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ACCESSIBILITY OF THE CENTRAL NERVOUS CONNECTIVES OF *ANODONTA CYGNEA* TO A COMPOUND OF LARGE MOLECULAR WEIGHT

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INTRODUCTION

Some previous studies from this laboratory have been concerned with the investigation of the ionic requirements for axonal function in the central nervous system of the freshwater lamellibranch, *Anodonta cygnea*, which appears to be the possessor of the most dilute blood so far described in the Animal Kingdom (44·0 m-osmoles) (Potts, 1954). It has been shown that, despite the low sodium level of the blood (*c.* 15 mM/l), the axons appeared to be 'conventional' in possessing a relatively low intracellular sodium concentration (8·6 mM/l) (Mellon & Treherne, 1969) and to function by sodium-mediated action potentials (Treherne, Mellon & Carlson, 1969).

It was also postulated, in some earlier publications, that there was relatively free movement of small water-soluble ions and molecules between the blood, or bathing medium, and the axon surfaces in the intact cerebro-visceral connectives of *A. cygnea*. This conclusion was based on the four following separate lines of evidence.

1. The observation that the slow component of the compound action potential (contributed by the numerous small axons, *c.* 0·1–0·3 μm in diameter) showed a relatively rapid decline in amplitude in intact preparations bathed in isotonic solutions of non-electrolytes (Treherne, Mellon & Carlson, 1969).

2. The rapid reduction in conduction velocity (in intact preparations bathed in isotonic non-electrolyte solutions) and the somewhat slower decline in amplitude (in intact preparations bathed in solutions in which sodium ions were replaced by those of tris or choline) of the fast action potentials, associated with the larger (2–4 μm diameter) axons (Treherne, Mellon & Carlson, 1969; Carlson & Treherne, 1969).

(3) The rapid exchange of ^{22}Na and ^{24}Na between the bathing medium and the tissues of intact cerbro-visceral connectives, the rapidly exchanging sodium fraction in those experiments comprising $74{\cdot}7 \pm 5{\cdot}5\%$ of the total tissue sodium, the exchange constant (K_{out}) being $3{\cdot}53 \pm 0{\cdot}74 \times 10^{-3}$ sec^{-1} (Mellon & Treherne, 1969).

(4) Ultrastructural observations showing the absence of any visible intercellular occlusions which would be likely to appreciably restrict diffusion of small inorganic ions from the blood, or bathing medium to the axon surfaces (Gupta, Mellon & Treherne, 1969).

A recent contribution to this journal (Twarog & Hidaka, 1972) has challenged the above interpretation and has advanced the view that in two American freshwater lamellibranchs (*Anodonta cataracta* and *Elliptio complanata*) the neural sheath func-

tions to severely restrict the access of sodium ions to the axon surfaces. In particular Twarog & Hidaka question the ultrastructural observations of Gupta *et al.* (1969) and suggest, in the absence of any ultrastructural data, that in *A. cygnea* intercellular diffusion could be restricted by occlusions between adjacent membranes of the peripheral layer of glial processes. The present investigation was undertaken to test this latter hypothesis by following the degree of penetration of the large molecules of horseradish peroxidase (M.W. 40,000) into the extracellular system of intact cerebrovisceral connectives of *A. cygnea*.

MATERIAL AND METHODS

The animals used in this investigation were adult specimens of the freshwater lamellibranch *Anodonta cygnea* (Linnaeus). The tissue studied was the cerebrovisceral nerve connectives; these were carefully dissected out from the surrounding kidney and cut into lengths of about 10 mm after being ligatured at either end. The connectives were maintained in *Anodonta* blood or Ringer (Potts, 1954) for brief periods before being incubated at room temperature in solutions of horseradish peroxidase (obtained from the Sigma Chemical Company) (2 or 6 mg/ml) made up in *Anodonta* blood or Ringer. Incubation was allowed to proceed for 1, 5, 16 or 24 h. Control connectives were incubated in *Anodonta* blood or Ringer without added peroxidase. Following this treatment the tissue was fixed in 2·5% glutaraldehyde in *Anodonta* blood for 15 min at room temperature and then transferred to 3% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, at 4 °C, or room temperature, for 90 min. (Gupta *et al.* 1969). Subsequently the connectives were washed in several changes of cacodylate buffer at 4 °C and then treated with a buffered solution of diamino-benzidine (DAB) according to the technique of Cotran & Karnovsky (1968). Further controls were made by incubating peroxidase-treated tissue in a buffered solution from which the DAB was omitted. A buffer wash followed, after which the material was postfixed in 1% osmium tetroxide in 0·1 M cacodylate buffer at 4 °C for 60 min. The tissues were then taken through an ascending series of ethanols to propylene oxide and embedded in Araldite. Unincubated preparations were fixed in 2·5% glutaraldehyde in *Anodonta* blood, transferred to 3% glutaraldehyde in cacodylate buffer, washed, post-osmicated and embedded in the usual fashion. 1–2 μm sections for light microscopical examination were cut on an LKB Ultrotome III and stained with 1% toluidine blue in 1% borax. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300.

RESULTS

Control tissues incubated in *Anodonta* Ringer or blood without added peroxidase contained no reaction product, indicating the absence of any endogenous peroxidase activity. Other control materials incubated in peroxidase solutions, but subsequently treated with a buffered solution lacking DAB, were similarly negative.

The fine structural details of the connectives studied in this report are similar to those described previously by Gupta *et al.* (1969). In summary, an acellular neural lamella ensheaths the nervous tissue (Pl. 1, fig. 1) and sends wedge-shaped radial

invaginations into the periphery. No perineurium exists, but a thin layer of glial processes underlies the outer lamella (Pl. 1, fig. 1). These attenuated glial processes (Pl. 1, fig. 1), characterized by their irregular outline, electron density and the possession of dense glial granules and masses of filaments, radiate inwardly from the neural lamella into the centre of the connective. These cells may also contain glycogen particles, lysosomes and large droplets that are probably lipid in nature. In some cases desmosomal junctions occur between neuroglial cells, especially between those near the neural lamella. The glia appear to be scattered at random among the axons and do not encapsulate them. The axons themselves are mostly relatively small in diameter (Pl. 1, fig. 1) and contain neurotubules, neurofilaments and occasionally dense neurosecretory granules. Extracellular spaces of varying sizes occur between the glia and axons (Pl. 1, fig. 1).

In the experimental connectives exposed to peroxidase, even after relatively short (60 min) incubations, reaction product for peroxidase is found throughout the neural lamella (Pl. 1, figs. 2, 3) except for that surface which had lain directly against the bottom of the incubation dish. Moreover, reaction product also occurs in the spaces between the axons and the glial cells (Pl. 1, figs. 2, 3). The desmosomes between glial cells seem to act as collection sites for the tracer molecule, as they are extremely dense after incubation (Pl. 1, fig. 3). The peroxidase adheres to the outer surface of the cell membrane of axons and glia and is usually not as concentrated in the extracellular spaces (Pl. 1, fig. 2). This may signify that some of these spaces are artifactual, possibly forming during fixation after the exogenous protein has been taken up. Perhaps bearing on this point is the fact that more and larger extracellular spaces are observed following more prolonged periods of incubation, such as 16 and 24 h. Reaction product can be found at varying depths throughout the connective, and this presumably is a function of the time for which the system has been exposed to the tracer molecule. At any rate, no restriction to the entry of a compound of such large molecular weight seems to be imposed by either the neural lamella or the glial cells, which indicates that the axonal surfaces must be readily accessible to substances in the bathing medium.

DISCUSSION

The above observations showing that molecules as large as those of peroxidase (M.W. 40,000) can penetrate the neural lamella and enter the extracellular fluid via the intercellular clefts between the peripheral glial cell membranes clearly does not accord with the assumption of Twarog & Hidaka (1972) that 'in *Anodonta cygnea* the surface layer of glial processes form junctions that occlude the extracellular space'.

Twarog & Hidaka's criticism of the observations of earlier observations (Gupta *et al.* 1969) largely rest on the superficial analogy which they draw between organization of the peripheral glial processes in *Anodonta cygnea* and that of the glial end-feet described by Peters (1962), which surround the capillaries in the optic nerve of the rat. In this early report Peters described 'quintupeal junctions' between adjacent glial membranes. Subsequent observations by a number of workers have shown that not all 'quintupeal' or 'pentalaminar' junctions observed in conventionally fixed tissues do, in fact, restrict the intercellular diffusion of electron-opaque makers (see Brightman & Reese, 1969). Furthermore, all these junctional complexes in animal tissues are

highly stable structures which resist considerable hyperosmotic stresses (Brightman & Reese, 1969) and can even be isolated from tissue homogenates (Goodenough & Revel, 1970). It is, therefore, extremely unlikely that if such junctions existed in the peripheral glia of the central nervous connectives in *A. cygnea* they would not have been preserved after the range of fixation procedures employed by Gupta *et al.* (1969). The latter authors' observations that zonulae adherentes, or 'intermediate junctions' (Farquhar & Palade, 1963; Brightman & Palay, 1963), are the only membrane associations in peripheral glia in *A. cygnea* is here confirmed by the rapid penetration of peroxidase macromolecules into the extracellular spaces in living central nervous connectives.

It is now relevant to consider the electrophysiological data of Twarog & Hidaka (1972), and their interpretation of it, in relation to the observations described above and the two concepts advanced in previous communications from this laboratory. The basis of Twarog & Hidaka's criticisms of the previous work on *A. cygnea* largely rests on their observation that in the freshwater lamellibranchs *Elliptio complanata* and *A. cataracta* the action potentials recorded in intact connectives in sodium-free solution persisted for longer periods than in the marine species, *Mytilus edulis*. The compound action potentials recorded by Twarog & Hidaka do, in fact, appear to be incomplete as they do not show the slow component of the action potentials described in lamellibranch nerves by both Nakajima (1961) and Treherne, Mellon & Carlson (1969). As has aready been emphasized it is this very large component of the action potential of *A. cygnea* (contributed to by many thousands of small axons as compared with the 70 or so large ones) which shows the most rapid response to reduction in the external sodium concentration, even with non-electrolyte solutions, and which thus affords the most sensitive indicator of sodium movements between the extracellular fluid and the bathing medium (Treherne, Mellon & Carlson, 1969). The responses of the fast action potentials to sodium-free conditions is complicated by the apparent presence of an extra-axonal sodium regulating system (Treherne, Carlson & Gupta, 1969; Carlson & Treherne, 1969). It should, therefore, be borne in mind that differences between the responses of the large axons to sodium-free conditions, recorded for individuals from different species and genera, might be a reflexion of variations in the degree of extra-axonal sodium regulation rather than of differences in the freedom of sodium ions to move between the bathing medium and the general extracellular fluid.

However, inspection of the data of Twarog & Hidaka (1972) shows that the amplitude of the fast action potentials recorded in intact connectives, after 1 h in sodium-free solution, had declined to approximately *one-third* of that recorded in normal Ringer solution – despite their claim that 'the action potential of the sheathed nerve altered very slightly after one hour of stimulation in sodium-free solution'. A change of this magnitude would obviously correspond to a substantial decline in the extra-axonal sodium level. The extent of the change in extra-axonal sodium level would depend upon the Nernst slope relating the amplitude of the action potential to sodium concentration. Such data does not appear to be available for any freshwater lamellibranch species. However, by analogy with the squid giant axon (Hodgkin & Katz, 1949) a proportional reduction of the action potential to one-third of its normal amplitude would be accompanied by an approximately fourfold to fivefold change in the extra-axonal sodium concentration. Probably a more appropriate Nernst slope is offered

by data from the neurones of another freshwater mollusc with dilute blood, *Lymnaea stagnalis*, in which the Nernst relation shows a 31·0 mV slope for 10-fold change in external sodium concentration (Sattelle, 1971). Using this value for the Nernst slope it can be calculated that a one-third change in amplitude of the action potential would correspond to a 17-fold change in the extra-axonal sodium concentration.

The present results contrast with those obtained with two insect species, *Periplaneta americana* (Lane & Treherne, 1969, 1970) and *Carausius morosus* (Lane & Treherne, 1971), in which it has been shown that the intercellular diffusion of peroxidase macromolecules into the central nervous connectives is restricted by occlusions at the inner margins of the perineurial clefts (Maddrell & Treherne, 1967). It has been postulated that in *Periplaneta americana* the relatively rapid exchanges of ^{22}Na between the bathing medium and the central nervous tissues (cf. Treherne, 1962) might represent a glial sodium fraction (Tucker & Pichon, 1972). The very small volume of the glial elements in *Anodonta cygnea* connectives (Gupta, Mellon & Treherne, 1969) and in *Cristaria plicata* (Nakajima, 1961) preclude this possibility in these lamellibranch species. It, therefore, seems reasonable to suppose that the relatively large rapidly exchanging sodium fraction observed in the connectives of *A. cygnea* by Mellon & Treherne (1969) represent rapid steady-state exchanges with the extracellular fluid via the relatively leaky neural lamella and superficial peripheral layer of glial cells.

The apparently free movement of water-soluble ions and molecules between the bathing medium and the extracellular fluid appears to be paralleled in the nervous systems of some other freshwater molluscs. In *Lymnaea stagnalis* and *Viviparus contectus* ultrastructural observations have revealed no potential structural barriers which would be likely to restrict diffusion to the neuronal surfaces (Sattelle & Lane, 1972). As with *Anodonta cygnea*, alterations in the ionic composition of the fluid bathing intact preparations were found to be reflected by rapid changes in the electrical responses of the nerves (Sattelle, 1971). The situation in *A. cygnea* would also appear to be essentially similar to that observed in a freshwater annelid, *Hirudo medicinalis*, in which there appears to be a ready movement of ions and molecules from the bathing medium to the axon surfaces via narrow intercellular channels (Kuffler & Potter, 1964; Nicholls & Kuffler, 1964).

SUMMARY

1. Ultrastructural observations on the uptake of an exogenous tracer substance, horseradish peroxidase (M.W. 40,000), have shown that this large molecule can penetrate the neural lamella in intact cerebro-visceral connectives of the lamellibranch, *Anodonta cygnea*.

2. Peroxidase molecules were also observed to penetrate between the intercellular clefts formed by adjacent membranes of the underlying peripheral glial cell layer and to move extensively into the underlying extracellular spaces.

3. These observations confirm the results of previous electrophysiological, radioisotopic and ultrastructural investigations indicating that a relatively rapid exchange of water-soluble ions and molecules occurs between the blood, or bathing medium, and the extracellular fluid bathing the axon surfaces in intact connectives.

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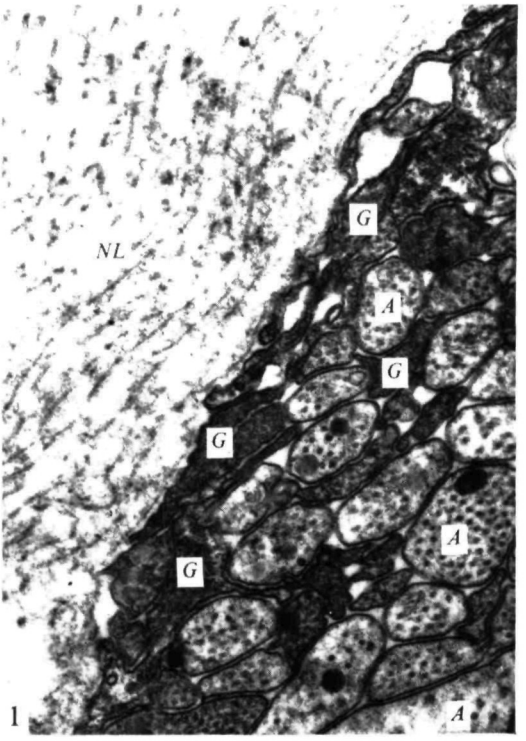
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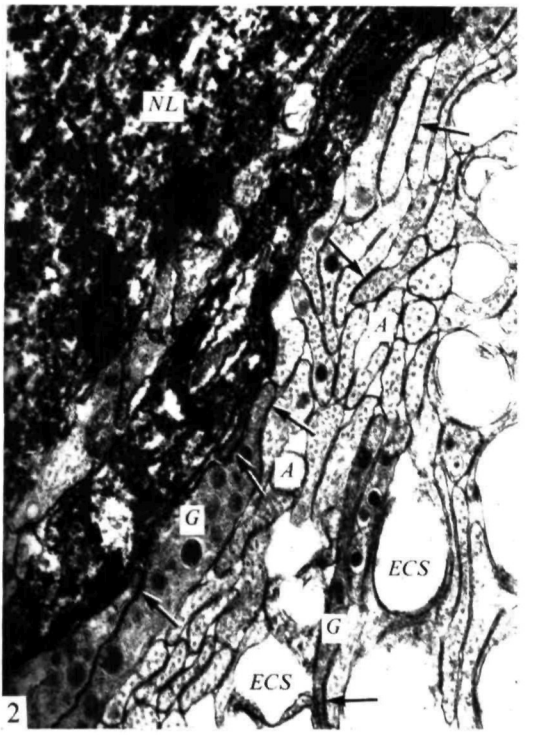
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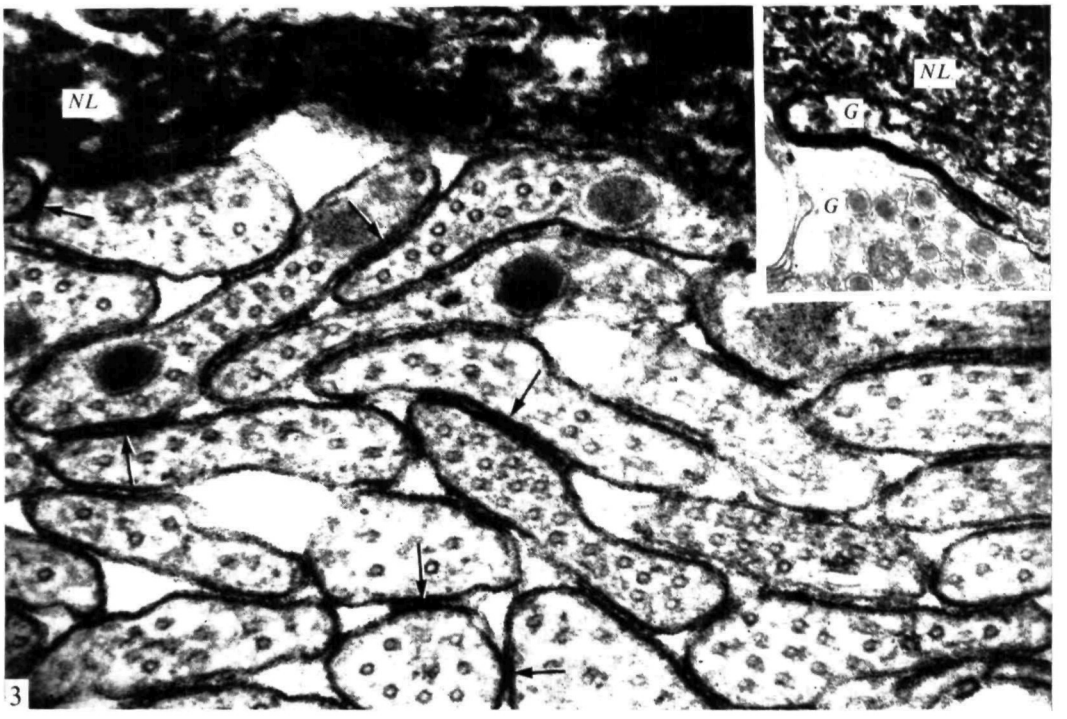
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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of a cross-section through an unincubated control connective of *Anodonta* showing the non-opaque neural lamella (*NL*) and irregular, attenuated, glial processes (*G*) which stain more intensely than the neurotubule-laden axons (*A*). × 24,080.

Fig. 2. Cross-section through an *Anodonta* connective that has been incubated in horseradish peroxidase for 60 min. Note the reaction product throughout the neural lamella (*NL*) as well as in the extracellular spaces (arrows) between neuroglia (*G*) and axons (*A*). Some of the extracellular spaces (*ECS*) are larger than in unincubated preparations. × 21,950.

Fig. 3. Higher magnification of a connective such as is shown in Fig. 2. The dense reaction product indicating sites of uptake of peroxidase occurs in the neural lamella (*NL*) and in the extracellular spaces. It is especially apparent where adjacent cells lie in intimate contact with one another (arrows). The insert shows peroxidase lying in a desmosomal junction between glial cells (*G*) beneath the neural lamella (*NL*). × 98,780: Insert, × 39,900.