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Lack of restriction at the blood–brain interface in *Limulus* despite atypical junctional arrangements

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Summary

Tracer and freeze-fracture techniques are used to evaluate the capacity of the central and peripheral nervous system of the horseshoe crab, *Limulus polyphemus* to admit or exclude molecular or ionic constituents of the blood intercellularly. Both the peripheral and central nervous systems are contained within blood sinuses so there is intimate contact between the haemolymph and the neural lamella. No discrete perineurium exists so any protection afforded to the nerve cells must be provided by the ensheathing glial cells and any junctions between them. Using ionic lanthanum as a pre-fixation incubation medium the system is seen to be completely 'open', with the tracer gaining access to all regions of the nervous tissue. Cellular associations in the peripheral nervous system, as revealed by thin-section and freeze-fracture, consist only of small scattered gap junctions between glial cells which afford no restriction to tracer entry. Gap junctions are again present between glial cells in the C.N.S. but here they are far more numerous, sometimes forming extensive sheets of almost continuous gap junctional plaques. Between certain glial cells there also exists a junctional system of linear PF ridges and complementary EF grooves; these may associate with or surround, often in discontinuous arrays, the gap junctional plaques. Given their characteristics and the freedom of tracer entry, they seem unlikely to represent either typical occluding tight junctions or septate junctions.

Introduction

The concept of the blood–brain barrier is well established for the vertebrate central nervous system (C.N.S.) (Reese & Karnovsky, 1967; Brightman & Reese, 1969; Brightman, 1977) as is the fact that the underlying morphological basis for the restriction to entry into the system is in the form of occluding tight junctional complexes between the capillary endothelial cells and the cells of the choroid plexus epithelium.

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Thus far, within the invertebrates, the only known blood–brain barrier occurs in the arthropods, and this is based on a different kind of structure from that of vertebrates. Here the barrier is located in the perineurial layer, a peripheral sheath of modified glial cells (Wigglesworth, 1959) that surround the C.N.S. (Lane & Treherne, 1972; Lane, 1978a). Disregarding the lower chordates (Lorber & Rayns, 1972, 1977; Georges, 1979), the other invertebrate phyla such as the molluscs and annelids (for references, see Lane & Skaer, 1980) possess a C.N.S. or brain which is not kept in a separate environment from the circulating haemolymph. In such systems no barrier is present and the body fluids are compatible, it seems, with the normal functioning of the nerve cells, any homeostasis apparently maintained by glial cell activity or the extracellular matrix acting as an ionic reservoir (Reinecke, 1976; Abbott & Treherne, 1977; Abbott *et al.*, 1977), or by the controlled exchange of ions across the axolemma (Skaer *et al.*, 1978).

Within the phylum Arthropoda, insects exhibit a well-characterized blood–brain barrier (Lane & Treherne, 1972; Treherne & Pichon, 1972; Lane, 1972; 1978a), as do spiders (Lane & Chandler, 1980), whereas the crustacean C.N.S. displays a delay to the entry of ions and molecules (Abbott, 1970, 1972; Lane *et al.*, 1977; Abbott *et al.*, 1977), although no ultimate restriction occurs, and ticks have no barrier at all (Binnington & Lane, 1980).

The question therefore arises as to whether a more primitive arthropod, the Xiphosuran chelicerate, *Limulus*, possesses a blood–brain barrier and occluding junctions like the insects and spiders or whether it more closely resembles the other arthropods thus far investigated. Earlier studies on thin sections of *Limulus* C.N.S. (Fahrenbach, 1976, 1979) made no reference to the existence of intercellular junctions. An analysis of the fine structure of both central and peripheral nervous systems in *Limulus* with reference to tracer uptake as well as by freeze-fracture has therefore been undertaken, with special concern for the existence and nature of any junctional complexes present. A parallel electrophysiological study (Willmer & Harrison, 1979) on the nerves of *Limulus* large walking legs indicated that the peripheral nervous system at least was accessible to any exogenous ions and molecules present in the circulating body fluids; this correlates with the lack of junctions reported in these peripheral nerves (Dumont *et al.*, 1965).

The present study reveals that, although tracers appear to enter the C.N.S. of *Limulus* freely, suggesting the absence of a barrier, junctional complexes do exist between the glial cells. These include not only gap junctions of quite remarkable size and number but also particle alignments with corresponding grooves on the complementary face. Although the latter bear some superficial resemblances to both tight junctions and septate junctions, they seem to represent a kind of junction not entirely typical of either.

Methods

Specimens of *Limulus polyphemus* obtained from Woods Hole, Massachusetts, U.S.A., were used in this investigation. They were maintained in tanks of aerated sea water at a temperature of 13–16° C. The tissues used, the nerve from the first walking leg and the cerebral ganglion, were treated in one of four ways: (1) normal fixation; (2) incubation in artificial sea water containing ionic lanthanum, prior to fixation; and (3) techniques for the analysis of junctional complexes by (a) freeze-fracture preparation or (b) treatment with colloidal lanthanum.

NORMAL FIXATION

The tissues were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, plus 0.4 M sucrose for from 1 up to 22 h at 4° C or room temperature (RT). The leg nerve was exposed and flooded with fixative. After 30 min the nerve was dissected out, cut into small pieces and placed in a fresh solution of fixative; the cerebral ganglion was removed and placed at once in the fixative. Following fixation, the samples were rinsed several times in changes of 0.1 M buffer, pH 7.4, containing 0.6 M sucrose, postfixed for 1 h with 1% osmium tetroxide in 0.1 M buffer ar RT, stained *en bloc* for 30 min in 2% uranyl acetate in sodium hydrogen maleate buffer (pH 6.2) or water, and then subsequently dehydrated through an ascending series of ethanols and propylene oxide prior to embedding in Araldite. Thin sections were cut with an LKB Ultrotome III, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope. One micrometre thick sections, which were stained with 1% toluidine blue, were prepared in parallel with the thin sections for examination by light microscopy.

INCUBATION IN IONIC LANTHANUM

The tissues were incubated for 1 h at RT with 10–50 mM lanthanum chloride in artificial sea water. After incubation the sea water solution was removed and the material was fixed in the normal fixative solution. The leg nerves were removed after 15 min and cut into short lengths; the ganglia were either left intact or cut into a maximum of four pieces. Processing of the tissues was then completed according to the schedule for normal fixation above. At least in the first instance, thin sections were often examined unstained for better visualization of tracer infiltration and staining.

TECHNIQUES FOR THE ANALYSIS OF JUNCTIONAL COMPLEXES

Freeze-fracture preparations

The C.N.S. and accompanying sheath of *Limulus* were dissected out in sea water as was the peripheral nerve. The tissues were then either placed unfixed in 25% glycerol in 0.1 M phosphate buffer, pH 7.4, plus 0.6 M sucrose, or immediately submerged in fixative for 30 min at RT. The fixative solution used was as described above. After fixing, the tissues were washed and placed in 25% glycerol made up in the same phosphate buffer as above. Equilibration proceeded for about 15–20 min after which both unfixed and fixed tissues were mounted in yeast paste in brass or nickel alloy holders. The tissues were rapidly frozen in Freon 22 cooled with liquid nitrogen and freeze-cleaved in a Balzers BA 360 M freeze-fracturing device. Fracturing took place in a vacuum of 1×10^{-6} Torr ($1.33 \times 10^{-4} N/m^2$) at a temperature of −100° C; shadowing was effected from a platinum/carbon or tungsten/tantalum source, followed by backing with carbon. The replicas were cleaned with sodium hypochlorite, sulphuric acid or dimethylformamide, rinsed with distilled water, mounted on coated grids and examined in a Philips EM300. The freeze-fracture micrographs are mounted so that the direction of shadowing is from the bottom or side.

Treatment with colloidal lanthanum

The fixative used was as above except that 0.1 M cacodylate buffer was substituted for phosphate buffer. One per cent lanthanum was added to the fixative in the form of colloidal lanthanum hydroxide prepared from lanthanum nitrate according to Revel & Karnovsky (1967). Following either short fixation at RT or overnight fixation at 4° C, the tissues were processed as in the normal fixation but again cacodylate buffer was used.

Observations

CENTRAL NERVOUS SYSTEM

Normal fixation

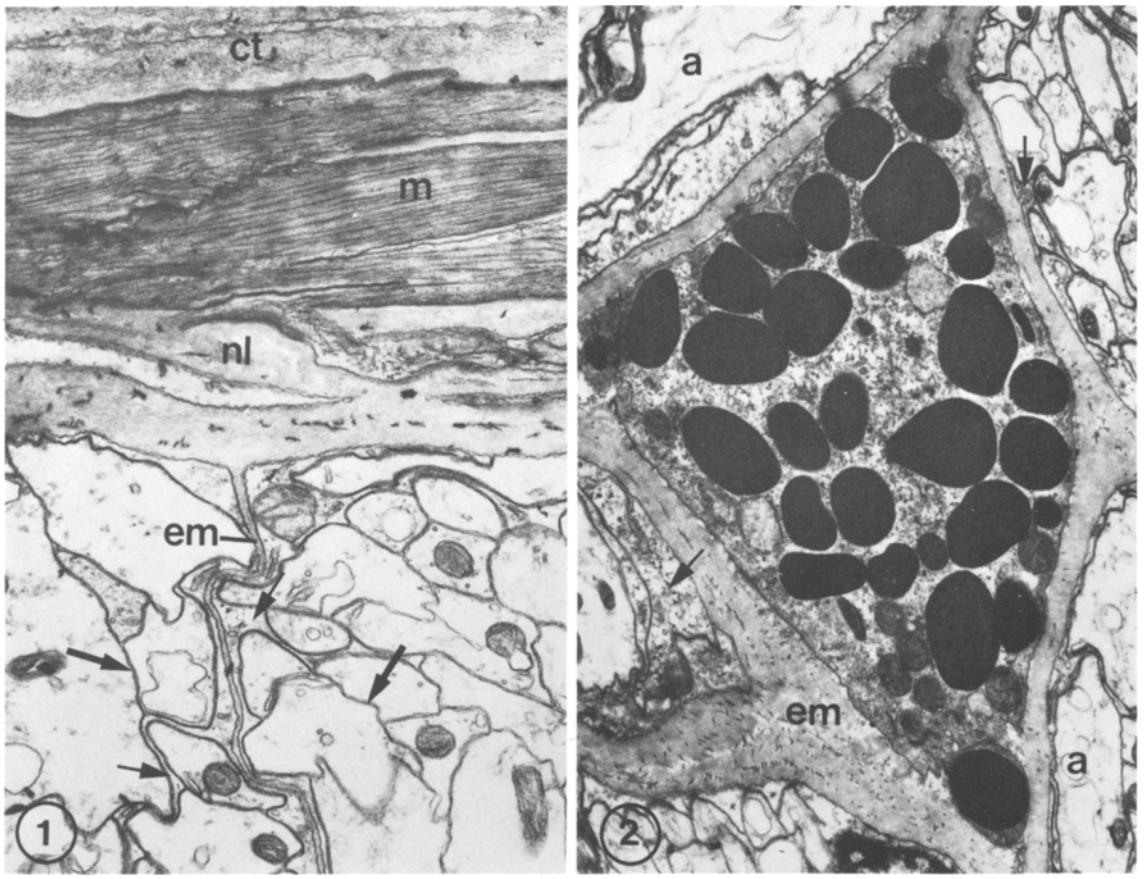
The entire central nervous system is contained within the blood vascular system which has a thick muscular and connective tissue wall closely applied to the nervous tissue (Fig. 1). Elongate cells are present within this vascular wall (Fig. 1) but a complete internal cellular lining is absent (Fahrenbach, 1976). The amoeboid mobility of the haemocytes (or amoebocytes, see Armstrong, 1979) of the vascular system and their ability to invade the extracellular spaces within the nervous tissue is suggested (Fig. 2) while trichromatic staining of histological sections demonstrates both haemocytes and haemocyanin (Fig. 3). There is no cellular lining around these extracellular spaces (Fig. 2).

The neural lamella, a thin extracellular layer consisting of a ground matrix containing collagen fibres, surrounds the ganglion and from it run the invasive arms of the extracellular matrix (or trabecular system) (see Figs. 1 and 2); the structure of the matrix is like that of the neural lamella in other arthropods (see Lane, 1974).

The gross morphology of the *Limulus* brain has been described (Fahrenbach, 1977, 1979) as has the distribution of the neuroglia (Fahrenbach, 1976) so only those features that relate to this study will be included. The terms peripheral and inner glia have been used to indicate the position of the glial cells under consideration. The C.N.S. is subdivided by a glial and matrix network (Figs. 1, 2). Those glial cells present around the perimeter of the ganglion and adjacent to the neural lamella we call peripheral glial cells; they are typically somewhat elongate in shape with adjacent cell boundaries being thrown into interdigitating folds (Fig. 4). These peripheral glial cells cannot be considered to form a true perineurium, as there is no discrete continuous ensheathment of the entire ganglion.

In the region of the circumoesophageal connectives axon to axon contact is common but attenuated 'inner' glial cell processes do pass between many axons (Fig. 1). In the corpora pedunculata are the very small Kenyon or globuli nerve cell bodies (Fahrenbach, 1979) which may have no glial investment at all.

Neurosecretory fibres are seen throughout the ganglionic masses, a large number occurring both by the outer neural lamella (Fig. 4) and by the extracellular matrix system (Fig. 7) where it abuts onto an internal blood space.



Figs. 1–14. Central nervous system.

Fig. 1. The vascular wall composed of connective tissue (ct) and muscle (m) is closely applied to the underlying central nervous system. An arm of the extracellular matrix system (em) can be seen moving from the neural lamella (nl) through the axonal and glial elements. Glial cells (small arrows) pass between many of the axons although axon to axon contact (thick arrows) is common. × 20 500.

Fig. 2. A haemocyte with its prominent granules is here seen lying deep within the ganglion, completely surrounded by arms of the extracellular matrix system (em) with no apparent cellular lining. a, axon; arrows, glial cells. × 12 000.

Incubation in ionic lanthanum to determine accessibility of nerve cells to tracer

After incubation of unfixed tissue in artificial sea water with ionic lanthanum, the electron-dense tracer appears intercellularly at all levels within the central nervous system. In the vascular wall it binds to the matrix and collagen fibres (Fig. 8). The tracer outlines the peripheral glial cells and reveals the many gap junctions between them; these are characterized by a narrowing of the intercellular space to a 2–3 nm gap within which the tracer becomes trapped (Fig. 9). In addition to these, similar

membrane associations also appear between the glia that surround axons in the interior (Fig. 13). Moving deeper into the nervous tissue the deposition of tracer is much more uneven, some areas showing heavy infiltration whilst others show very little. There appears, however, to be no tracer exclusion by any part of the nervous tissue; all parts of the system examined show some degree of lanthanum penetration (Figs. 4–6, 8–12, 14).

The neurosecretory terminals which lie adjacent to and beneath the acellular neural lamella and matrix (Figs. 4, 7) are also surrounded by lanthanum. There also appear to be some close membrane appositions between the axolemma and the adjacent glial cell membranes (Fig. 14).

Analysis of junctional structures by freeze-fracture and colloidal lanthanum

Freeze-fracture. After freeze-fracturing, the relationships between nerve cells and neuroglia appear to be no different from the sectioned material. The glial cells are characterized by conventional arthropod, inverted, gap junctions. These consist of macular plaques of 13 nm particles which cleave preferentially onto the E face of the membrane (Fig. 15). The plaques are fairly large and often circular or ovoid in outline (Fig. 15); they may also be numerous and clustered closely together, covering a large proportion of the surface membrane. In such cases, they are either separated by flat membrane areas (Fig. 15) or by regions in which the membrane between them appears pushed up or buckled into broad EF ridges (Figs. 16, 18) with complementary PF troughs (Fig. 16), so closely are the junctions packed together.

The gap junctional EF particles display presumed complementary pits in the P face of the membrane (Fig. 16) and the intercellular cleft at the points where the fracture plane moves from EF to PF is reduced (Fig. 16). Slight hollows or pits are to be seen in the centre of the EF junctional particles; these are especially clear only in certain particles (insert in Fig. 15).

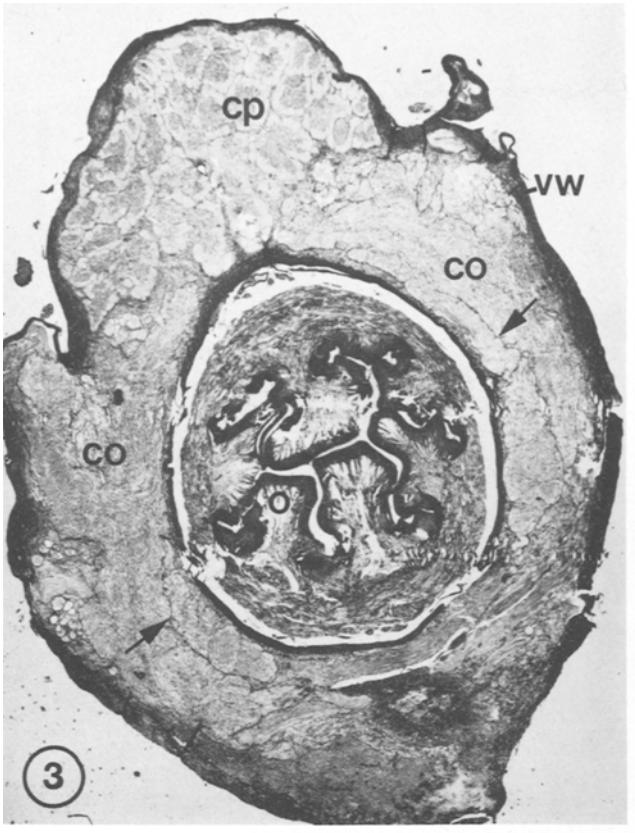
Fig. 3. Light micrograph of transverse section through the circumoesophageal (co) and corpora pedunculata (cp) regions of the brain. The oesophagus (o) passes through the ganglionic mass. The vascular wall (vw) (here for the most part dissected away) is in intimate contact with the underlying nervous tissue. Blood cells or haemocyanin (arrows) can be seen deep within the nervous system. Heidenhain's Azan stain. × 10.

Fig. 4. Neurohaemal areas where neurosecretory fibres terminate at the neural lamella on the outer surface of the ganglion. Interdigitating glial cells (g) can be seen beside them. × 28 600.

Fig. 5. Kenyon nerve cells within the corpora pedunculata (see Fahrenbach, 1977, 1979). Ionic lanthanum is present (arrows) in the extracellular spaces between the cells. × 6200.

Fig. 6. Hexagonally shaped axons within the peduncular neuropil. The size of the axons varies greatly from 1 μm to as small 0.1 μm (inset). The lanthanum tracer can be clearly seen between the axons. × 38 400; inset, × 23 500.

Fig. 7. Neurosecretory terminals either close to (small arrow) or abutting onto (large arrow) the internal extracellular matrix in the C.N.S. g, glial cell. × 18 800.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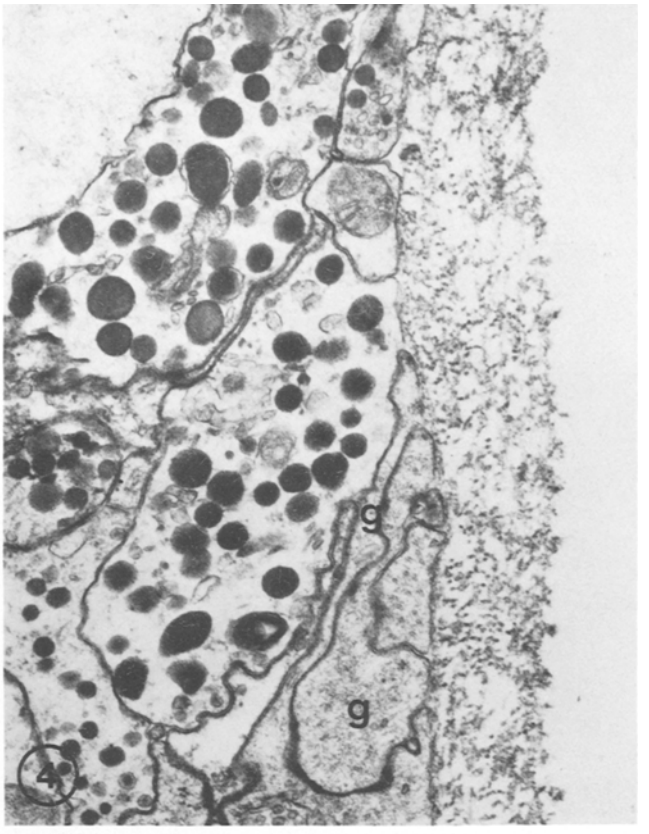
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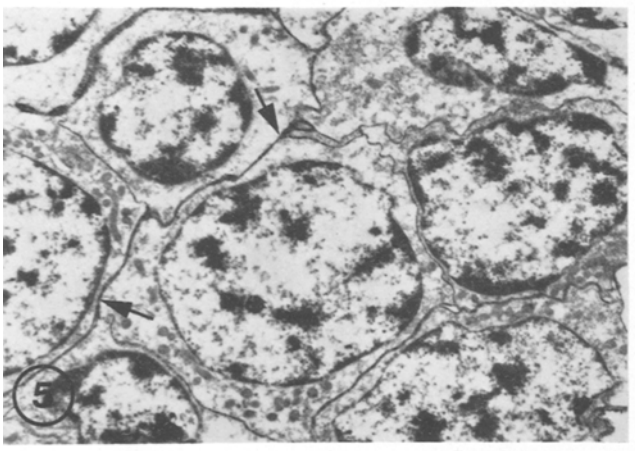
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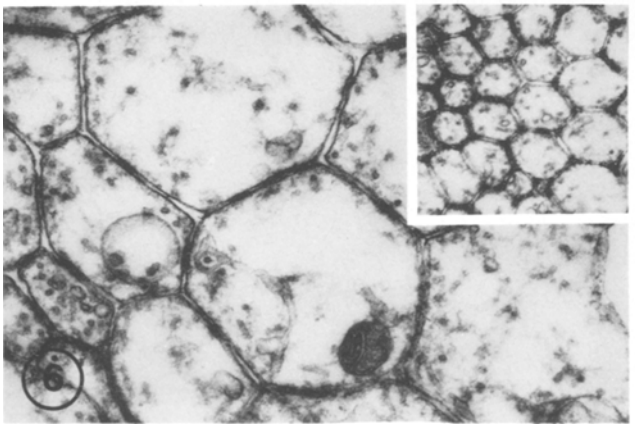
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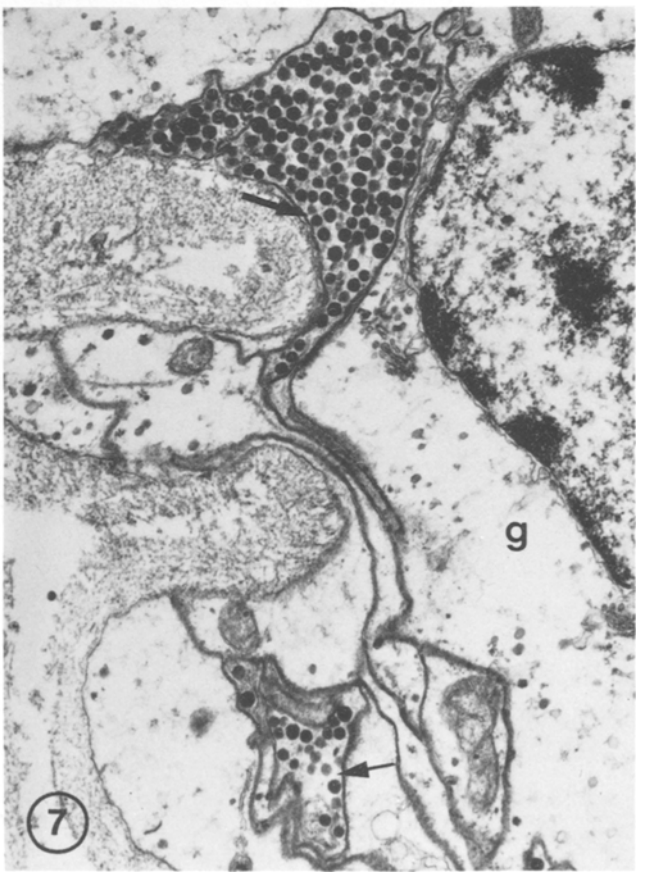
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In some cases the packing of component junctional particles in the glial gap junctions exhibit a degree of regularity (as in Fig. 24) but more frequently they display no consistent centre-to-centre spacing. Indeed, they sometimes become so loose that the macular arrangement is disrupted and the particles are spread out over the membrane face (Figs. 16, 17). This apparently takes place at random so that there is no way of predicting where it will occur but it has always been found near other fully formed junctional plaques and, from their size and fracturing characteristics, it seems that the loose particles are gap junctional in nature. This appearance is reminiscent of forming or disaggregating junctions (Lane, 1978b; Lane & Swales, 1978a, b, 1979, 1980; Lane & Skaer, 1980).

Close to the clustered arrays of glial gap junctions in the C.N.S. are frequently found linear configurations of pits or particles (Figs. 18–20). These are particles of about 12–14 nm in diameter which are irregularly aligned into structures that are not continuous ridges; the particles do not always appear to fuse together, but often lie in linear arrays of 2 or 3 that make up disrupted or discontinuous ridges (Fig. 19). These PF linear particle rows often run directly between the regions of gap junction pits (Fig. 19) and in some cases, surround the junctional plaques. This is also found in the EF where the gap junctional particle clusters are encroached upon or partly circumscribed by linear grooves (Fig. 20) which appear to be complementary to the rows of PF particles. There does not always appear to be a significant reduction in the intercellular cleft when the fracture plane cleaves from EF grooves across to PF particle rows, but they do not appear to be comparable to septate junctions.

Fig. 8. Ionic lanthanum can be seen around the cells (arrows) in the vascular wall surrounding the central nervous system. Binding of lanthanum by the collagen fibres and muscle may also occur. × 9000.

Fig. 9. Gap junctions (arrows) between three peripheral glial cells in the ganglion each exhibiting the characteristic 2–3 nm reduced intercellular space. × 98 000.

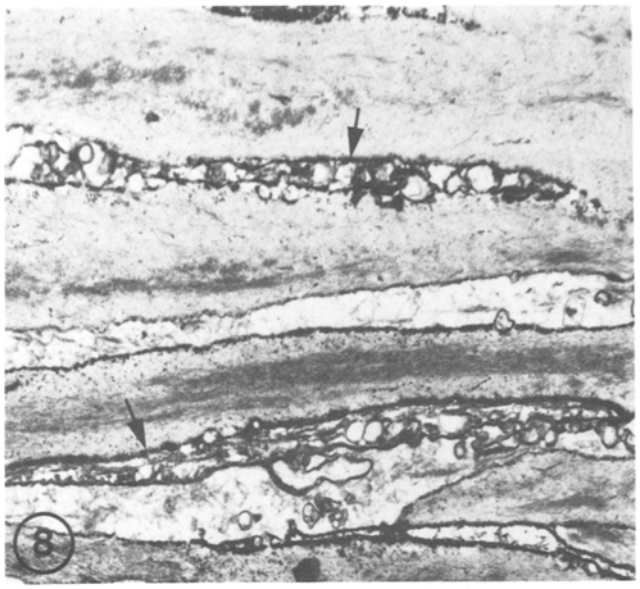
Fig. 10. Axons within the ganglion surrounded by dense ionic lanthanum tracer which has free access to these intercellular spaces. × 81 500.

Fig. 11. Kenyon nerve cells (k) of the corpora pedunculata with a tract of axons running between them. One larger axon (a) contains neurosecretory granules. Ionic lanthanum tracer can be observed to have penetrated the extracellular spaces. × 14 200.

Fig. 12. Globular neuropil of the corpora pedunculata with swirls of glial cells (g) surrounding at least one axon (a). A connective tissue partition (ct) can be seen between the neuropil and some Kenyon cells (k). Extracellular ionic lanthanum is evident throughout the tissue. × 8100.

Fig. 13. Glial processes (g) can be seen running between axons (a) in the interior of the central nervous tissue. Narrowing of the extracellular space between the glial cells indicates a large number of gap junctions (arrows). × 52 300.

Fig. 14. A neurosecretory terminal lies adjacent to the extracellular matrix (em) of the ganglion. Lanthanum tracer can be seen between glial cells (g). There is some suggestion of gap junctions being present between the terminal and an adjacent glial cell (large arrows) as well as between the glial cells (small arrow). × 31 800.



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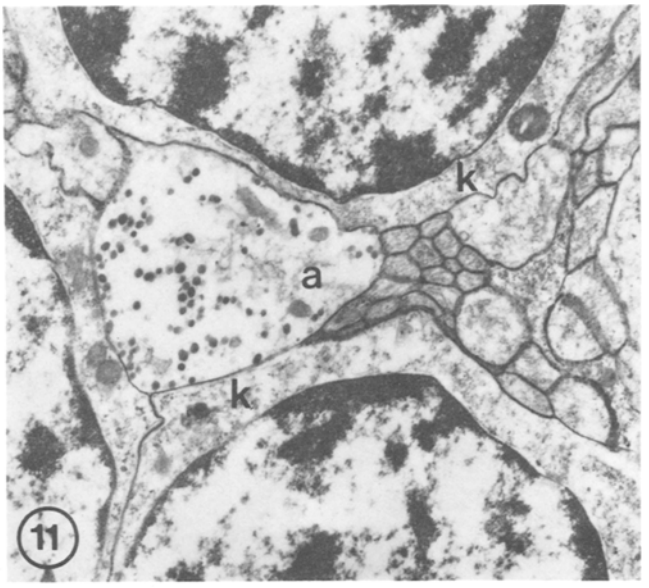

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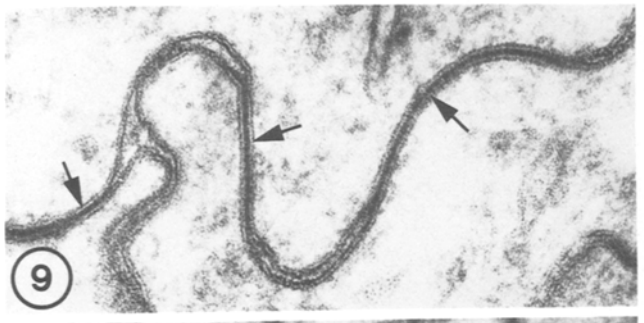

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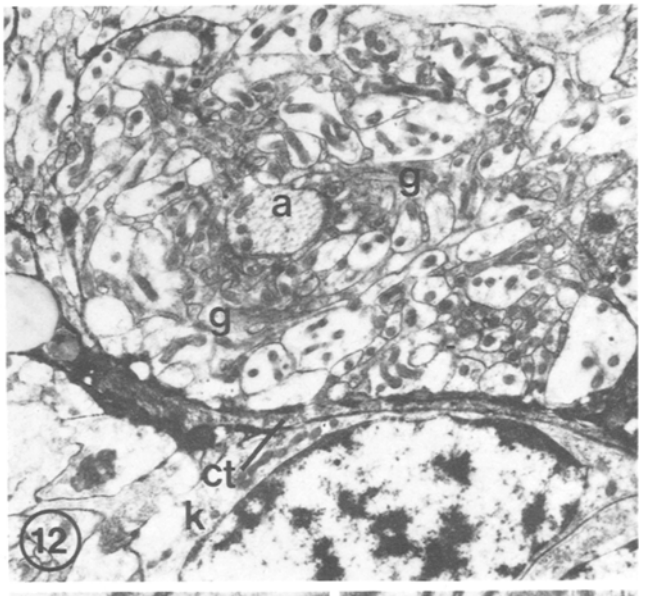

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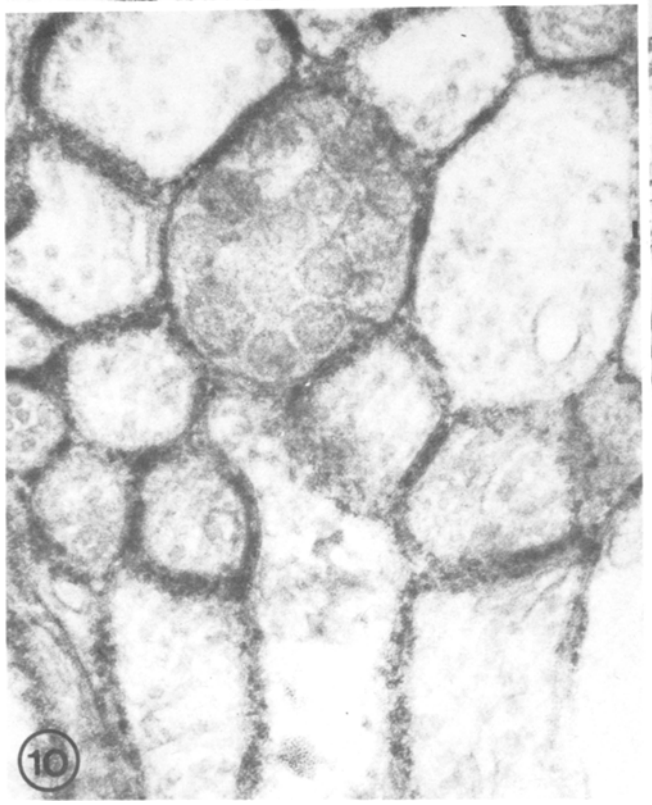

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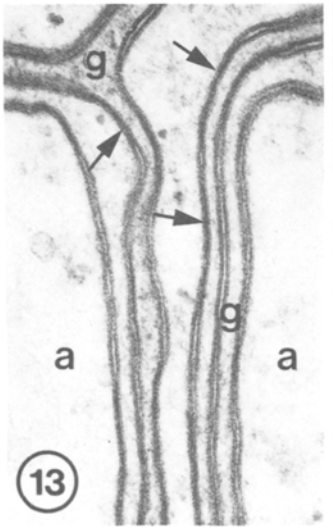

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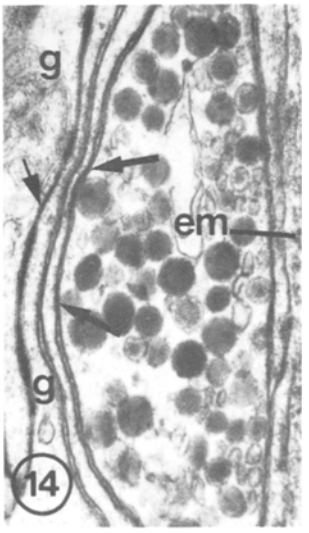

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Treatment with colloidal lanthanum. Oblique sections through the apposed glial membranes comprising the gap junctions show, in *en face* views, the particle arrangement typical of such junctions (Figs. 24, 25). Although the distribution of these particles within the plaques is often apparently random on freeze-fracture replicas (Figs. 15, 16), they often appear to be packed into rows in the lanthanum infiltrated thin-sectioned images. This is revealed as a banding of lanthanum with one of two periodicities; the smaller periodicity of approximately 6.25 nm (Fig. 21) is half that of the other (12.5 nm) (Figs. 22, 23). The 12.5 nm periodicity observed is assumed to be a measure of the spacing of the junctional particles which in some cases is fairly regular. This ordered arrangement is more likely to be due to hexagonal packing of the particles, rather than a linear distribution, since variations in the plane of section could then account for both the 12.5 and the 6.25 nm spacing observed. However, it should be noted that hexagonal packing of gap junctional particles is atypical of arthropods (see Lane & Skaer, 1980), although common in vertebrate tissues (Revel & Karnovsky, 1967).

PERIPHERAL NERVOUS SYSTEM

Normal fixation

The neural lamella consists of a thin layer of extracellular connective tissue which ensheathes the nerve but no outer continuous, perineurial-like glial cell layer exists to ensheath the peripheral nerves. An extracellular matrix subdivides the nerve into

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Figs. 15–20. Freeze-fracture replicas from *Limulus* C.N.S.

Fig. 15. Fracture plane showing the numerous macular arrays of closely clustered EF particles that comprise the glial gap junctions. Inset at higher magnification shows the channels present in the particles. × 37 400; inset, × 185 900.

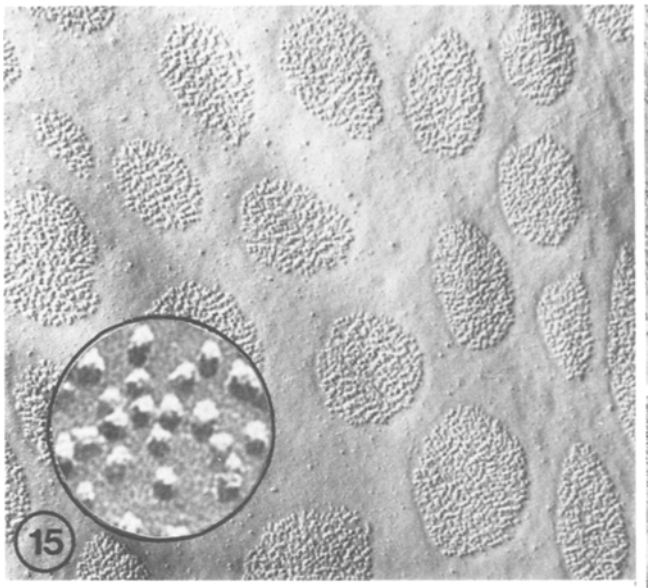
Fig. 16. Gap junctions packed so closely that the fracture face between them exhibits either an E-face (EF) hump (*) or a complementary P-face (PF) depression (*). The EF particles and complementary PF pits of the gap junctions (gj) are separated by a reduced intercellular cleft. In some regions the junctions appear rather loosely clustered. × 41 900.

Fig. 17. Gap junctional plaques displaying a local disaggregation of particles which could relate to the extent to which they are coupled, or to stages in junctional breakdown or formation. × 41 000.

Fig. 18. Low-power view to demonstrate the close packing of numerous glial gap junctional plaques with humped EF ridges between them. Note to the right the encroachment of linear grooves (arrows) into the gap junctional area. × 26 000.

Fig. 19. P-face showing the gap junctional pits (gj) and an extensive array of PF ridges around them, rather in the manner of vertebrate gap junctions and intercalating tight junctional ridges. These ridges are composed of aligned particles which are often fused (at arrows). × 42 600.

Fig. 20. Higher power of an E face to show the linear furrows that may circumscribe the gap junctional (gj) particle plaques. They may contain an occasional particle near the base of the groove (arrows) which may have fractured off the complementary P face ridge of particles (illustrated in Fig. 19). × 47 100.



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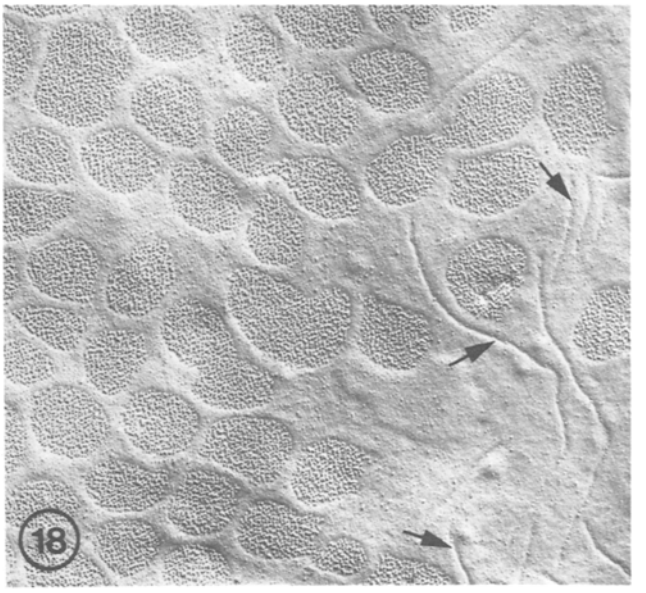

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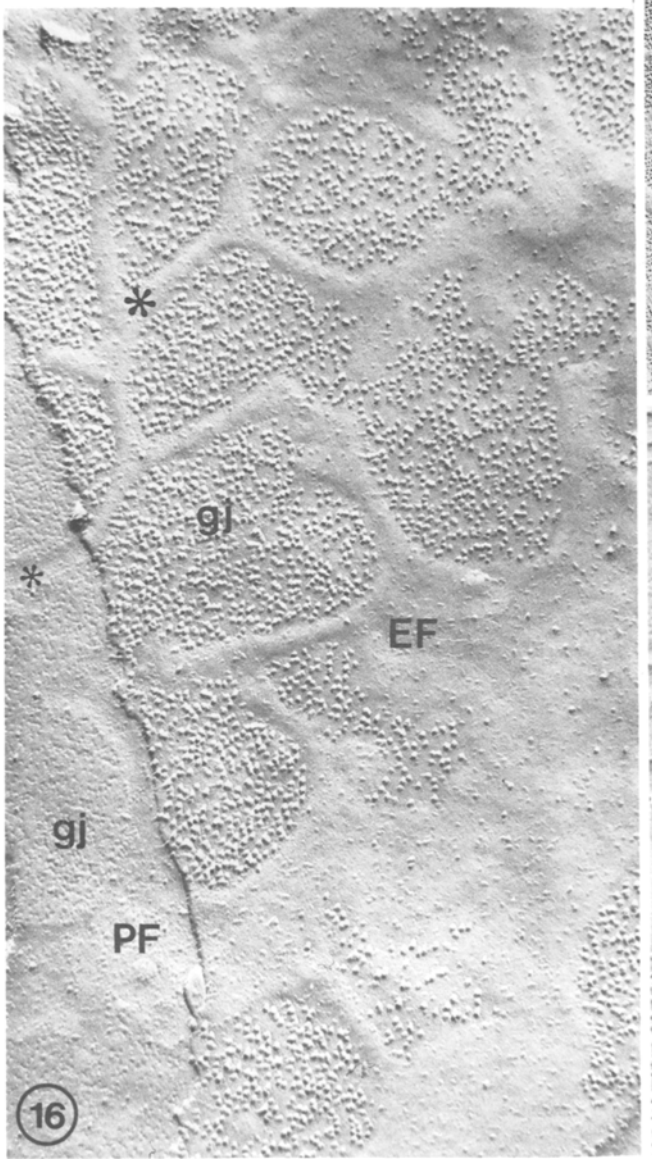

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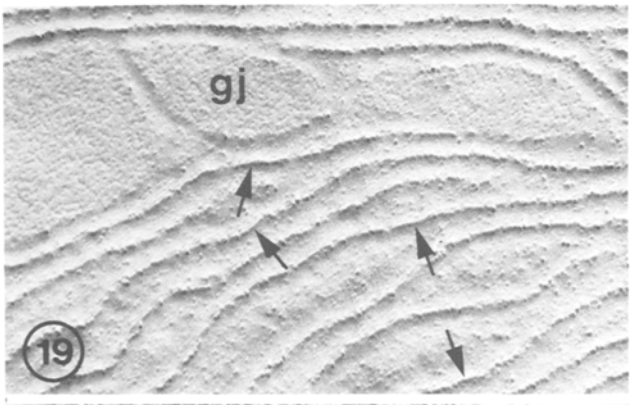

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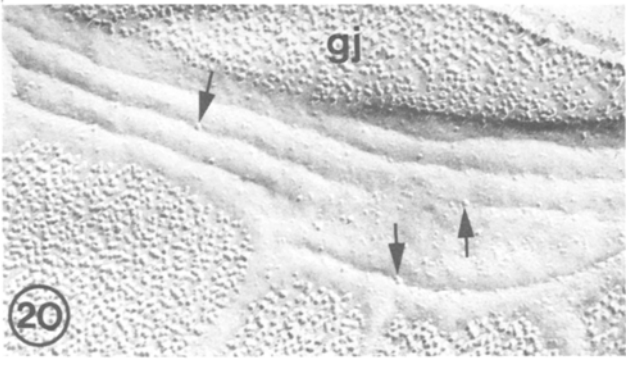

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axon bundles. The axons may be classified into three groups according to their size, similar-sized axons associating together (Fig. 26). These axons are often separated from each other by attenuated glial cell processes, but axon to axon contact is not unusual (Fig. 27).

Incubation in ionic lanthanum

Ionic lanthanum is seen to bind particularly heavily to the collagen fibrils (Fig. 28) both in the neural lamella and in the extracellular matrix surrounding the axons

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Figs. 21–25. Glial cells of the central nervous tissue incubated in colloidal lanthanum.

Fig. 21. Gap junction sectioned at right angles showing cross-striation (arrows) with a periodicity of 6.25 nm. × 109 000.

Fig. 22. Gap junction sectioned at right angles showing cross-striations (arrows) with a periodicity of 12.5 nm. × 135 500.

Fig. 23. Gap junction showing a 12.5 nm periodicity of banding. The plane of section to the membranes passes from being at right angles (on the left) to partly *en face* (on the right). The component particles of the gap junction possess a somewhat linear alignment (arrow). × 122 500.

Fig. 24. *En face* view of a gap junction. The particles are arranged in rows with a central pore visible in some of them (arrows). × 151 300.

Fig. 25. A vast expanse of gap junctional membrane is here shown *en face* which correlates with the extensive freeze-fracture images (as in Fig. 16). Little organization of the particles is apparent although some regularity can be seen (asterisk) in one area. The central channels of the particles are clearly visible (arrows). × 108 300.

Figs 26–31. Micrographs of the peripheral leg nerve of *Limulus.*

Fig. 26. Light micrograph of leg nerve sectioned transversely and stained with 1% toluidine blue. The vascular wall around the nerve is distended at one side to produce a longitudinally running blood channel (asterisk). Haemocytes may be frequently observed within the channel and also permeating the nervous tissue (short arrows). Three groups of axons (according to size) are visible (a, b, c). The two largest groups frequently contain axons with small, centrally situated mitochondria (long arrows). × 100.

Fig. 27. Elliptically shaped bundles of glia (g) and axons (a) ensheathed by the extracellular matrix (em). Attenuated glial cell processes are seen to pass between many axons (small arrows) but axon to axon contact is frequently encountered (large arrows). × 11 300.

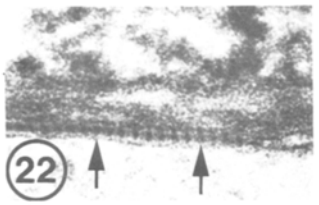
Fig. 28. Ionic lanthanum between the ensheathing vascular elements and bound to the collagen fibres of the vascular system, the neural lamella (nl) and extracellular matrix system (em). × 10 100.

Fig. 29. Ionic lanthanum is heavily bound to the extracellular matrix and is also present extracellularly around the axonal and glial elements. × 40 300.

Fig. 30. In this colloidal lanthanum preparation relatively short gap junctions (arrows) are present between glial cells. × 140 000.

Fig. 31. E-face from a glial cell of the leg nerve. The gap junctions are almost invariably small and often elliptical in outline. × 41 600.


21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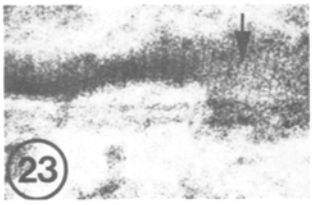
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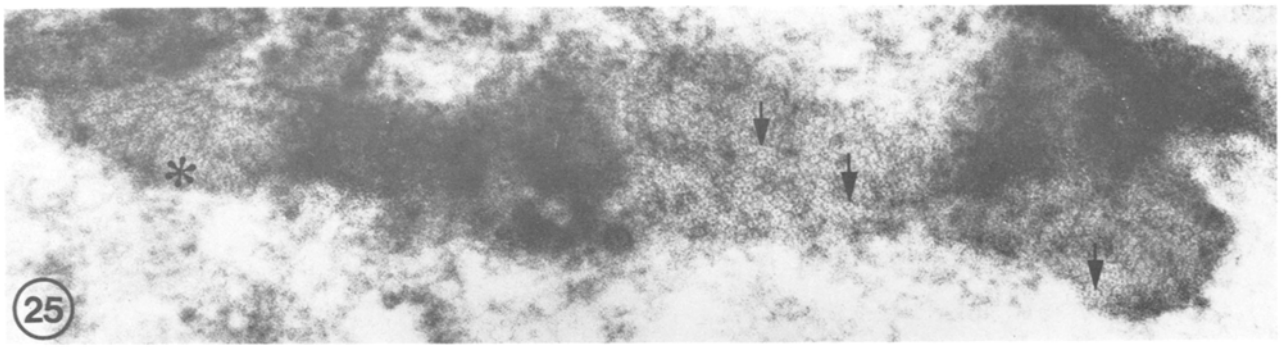
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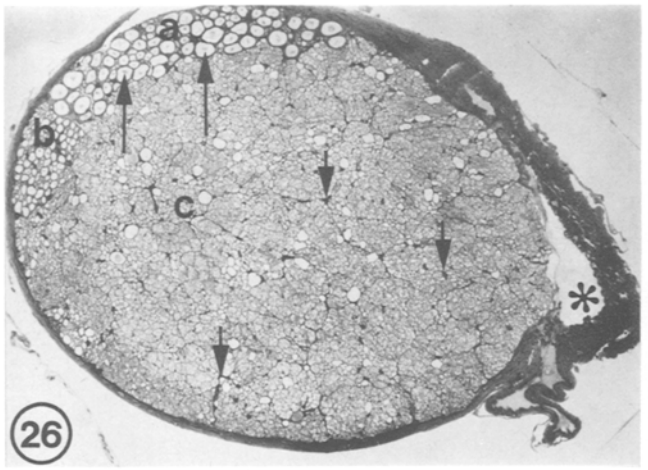
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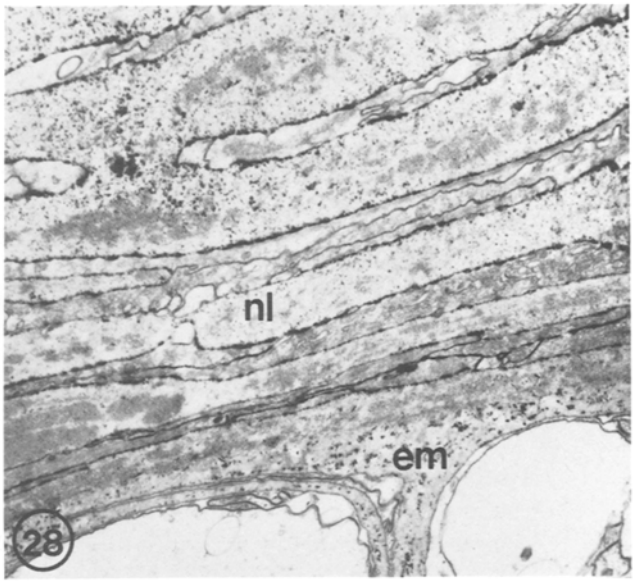
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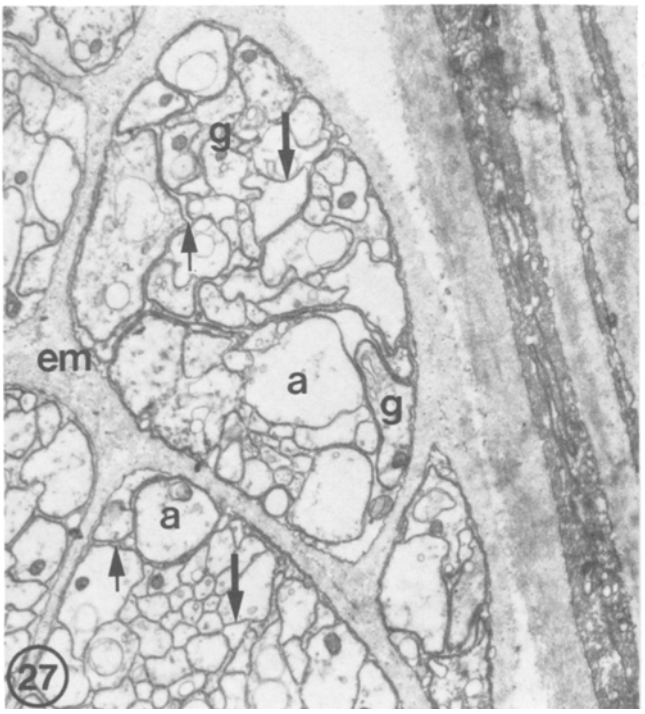
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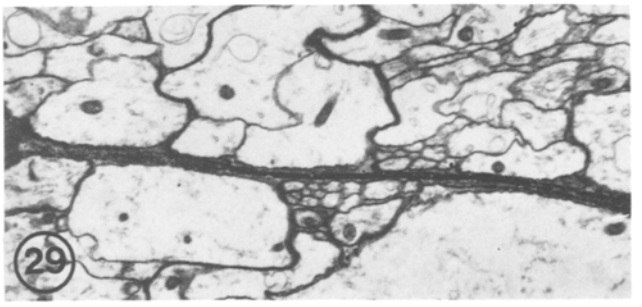
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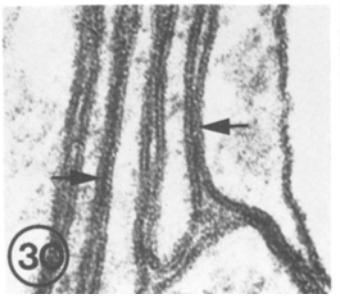
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(Fig. 29). Tracer deposition is also seen in the extracellular space between axons (Fig. 29) and there is no restriction to its entry throughout the whole of the nerve.

Treatment with colloidal lanthanum and freeze-fracture

In the peripheral nerve, like the C.N.S., the only cell-to-cell associations in thin-sections are gap junctions between the glial cells; in the sparse glial ensheathment of the large axons junctional complexes are rarely seen, presumably because of the infrequent contact areas between glia. The gap junctions exhibit the characteristic 3 nm gap between adjacent membranes (Fig. 30). In freeze-fractured replicas they tend to be rather small plaques consisting of relatively few particles; the junctional plaques are often elliptically shaped (Fig. 31).

Discussion

Absence of a blood–brain barrier

Both the peripheral and central nervous system in *Limulus* are here seen to be accessible to ionic lanthanum and hence are presumably open to molecules of a comparable size in the circulating haemolymph. The blood channels which permeate the C.N.S. of *Limulus* are unlike those of vertebrates; the latter are vessels lined by endothelial cells which are associated laterally by tight junctions (Reese & Karnovsky, 1967). In the horseshoe crab the blood channels are spaces, lined only by the extracellular matrix, while the interglial clefts next to them, ensheathing the adjacent neurons, are patent to tracers. The blood–brain interface, therefore, is an open, rather than a closed one. The junctions that occur between the glial cells are not constructed so as to restrict the free intercellular entry of ions and molecules.

Since the organism is marine and a rather primitive arthropod, these results are not entirely unexpected because other marine invertebrates such as serpulid annelids (Skaer *et al.*, 1978) possess no blood–brain barrier. Even certain freshwater invertebrates, such as lamellibranch molluscs (Lane & Treherne, 1972) and crustaceans (Abbott, 1972; Lane *et al.*, 1977) exhibit no ultimate restriction to the entry of substances from the circulating haemolymph to the axonal surfaces. In the case of terrestrial arthropods although some, such as the ticks (Binnington & Lane, 1980), do not possess a blood–brain barrier, others, for example, insects (Lane, 1978a; Lane & Skaer, 1980) and spiders (Lane & Chandler, 1980), do have a blood–brain restriction. It would appear that a homeostatic microenvironment is particularly important for neuronal function in these latter cases, while in organisms such as *Limulus* and the crayfish, any regulation which does occur to maintain homeostasis, must take place directly at the level of the inner glial–neuronal interface (Abbot *et al.*, 1977; Abbott & Treherne, 1977).

Linear particle alignments

The linear junctional ridge/groove system present in certain glial cells in *Limulus* C.N.S. bears some similarities to tight junctions by freeze-fracture criteria. However,

their constituent particles are larger, they are not always fused laterally into ridges but often remain as separate particles, and the intercellular clefts between their complementary ridges and grooves rarely seem to be obliterated. They have few or no anastomosing strands connecting adjacent parallel ridges and in this respect resemble the tight junctions of certain vertebrate cell types (Nagano & Suzuki, 1976; Simionescu & Simionescu, 1977). Any similarity they may display to septate junctions is only superficial for no junctional areas exhibiting obvious septa in thin sections or in lanthanum-impregnated tissue have been found. In replicas, the particles are more disordered than those of septate junctions and are not precisely aligned into rows; in addition, their centre-to-centre particle spacing is uneven. Moreover, the particles are of a somewhat different size (about 13 nm) from those characterizing septate junctions (about 8 nm). However, these linear junctions do maintain a close spatial association with the gap junctions which in arthropods is a feature common to both septate junctions (see Lane & Skaer, 1980) and tight junctions (Lane & Chandler, 1980). In spite of their similarity in diameter, the particles cannot represent degenerating or forming gap junctions (Lane & Swales, 1980) because their component particles are on the P-face rather than the E-face, which is the fracture face characteristic of gap junctional particles in other tissues of *Limulus* as well as other arthropods (Flower, 1972; Johnson *et al.*, 1973; Lane, 1978b; Lane & Harrison, 1978; Lane & Skaer, 1980). The physiological significance of their association with gap junctions is not yet clear, but they may serve as intercellular attachments, as suggested elsewhere for rather comparable fasciar structures (Simionescu & Simionescu, 1977).

No exactly comparable linear arrays of partially fused particles have been observed in other arthropod tissues, although in ticks, occasional EF particle rows are encountered in the perineurium (Binnington & Lane, 1980). In insects, the basal tight junctions observed in the C.N.S. display fused PF particle rows (Lane & Skaer, 1980) much simpler than those of vertebrate tight junctions (Claude & Goodenough, 1973; Brightman 1977; Van Deurs & Koehler, 1979). The junctions in spider C.N.S. are more complex and similar to the vertebrate type (Lane & Chandler, 1980). These tight junctions of the spider C.N.S. differ from the junctions described here in that not only are their component particles smaller and fused laterally into linear ridges, but there is also distinct membrane fusion at the points of cell to cell association so that permeability barriers are established. No such barrier can be observed here in the horseshoe crab, nor does it occur in the tick C.N.S. (Binnington & Lane, 1980). Clearly, further studies to elucidate the nature of these particle alignments are necessary.

Gap junctions

The freeze-fracture and negatively stained preparations both suggest the presence between glial cells of junctional plaques which occasionally appear to be in the act of forming or disaggregating (as in Fig. 17). This may mean that the glial cells are undergoing turnover, or some other change of intercellular associations (Lane, 1978b;

Lane & Swales, 1980). The function of so many gap junctions clustered together on relatively small areas of membrane face (as in Figs. 15–18) is obscure, unless cell-to-cell exchange of molecules is required to occur at massive levels. Although in those areas where they are most numerous they must produce on the whole a very restricted intercellular cleft, they appear not to impede entry of molecules into the C.N.S.

Concluding remarks

It is clear from these results that the more primitive marine arthropod *Limulus* possesses a less specialized nervous system, in terms of the mechanisms present for maintaining homeostasis, than the advanced terrestrial arthropods. This is no doubt partly due to the relatively stable ionic environment enjoyed by *Limulus* in comparison with the rigours of a dilute or fluctuating haemolymph composition as occurs in fresh water or non-aquatic organisms. Under these latter conditions, a partial or complete blood–brain barrier has evolved as, for example, in crustaceans (Abbot *et al.*, 1977), insects (Treherne & Pichon, 1972) and spiders (Lane & Chandler, 1980). In *Limulus*, however, the C.N.S. is accessible to ions or molecules present in the circulating body fluids; since it is also conventional in its cation requirements (Willmer & Harrison, 1979), it can function in the constant environment of its blood, the ionic composition of which is like that of sea water and so compatible with its normal mode of nervous conduction.

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References

ABBOTT, N. J. (1970) Absence of blood–brain barrier in a crustacean, *Carcinus maenas* L. *Nature* **225**, 291–3.

ABBOTT, N. J. (1972) Access of ferritin to the interstitial space of *Carcinus* brain from intracerebral blood vessels. *Tissue and Cell* **4**, 99–104.

ABBOTT, N. J., PINCHON, Y. & LANE, N. J. (1977) Primitive forms of potassium homeostasis: observations on crustacean central nervous system with implications for vertebrate brain. *Experimental Eye Research* Suppl. **25**, 259–71.

ABBOTT, N. J. & TREHERNE, J. E. (1977) Homeostasis of the brain microenvironment: a comparative account. In *Transport of Ions and Water in Animals* (edited by GUPTA, B. L., MORETON, R. B., OSCHMAN, J. L. and WALL, B. J.), pp. 481–510. London: Academic Press.

ARMSTRONG, P. B. (1979) Motility of the *Limulus* blood cells. *Journal of Cell Science* **37**, 169–80.

BINNINGTON, K. C. & LANE, N. J. (1980) Perineurial and glial cells of the tick nervous system: a tracer and freeze-fracture study. *Journal of Neurocytology* **9**, 343–62.

BRIGHTMAN, M. W. (1977) Morphology of blood–brain interfaces. *Experimental Eye Research* Suppl. **25**, 1–25.

BRIGHTMAN, M. W. & REESE, T. S. (1969) Junctions between intimately apposed cell membranes in the vertebrate brain. *Journal of Cell Biology* **40**, 648–77.

CLAUDE, P. & GOODENOUGH, D. A. (1973) Fracture faces of *zonulae occludentes* from 'tight' and 'leaky' epithelia. *Journal of Cell Biology* **58**, 390–400.

DUMONT, J. N., ANDERSON, E. & CHOMYN, E. (1965) The anatomy of the peripheral nerve and its ensheathed artery in the horseshoe crab, *Xiphosura* (*Limulus*) *polyphemus*. *Journal of Ultrastructure Research* **13**, 38–64.

FAHRENBACH, W. H. (1976) The brain of the horseshoe crab (*Limulus polyphemus*) I. Neuroglia. *Tissue and Cell* **8**, 395–410.

FAHRENBACH, W. H. (1977) The brain of the horseshoe crab (*Limulus polyphemus*) II. Architecture of the corpora pedunculata. *Tissue and Cell* **9**, 157–66.

FAHRENBACH, W. H. (1979) The brain of the horseshoe crab (*Limulus polyphemus*) III. Cellular and synaptic organisation of the corpora pedunculata. *Tissue and Cell* **11**, 163–200.

FLOWER, N. E. (1972) A new junctional structure in the epithelia of insects of the order, *Dictyoptera*. *Journal of Cell Science* **10**, 683–91.

GEORGES, D. (1979) Gap and tight junctions in tunicates. Study in conventional and freeze-fracture techniques. *Tissue and Cell* **11**, 781–92.

JOHNSON, R. G., HERMAN, W. S. & PREUS, D. M. (1973) Homocellular and heterocellular gap junctions in *Limulus:* a thin section and freeze-fracture study. *Journal of Ultrastructure Research* **43**, 298–312.

LANE, N. J. (1972) Fine structure of a lepidopteran nervous system and its accessibility to peroxidase and lanthanum. *Zeitschrift für Zellforschung und mikroskopische Anatomie* **131**, 205–22.

LANE, N. J. (1974) The organisation of the insect nervous system. In *Insect Neurobiology* (edited by TREHERNE, J. E.), pp. 1–71. Frontiers of Biology, Vol. 35, Amsterdam, New York: North Holland.

LANE, N. J. (1978a) Intercellular junctions and cell contacts in invertebrates. In *Electron Microscopy 1978,* Vol. 3, *State of the Art, Symposia* (edited by STURGESS J. M.), pp. 673–691. Toronto: The Imperial Press Ltd.

LANE, N. J. (1978b) Developmental stages in the formation of inverted gap junctions during turnover in the adult horseshoe crab, *Limulus*. *Journal of Cell Science* **32**, 293–305.

LANE, N. J. & CHANDLER, H. J. (1980) Definitive evidence for the existence of tight junctions in the invertebrates. *Journal of Cell Biology* **86**, 765–74.

LANE, N. J. & HARRISON, J. B. (1978) An unusual type of continuous junction in *Limulus*. *Journal of Ultrastructure Research* **64**, 85–97.

LANE, N. J. & SKAER, H. le B. (1980) Intercellular junctions in insect tissues. *Advances in Insect Physiology* **15**, 35–213.

LANE, N. J., SKAER, H. le B. & SWALES, L. S. (1977) Intercellular junctions in the central nervous system of insects. *Journal of Cell Science* **26**, 175–99.

LANE, N. J. & SWALES, L. S. (1978a) Changes in the blood–brain barrier of the central nervous system in the blowfly during development, with special reference to the formation and disaggregation of gap and tight junctions. I. Larval development. *Developmental Biology* **62**, 389–414.

LANE, N. J. & SWALES, L. S. (1978b) Changes in the blood–brain barrier of the central nervous system in the blowfly during development, with special reference to the formation and disaggregation of gap and tight junctions. II. Pupal development and adult flies. *Developmental Biology* **62**, 415–31.

LANE, N. J. & SWALES, L. S. (1979) Intercellular junctions and the development of the blood–brain barrier in *Manduca sexta*. *Brain Research* **169**, 227–45.

LANE, N. J. & SWALES, L. S. (1980) Dispersal of gap junctional particles, not internalization, during the *in vivo* disappearance of gap junctions. *Cell* **19**, 579–86.

LANE, N. J., SWALES, L. S. & ABBOTT, N. J. (1977) Lanthanum penetration in crayfish nervous system; observations on intact and desheathed preparations. *Journal of Cell Science* **23**, 315–24.

LANE, N. J. & TREHERNE, J. E. (1972) Studies on perineurial junctional complexes and the sites of uptake of microperoxidase and lanthanum in the cockroach central nervous system. *Tissue and Cell* **4**, 427–36.

LORBER, V. & RAYNS, D. G. (1972) Cellular junctions in the tunicate heart. *Journal of Cell Science* **10**, 211–27.

LORBER, V. & RAYNS, D. G. (1977) Fine structure of the gap junction in the tunicate heart. *Cell and Tissue Research* **179**, 169–75.

NAGANO, T. & SUZUKI, F. (1976) Freeze-fracture observations on the intercellular junctions of Sertoli cells and of Leydig cells in the human testis. *Cell and Tissue Research* **166**, 37–48.

REESE, T. S. & KARNOVSKY, M. J. (1967) Fine structural localization of a blood–brain barrier to exogenous peroxidase. *Journal of Cell Biology* **34**, 207–17.

REINECKE, M. (1976) The glial cells of the cerebral ganglia of *Helix pomatia* L. (Gastropoda Pulmonata) II. Uptake of ferritin and ^{3}H-glutamate. *Cell and Tissue Research* **169**, 361–82.

REVEL, J.-P. & KARNOVSKY, M. J. (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *Journal of Cell Biology* **33**, C7–C12.

SIMIONESCU, M. & SIMIONESCU, N. (1977) Organisation of cell junctions in the peritoneal mesothelium. *Journal of Cell Biology* **74**, 98–110.

SKAER, H. le B., TREHERNE, J. E., BENSON, J. A. & MORETON, R. B (1978) Axonal adaptations to osmotic and ionic stress in an invertebrate osmoconformer (*Mercierella enigmatica* Fauvel) I. Ultrastructural and electrophysiological observations on axonal accessibility. *Journal of Experimental Biology* **76**, 191–204.

TREHERNE, J. E. & PICHON, Y. (1972) The insect blood–brain barrier. In *Advances in Insect Physiology*. Vol. 9. (edited by TREHERNE, J. E., BERRIDGE, M. J. and WIGGLESWORTH, V. B.), pp. 257–313. London: Academic Press.

VAN DEURS, B. & KOEHLER, J. K. (1979) Tight junctions in the choroid plexus epithelium. A freeze-fracture study including complementary replicas. *Journal of Cell Biology* **80**, 662–73.

WILLMER, P. G. & HARRISON, J. B. (1979) Cation accessibility of the peripheral nervous system in *Limulus polyphemus:* an electrophysiological study. *Journal of Experimental Biology* **82**, 373–6.

WIGGLESWORTH, V. B. (1959) The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera) II. The central ganglia. *Quarterly Journal of Microscopical Science* **100**, 299–313.