

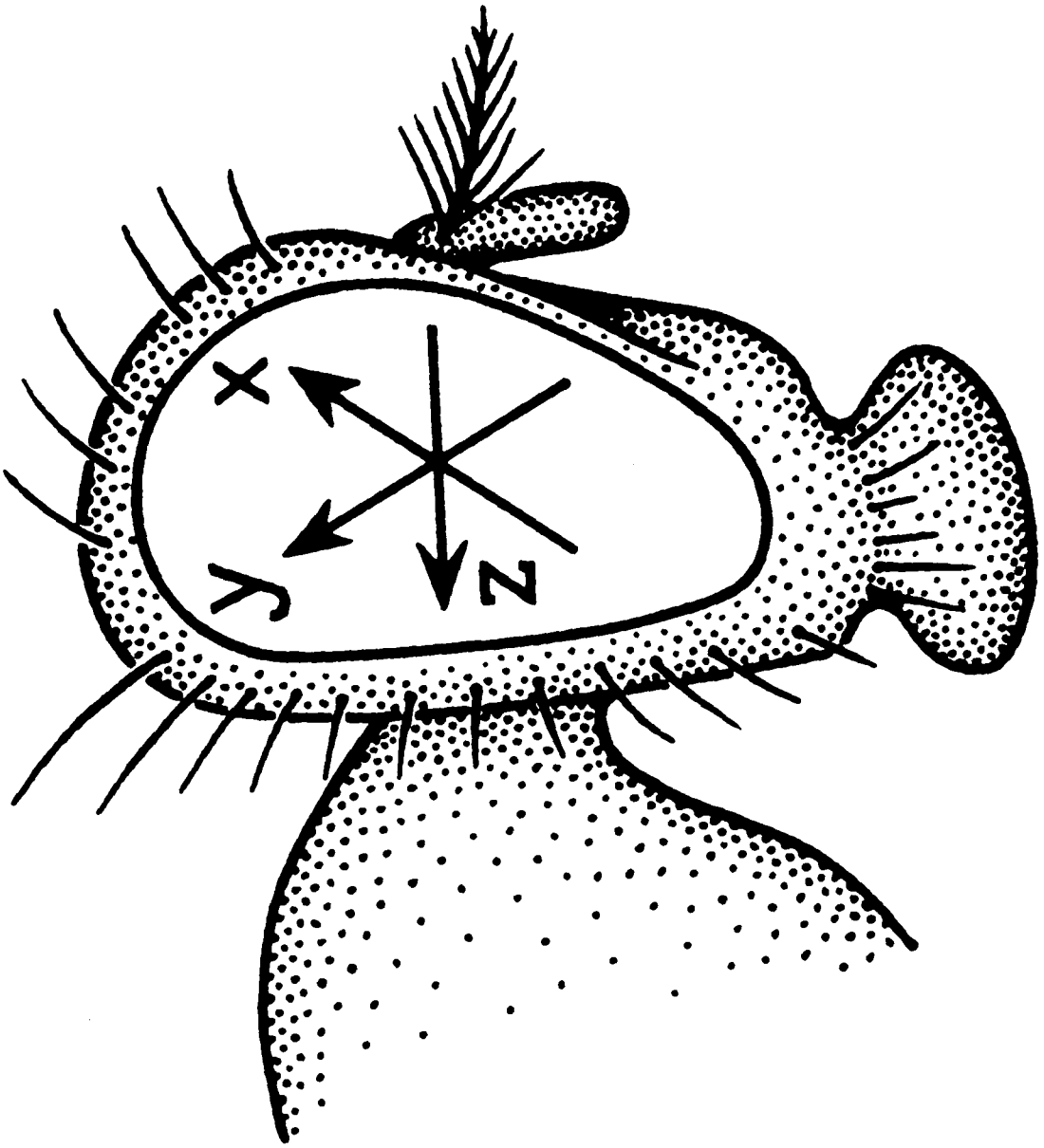


FIG. 6b. Orientation of the hexagonal array of ommatidia at the surface of the eye.

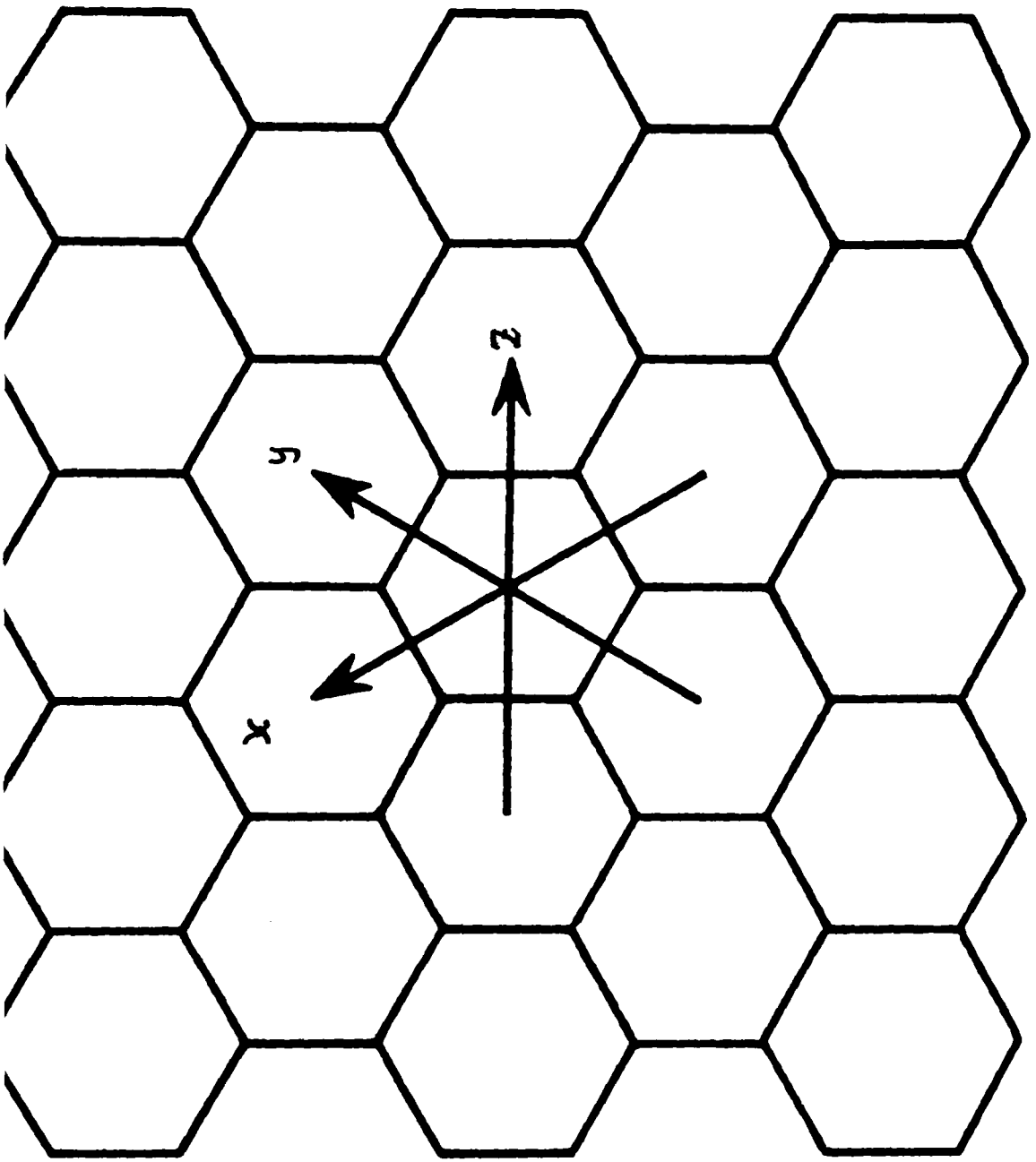


FIG. 7. The orientation of the hexagonal array in the medulla.

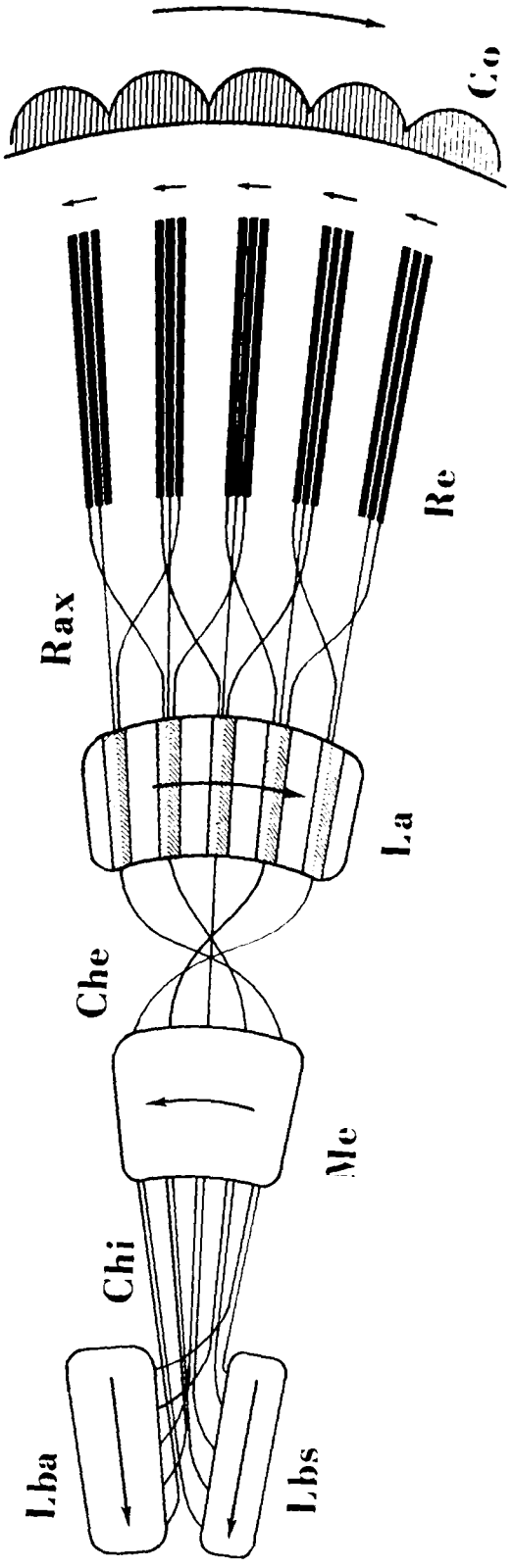


FIG. 8. The visual system of the fly: Co=Cornea, Re=Retina, Rax=crossing over of the retinular axons, La=Lamina, Che=Chiasma, Me=Medulla, Chi=chiasma interna, Lba and Lbs=Lobula and Lobula plate.

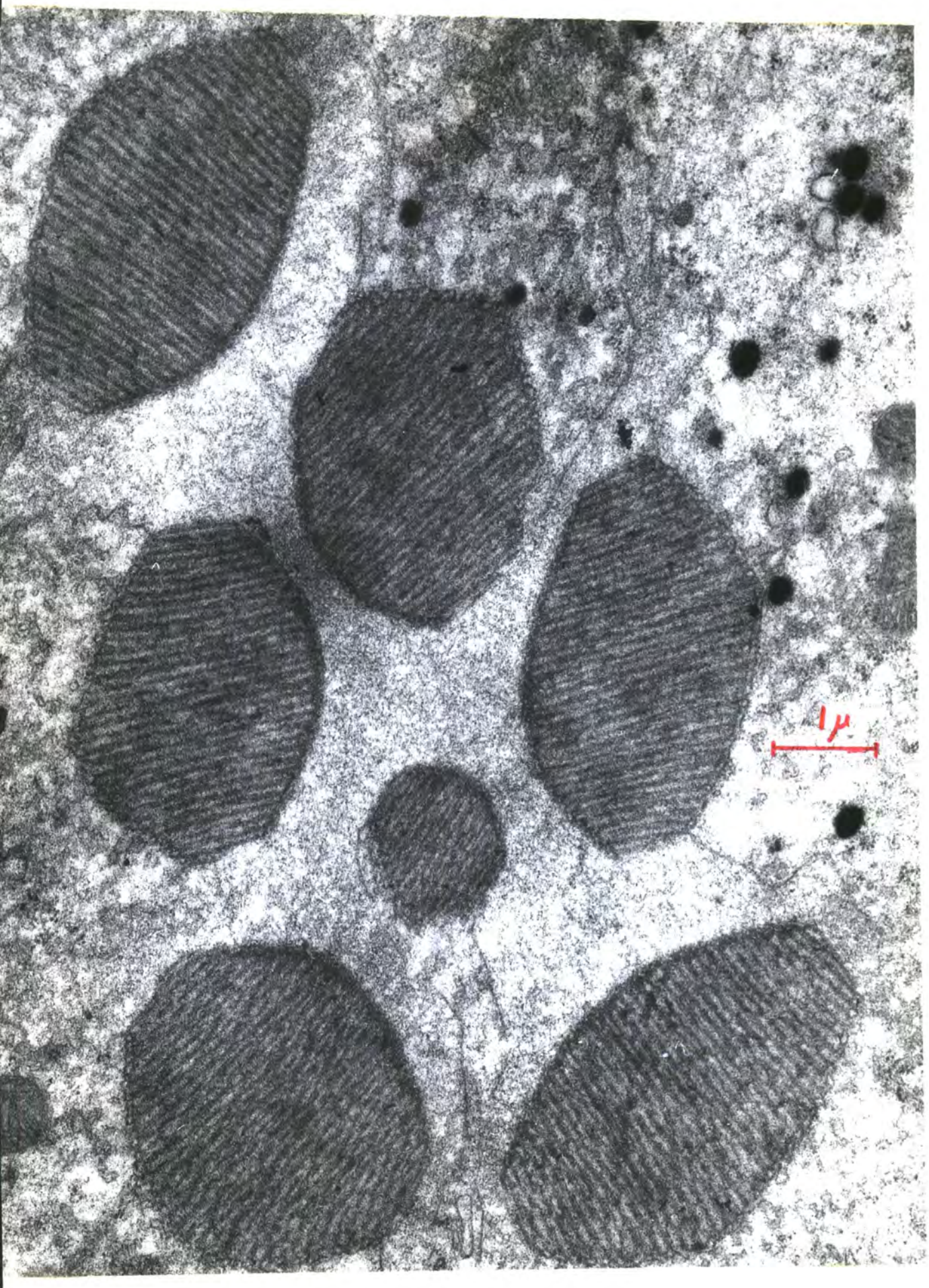


FIG. 9. Electron micrograph of the retina of Musca, TS. Shows the orientations of the rhabdomere microvilli. (Compare ref.2).

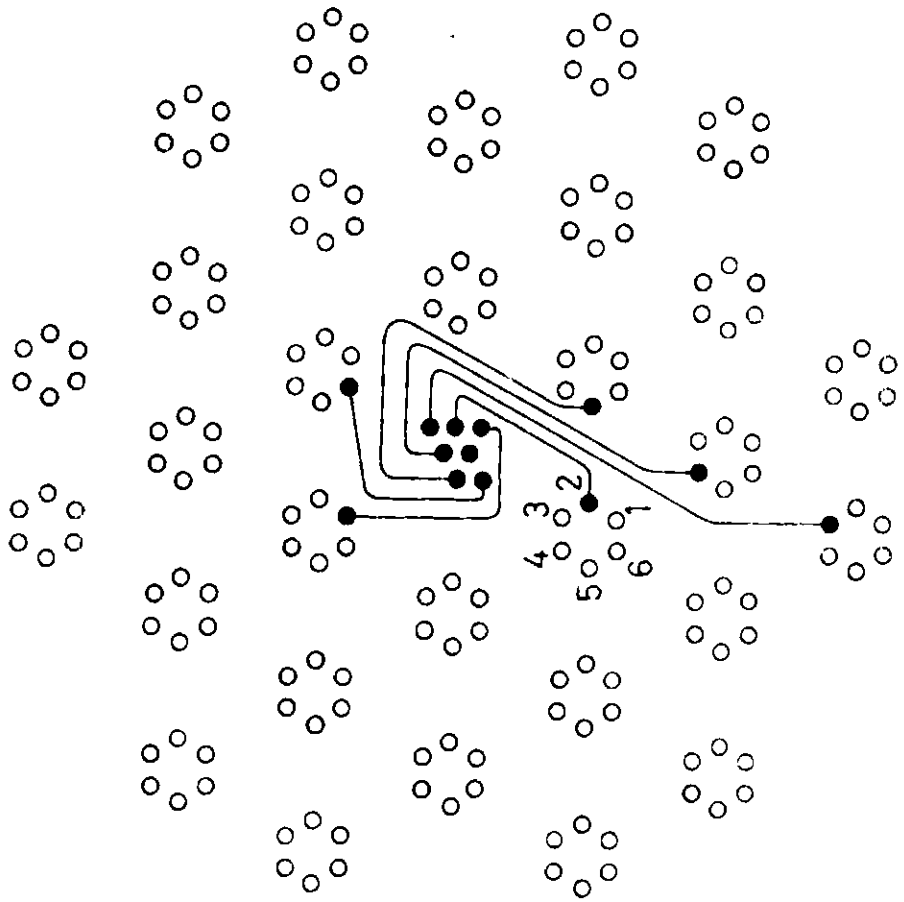
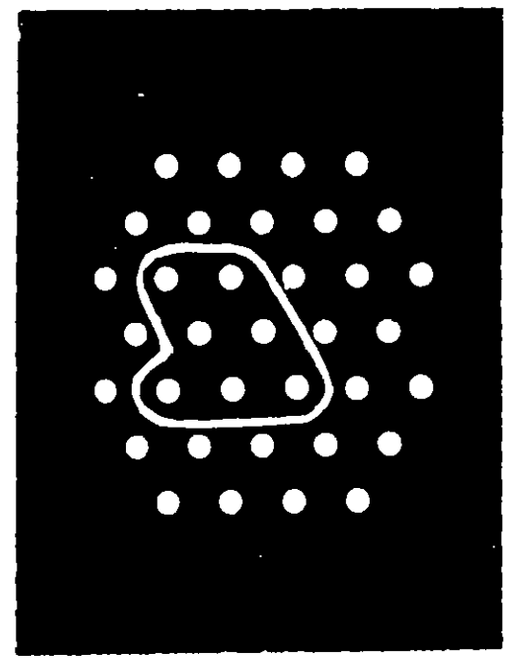
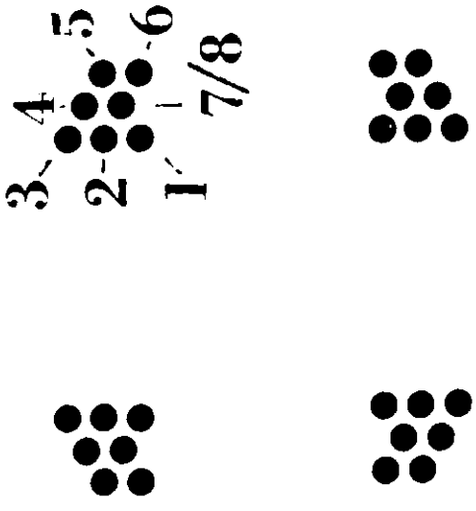


FIG. 10. The projection of retinular cell axons from seven different rhabdomes (white circles) onto one lamina cartridge (black circles). Because the rhabdome is an unsymmetrical shape the rhabdomeres can be individually identified and numbered (from 3).



b



a

FIG. 11. a) gives the numbering of the individual rhabdomeres; the two halves of the eye are mirror images, as are the two different eyes. b) outlines the seven rhabdomes of the retina which are "looking" at one point in the visual environment (Compare Figs. 7 and 8 of ref. 10).

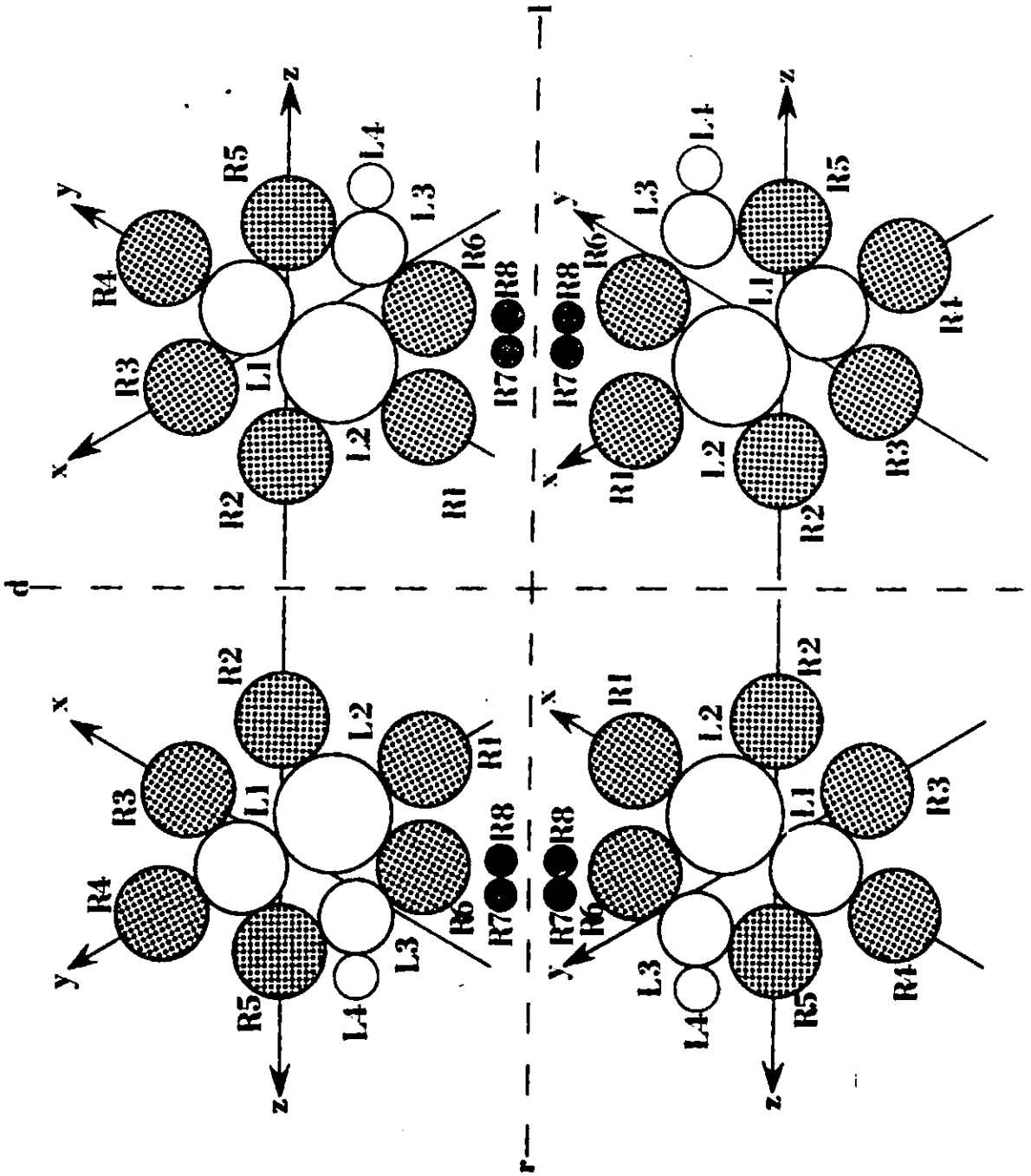


FIG. 12. Diagrammatic representation of the spatial relationships within the lamina cartridge; R1-R8=Retinular cell axons, L1-L4=lamina neurons, x,y,z are the co-ordinate axes. The figure shows the mirror imageing between the eyes and the halves of each eye (from ref. 3).

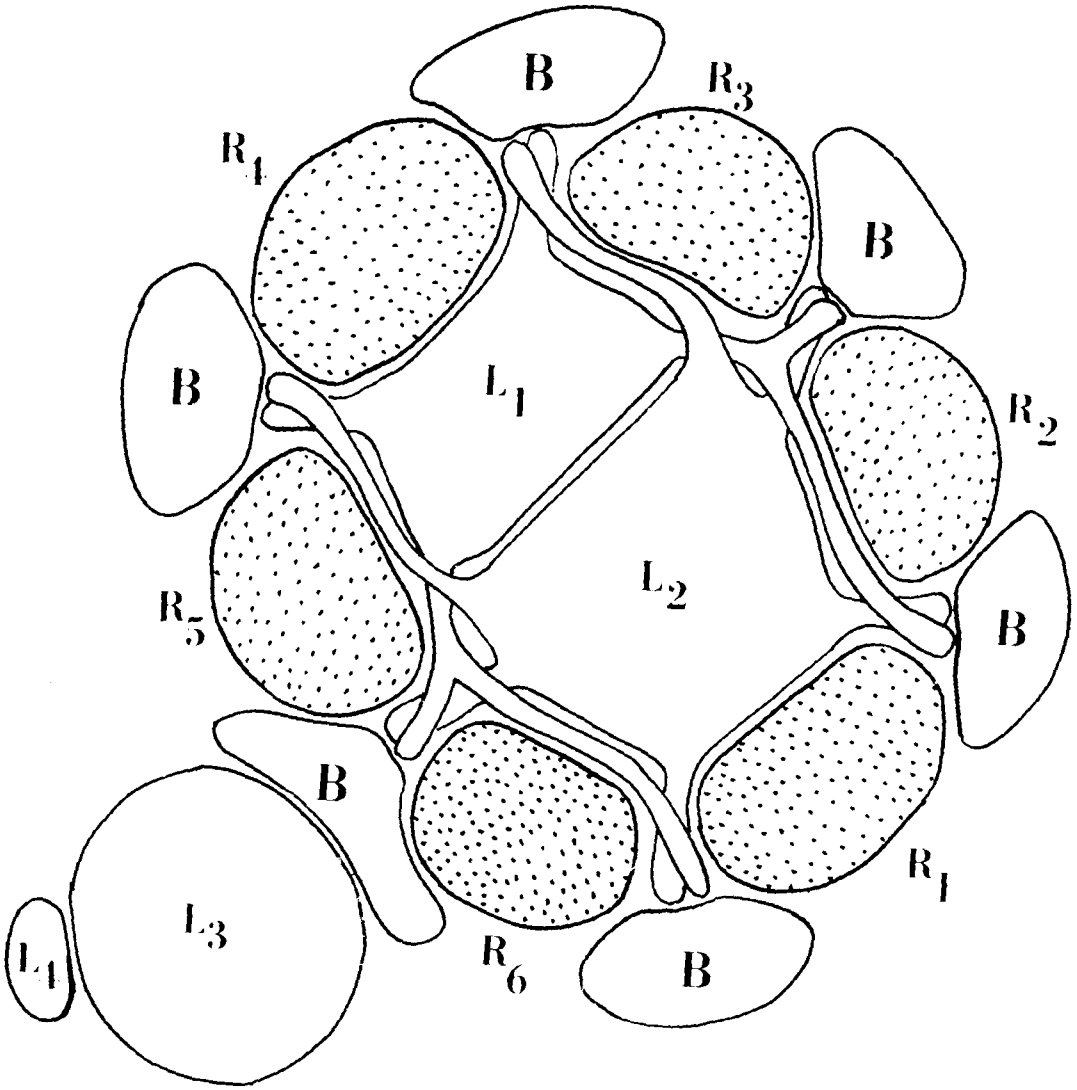


FIG. 13. Semi-diagrammatic representation of the lamina cartridge. Compare Fig. 12 and photograph 1a) of the series.

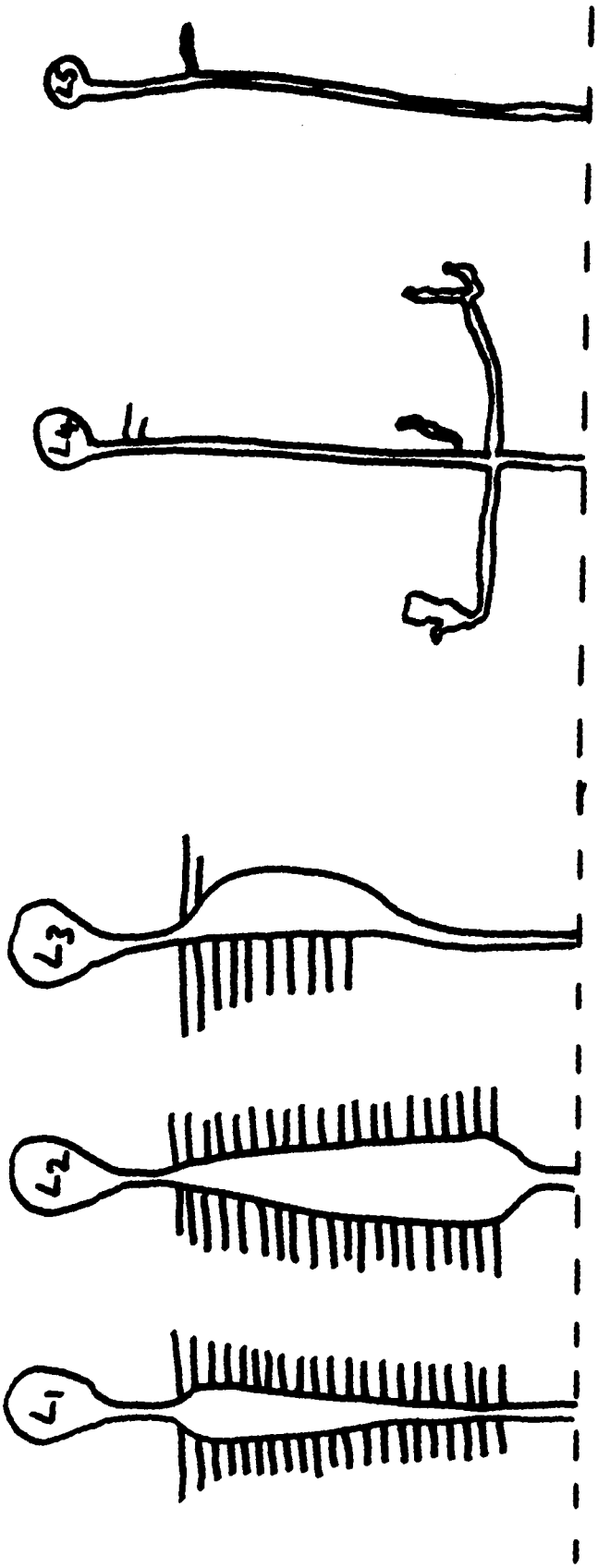


FIG. 14. Branching patterns of L1-L5 neurons in the lamina. The dotted line represents the lamina-chiasma boundary; each of the neurons projects an axon into the chiasma.

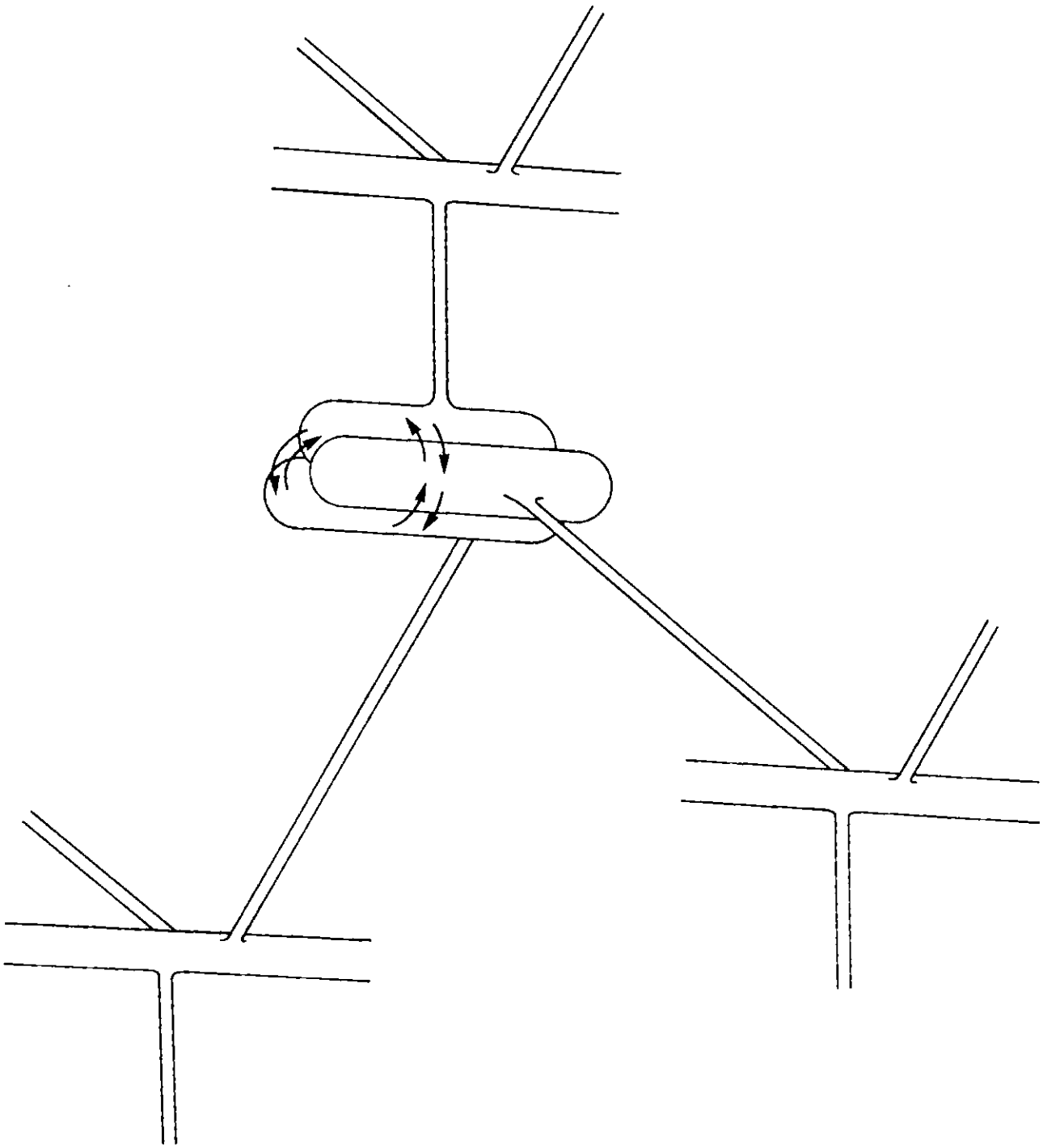


FIG. 15. The cluster of three L4 collaterals which make contact within a single cartridge, the arrows representing synaptic contacts between the collaterals (from ref. 6).

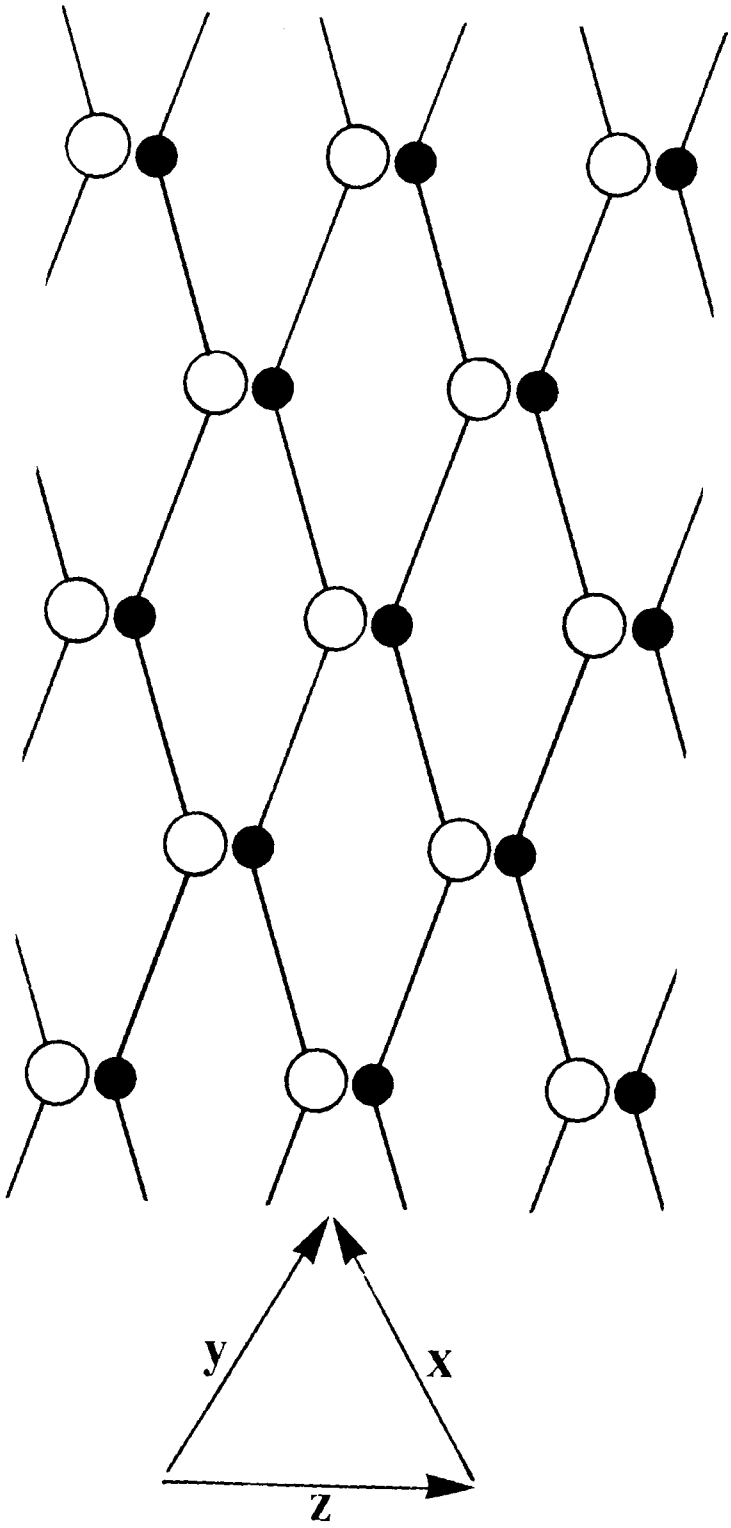


FIG. 16. Directions of the L4 collaterals (black lines) with respect to the cartridges (black circles) and co-ordinate axes of the lamina. White circles represent L4 stem axons, (from ref.6)

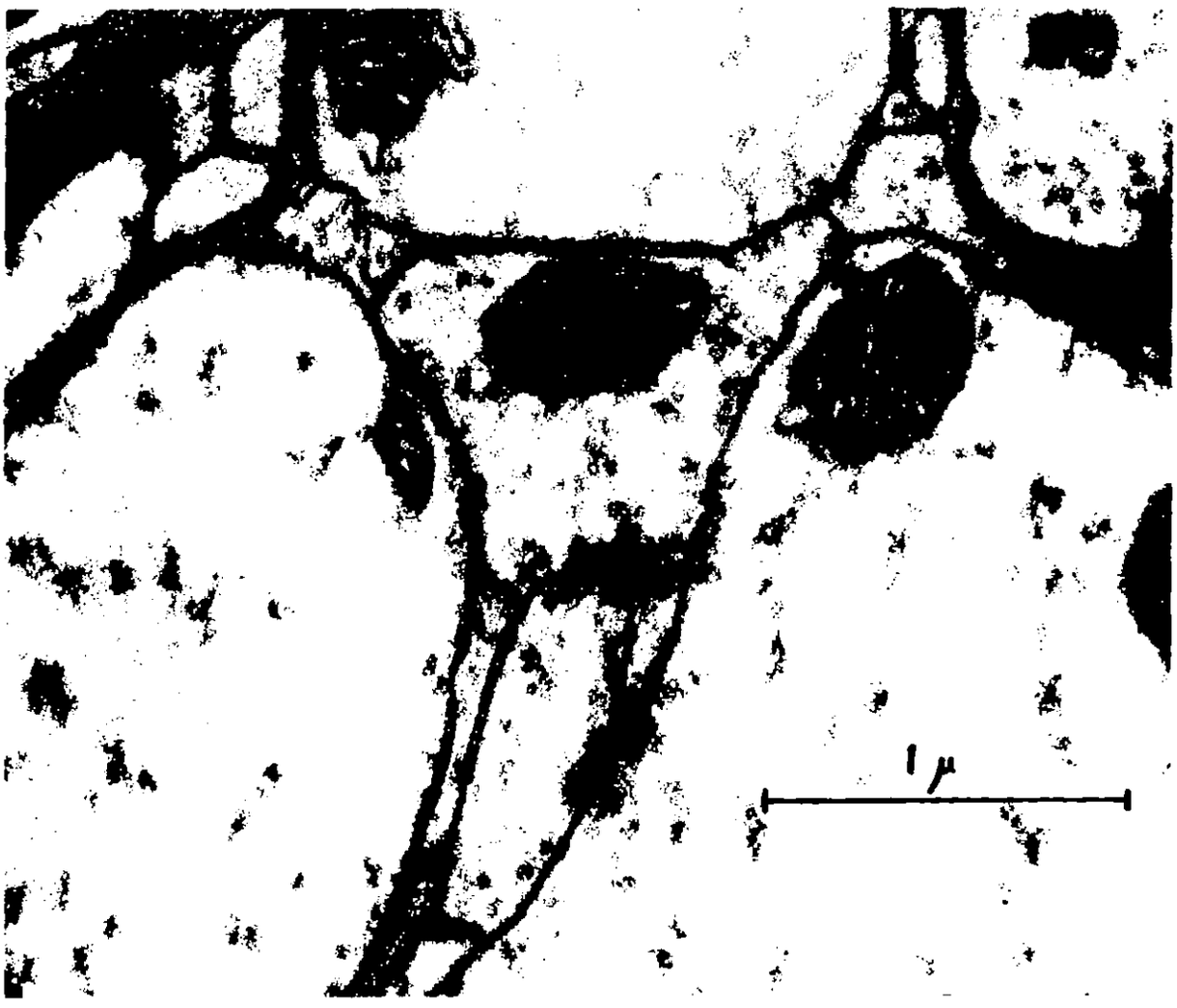


FIG. 17. Electron micrograph showing three L4 collateral profiles between L1 and L2 profiles. Large L4 profile is presynaptic to the other two L4 profiles; presynaptic structure is T-shaped, and the postsynaptic structures are rectangular in L4 (from ref.6).

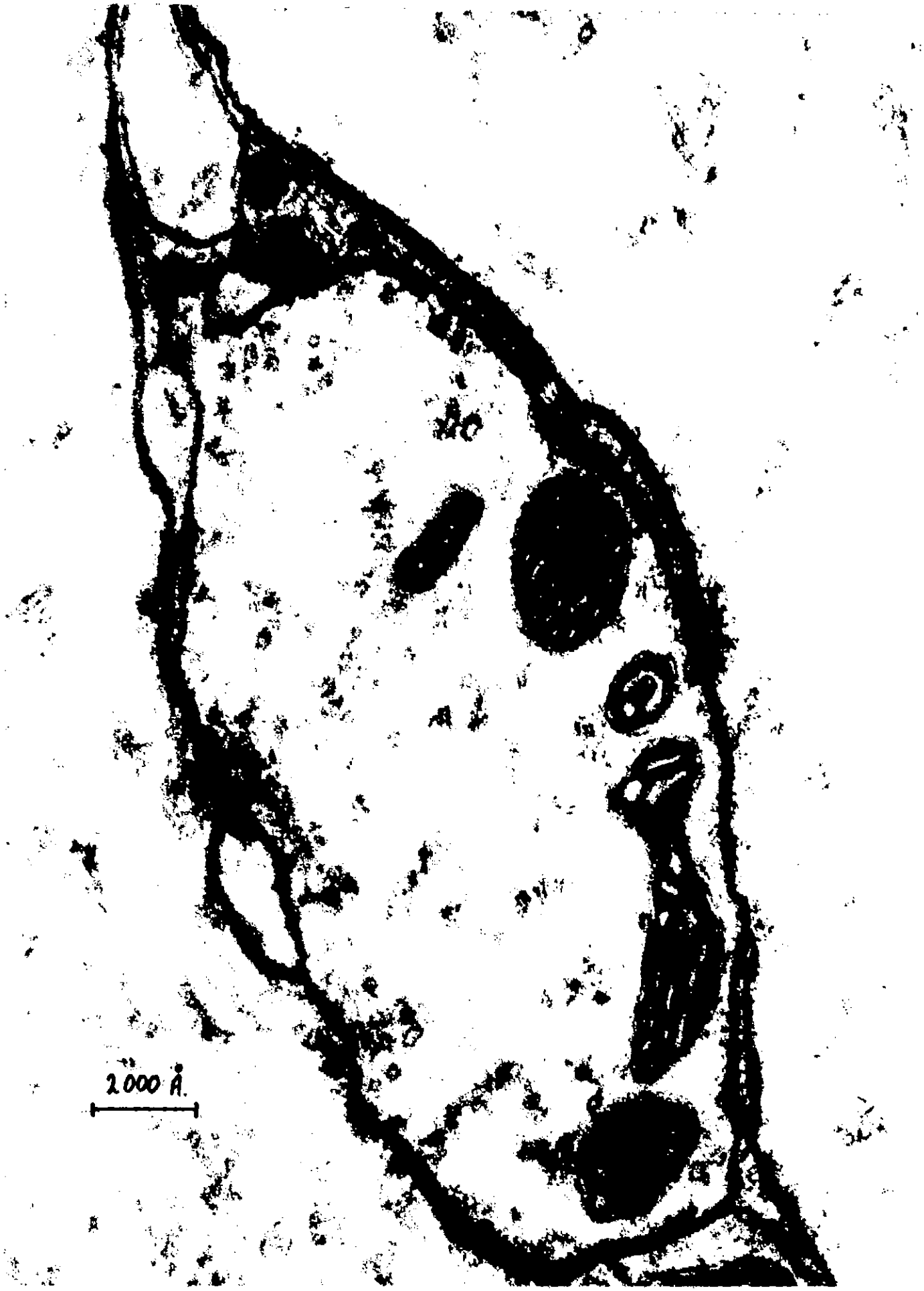


FIG. 18. L4 profile presynaptic to L2 and another L4 profile. Apposed to the T-shaped presynaptic structure, in L2 is a straight bar as postsynaptic structure, and in L4 is a fuzzy rectangular postsynaptic structure.

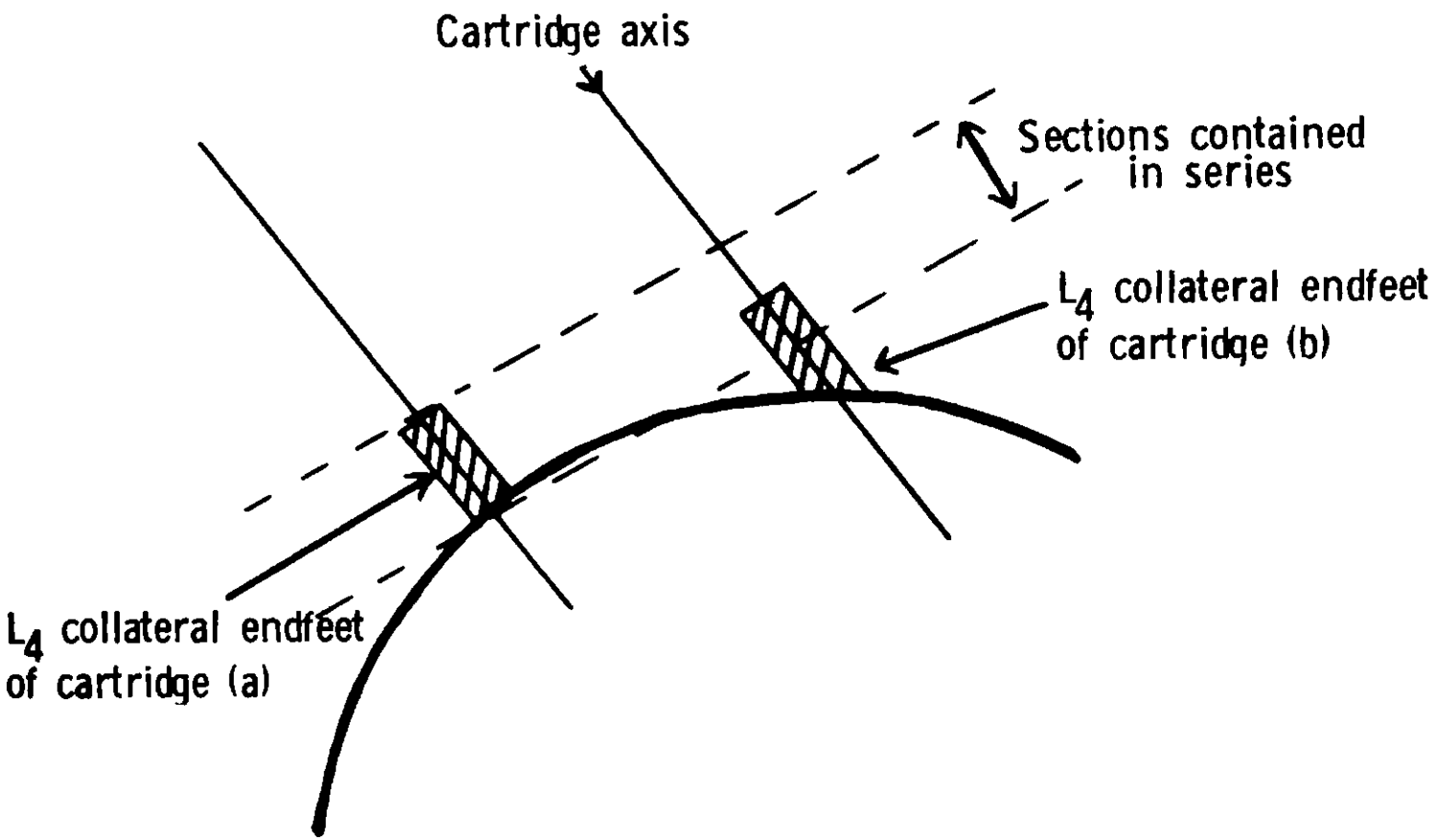


Fig. 19 For ease of interpretation the sections should be cut normal to the curved plane of the lamina. This has the disadvantage that a series sufficient to contain the area of interest in cartridge (a) will be too short to contain the whole of the corresponding area in cartridge (b).

FIG. 19. Curvature of the lamina, in text p. 60.

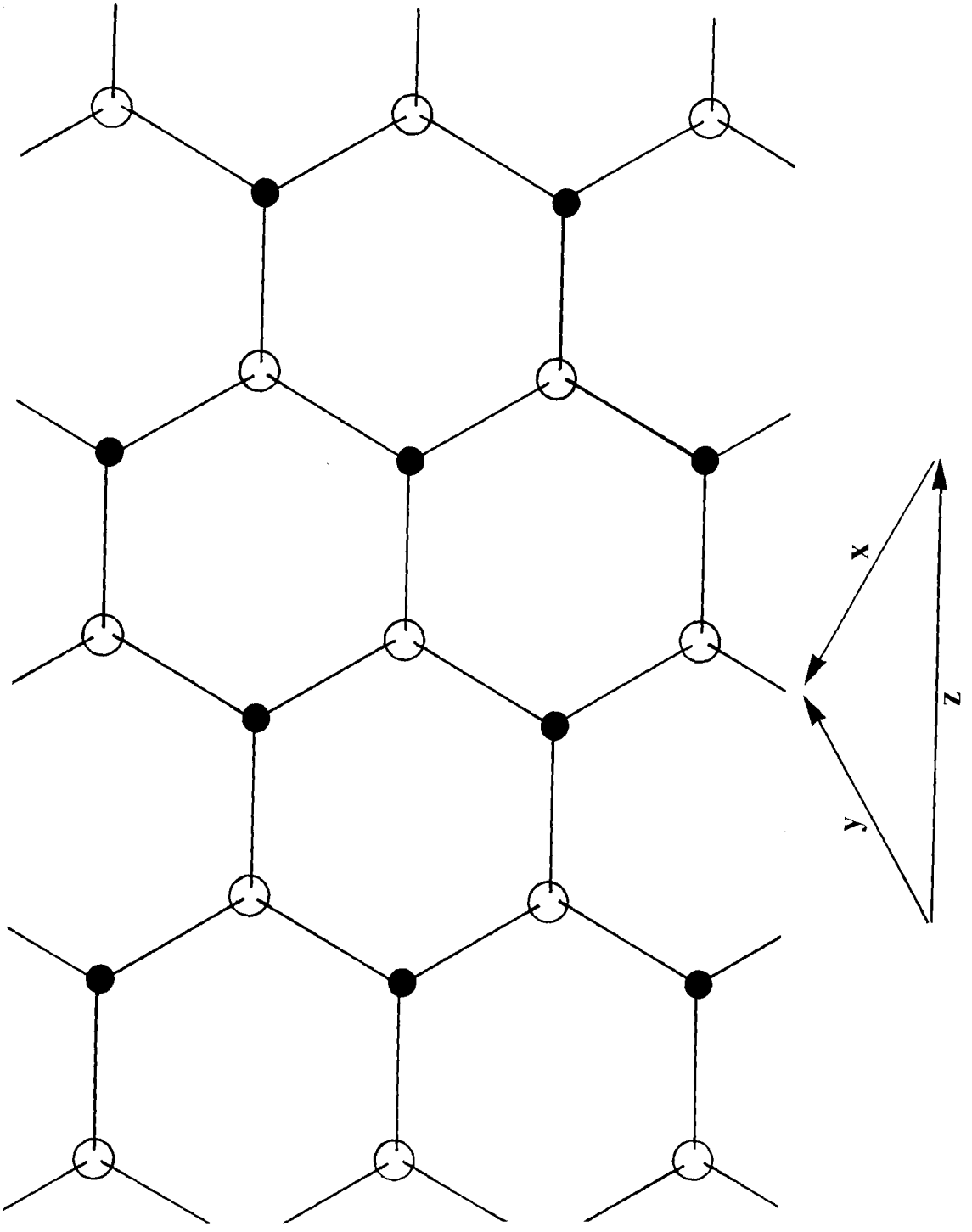


FIG. 20. Black circles: L4 axons. Black lines: L4 collaterals. White circles: cartridges. Figure shows schematically how each L4 cell sends collaterals to three different cartridges (from ref. 6).

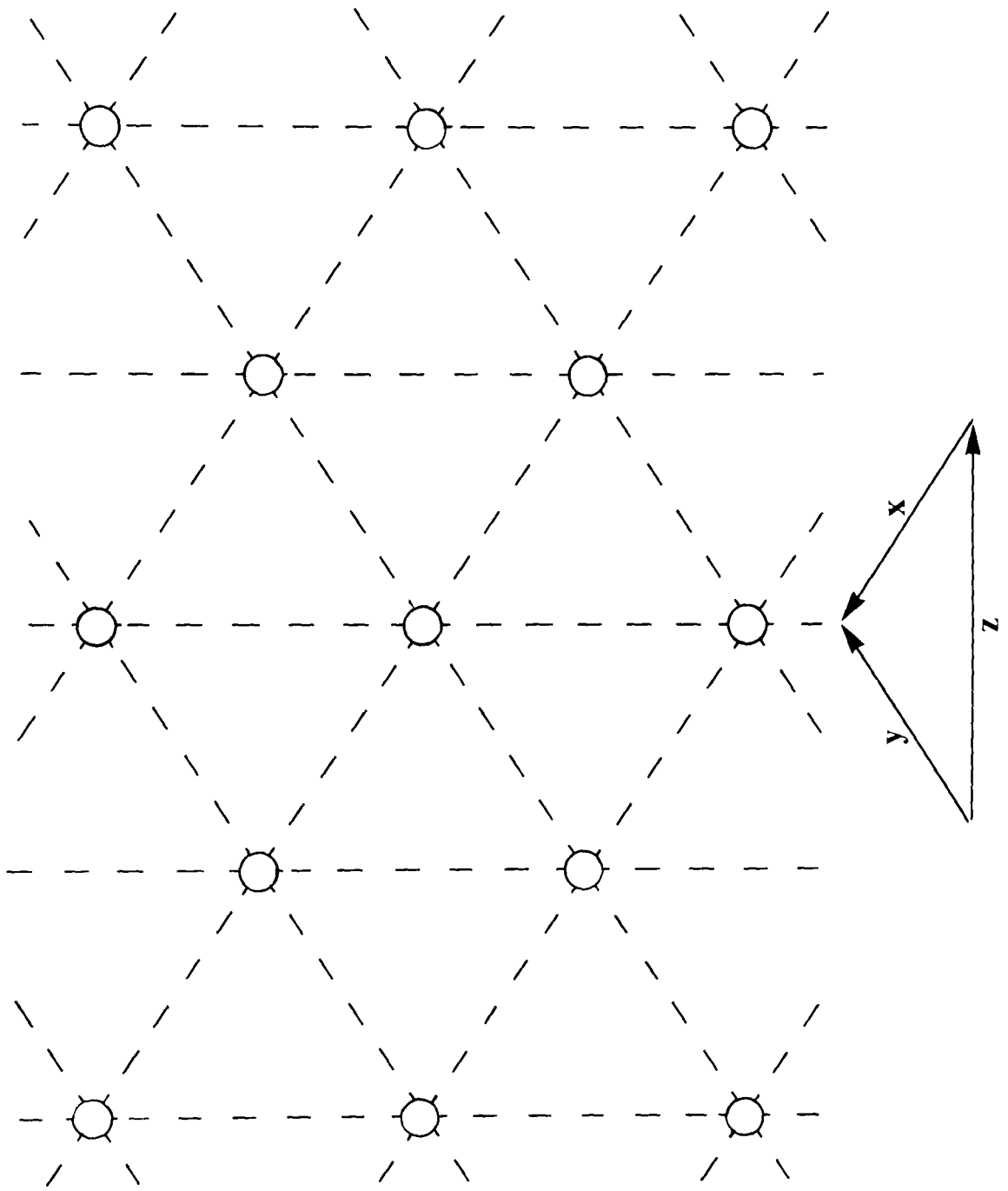
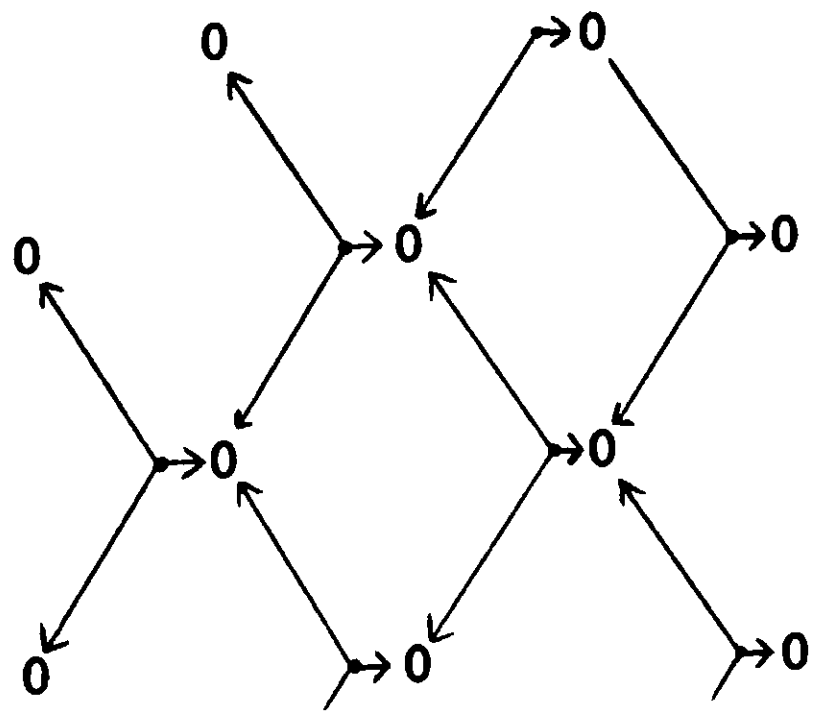


FIG. 21. Simplest scheme of connections possible in a hexagonal array (from ref. 6).



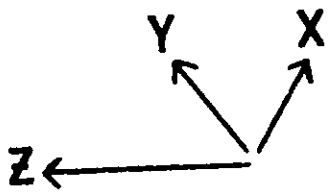


FIG. 22. Directional flow to the collateral clusters produces a sum effect along the +z direction.

TABLE 1.

Presynaptic / Postsynaptic	x	y	o	R
x		1	1	2
y	2		-	-
o	1	2		-
L2	7	2	-	Not observed
R	1	1	-	

(Table 1 is a summation, compiled from a survey of 20 cartridges, northern hemisphere of left eye of an old ♂ fly).

TABLE 2.

Presynaptic / Postsynaptic	x	y	o	R
x		2	-	-
y	1		-	-
o	1	1		-
L2	1	1	1	Not observed
R	-	-	-	
?	1	?		

(Table 2 is the results of observing a series of 23 silver sections, and represents the synapses made within one cartridge in that series. Same fly, same area of eye as Table 8).

TABLE 3.

Presynaptic / Postsynaptic	x	y	o	R
x		2	2	-
y	1		-	-
o	1	1		-
L2	1	2	1	Not observed
R	1	2	3	

Table 3. Synapses observed in one cartridge, in a series of 50 sections. ♂ fly, 4 days old, northern area of right eye.

TABLE 4.

Presynaptic / Postsynaptic	x	y	o	R
x		-	1	-
y	1		-	-
o	1	2		-
L2	Not observed	Not observed	Not observed	Not observed
R	Not observed	Not observed	Not observed	

Table 4. Synapses observed in one cartridge, in a series of 30 sections. ♂ fly, 4 days old, northern area of right eye.

TABLE 5.

Presynaptic / Postsynaptic	x	y	o	R
x		-	3	-
y	1		2	-
o	1	1		-
L2	Not observed	Not observed	Not observed	Not observed
R	Not Observed	Not observed	Not observed	

Table 5. Synapses observed in one cartridge, in a series of 60 sections. ♂ fly, 4 days old, northern area of right eye.

TABLE 6.

Presynaptic / Postsynaptic	x	y	o	R
x		4	6	-
y	4		2	-
o	4	5		-
L2 (Tables 2 + 3 only)	2	3	2	Not observed
L1	-	-	-	Not observed
R (Table 3 only)	1	2	3	

Table 6. Sum of Tables 2-5. Very similar to Table 5.

GRIDBOX 27295

	1	2	3	4	5	NOTES
A	1 Silver	10 Silver	1 Gold 1 Grey 3 Silver 1 Grey	7 Silver	1 Silver 1 Grey 2 Silver	Groups out of order here
B	2 Silver	1 Silver	1 Silver 1 Grey 14 Silver	1 Gold 1 Grey 2 Silver 1 Gold 1 Silver	14 Silver	
C	1 Silver 1 Gold 4 Silver	1 Gold 6 Silver	18 Silver	14 Silver 1 Blue	10 Silver 1 Grey	C4=2 last ones partly. C5=1 off edge

TABLE 7.

Transcript of notes made during serial sectioning. The rows A, B etc. represent rows of the gridbox, the columns 1-5 represent columns of the gridbox. Such notes were routinely made during sectioning.

TABLE 8. The following 11 pages are photocopies of the EM-95 logbook. They contain information necessary for the reconstruction of the series from the individual EM pictures, and are annotated for this purpose.

S'36 Almost 100% dorsal, 100% distal, retina Genis.
S'37 Next cannabinum proximal.
S'38
S'39
S'40
S'41
S'42
S'43
S'44

Ip=42

1 row.

S'45 Almost 100% ventral, 100% distal.
S'46 Next cartridge proximal.
S'47

1 row. Ip=42

S'48 Next cartridge dorsal to S'47
S'49 S'48
S'50 S'49
S'51 S'50
S'52 S'51
S'53 S'52
S'54 S'53

1 row.
Ip=42.

S'55
S'56 Pacifastacus M1
S'57 "
S'58 "
S'59 " P1. Ip=34.

Pseudocartridges PD & PV

~~S'60 discarded~~ 1/2, cartr S'51 Kontr 3 A1 27295
S'61 2 A1
S'62 2 3 A1
~~S'63 discarded~~ 2 A1

1b
1a

PD

A_2	Contr 1	sect. 1	contr 3	2a	24.7.74.
A_2	" "	sect. 2	"	3a	
A_2	" "	sect 3	"	4a	
A_2	" "	sect 4	"	5a	
A_2	" "	sect 5	"	6a	
A_2	" "	sect 6	"	7a	
A_2	" "	sect 7	"	8a	
A_2	" "	sect 8	"	9a	
A_2	" "	sect 9	"	10a	
A_2	" "	sect 10	"	11a	
A_2	contr. 2	sect 1	"	2b	
A_2	contr. 2	sect 2	"	3b	
A_2	"	sect 3	"	4b	
A_2	"	sect 4	"	5b	
A_2	"	sect 5	"	6b	
A_2	"	sect. 6	"	7b	
A_2	"	sect 7	"	8b	
A_2	"	sect 8	"	9b	
A_2	"	sect 9	"	10b	

Plates changed. 60 inserted. (SSS-6, no plate) 25.7.74.

A_2	contr 2	sect 10	contr 3	11b	
A_2			4	test, (Neutral Sinus 1+6)	
A_2			5		
A_2			6		
A_2			7		
A_2			8		

no intermediate diaphragm used

(P2)

595	Cantr	1	A_3	sect	1	contr	3	28a	M_2	PD
596	Cantr	2	A_3	sect	1	"	"	28b		
597	Cantr	1	A_3	sect	2	"	"	27a		
598	Cantr	2	A_3	"	"	"	"	27b		
599	"	1	A_3	"	3	"	"	26a		
600	"	2	A_3	"	3	"	"	26b		
601	"	1	A_3	"	4	"	"	25a		
602	"	2	A_3	"	4	"	"	25b		
603	"	1	A_3	"	5	"	"	24a		
604	"	2	A_3	"	5	"	"	24b		
605	"	1	A_3	"	6	"	"	23a		
606	"	2	A_3	"	6	"	"	23b		

607	M_2	Pacfastacus	pseudocambridge
608	M_1	"	cambridge
609	M_1	"	"
610	M_1	"	"

PD

611	Cantr	2	A_4	sect	1	contr	3	M_2	12b
612	"	1	A_4	"	"	"	"	"	12a
613	"	1	A_4	"	2	"	"	"	13a
614	"	2	A_4	"	2	"	"	"	13b
615	"	1	A_4	"	3	"	"	"	14a
616	"	2	A_4	"	3	"	"	"	14b
617	"	1	A_4	"	4	"	"	"	15a
618	"	2	A_4	"	4	"	"	"	15b
619	"	1	A_4	"	5	"	"	"	16a
620	"	2	A_4	"	5	"	"	"	16b
621	"	1	A_4	"	6	"	"	"	17a
622	"	2	A_4	"	6	"	"	"	17b
623	"	1	A_4	"	7	"	"	"	18a
624	"	2	A_4	"	7	"	"	"	18b

PD

20 Platten eingelegt. PD.

3 Lamina Kombination. T. 29.7.74

Lipi Frozen

Box 36629 Sec Q5

M_2	Contr 1	As sect 1	Contr 3	19a	PD
"	Contr 2	As " 1	" "	19b	
"	" 1	As sect 2	" "	20a	
"	" 2	As " 2	" "	20b	
"	" 1	As sect 3	" "	21a	
"	" 2	As " 3	" "	21b	
"	" 1	As " 4	" "	22a	
"	" 2	As " 4	" "	22b	

} Scanning mosaic Genis ret, lam, med. PD + KW

M_1 Genis lam
" " " ← from 652
" " " → " 653
" " " ← from 654
" " " ← " 655
" " " ↓ 656.
" " " → " 657.

M_1 Prij. trans lam PD + DN
" " " " "
" " " " "
" " " " "

59 Platten eingelegt. PD. 30.7.74

								15	
2663	M_2	Cuber	1	B_1	sect	1	Cuber 3	30a	PD 3
2664	"	"	2	B_1	"	1	" "	30b	
2665	"	"	1	B_1	"	2	" "	29a	
2666	"	"	2	B_1	"	2	" "	29b	
2667	"	"	1	B_2	"	1	" "	31a	
2668	"	"	2	B_2	"	1	" "	31b	
2669	"	"	1	B_3	"	1	" "	47a	
2670	"	"	2	B_3	"	1	" "	47b	
2671	"	"	1	B_3	"	2	" "	46a	
2672	"	"	2	B_3	"	2	" "	46b	
2673	"	"	1	B_3	"	3	" "	45a	
2674	"	"	2	B_3	"	3	" "	45b	
2675	"	"	1	B_3	"	4	" "	44a	
2676	"	"	2	B_3	"	4	" "	44b	
2677	"	"	1	B_3	"	5	" "	43a	
2678	"	"	2	B_3	"	5	" "	43b	
2679	"	"	1	B_3	"	6	" "	42a	
2680	"	"	2	B_3	"	6	" "	42b	
2681	"	"	1	B_3	"	7	" "	41a	
2682	"	"	2	B_3	"	7	" "	41b	
2683	"	"	1	B_3	"	8	" "	40a	
2684	"	"	2	B_3	"	8	" "	40b	
2685	"	"	1	B_3	"	9	" "	39a	
2686	"	"	2	B_3	"	9	" "	39b	
2687	"	"	1	B_3	"	10	" "	38a	
2688	"	"	2	B_3	"	10	" "	38b	
2689	"	"	1	B_3	"	11	" "	37a	
2690	"	"	2	B_3	"	11	" "	37b	
2691	"	"	1	B_3	"	12	" "	36a	
2692	"	"	2	B_3	"	12	" "	36b	
2693	"	"	1	B_3	"	13	" "	35a	
2694	"	"	2	B_3	"	13	" "	35b	

M_2	Contr 1	B_3	sect 14	Contr 3	34a PD
"	" 2	B_3	" 14	" "	34b
"	" 1	B_3	" 15	" "	33a
"	" 2	B_3	" 15	" "	33b
"	" 1	B_3	" 16	" "	32a
"	" 2	B_3	" 16	" "	32b

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M_2	Contr 1	B_4	sect 1	Contr 3	48a PD 31.7.74
"	" 2	B_4	" 1	" "	48b
"	" 1	B_4	" 2	" "	49a
"	" 2	B_4	" 2	" "	49b
"	" 1	B_4	" 3	" "	50a
"	" 2	B_4	" 3	" "	50b
"	" 1	B_4	" 4	" 3	51a
"	" 1	B_4	" 4	" 6	51a (Stripe)
"	" 2	B_4	" 4	" 3	51b
"	" 1	B_4	" 5	" "	52a
"	" 2	B_4	" 5	" "	52b
"	" 1	B_4	6	" "	53a
"	" 2	B_4	6	" "	53b

M_1	Genis dorsal retina VS distal			Contr 3	PD e KW
"	" " " " prox.			" "	
"	" ventr-lat. " VS distal			" "	
"	" " " " " " "			" "	mosaic
"	" subretinaler space (ventr lat)			" "	mosaic
"	" proj'n inde lam. " " "			" "	mosaic

M_2	Contr 1	B_5	sect 1	Contr 3	54a PD 31.7.74
M_2	" 2	B_5	" 1	" "	54b
"	" 1	B_5	" 2	" "	55a
"	" 2	B_5	" 2	" "	55b
"	" 1	B_5	" 3	" "	56a
"	" 2	B_5	" 3	" "	56b

2727	M_2	Contr 1	Bs	sect 4	Contr 3	57a
2728	"	" 2	Bs	" 4	" "	57b
2729	"	" 1	Bs	" 5	" "	58a
2730	"	" 2	Bs	" 5	" "	58b
2731	"	" 1	Bs	" 6	" "	59a
2732	"	" 2	Bs	" 6	" "	59b
2733	"	" 1	Bs	" 7	" "	60a
2734	"	" 2	Bs	" 7	" "	60b
2735	"	" 1	Bs	" 8	" "	61a
2736	"	" 2	Bs	" 8	" "	61b
2737	"	" 1	Bs	" 15	" "	68a
2738	"	" 2	Bs	" 15	" "	67b
2739	"	" 1	Bs	" 14	" "	67a
2740	"	" 2	Bs	" 14	" "	66b
2741	"	" 1	Bs	" 13	" "	66a
2742	"	" 2	Bs	" 13	" "	65b
2743	"	" 1	Bs	" 12	" "	65a
2744	"	" 2	Bs	" 12	" "	64b
2745	"	" 1	Bs	" 11	" "	64a
2746	"	" 2	Bs	" 11	" "	63b
2747	"	" 1	Bs	" 10	" "	63a
2748	"	" 2	Bs	" 10	" "	62b
2749	"	" 1	Bs	" 9	" "	62a
~~2750~~	missing	" 2	Bs	" 9	" "	/
2751	"	1	Bs	15	" "	68a
2752	"	2	Bs	15	" "	67b
2753	"	1	Bs	14	" "	67a
2754	"	2	Bs	14	" "	66b

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2755	M_2	Contr 1	C_1	sect 1	Contr 3	69a
2756	"	" 2	C_1	" 1	" "	68b

7	M_2	Cartr 1	C_1	sect 2	cartr 3	70a	PD	1·8·7·4
8	"	" 2	C_1	" 2	" "	69b		
9	"	" 1	C_1	" 3	" "	71a		
0	"	" 2	C_1	" 3	" "	70b		
1	"	" 1	C_1	" 4	" "	72a		
2	"	" 2	C_1	" 4	" "	71b		
3	"	" 1	C_1	" 5	" "	73a		
4	"	" 2	C_1	" 5	" "	72b		
5	"	" 1	C_1	" 6	" "	74a		
6	"	" 2	C_1	" 6	" "	73b		
~~7~~	discarded	" 1	C_2	" 1 (thin grey)	" "			
~~8~~	discarded	" 2	C_2	" 1	" "			
9	"	" 1	C_2	" 2	" "	75a		
0	"	" 2	C_2	" 2	" "	74b		
1	"	" 1	C_2	" 3	" "	76a		
2	"	" 2	C_2	" 3	" "	75b		
3	"	" 1	C_2	" 4	" "	77a		
4	"	" 2	C_2	" 4	" "	76b		
5	"	" 1	C_2	" 5	" "	78a		
6	"	" 2	C_2	" 5	" "	77b		
7	"	" 1	C_2	" 6	" "	79a		
8	"	" 2	C_2	" 6	" "	78b		
9	"	" 1	C_2	" 7	" "	80a		
80	"	" 2	C_2	" 7	" "	79b		
81	"	" 1	C_2	" 8	" "	81a		
82	"	" 2	C_2	" 8	" "	80b		

discarded } wrong cartridge.

19

15.11.74.

PD

2889 / 2910. Genis Frontal lam. M, Mosaic. (2889 is the small nerve bundle near the lamina. 2910 is the proj'n cut across.)

Grid G1. Box 2738).

(sect 1 missing)

2911	M_2	Contr 2	C_3	sect	2	contr 3	81b	15.11.74
2912	"	"	C_3	"	3	"	82b	
2913	"	"	C_3	"	4	"	83b	
2914	"	"	C_3	"	5	"	84b	
2915	"	"	C_3	"	6	"	85b	
2916	"	"	C_3	"	7	"	86b	
2917	"	"	C_3	"	8	"	87b	
2918	"	"	C_3	"	9	"	88b	
2919	"	"	C_3	"	10	"	89b	
2920	"	"	C_3	"	11	"	90b	
2921	"	"	C_3	"	12	"	91b	
2922	"	"	C_3	"	13	"	92b	Wrong cartridge
2923	"	"	C_3	"	14	"	93b	
2924	"	"	C_3	"	15	"	94b	
2925	"	"	C_3	"	16	"	95b	
2926	"	"	C_3	"	17	"	96b	
	Section 18 missing.							
2927	"	Contr 1	C_3	"	2	"	82a	
2928	"	"	C_3	"	3	"	83a	
2929	"	"	C_3	"	4	"	84a	
2930	"	"	C_3	"	5	"	85a	
2931	"	"	C_3	"	6	"	86a	
2932	"	"	C_3	"	7	"	87a	
2933	"	"	C_3	"	8	"	88a	

34	M_2	Contr 1	C_3	Section 9	Contr 3	89a	PD
35	"	"	C_3	" 10	"	90a	
36	"	"	C_3	" 11	"	91a	

sections 12-18 not yet taken.

60 Platten in EM. PD. 21.11.74.

22.11.74.
PD.

137 ↓ Mosaic M, Genis lam horizontal.
157 ↓
158 } Series Genis lam M, frontal
↓
160 }

22.11.74

161	M_2	Contr 2	C_4	Sect 1 (blue)	Contr 3		PD
162	"	" 1	C_4	" 1	"	"	
163	"	" 2	C_4	" 2	grey	"	
164	"	" 2	C_4	" 3		"	
165	"	" 2	C_4	" 4		"	
166	"	" 2	C_4	" 5		"	
967	"	" 2	C_4	" 6		"	
968	"	" 2	C_4	" 7		"	
969	"	" 2	C_4	" 8		"	
970	"	" 2	C_4	" 9		"	
971	"	" 2	C_4	" 10		"	
972	"	" 2	C_4	" 11		"	
973	"	" 2	C_4	" 12		"	

next 3 sections off edge of grid.

974	M_2	Contr 2	C_5	Sect 1	Contr 3
975	M_2	" 2	C_5	" 2	"

III

2976	M_2	Centr 2	Cs	Sect 2	Centr 3	22.11.74. PD
2977	M_2	Centr 2	Cs'	" 3	" "	
2978	"	" 2	Cs'	" 4	" "	
2979	"	" 2	Cs'	" 5	" "	
2980	"	" 2	Cs'	" 6	" "	
2981	"	" 2	Cs'	" 7	" "	
2982	"	" 2	Cs'	" 8	" "	
2983	"	" 2	Cs'	" 9	" "	
2984	"	" 2	Cs'	" 10	" "	

grey section impossible to photograph.

40 Platten eingelegt. PD. 22.11.74.

2985 Doos. [illegible] JC 114-271177-01
2994 [illegible] [illegible] 6.12.74 [illegible]

2995 9.12.74 Preißler
3039 Golubev
↓ kei 10.12.74 Preißler
3080 "
| 11.12.74 Preißler
3123 "

TABLE 9.

Plate no. (EM)	Grid no.	Serial no.	Orientation of series in lamina
CARTRIDGE a)			
2927 → 2936	C3	82a → 91a	Proximal
2781 → 2721	C2→C1→B5	81a → 54a	
2712 → 2701	B4	53a → 48a	
2669 → 2699	B3(backwards)	47a → 32a	
2667 → 2663	B2→B1	31a → 29a	
2595 → 2605	A3(backwards)	28a → 23a	
2648 → 2642	A5	22a → 19a	
2623 → 2612	A4	18a → 12a	
2573 → 2562	A2→A1	11a → 1a	Distal
CARTRIDGE b)			
2917 → 2911	C3	87b → 81b	Proximal
2782 → 2754	C2→C1→B5	80b → 66b	
2740 → 2748	B5	66b → 62b	
2736 → 2722	B5	61b → 54b	
2713 → 2702	B4	53b → 48b	
2670 → 2700	B3	47b → 32b	
2668 → 2666	B2→B1	31b → 29b	
2596 → 2606	A3	28b → 23b	
2649 → 2643	A5	22b → 19b	
2624 → 2611	A4	18b → 12b	
2587 → 2575	A2	11b → 2b	
2561	A1	1b	Distal

TABLE 9. This index provides a cross-reference between the original ultra-thin sections, the EM pictures and the final serial reconstruction. It was a necessary step in the interpretation of the serial sections.

TABLE 10.

Presynaptic / Postsynaptic	x	y	o	alpha
x				
y				
o		82b, 83b		3-5b, 14-17b, 29-30b.
L1				
L2	74b?	82b, 83b		

TABLE 10. Synapses observed in cartridge "b". (87 sections, 4-day-old male fly, right eye near northern perimeter).

TABLE 11. Presentation of synapses observed on each picture of serial section through cartridge "a". (91 sections, 4-day-old male fly, right eye near northern perimeter). This was a necessary step in the interpretation of the serial section.

TABLE 11

	x ↓ y	x ↓ o	y ↓ x	y ↓ o	o ↓ x	o ↓ y	x ↓ L2	y ↓ L2	o ↓ L2	x ↓ L1	y ↓ L1	o ↓ L1	α ↓ L4(o)	o ↓ R5
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														?
14												?		
15														
16														
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														
27														

TABLE 11 Cont'd.

	x ↓ y	x ↓ o	y ↓ x	y ↓ o	o ↓ x	o ↓ y	x ↓ L2	y ↓ L2	o ↓ L2	x ↓ L1	y ↓ L1	o ↓ L1	α ↓ L4(o)	o ↓ R5
28														
29														
30														
31														
32														
33														
34		?												
35		?												
36														
37														
38														
39														
40					?									
41														
42														
43														
44														
45														
46														
47														
48							?							
49							?							
50							?							
51														
52														
53														
54														

TABLE 11 Cont'd

	x ↓ y	x ↓ o	y ↓ x	y ↓ o	o ↓ x	o ↓ y	x ↓ L2	y ↓ L2	o ↓ L2	x ↓ L1	y ↓ L1	o ↓ L1	α ↓ L4(o)	o ↓ R5
55														
56												?		
57														
58														
59														
60														
61														
62														
63														
64														
65														
66														
67														
68														
69														
70				?				?						
71				?				?						
72				?				?						
73				?				?						
74				?				?						
75														
76														
77														
78														
79														
80														

TABLE 12.

Presynaptic / Postsynaptic	x	y	o	α
x		2	3	
y	1		2	
o	1+?	2		-
L1	-	-	?	
L2	3	3+?	1+?	

TABLE 12. Summary of the synapses found in cartridge "a", compiled from Table 11.

TABLE 13.

Presynaptic / Postsynaptic	x	y	o
o+L2	19-24a (No.1) visible	38-40a (No.4) very clear / 71-77a (No.10) indistinct	
y+L2	33-35a (No.2) very clear		43-44a (No.5) visible
x+L2		43-44a (No.6) visible	35-37a (No.3) indistinct
β+L2	47-53a (No.7) indistinct		
x+y			54-57a (No.8)
x+?		?-67a (No.9) visible	

TABLE 13. Presentation of the different types of synapses found in cartridge "a". The entries refer to the number of the pictures in the Series a) in which the synapses are evident.