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Cell Junctions in the Subcommissural Organ of the Rabbit as Revealed by Use of Ruthenium Red*

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Summary. Tight junctions were found in the apical junctional complex of the adult rabbit subcommissural organ (SCO) in addition to zonulae adhaerentes and gap junctions of typical ependymal cells. Ventricular perfusion of ruthenium red before fixation was found to give excellent results for distinguishing between gap and tight junctions at the ependymal surface. The implication of tight junctions as a mechanical means of sealing off the SCO area from the cerebrospinal fluid and the use of ruthenium red as a tracer substance are discussed.

Key words: Subcommissural organ (rabbit) — Ependyma — Cell junctions — Ruthenium red.

Introduction

The subcommissural organ (SCO) consists of a single layer of high columnar ependymal cells covering the anterior-inferior surface of the posterior commissure and extending from the pineal recess in the third ventricle to the mesocoelic recess in the cerebral aqueduct. The SCO is included in a group of specialized areas of the ependyma which are collectively called circumventricular organs. The function of the SCO remains unclear. The fact that it secretes Reissner's fiber into the ventricle has been substantiated by autoradiographic studies. The progress of cystine-S^{35} was followed from its incorporation into the cells of the SCO to its destination in the Reissner's fiber (Sterba *et al.*, 1967; Ermisch *et al.*, 1971). An apical absorptive property has also been shown for the cells of the SCO by the observation of their active uptake of ferritin particles from the cerebrospinal fluid (CSF) (Smith, 1970). The question of a basal secretion and/or absorption remains open. Histochemical and morphological evidence suggests that an interchange occurs between the SCO and plasma (Møllgaard, 1972). Gomori-positive granules characterize the SCO cells of all vertebrates investigated (Stutinsky, 1950; Bargmann and Schiebler, 1952; Wislocki and Leduc, 1952a; Wingstrand, 1953). Moreover, the SCO cells send basal processes down to capillaries which exhibit a widened perivascular space (Papacharalampous *et al.*, 1968; Rodríguez, 1970).

The normal ependymal cell barrier allows uninhibited entrance of proteinaceous material from CSF to the extracellular fluid of the neuropil through gap junctions and zonulae adhaerentes (Brightman and Reese, 1969). This free passage

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is effective throughout the brain except in a few areas which lack a blood-brain barrier, notably in two of the circumventricular organs, the median eminence and area postrema. It was found that in these areas tight junctions seal off the ependymal region from the CSF (Reese and Brightman, 1968). In view of the postulated interchange of materials between SCO and plasma it is a logical step to investigate the possibility that tight junctions also isolate the SCO from the CSF.

Materials and Methods

Eight male rabbits weighing 2.5–3.0 kg were anaesthetized with Nembutal® (30 mg/kg), curarized with Gallamonium iodide (Relaxan®) and artificially respirated with a small animal respirator (Harvard apparatus).

In four rabbits a catheter was placed in the abdominal aorta, and the brains were fixed by perfusing a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4) through the aortic catheter at a constant pressure of 150 mm Hg. In the other four rabbits the ventricular system was perfused from one lateral ventricle to the cisterna magna. The ventriculo-cisternal perfusion was performed according to the method described by Oldendorff and Davson (1967). Intracranial pressure and arterial blood pressure were monitored continuously in order to assure that these parameters remained normal during the ventriculo-cisternal perfusion. In two of these rabbits the ventricular system was first perfused with mock cerebrospinal fluid (CSF) at a rate of 0.35 cc/min for 1 hour. Thereafter, the brains were fixed by ventriculo-cisternal perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4). In two other rabbits the ventricular system was perfused for 1 hour with mock CSF containing ruthenium red (1000 p.p.m.), and the brains were thereafter fixed by ventriculo-cisternal perfusion with a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer and ruthenium red (1000 p.p.m.) as during the previous perfusion with mock CSF.

The osmolarities of the above-mentioned perfusates were as follows: mock CSF, 294 m.osm.; mock CSF + ruthenium red (1000 p.p.m.), 303 m.osm.; glutaraldehyde + buffer, 526 m.osm.; glutaraldehyde + buffer + ruthenium red (1000 p.p.m.), 505 m.osm.

Following fixation by intra-arterial or ventriculo-cisternal perfusion, the brains were immersed for 8 hours in the fixative. Tissue blocks containing the subcommissural organ and median eminence were isolated and postfixed for 2 hours in 2% OsO_4 in 0.1 M cacodylate buffer. The osmium solutions used for the tissues which had been exposed to ruthenium red contained ruthenium red in the same concentration as in the perfusate and the fixative. Specimens were block-stained with uranyl acetate for 1 hour, quickly dehydrated in increasing concentrations of ethanol and embedded in Epon®. Areas suitable for electron microscopy were localized in 1 micron sections stained with toluidine blue. Silver-to-grey thin sections were stained with uranyl acetate and lead citrate. Some grids with sections of ruthenium red treated tissue were examined without poststaining.

Results

Ependymal cells are joined by multiple zonulae adhaerentes (intermediate junctions) which exhibit a typical dense filamentous network on the cytoplasmic surface of the junction. The intercellular substance sometimes can be seen as filamentous cross bridges extending from one plasma membrane to the next (Fig. 1). The intercellular space between sequential zonulae adherentes appears to bulge, whereas the space within the junction remains at a fairly constant width of about 200 Å. In addition to multiple zonulae adhaerentes, the junctional complex frequently includes a junction exhibiting closely apposed membranes running strictly parallel to each other. The overall width of these appositions measured from cytoplasm to cytoplasm is about 200 Å, indicating that the intercellular

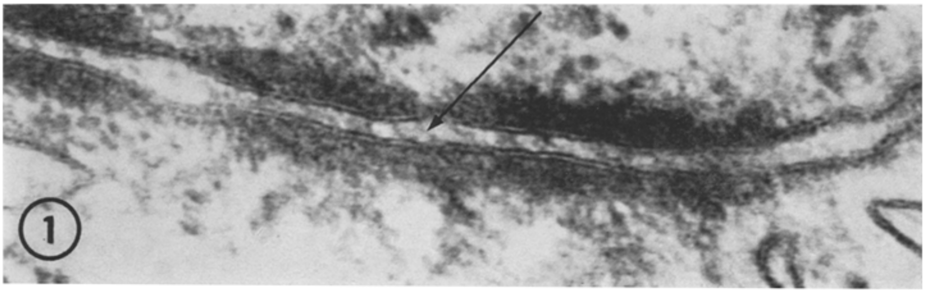


Fig. 1. A typical zonula adherens (intermediate junction) joining ependyma of the cerebral aqueduct. Note the membrane-to-membrane cross bridges of which one is indicated by an arrow. The width of a crossbridge is about 40 Å while the length varies from 190–220 Å. A certain regularity can be seen in the spacing between these filaments. $\times 138000$

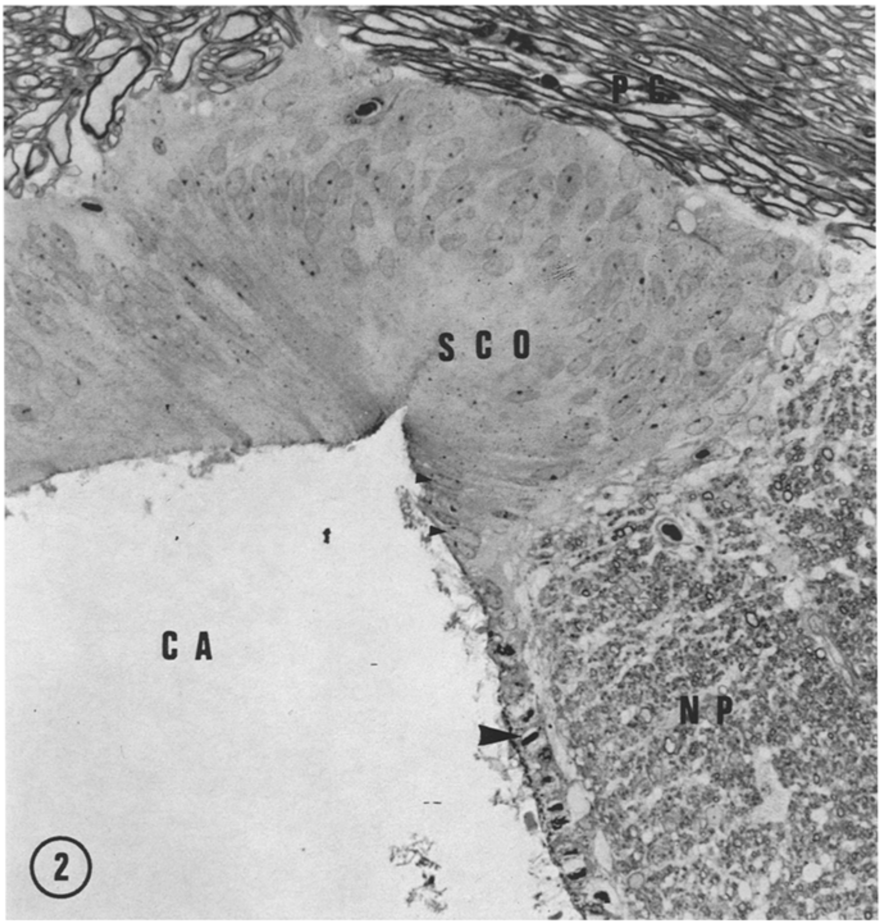


Fig. 2. Light microscopy shows the marked contrast between the preservation of SCO ependyma and the adjacent ependyma after treatment with ruthenium red. This dramatic difference is also reflected in the neuropil underlying each area. Compare in particular the morphology of the ependymal nuclei (*large arrow head*) with SCO nuclei (*small arrow heads*), both of which, in the examples indicated, lie an equal distance from the ependymal surface. Tight junctions between SCO cells have apparently kept the damaging ruthenium red solution out of the area. *CA* cerebral aqueduct; *NP* neuropil; *PC* posterior commissure. $\times 375$

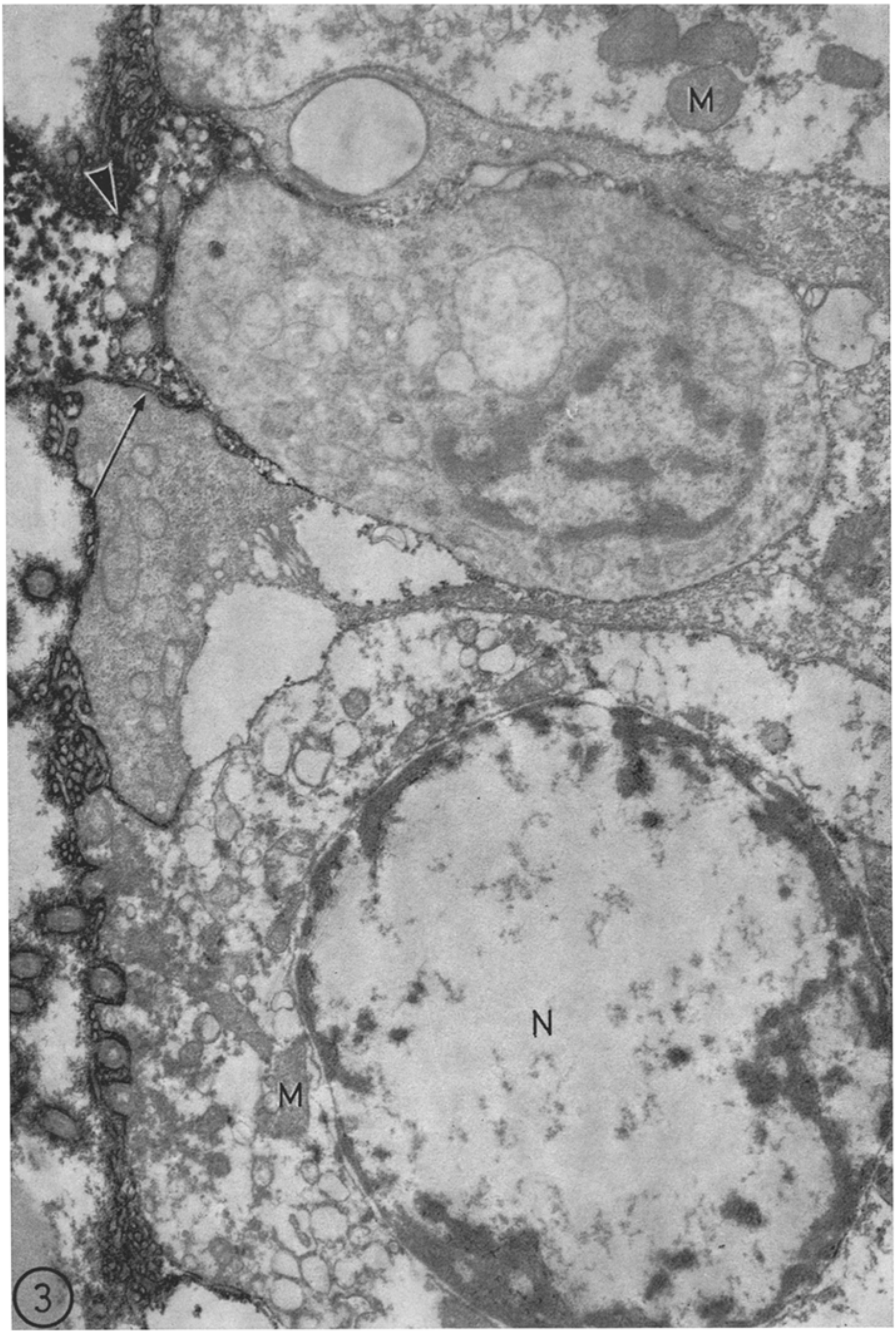


Fig. 3. Ruthenium red-treated normal ependyma after staining with uranyl acetate and lead citrate. The chromatin in the nuclei (N) is unusually condensed. The mitochondria (M) are recognizable only by size and shape. *Large arrowhead* marks an apical plasma membrane which has broken, releasing the contents of the cell. *Arrow* shows a junctional complex which holds together despite the condition of the cells' intracellular components on either side. $\times 13200$

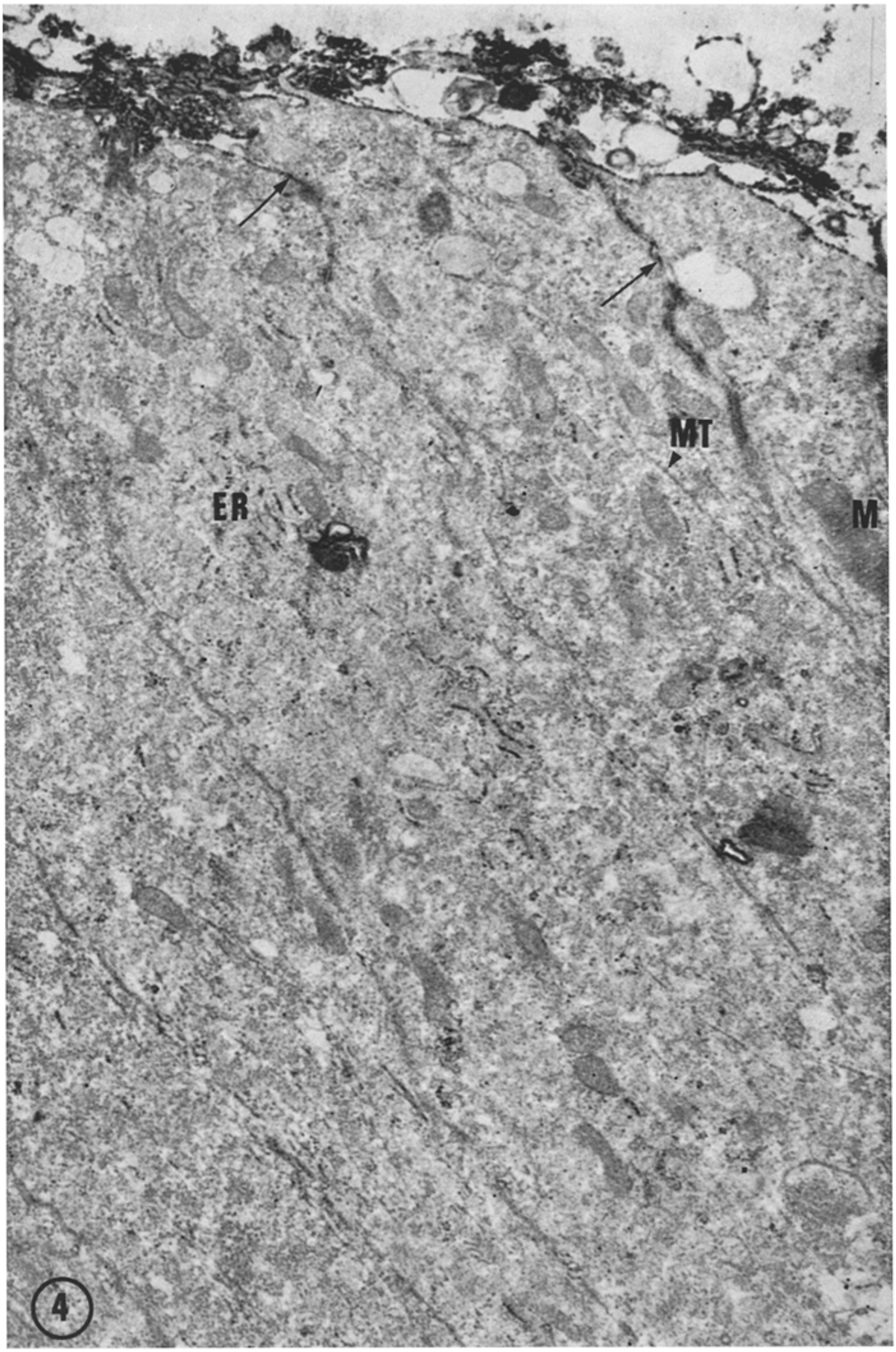


Fig. 4. Ruthenium red-treated SCO ependyma (apical processes) without post-staining. The cells are characterized by an extremely granular cytoplasm. The fine structure, however, is not entirely lost. Note endoplasmic reticulum (*ER*), microtubules (*MT*) and mitochondria (*M*). Faint staining of intracellular components is due to osmium tetroxide and uranyl acetate block staining. Ruthenium red is prevented from penetrating by tight junctions (*arrows*). $\times$ 13200

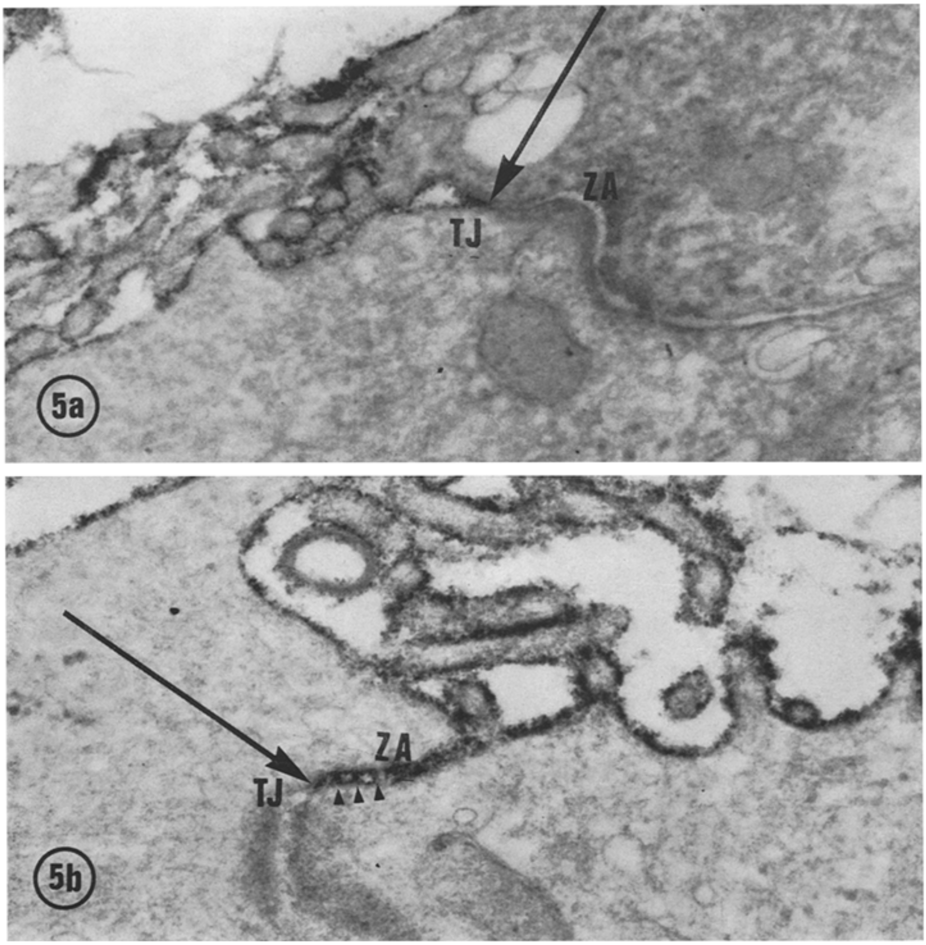
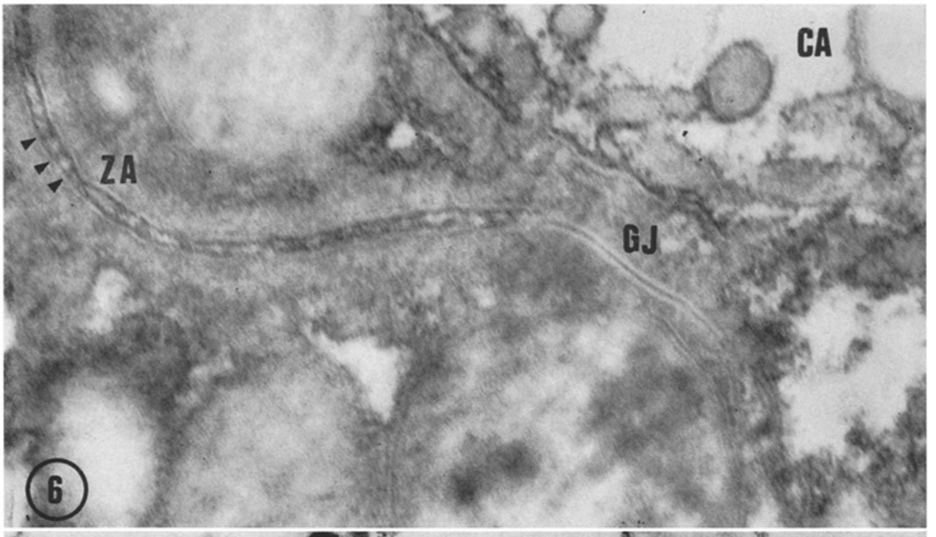
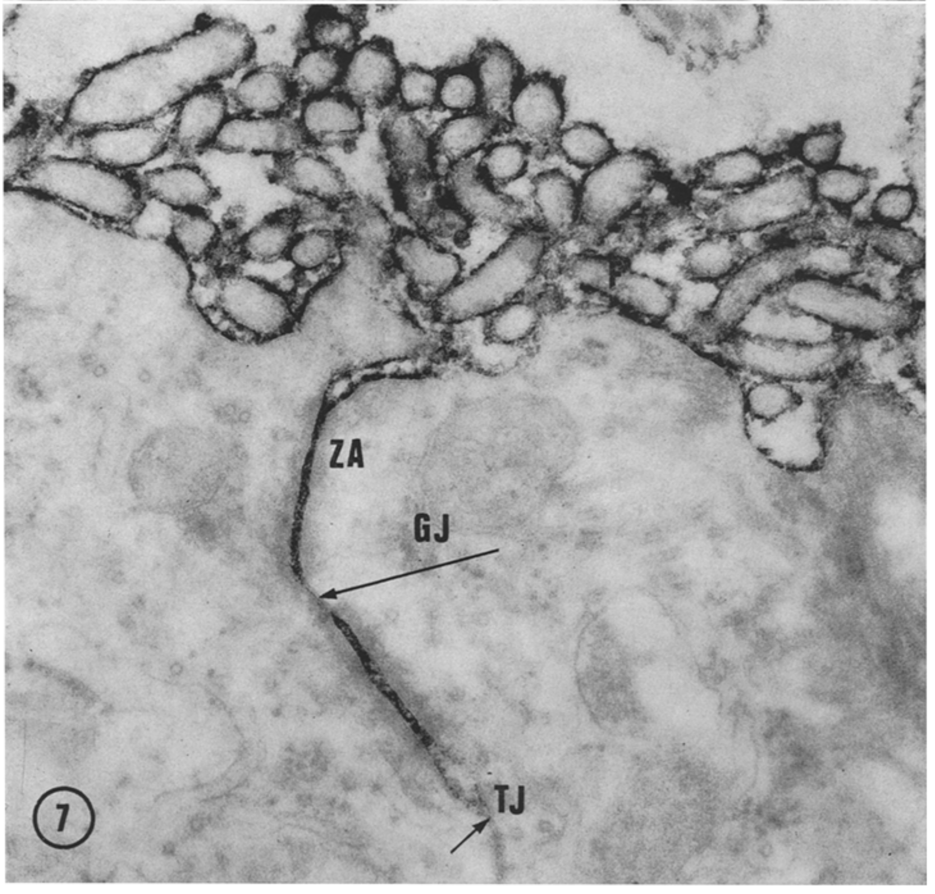


Fig. 5a and b. Tight junctions (*TJ*) in the SCO as revealed by ruthenium red. Both sections are unstained. a) ruthenium red stopped at a tight junction (*arrow*) located apical to a zonula adhaerens (*ZA*). × 57000. b) ruthenium red stopped at a tight junction (*arrow*) just below a zonula adherens. Ruthenium red binds to the cross bridges in the zonula adhaerens (*arrowheads*). × 57000

Fig. 6. Unstained section of a typical junctional complex between two normal ependymal cells after treatment with ruthenium red. The outline of a gap junction (*GJ*) here is 40 Å. Note also regular membrane-to-membrane densities (*small arrowheads*) in the zonula adherens (*ZA*). These are probably analogous to the cross bridges seen in Fig. 1. The lack of preservation of cellular fine structure in these cells should be contrasted with the relatively good preservation of SCO cells in Fig. 7. × 102000

Fig. 7. Unstained section of a junctional complex between two SCO ependymal cells after treatment with ruthenium red. The outline of the gap junction (*GJ*) is here 30 Å in width. A tight junction (*TJ*) again stops further penetration of ruthenium red (*arrow*). Note also the fine structure in the adjacent cytoplasm; eg. the well-preserved endoplasmic reticulum and microtubules. *ZA* zonula adherens. ×57000





Figs. 6 and 7

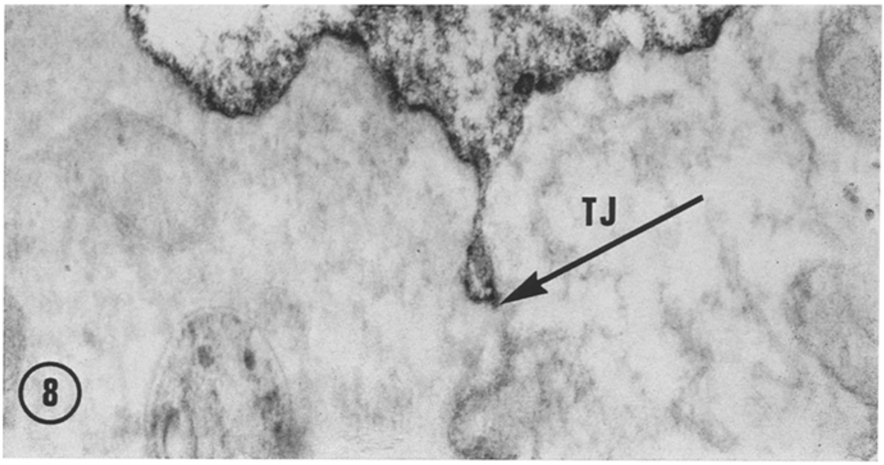


Fig. 8. Tight junction (*TJ*) in the median eminence as revealed by ruthenium red. ×48000

cleft is only approximately 30 Å. These junctions appear heptalaminar at high magnification and are, therefore, considered to be gap junctions.

Subcommissural ependymal cells are similarly joined by multiple zonulae adherentes and gap junctions as described above. In addition, punctate close appositions of the membranes are found in every junctional complex. These punctate junctions have an overall width of approximately 150 Å measured from cytoplasm to cytoplasm and exhibit a pentalaminar appearance in uranyl acetate block-stained tissue. We therefore consider these junctions to be tight junctions. We never saw a long continuous fusion of the membranes.

The tight junctions are found either where the intercellular cleft opens into the ventricle or between two apical zonulae adhaerentes. Typically one or two tight junctions are found between adjacent cells, but we have seen up to seven tight junctions alternating with zonulae adhaerentes in one junctional complex.

A striking difference between the subcommissural organ and normal ependyma is observed after ventriculo-cisternal perfusion with ruthenium red. On one micron sections the subcommissural organ seems well preserved, whereas the ependyma from the cerebral aqueduct is vacuolated and in some areas nearly disintegrated (Fig. 2). A clear difference between the two types of cells is also observed at the electron microscopic level, though at this magnification it is clear that the cells in the subcommissural organ have also suffered. In normal ependymal cells only the skeletons of nuclei and mitochondria remain; plasma membranes are sometimes disrupted and finer constituents of the cell are no longer recognizable (Fig. 3). In cells in the subcommissural organ a vacuolation is seen, and the cytoplasm shows a coarse granularity. However, such fine elements as microtubules, microfilaments and endoplasmic reticulum can still be distinguished (Fig. 4). Furthermore, the nuclei appear to be normal.

Unstained thin sections show very clearly that ruthenium red has penetrated between the ependymal cells of the cerebral aqueduct to reach subependymal glial layers and underlying nervous tissue. In contrast, ruthenium red is stopped from penetration beyond the apical area of the subcommissural organ by a narrowing of the intercellular cleft (Figs. 5a, b). Both in the subcommissural organ and in normal ependyma, the ruthenium red lining of the intercellular space can sometimes be seen to narrow to 30–40 Å corresponding to gap junctions (Figs. 6, 7). Thus, the pentalaminar junctions seen in uranyl acetate block-stained sections from the subcommissural organ must prevent penetration of ruthenium red, and they must be considered tight.

Sections from the median eminence show that ruthenium red is also stopped within the apical junctional complex of these ependymal cells (Fig. 8).

Discussion

Junctions formed by closely apposed membranes, formerly thought to be one type of junction known as the zonula occludens or tight junction, have been shown to include two types of junctions with very different properties. One type is still called a tight junction and is characterized by a pentalaminar appearance in uranyl acetate block stained tissue, indicating a fusion of membranes. The other type is called a gap junction and is characterized by a heptalaminar appearance when treated similarly, thus exhibiting a narrow gap between the apposed trilaminar membranes (Brightman and Reese, 1969). An important difference between tight and gap junctions is that tight junctions encompass the cell by a continuous fusion of the membranes, whereas gap junctions exist as rounded plaques, and thus, are discontinuous. Electron dense tracers like lanthanum and horseradish peroxidase have proven very useful in distinguishing between these two types of junctions. These tracers pass through and outline gap junctions, whereas they are blocked by tight junctions. The effectiveness of the tight junctions as a barrier depends not only on the fusion of the membranes, but also on the continuity of the occlusion around the entire circumference of the cell. It is not known how permeable tight junctions are to smaller organic molecules and ions, but it seems reasonable to deduce from the studies performed with electron dense tracers that the tight junctions are impervious to physiological proteinacious substances. This deduction is also supported by the fact that in certain areas of the brain, which are known to lack a blood-brain barrier and which may be involved in the secretion of large molecular weight substances, e.g. hormones, the ependymal cells are connected by tight junctions which at least partially seal off the secreting cells from the ventricular system. Typical ependymal cells do not exhibit tight junctions.

Gap junctions can be distinguished from tight junctions (both of which appear as a close apposition of membranes) by the use of an electron dense tracer of small molecular size. Colloidal lanthanum has been used for this purpose (Revel and Karnovsky, 1967), but its toxicity has limited its application to *in vitro* studies. Ruthenium red, on the other hand, can be used *in vivo,* even though it also is toxic to the tissues. Thus, we found that the intracranial pressure and blood pressure stayed normal during ventriculo-cisternal perfusion with ruthenium red for one hour.

In contrast to lanthanum and horseradish peroxidase which fill up intercellular spaces, ruthenium red which is a hexavalent cation, binds to the extracellular coat of animal tissues (Luft, 1964) thereby outlining the outer edge of the plasma membrane. When the outer leaflets of the plasma membrane are fused together, ruthenium red cannot bind, nor can it pass such a fusion. However, due to its diameter of only 11.3 Å (Luft, 1971), it can pass into the 20 Å gap junctions. Use of ruthenium red as a tracer, also has the advantage that it is not washed out by fixative, probably because it is bound to the plasma membrane. In contrast lanthanum may be washed out during the fixation procedure (Revel and Karnovsky, 1967).

We found that ruthenium red damages the ependyma even though the animals did not show any sign of cerebral edema or circulatory disturbances. Very few of the intracellular components were intact when ruthenium red could surround the cell. In contrast, when only the apical plasma membrane was exposed to ruthenium red, as in the subcommissural organ and the median eminence, most of the finer constituents of the cell could be clearly identified. The mechanism of the damaging effect of ruthenium red is not known. It should be noted that the damage cannot be attributed to osmotic stress, because the osmolarity of the ruthenium red-mock CSF solution was found to be nearly the same as the osmolarity of the mock CSF alone. It must be emphasized that we never saw interruption of the junctional complexes in tissues which had been exposed to ruthenium red.

Ruthenium red has previously been used in studies of septate junctions (Gilula *et al.*, 1970) and tight junctions of endothelial cells (Luft, 1966). In reference to the latter, doubt has been cast on the reliability of the results (Luft, 1971) on the grounds that the diffusion time may have been too short or the concentration not high enough to insure the furthest possible penetration. Gap junctions have been outlined by ruthenium red between ependymal cells (Westergård, 1972) and between astrocytes (Tani and Ametani, 1972). It should be noted that in the above studies ruthenium red was administered in combination with fixation, either by immersion fixation or vascular perfusion followed by immersion.

In the present study, which utilized ventriculo-cisternal perfusion, the ruthenium red was perfused in a solution of mock CSF which was osmotically comparable to mock CSF alone for one hour before fixation. The ruthenium red was found to penetrate both zonulae adherentes and gap junctions between normal ependymal cells, and thereby to reach subependymal layers. Both in the median eminence where the presence of tight junctions was established previously by the use of horseradish peroxidase (Reese and Brightman, 1968) and in the subcommissural organ the passage of ruthenium red was stopped at the first tight junction, and gap junctions were outlined only if there was not a preceeding tight junction more apically. This shows that ruthenium red may be used to trace junctions.

The existence of cross bridges from plasma membrane to plasma membrane in the zonulae adherentes was also substantiated by the experiments where ruthenium red was used. Zonulae adhaerentes do not reveal distinguishing modifications in freeze-etch preparations (Friend and Gilula, 1972), which may mean that the anchoring of such filaments in the plasma membrane is susceptible to easy disruption. It is possible that the ruthenium red binding may have stabilized these filaments.

Tight Junctions in the Subcommissural Organ

Tight junctions between ependymal cells have so far been demonstrated in those regions in which the capillaries are permeable to trypan blue, and exhibit fenestrations and a widened collagen-containing space. These regions include choroid plexus (Brightman and Reese, 1969), median eminence and area postrema (Reese and Brightman, 1968). Furthermore, it has generally been accepted (e.g. Brightman *et al.*, 1970; Pappas, 1970) that the presence of capillaries with an enlarged perivascular space, in the mammalian brain, is indicative of an absence of the blood-brain barrier within that region. Tight junctions in these specialized ependymal regions could function in insulating the area from the cerebrospinal fluid.

The question of whether or not the SCO is outside the blood-brain barrier has been discussed previously. Wislocki and Leduc (1952a, b) do not include the subcommissural organ in their list of areas outside the blood-brain barrier after an extensive study using trypan blue and silver nitrate, but they found that the cells of the subcommissural organ react histochemically like regions which are outside the barrier. Löfgren (1965), on the other hand, found that the subcommissural organ is stained faintly after intravenous injection of trypan blue, but it is stained much less than the median eminence.

Recent electron microscopic studies have shown that the perivascular space around capillaries in the subcommissural organ often expands into collagen-filled sacs in the rat (Papacharalampous *et al.*, 1968) and toad (Rodríguez, 1970), which together with the presence of tight junctions between the ependymal cells suggests that the subcommissural organ lacks a blood-brain barrier.

The extent to which these tight junctions in the SCO permit passage to ions smaller than ruthenium red is unknown, but it has been postulated that tight junctions are the morphological site of ion diffusion across an epithelial barrier (Frömter and Diamond, 1972; Sørensen, 1972). Whether this is the case in the ependyma is not known. However, the influence of the tight junctions upon the flow of ions must depend on physical and chemical factors characterizing the fusion of membranes which may well vary from epithelium to epithelium.

The main conclusion of the presence of tight junctions in the ependyma of the SCO is that the region is, to a large extent, sealed off from the CSF.

We suggest that the subcommissural organ might be a secretory organ, which secretes substances into blood.

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