Tight Junctions of Brain Endothelium *in vitro* Are Enhanced by Astroglia

Jung-Hwa Tao-Cheng, Zoltan Nagy,^a and Milton W. Brightman

Laboratory of Neurobiology, NIH/NINCDS, Bethesda, Maryland 20892

The belts of endothelial tight junctions, which impede diffusion between blood and brain, were reduced to fragmentary, small junctions in subcultured brain endothelium. When cocultured with the capillaries' nearest neighbor, the astrocytes, these endothelial tight junctions were enhanced in length, width, and complexity, as seen by *en face* views of the cell membranes with freeze-fracture electron microscopy. Gap junctions, common in brain endothelium *in vitro* but absent in mature brain capillaries *in vivo*, were markedly diminished in area from among the enhanced tight junctions of the cocultures. Thus, astrocytes *in vitro* play a role in the formation, extent, and configuration of the junctional complexes in brain endothelium, whose diffusion barrier may likewise be influenced by astrocytes *in vivo*.

Brain capillaries have zonular tight junctions (TJs) impermeable to hydrophilic solutes (Reese and Karnovsky, 1967; Brightman and Reese, 1969). In freeze-fracture replicas of TJs, the constituent strands are continuous, anastomose extensively without free ends, and are devoid of gap junctions (Connel and Mercer, 1974; Dermietzel, 1975; Shivers, 1979; Nagy et al., 1984). The ability of brain endothelial cells to form this barrier depends on the environment in which they grow (Stewart and Wiley, 1981). The astrocyte, as the cell immediately ensheathing brain capillaries, is the most likely candidate to affect the capillaries' passive permeability by modulating the formation and maintenance of the TJs between their endothelial cells. To test this notion, we have cultured beef brain microvessel endothelium alone or together with rat astroglial cultures, and examined the endothelial TJ with freeze-fracture electron microscopy. In thin plastic sections, tight junctions appear as fused spots between 2 cell membranes, and it is very difficult to derive any quantitative information from these cross-sectional views of the TJs. The freeze-fracture technique displays the *en face* vista of the interior of the cell membrane (Branton, 1966), where the TJs appear as strands of particles in a 2-dimensional plane. Furthermore, the length and width of the TJ strands can be measured morphometrically. Preliminary results of the present work have been published as an abstract (Tao-Cheng et al., 1986).

Materials and Methods

Bovine brain endothelial cultures. Primary cultures were prepared according to the methods of Bowman et al. (1983). The endothelial culture medium consisted of equal volumes of minimum essential medium and nutrient F12 (Gibco) with 5% plasma-derived horse serum (P. D. Bowman et al., personal communication), 10 mM HEPES, 13 mM sodium bicarbonate, 100 μ g/ml heparin, 20 μ g/ml endothelial cell growth supplement (Sigma), 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B. After the cells were confluent at 7–20 d *in vitro*, 0.05% trypsin and 0.02% EDTA in Puck's saline solution without Ca^{2+} or Mg^{2+} were used to dissociate the cells for subculturing. In the present study, only the subcultures were examined because (1) primary cultures require laborious procedures for each preparation, and (2) the subcultured cells were more homogeneous as dissociated single cells than as the capillary fragments and cell clumps that were often present in primary cultures. Our brain endothelial subcultures were highly enriched, with more than 90% of the cells taking up fluorochrome-labeled, acetylated low-density lipoprotein (acet-LDL; Biomedical Technologies, Cambridge, MA), a marker for live endothelial cells (Fig. 1; Voyta et al., 1984).

Rat astroglial cultures. Primary (Anders and Brightman, 1982) and secondary enriched astroglial cultures (McCarthy and de Vellis, 1980) were derived from 2-d-old rats and grown on Lux (Miles Laboratory) plastic coverslips. The astroglial culture medium consists of Eagle's basal medium (BME) with Earle's salts, 26 mM sodium bicarbonate, 10% fetal calf serum, 4% BME vitamins, 2% BME amino acids, 1% penicillin-streptomycin, and 1% 7 M glucose. Astrocytes were labeled with fluorescent antibody to glial fibrillary acidic protein (GFAP; Bignami et al., 1972) and oligodendrocytes with antibody to galactocerebroside (GC; Raff et al., 1978). The percentage of GFAP+ cells was 60–85% in our primary cultures and 75–90% in our enriched secondary astroglial cultures.

Endothelial and astroglial cocultures. Dissociated, subcultured brain endothelial cells were seeded on a confluent bed of astroglia (5–12 d *in vitro*) at a density of 1.0×10^5 cells/mm². Cocultures were maintained in the same medium used for endothelial cultures alone. Endothelial cells from the same batches were seeded at the same density to be grown alone as controls. Some of the astroglial cultures from the same batches were grown as independent cultures and transferred to endothelial culture medium at the time of coculturing in order to serve as additional controls for the effect of endothelial culture medium on the astroglial cultures.

A second coculture method was to place a coverslip of confluent endothelial cells (7–10 d *in vitro*) so that they faced a confluent astroglial bed (5–12 d *in vitro*; cf. DeBault and Cancilla, 1980). Controls for this group were the sister solo endothelial (E) cultures maintained on coverslips. However, there were 3 technical disadvantages of this method: (1) in endothelial-astroglial (E + A) cocultures, when the coverslips of endothelial cells were removed for processing at the end of the coculture period, the cells sometimes became detached from them (perhaps remaining attached to the astroglial bed), so the yield was much lower with this method; (2) the contact between the 2 cell types with this method may have been uneven, owing to the inherently variable thicknesses of the 2 confluent cultures; and (3) the coverslips were easily displaced by even slight disturbances of the petri dishes. These perturbations, which unavoidably accompanied changes of medium every 3–4 d, made it impossible to sustain continuous contact between the 2 cell types in coculture for longer periods. As a result, data from this

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Correspondence should be addressed to Dr. Jung-Hwa Tao-Cheng, NIH, Building 36, Rm. 3B26, Bethesda, MD 20892.

^a Present address: Department of Psychiatry, Semmelweis Medical University, Budapest, Hungary.

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Figure 1. Enrichment of beef brain endothelial cultures. *a*, Fluorescent light micrograph. Over 90% of the cells incorporated fluorochrome-conjugated acetylated LDL, a marker for live endothelial cells. *b*, Phase-contrast light micrograph of the same field as in *a*.

method were limited; therefore, all of the descriptions and morphometric analyses were performed on cocultures prepared by the first method, unless otherwise indicated.

Conditioned medium from astroglial cultures. Fresh endothelial medium was fed to astroglial cultures between 10 and 24 d *in vitro* and then collected at 3–4 d intervals as conditioned medium. This conditioned medium was fed to the confluent endothelial cultures (7–10 d *in vitro*) for 3–17 d. A second method of bathing endothelial cells in conditioned astroglial medium was to place coverslips of confluent endothelial cells in a dish of astroglial bed, but facing away from the astroglia. This arrangement also provided an additional control for the second coculture method. Again, endothelial culture medium was used when the 2 cell types were grown in the same dish.

Control cultures. In tests of whether cells other than astrocytes affect the structure of cerebral endothelium, 3 types of control cultures were grown to confluency, at which time dissociated beef brain endothelial cells were seeded on top. The control cultures were (1) rat fibroblast prepared by dissociating subcutaneous and perimuscular connective tissues from the hind legs of 2-d-old rats, and grown in 10% fetal calf serum in Eagle's basal medium, (2) beef fibroblast-like cells from trachea obtained from the American Type Culture Collection (ATCC, Rockville, MD), and (3) smooth muscle cells derived from beef aorta.

In tests of whether astrocytes affect the junctions of noncerebral endothelium, endothelial cultures from beef aorta, human umbilical vein, and beef pulmonary artery (ATCC) were seeded onto a confluent astroglial bed.

Freeze-fracture. The cultures were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at room temperature for 30 min to several hours, washed, and stored in buffer. For cultures grown on plastic coverslips, 3 × 4 mm rectangles were cut out of the coverslips before further processing. For cultures grown on petri dishes, the confluent cell layers were scraped off and cut into 2 mm² pieces. The samples were glycerinated in 10, 20, and 30% glycerol in buffer for 10 min each, then mounted onto specimen carriers for Balzers' double-replication device with a drop of 5% polyvinyl alcohol in 30% glycerol (Pauli et al., 1977). The specimens were then frozen in liquid freon 22, cooled by liquid nitrogen, stored in liquid nitrogen, fractured at –120°C, and

replicated with platinum and carbon in a Balzers 301 freeze-fracture unit.

Morphometry. In the experimental groups used for morphometry, every TJ encountered was photographed. Two parameters, TJ length and width, were measured. The overall length of the TJ is the most important parameter for assessing the continuity of the TJ. The overall length most likely corresponds to the circumferential dimension of the barrier *in vivo*, while the width of the TJ represents the depth of the diffusion barrier. TJ length was measured along its long axis. For TJs shorter than 4 μm, the width was measured at the midpoint of the length, perpendicular to the long axis. For TJs longer than 4 μm, the width was measured every 2 μm along the long axis and then averaged. Partially exposed TJs were omitted if they were shorter than 1 μm. Gap-junctional areas were traced and measured on micrographs with a Bioquant II digitizer in conjunction with an Apple II+ computer.

Results

Solo endothelial cultures (E)

In thin sections, the brain endothelial cultures were not monolayers, but consisted of several layers of overlapping processes. The number of layers was variable, generally increasing with the age of the cultures.

In freeze-fractured replicas, the extent of the TJs in the subcultured brain endothelium was greatly reduced from that of their counterpart *in vivo*, i.e., the beltlike TJ in brain capillaries. TJs were infrequent and fragmentary in all 25 different endothelial subcultures from 6 adult beef brains, with the number of passages ranging from 1 to 6, and the days in culture ranging from 3 to 21. One of the 6 brains yielded extremely few TJs, and in the other 5 brains only about 5–10% of the membrane profiles in the freeze-fractured replicas had TJs. At 6–10 d *in vitro*, the TJs usually consisted of short (1–2 μm) patches of anastomosing strands, sometimes with free ends, and enclosed many gap junctions (Fig. 2*a*). The frequency and extent of the TJs usually increased with time (Figs. 2, *a* vs *c*; 3, *a* vs *e*), but the patches remained separate and were thus very different from *in vivo* brain TJs, which are always beltlike and continuous. A second *in vitro* deviation was the prevalence of gap junctions among TJ strands at all ages of solo endothelial cultures (GJ in Fig. 2, *a* and *c*). In contrast, mature brain capillaries are devoid of gap junctions *in vivo* (Nagy et al., 1984).

Endothelial and astroglial cocultures (E + A)

To see whether astroglia influences TJ formation in the brain endothelial cultures, we cocultured the 2 groups of cells. Cocultures were made with various endothelial subcultures derived from 4 beef brains, whose number of passages ranged from 1 to 5. Typically, thin sections of cocultures showed several layers each of overlapping endothelial and astroglial processes. Sometimes a basal lamina was intercalated between processes from the 2 cell types.

In freeze-fractured replicas, the very low number of endothelial TJs from the one "refractory" brain was not significantly increased, even in coculture with astroglia. However, endothelial cells from the other 3 brains usually yielded much higher

Figure 2. *a*, Typical examples of TJs from solo beef brain endothelial culture (E) 6 d *in vitro*. Tight junctions were short, fragmented, with free ends (arrows), and enclosed many gap junctions (GJ). ×43,000. *b*, The same batch of beef brain endothelial cells as in Figure 2*a*, but cocultured with rat astroglia for 6 d (E + A). tight junctions (TJ) were longer, broader, with far fewer gap junctions, and thus resemble those of brain capillaries *in vivo*. This enhanced TJ was 15 μm long and 2 μm wide. ×43,000. In comparison, the longest TJ in the sister solo E culture is only 4.3 μm long and 0.4 μm wide. When the total length of the component TJ strands were summed in these 2 examples, the difference was even more striking, at 165 vs 13 μm for E + A and E, respectively. *c*, Solo beef brain endothelial culture (E) 17 d *in vitro*. Tight junctions were still in separate patches and are associated with many gap junctions (GJ). ×34,000. *d*, Beef brain endothelium cocultured with rat astroglia (E + A) for 14 d. An astrocyte,

distinguished from other cell types by its orthogonal arrays of particles (arrows in inset, enlarged $\times 62,500$), was next to this endothelial cell, in which the enhanced TJ was $30\ \mu\text{m}$ long and about $3\ \mu\text{m}$ wide. $\times 22,000$.

Figure 3. *a–d*, Histograms of TJ length and width in the same batch of cerebral endothelial cells cultured alone (*E*) or with astroglia (*E* + *A*) for 6 d. There is a distinct population of longer and wider TJs in

Figure 4. Percentage area of junctional complex occupied by gap junctions (*GJ*) was much less in the same batches of brain endothelium cocultured with astroglia (*E* + *A*) than when grown alone (*E*) for 17 d. The number of junctional complexes measured for *E* and *E* + *A* was 38 and 35, respectively. The *E* and *E* + *A* distributions were significantly different ($p < 0.005$) by the Smirnov test.

frequencies of TJ when they were cocultured with astroglia. For example, in one pair of *E* + *A* versus *E* sister cultures, the number of TJs encountered in a given replica area in the *E* + *A* coculture was more than twice that in the solo *E* culture (74 vs 34). The number of TJs displayed was greater in *E* + *A* despite the decreased chance of cleaving endothelial cell membranes in the cocultures where there was a mixture of endothelial and astroglial membranes.

In 11 out of 14 *E* + *A* cocultures from the 3 “responsive” brains, there were greatly enhanced TJs, with markedly increased lengths, widths, and complexity (Figs. 2, *b*, *d* vs *a*, *c*). An enhanced TJ in the cocultures is one in which the TJ is longer or wider than the largest TJ in the same batch of endothelial cells grown alone. Thus, in the TJs derived from a given subculture, any TJ in *E* + *A* coculture with a greater length or width than that in the corresponding solo *E* culture was designated as “enhanced”. For example (Fig. 3, *e–h*), in 2 similar solo *E* subcultures, the longest TJ was 9 μm (Fig. 3*e*), and the widest, 1.2 μm (Fig. 3*f*). In the sister *E* + *A* cocultures, those TJs with dimensions greater than these 2 values were classified as enhanced. Accordingly, about 20–30% of the TJs in the cocultures shown in Figure 3, *g* and *h*, were enhanced. The percentage range of enhanced TJs in the present study was 15–35%. It should be noted that the majority (~70%) of the “longer” TJs were also “wider,” and that they were usually enhanced in both dimensions.

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E + *A* cocultures (*c*, *d*) that is not present in the solo *E* cultures (*a*, *b*). For this sister pair of cultures, the same amount of replica areas was examined in *E* and *E* + *A*, and the number of TJs measured for *E* and *E* + *A* was 34 and 74, respectively. *e–h*, Histograms of TJ length and width in the same batches of cerebral endothelial cells cultured alone (*E*) or with astroglia (*E* + *A*) for 14–16 d. The data here are pooled from 2 similar experiments because there is no significant difference between the 2 solo *E* cultures and the 2 *E* + *A* cocultures by the Smirnov test. In solo *E* cultures, there were many more shorter and narrower TJs, the longest being at 9 μm (*e*) and the widest at 1.2 μm (*f*). In *E* + *A* cocultures, 20% of the TJs were longer than 9 μm (*g*) and 30% were wider than 1.2 μm (*h*), and these were termed “enhanced” TJs. The *E* and *E* + *A* distributions were significantly different ($p < 0.005$) for both length and width (Smirnov test). The number of TJs measured for *E* and *E* + *A* was 136 and 76, respectively, and the number of membrane profiles from which the TJs were measured was 68 and 48, respectively. About 4 times as many replica areas were scrutinized in *E* as in *E* + *A* to confirm the absence of enhanced TJ in the *E* group. The proportion of partially revealed TJs in *E* and *E* + *A* was ~10 and 30% (80% of the latter were enhanced TJs).

The enhancement of TJs was first evident at 6 d (Figs. 2, *a* vs *b*; 3, *a* vs *c*, *b* vs *d*) with the first coculture method, where dissociated endothelial cells were seeded on top of the astroglial bed. The TJ enhancement was apparent at 4 d in the case of the second coculture method, where confluent endothelial cultures were placed facing the astroglial bed. The degree of enhancement increased with time (Fig. 3, *c* vs *g*, *d* vs *h*) up to 17 d, the longest period that the cocultures were maintained. Significantly, these augmented TJs in cocultures formed extensive, long networks rather than separate patches. This trend toward continuity is what would be expected of a blood–brain barrier seal. Moreover, the much more elaborate TJs of cocultures had far fewer gap junctions (Fig. 2, *b*, *d* vs *a*, *c*; E + A vs E in Fig. 4), and thus more closely resembled the TJs of brain capillaries *in vivo*.

In some fortuitous fractures, astroglial membranes, identified by their orthogonal array of particles termed “assemblies” (Landis and Reese, 1974), were next to the endothelial membranes containing greatly enhanced TJs (Fig. 2*d*). However, it should be stressed that no junctional specialization has ever been found between an endothelial cell and an astrocyte.

Conditioned medium

Conditioned medium from astroglial cultures did not enhance TJs in brain endothelial cultures. Coverslips of confluent endothelial cells placed in the same dish as the astroglial bed, but facing away from the astroglia, did not display enhanced TJs.

Controls

Enhanced TJ was never found in control solo astroglial cultures that were grown in astroglial culture medium and then incubated in endothelial culture medium at the time their sister cultures were cocultured with brain endothelium.

Fibroblasts from rat peripheral connective tissues and beef trachea, and smooth muscle cells from beef aorta, were used as substitutes for astroglia in the cocultures for 2, 6, and 5 trials, respectively. These controls were all derived from peripheral tissues to avoid contamination by astroglia. In more than 300 examples of TJs, none of the control cells enhanced the endothelial TJs. Significantly, in 2 cases each, the same batches of brain endothelial cells that had greatly enhanced TJs when cocultured with astroglia were unaffected when concurrently cocultured with beef fibroblasts or smooth muscle cells. Conversely, astroglia did not induce TJ formation when cocultured with subcultured endothelial cells from beef aorta, beef pulmonary artery, or human umbilical vein in 3, 4, and 2 trials, respectively.

Discussion

Enhanced tight junctions with long and broad networks of particle strands were found only in brain endothelial cells cultured in the company of astrocytes. We conclude that these enhanced TJs in our cocultures were endothelial and not astroglial because enhanced TJs were never found in the same membrane as the orthogonal arrays of particle assemblies characteristic of astroglial membranes. Moreover, the enhanced TJs were not formed by the contaminants in the astroglial cultures because (1) the major cell type in astroglial cultures capable of forming TJs was oligodendroglial, whose TJs are distinct from those of brain endothelium (Massa and Mugnaini, 1982, 1985); (2) although *rat* endothelial cells might have been included in our astroglial cultures, they were not the source of the enhanced TJs,

since solo astroglial cultures, even when incubated in endothelial culture medium, never contained any enhanced TJ; (3) leptomeningeal cells, which have tight and gap junctions, and which might also have been a minor contaminant of the astroglial cultures, were not the source of the enhanced TJs, since solo astroglial cultures never contained any enhanced TJ.

As our enriched astroglial cultures were still 10–20% contaminated by other cell types, it is conceivable that the effects on the brain endothelial junctional complex were exerted not by astroglia alone, but together with other cell types. Oligodendroglia was most likely to have been the other cell type because it was the major contaminant (5–10%), it is ubiquitous in the brain, and it may communicate with astroglia via gap junctions (Massa and Mugnaini, 1982, 1985). Oligodendroglia could, therefore, concurrently affect the endothelium by acting through astroglia. Since oligodendroglial cultures (McCarthy and de Vellis, 1980), in turn, always included astroglia, their effect on the TJs has not yet been assessed. All other contaminants in astroglial cultures were too small in number to have affected the TJs. Janzer and Raff (1987) have recently reported that dissociated astrocytes, when injected into the anterior chamber of the eye, can induce the invading, permeable blood vessels to become, like CNS vessels, impermeable to Evans blue. Our experiments further define the hypothesis that the milieu of a region being vascularized determines the characteristics of the ingrowing blood vessels (Stewart and Wiley, 1981). It is the astrocytic component of the CNS milieu that affects a specific morphological feature: the junctional complex between endothelial cells.

The mechanism of this astrocytic effect of enhancing brain endothelial TJ is unknown. Indeed, not all TJs in our endothelial–astroglial cocultures were augmented; the percentage of enhanced TJs was between 15 and 35%. These percentages may be underestimates because we used the values of the greatest TJ lengths and widths from the solo E cultures as the defining criteria for an enhanced TJ in the E + A cocultures. The junctional response is, nevertheless, variable. One possible reason for this variability is that only a subpopulation of endothelial cells, or perhaps cells at a certain stage of the mitotic cycle, are responsive. Another possible reason for the variable response is that only the TJs at the endothelial–astroglial interface were enhanced, while the rest of the TJs formed by the endothelial layers farther from the interface were not affected. Proximity between the 2 cell types may be required for the junction responses. This explanation is in agreement with the observation that enhanced TJs were found only when endothelial cells grown on coverslips were placed facing the astroglial bed (the second coculture method), but not when they were facing away. Moreover, conditioned medium from glial cultures did not enhance the endothelial TJ. Therefore, the junctional responses were probably not brought about by substances that were secreted into the medium by astrocytes and that were freely diffusible across 2–3 layers each of astroglia and endothelium to reach the endothelial layers farther from the interface. Since no intercellular junction has been found between the 2 cell types *in vivo* or *in vitro*, the astrocytes' effect on endothelial TJs is very likely mediated by factor(s) secreted into and perhaps even concentrated within the basal lamina. A basal lamina is always present between the capillary endothelial cells and the perivascular astrocytic endfeet *in vivo*, and, sometimes, between the 2 cell types in our cocultures, and might be involved in coupling the action of astroglia on endothelium.

Interestingly, enhanced TJs were detected earlier (4 vs 6 d of coculture) with the second coculture method, in which confluent endothelial cells grown on coverslips (7–10 d *in vitro*) were placed facing the astroglial bed, than with the first coculture method, where dissociated endothelial cells were seeded on top of the astroglial bed. This earlier appearance is not surprising, since the endothelial cell in the second coculture method had a 7–10 d head start in development. However, it is still not clear whether the enhanced TJs in E + A cocultures were newly formed in their entirety or were added to pre-existing patches of TJ in the solo E cultures.

Endothelial TJs from 1 out of 4 beef brains in the present study were nonresponsive when cocultured with astroglia. It is possible that an error was made in the isolation procedure, selectively eliminating the responsive subpopulation of endothelial cells from this brain. In the other 3 brains, enhanced TJs were found in 11 out of 14 coculture experiments. The 3 failures may be due to insufficient sampling of limited numbers of replicas in some cases, or to errors made in the preparation of the astroglial or endothelial subcultures.

TJs can proliferate in various systems in response to enzymatic action (Orci et al., 1973; Polak-Charcon et al., 1978), regeneration (Huttner et al., 1985), and as-yet-unknown stimuli (Kachar and Pinto da Silva, 1981). Our study has demonstrated that one group of cells can influence the assembly of TJs in another cell type. Furthermore, this effect is exerted on the brain endothelium of a species different from that of the astroglial donor, and may therefore be a fundamental property of astroglia. Another interspecies effect is the *in vitro* re-expression of gamma-glutamyl transpeptidase in brain endothelium of the mouse by C₆ glioma cells of the rat (DeBault and Cancilla, 1980).

Primary cultures of brain endothelium alone can form TJs (Bowman et al., 1983), some of which are described as halting the diffusion of horseradish peroxidase (Dorovini-Zis et al., 1984). However, no morphometric data were available as to the frequency or complexity of the TJs in the primary cultures. In the present study, we examined only subcultures because they were homogeneous, previously isolated, and thus ready for replating. After subsequent passages, the endothelial TJs became few and fragmentary, and therefore distinctly different from their counterparts *in vivo*, the TJs of brain capillaries, which are always beltlike and continuous. Thus, the unique attribute of brain capillaries, the barrier to the passive, intercellular flow of solutes, was all but lost upon subculturing of the endothelial cells. The presence of astroglia largely restored the morphological properties of barrier TJs, that is, broad arrays of long, uninterrupted strands. The morphological complexity of the TJ is related to the impermeability of tight junctions in certain systems (Easter et al., 1983; Okuda and Yamamoto, 1983), but probably not in others (Martinez-Palomo and Erlij, 1975; Mollgard et al., 1976). Whether the enhanced TJs in our cocultures are functionally tight is currently being investigated in our laboratory. Measurements of electrical resistance across confluent monolayers grown on a permeable support membrane in bipartite chambers are correlated with the impermeability of the cultures in many epithelial cells (e.g., Misfeldt et al., 1976). Likewise, a preliminary report indicated a higher electrical resistance in cocultures of mouse brain endothelium and rat C₆ glioma than in the solo endothelial culture (Cancilla, 1986).

A second important astroglial effect on the endothelial junctional complex is the marked reduction of gap junctions in the enhanced TJ. Endothelial gap junctions are common in embry-

onic chick telencephalon, but decrease in number as development progresses (Delorme et al., 1970). Thus, gap junctions in solo endothelial cultures may indicate a less mature state of the junctional complexes, while the addition of astrocytes facilitates the maturation of the TJs. Our conclusion, therefore, is that astrocytes “normalize” the morphology of the brain endothelial tight junction *in vitro*, and, likewise, may influence the formation and maintenance of zonular tight junctions in vessels of the brain.

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