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LOCALIZATION OF SYNAPSES IN RAT CORTICAL CULTURES

K. M. HARRIS* and P. A. ROSENBERG

Department of Neurology, Children's Hospital and The Program in Neuroscience at Harvard Medical School, Boston, MA 02115, U.S.A.

Abstract—Astrocyte-rich and astrocyte-poor cultures derived from embryonic rat cerebral cortex were compared to determine whether differences in the location of neuronal somas, dendrites, axons, synapses or astrocytes, relative to the bulk culture medium, could help to explain the large difference in neuronal susceptibility to glutamate toxicity between the two culture systems. The cultures were processed for electron microscopy, thin sectioned across their depths, and photomontaged. In astrocyte-rich cultures, most of the dendrites, axons and synapses were sequestered from the medium by a nearly continuous layer of astrocyte cell bodies and processes. In contrast, astrocytes did not cover the synapses or neuronal processes in astrocyte-poor cultures. In neither culture system were neuronal cell somas covered by glia. Since neuronal cell somas are freely exposed to the medium in both culture conditions, it seems unlikely that receptors on the somal membrane mediate the greater susceptibility of neurons in astrocyte-poor cultures to glutamate toxicity. The layer of astrocytes in the astrocyte-rich cultures may provide a physical buffer that could hinder diffusion of substances from the medium to the interstitium of the neuropil. This physical buffer combined with avid glutamate uptake mechanisms might allow astrocytes to maintain a sufficiently low concentration of glutamate in the local extracellular space to protect dendrites and synapses in the astrocyte-rich, but not in the astrocyte-poor cultures, from the excitotoxic effects of glutamate.

The results of this study demonstrate that local sequestering of neurites and synapses by a physical buffer of astrocytes may help to explain the relative resistance of neurons cultured with astrocytes to glutamate toxicity. A similar physical sequestering by astrocytes, of sensitive regions of neurons in the brain, may help protect neurons from glutamate toxicity *in vivo*.

The use of *in vitro* model systems has permitted a cellular approach to elucidate multiple molecular and physiological events that involve interactions between neurons and glia.[3] Glia may influence neuronal activity directly through regulation of the concentration of extracellular cations, the concentration of neurotransmitters, especially glutamate, and the release of neurotransmitters or other molecules that modulate or even mediate the action of neurotransmitters. An indirect influence may arise *in vivo* through the regulation by glia of blood flow, thereby modulating the availability of nutrients to neurons. The morphological characteristics of astrocytes can be regulated by the presence of neurons *in vitro*,[13,19] and there is compelling evidence from *in vivo* observations that synapses cluster in the vicinity of glial cells.[24]

Culture systems have been extensively utilized to understand mechanisms of glutamate toxicity, and have significantly furthered our understanding of its potential importance not only in stroke but also in a variety of neurodegenerative diseases.[18] One obstacle to accepting the hypothesis that glutamate toxicity may cause nerve cell death had been the finding that glutamate itself is a relatively weak neurotoxin when it is infused into an otherwise normal brain.[9,16] This problem was overcome when glutamate was demonstrated to be a potent neurotoxin with an EC_{50} of approximately 50–200 μM in dissociated cultures of neocortex or hippocampus.[8,14] The difficulty in demonstrating the toxicity of glutamate *in vivo* and the ease of demonstrating its toxicity *in vitro* were explained by the operation of an avid uptake system for glutamate in the *in vivo* experiments, and the rendering of this uptake inconsequential by the geometry of the tissue culture system in which it was generally thought that neurons were growing on top of a bed of confluent astrocytes.

More recently it has been shown that neurons are approximately 40–100-times more sensitive to glutamate toxicity in neuron-enriched astrocyte-deficient cultures (astrocyte-poor), than in the cultures with a confluent layer of astrocytes (astrocyte-rich).[27,28] The resistance to glutamate toxicity observed in the astrocyte-rich cultures might be due to: (i) a decreased sensitivity of neuronal glutamate receptors; (ii) a decreased access of glutamate to neurons in the astrocyte-rich cultures; and/or (iii) a change in the secondary consequences of glutamate receptor stimulation.

The possibility that neurons in astrocyte-poor cultures are more sensitive to stimulation by glutamate at *N*-methyl-D-aspartate receptors was excluded in previous experiments in which membrane responses

*To whom correspondence should be addressed at: Department of Neurology, Children's Hospital, Enders 260, 300 Longwood Avenue, Boston, MA 02115, U.S.A.

to *N*-methyl-D-aspartate in the two culture types were found to be similar.[27] Recent work[28] has demonstrated the importance of glutamate uptake in protecing neurons in astrocyte-rich cultures, as predicted by an earlier study of cerebellar cells.[12] Even though an unstirred layer of medium immediately above the culture might with time have a lower concentration of glutamate,[32] the rapidity of the onset of glutamate-mediated injury[7] suggests that uptake into astrocytes could not clear the extracellular medium rapidly enough to protect neurons if the site of glutamate action on these neurons was freely exposed to the extracellular medium. Since glutamate receptors have been found concentrated in postsynaptic densities of excitatory synapses,[11] we predicted that most if not all of the excitatory synapses in astrocyte-rich cultures must be sequestered from the extracellular medium by the astrocytes in order for the glutamate uptake system to be sufficient to protect the neurons.

There is a paucity of morphological studies characterizing the location of synapses in relation to glia in tissue culture. Several studies have examined the morphology of neuronal processes in astrocyte-poor hippocampal cultures,[1,2,4,5] astrocyte-rich hippocampal cultures,[22] astrocyte-rich spinal cord cultures,[20,21] and astrocyte-rich neocortical cultures,[15] without specifically addressing the issue of their location with respect to glia. Here we have compared astrocyte-rich and astrocyte-poor neocortical cultures to ascertain whether differences in the location of neurons, neurites, synapses, and astrocytes relative to the extracellular medium could help to explain the differences between the two culture systems in the sensitivity of the neurons to glutamate. These anatomical observations may also be applicable in elucidating the location of action and effective concentration of any substance *in vitro*.

EXPERIMENTAL PROCEDURES

Preparation of astrocyte-rich and astrocyte-poor cortical tissue cultures

Astrocyte-rich and astrocyte-poor cultures, derived from embryonic (fetal day 16) Sprague–Dawley (Charles River) rat cerebral cortex, were prepared by methods previously described.[26–28] Briefly, dissociation was facilitated by exposure to 0.027% trypsin and the cells were plated on poly-L-lysine (astrocyte-poor) or collagen and poly-L-lysine (astrocyte-rich)-coated glass coverslips in Dulbecco's modified Eagle's medium/Ham's F-12/heat inactivated iron supplemented calf serum (HyClone) 8:1:1 (DHS).

The conventional astrocyte-rich culture system used in this study is what is commonly referred to as cerebral cortex in dissociated cell culture.[10,29] These cultures were exposed to 5 μM cytosine arabinoside at 15 days *in vitro*, for 72 h, after the glial layer had become confluent. These cultures were maintained using three medium changes per week with heat-inactivated iron-supplemented calf serum. Astrocyte-poor cultures were produced by exposure to 5 μM cytosine arabinoside for 48 h, beginning at four days *in vitro*. After inhibition, medium was replaced with Dulbecco's modified Eagle's medium/Eagle's Minimal Essential Medium/F12 (4:5:1) with the N2 supplements of Bottenstein and Sato,[6a] penicillin and streptomycin, 2 mM glutamine, catalase 1 μg/ml,[33] plus 1% serum. Medium was not subsequently replaced because regular medium changing was associated with diminished survival of neurons in the astrocyte-poor cultures.[26,27] Astrocyte-rich and astrocyte-poor cultures from a single plating were processed for electron microscopy at five weeks after plating.

Fixation and processing of tissue cultures for electron microscopy

Five coverslip cultures each from the astrocyte-rich and astrocyte-poor conditions were fixed for 1 h in 2% paraformaldehyde, 2.5% glutaraldehyde, 2 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.35) at room temperature ($\sim$25°). Fixative was added slowly to the side of the culture dish to avoid mechanical disruption of the cells in the cultures. The fixative was then pipetted off from the edge of the culture dish, and the cultures were rinsed five times over 10 min with gentle agitation in 0.1 M cacodylate buffer, immersed for 1 h in 1% OsO_4 with 1.5% $K_4Fe(CN)_6$–$3H_2O$ and 0.1 M cacodylate buffer, rinsed five times over 10 min in 0.1 M caco-

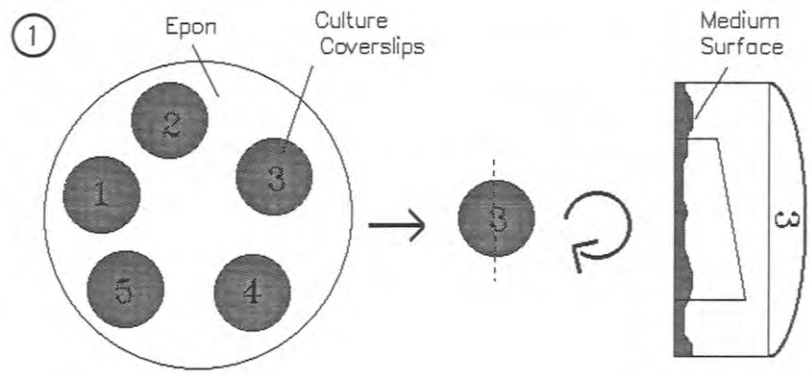

Fig. 1. Orientation of cortical tissue cultures for thin sectioning. The coverslips with cortical tissue cultures were processed for electron microscopy and embedded in Epon. Then the glass was removed with hydrofluoric acid and the block was cut through its middle. Finally, a trapezoid was trimmed such that the cultures were sectioned perpendicularly to the culture medium surface. This orientation provided long stretches of a smooth culture–medium interface along the middle of the section with minimal chatter at the top and bottom surfaces of the trapezoid. When test blocks were trimmed to be sectioned with the trapezoid oriented 90° perpendicular to the illustrated orientation, the culture itself had considerable chatter and often was lost at the knife edge. The total height of the trapezoid was less than 1 mm with a width of about 0.2–0.5 mm such that one section fitted on a slot grid.

dylate buffer, and rinsed twice briefly with distilled water. The cultures were dehydrated for 5 min in 50% EtOH, for 10 min in 70% EtOH with 1% uranyl acetate, for 5 min each in 80, 90 and 95% EtOH, for three times over 15 min in 100% EtOH, and for two brief immersions in 1:1 100% EtOH/Epon mixture. The culture coverslips were positioned around the bottom of two aluminum weigh boats, one labeled for each condition (Fig. 1). A 1:3 100% EtOH/Epon with DMP-30 mixture was added slowly to the side of the weigh boats and these were left overnight at room temperature. This EtOH/Epon mixture was removed the next morning, replaced by fresh Epon with DMP-30 to 5 mm depth, and cured at 60°C for 48 h.

The Epon molds were removed from the aluminum weigh boats, heated to 60°C, individual coverslips were carved free from one another, and the film of polymerized Epon on the

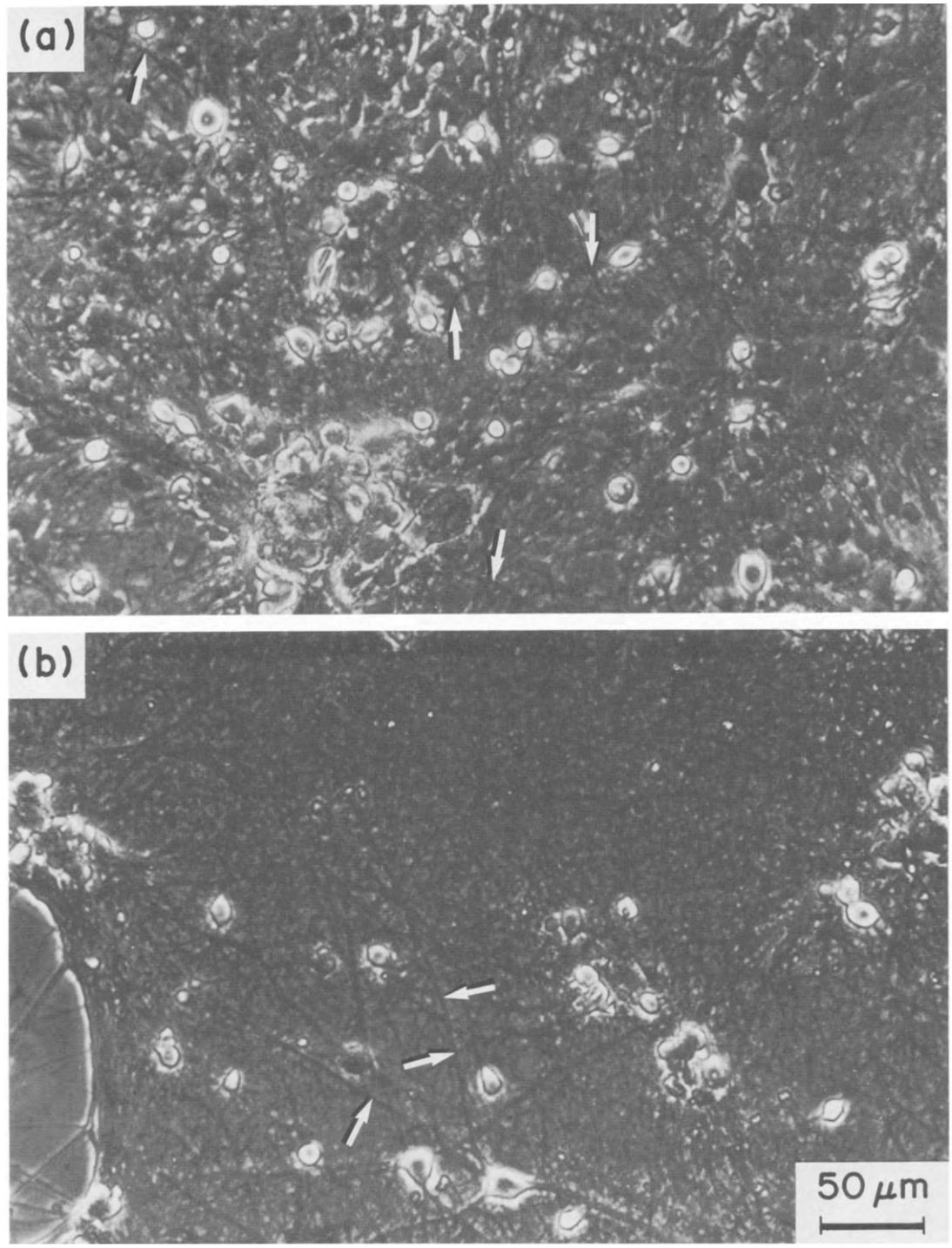

Fig. 2. Phase-contrast light microscopy of the surface of astrocyte-rich (a) and astrocyte-poor (b) cortical cultures. Neurons are the round phase-bright cells. White arrows indicate processes or bundles of processes which are presumed to be neurites. Scale bar in b also applies to a.

glass surface was scraped off with a razor blade. The glass was then swabbed with acetone, and the coverslip was etched away from the epon-embedded cultures with concentrated hydrofluoric acid which was subsequently neutralized in saturated sodium bicarbonate solution and water. Complete removal of the glass was determined under a dissecting scope and usually required 20–30 min of exposure to hydrofluoric acid.

Thin sectioning

Each Epon block was heated to 60°C and cut with a razor blade perpendicularly to the depth of the culture (Fig. 1). Three coverslips each from the astrocyte-rich and astrocyte-poor conditions were then used for subsequent processing and analyses. The resulting semicircular block was clamped in a Reichert Ultracut E ultramicrotome and a trapezoid was glass-trimmed with the transected culture on the left side of the block (Fig. 1). Thin sections were cut at a thickness of 90 nm on the ultramicrotome, mounted on pioloform-coated Synaptek slot grids (Pella), and stained for 5 min with Reynolds' lead citrate.[25] The grids were then mounted in grid cassettes (JEOL SRH-10Mod for side-entry electron microscopes) and stored in labeled gelatin capsules.

Photography and length measurements

The entire length of each culture was photomontaged at 4000–5000 magnification in a JEOL 1200EX electron micro-

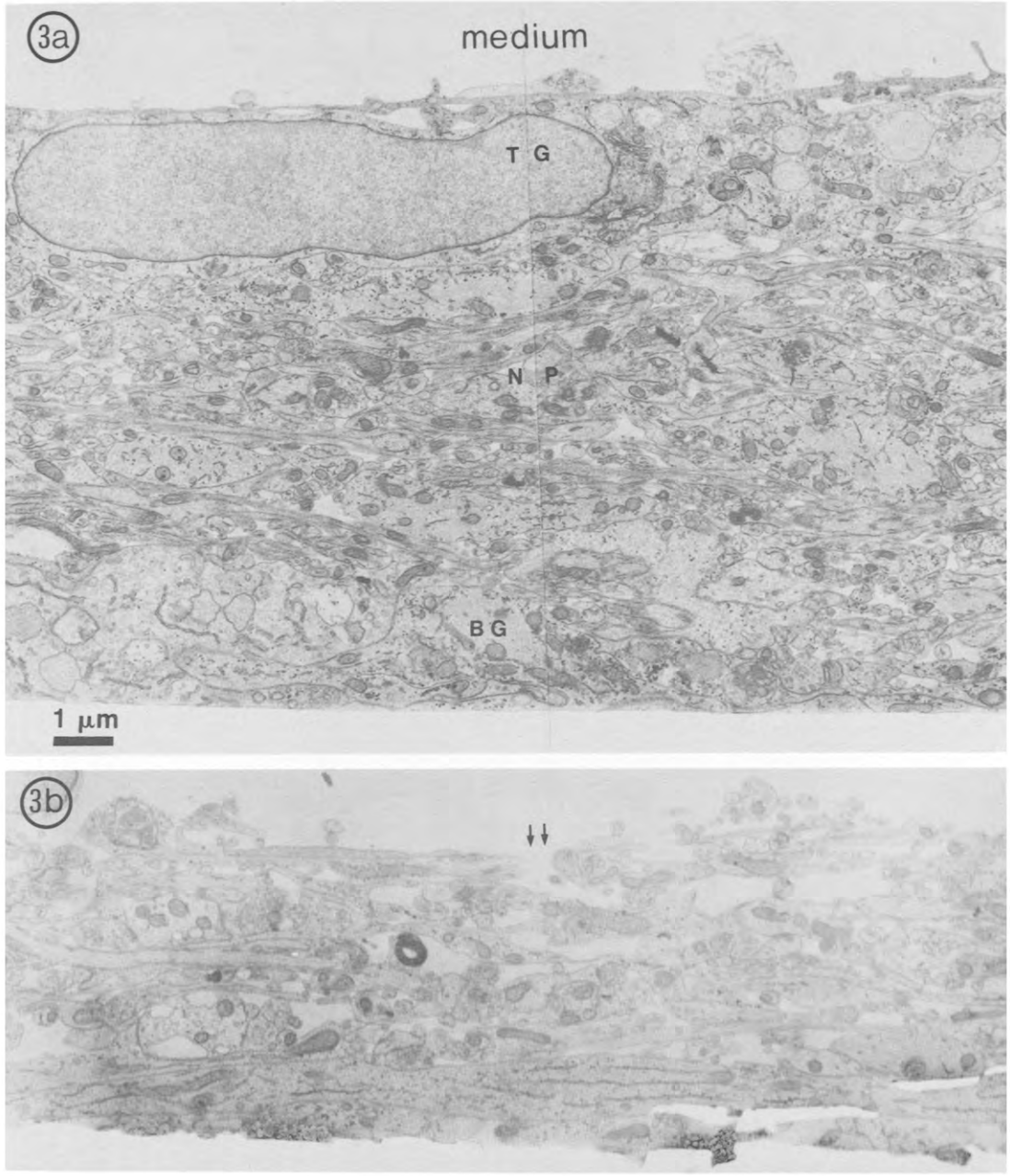

Fig. 3. The full depth from the medium surface (at top) to the coverslip surface (at bottom) of astrocyte-rich cultures. (a) The top glial (TG), middle neuropil (NP) and bottom glial (BG) layers are all relatively thick. (b) The top glial layer is thin and discontinuous (double arrows) and the underlying neuropil layer is thinner than in a. Scale bar in a also applies to b.

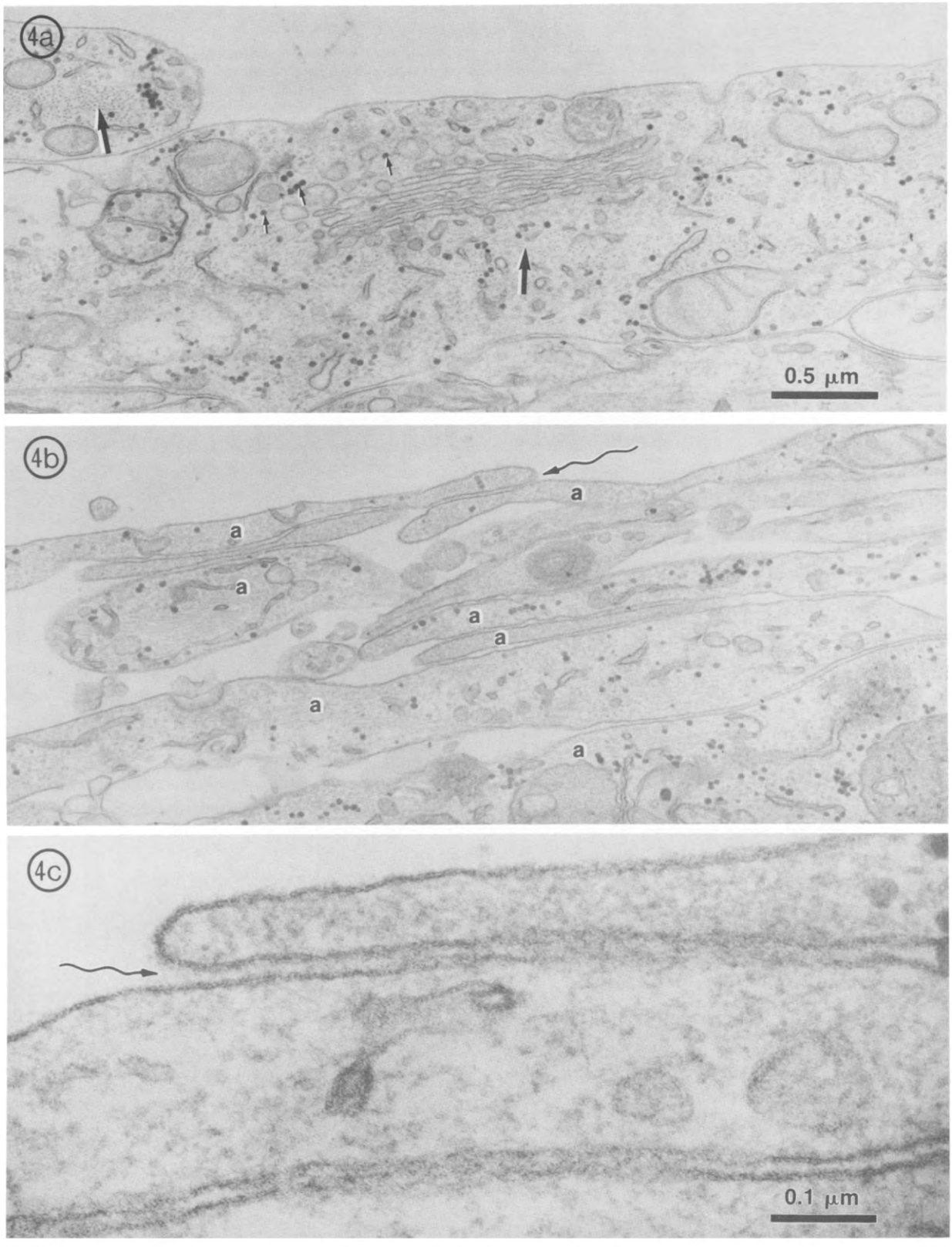

Fig. 4. Characteristics of astrocytes in the top glial layer. (a) Astrocytes in the top glial layer were identified by the presence of bundles of fibrils (large arrows) and glycogen granules (small arrows). (b) Overlapping adjacent astrocytic (a) processes of the top glial layer physically sequester the underlying neuropil from the medium (wavy arrow). (c) Electron-dense staining material occurred between adjacent astrocytic processes. Scale bar in a also applies to b.

scope. The negatives were printed to contain the full depth of the culture on multiple sheets 8 × 10″ photographic and viewed through an illuminated magnifying ring. Some regions of interest were photographed at higher magnifications. All culture lengths and depths were measured by superimposing a photograph of a calibration grid (0.463 μm per grid length) on the phtomicrographs of the cultures.

Quantitative descriptions of the components of astrocyte-rich and astrocyte-poor cultures

For the astrocyte-rich cultures 145 micrographs spanning 1850 μm, and for the astrocyte-poor cultures 134 micrographs spanning 1980 μm of culture–medium interface were evaluated. The number of neuronal cell bodies, neurite clusters, and synapses directly exposed to the medium with

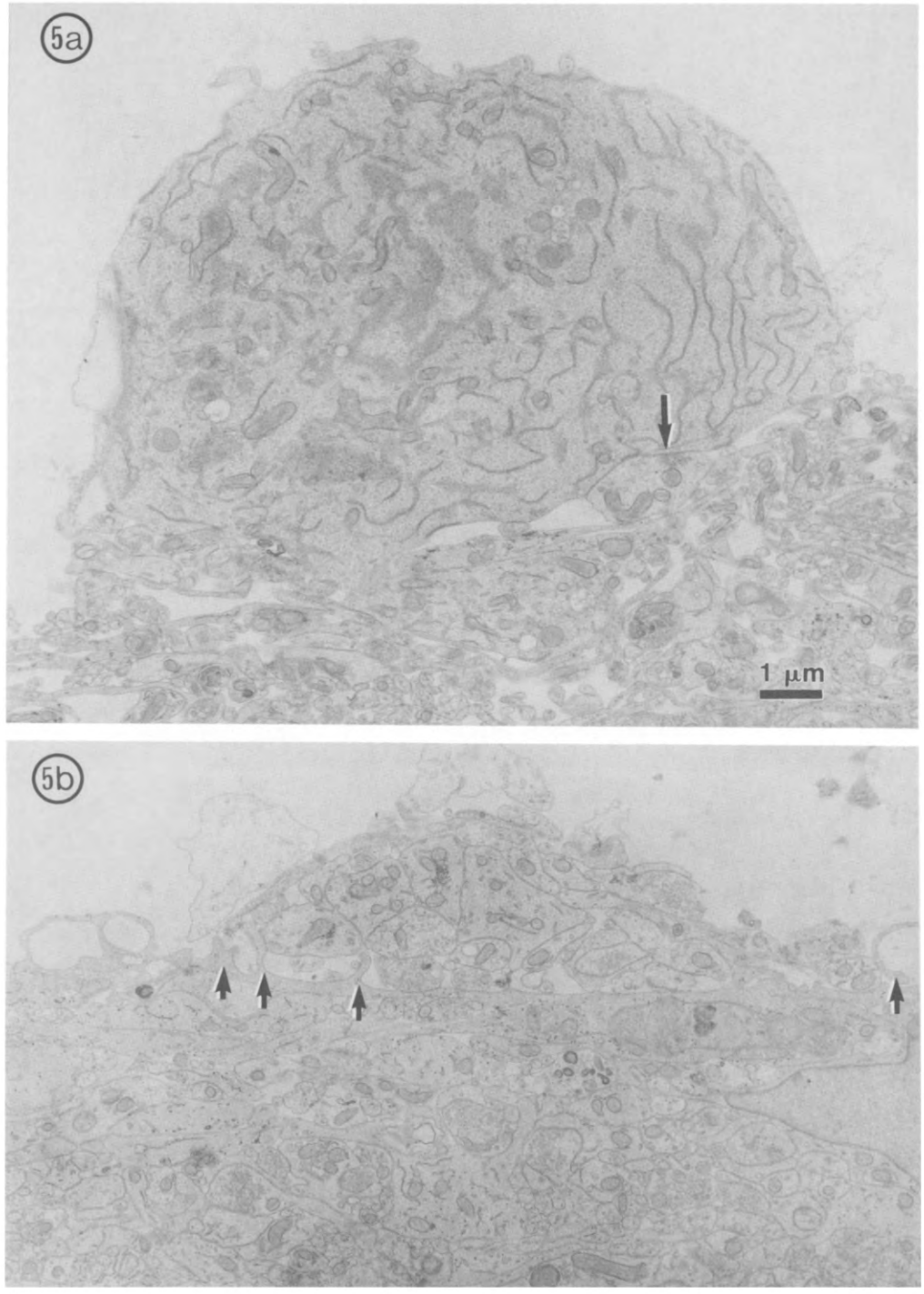

Fig. 5. (a) Neuron at the culture–medium interface of an astrocyte-rich culture with a synapse at the neuron–culture interface (arrow). (b) Neurite cluster at culture–medium interface of an astrocyte-rich culture with astrocytic finger-like processes (arrows) penetrating the cluster or occurring at its edge. Scale bar in a also applies to b.

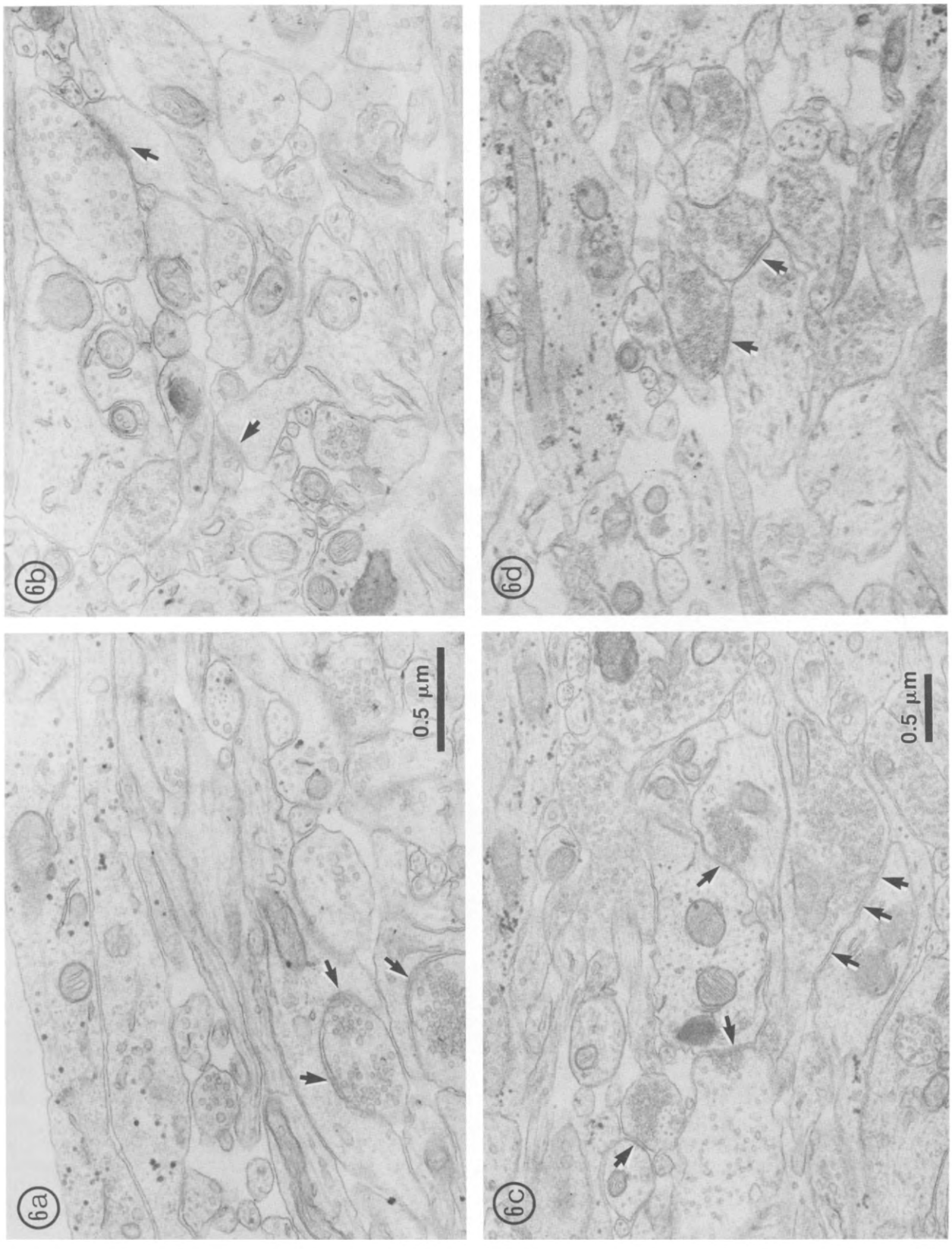

Fig. 6. Synapses beneath the top glial layer of astrocyte-rich cultures. (a) Synapses on dendritic spines (arrows) in the neuropil layer near to the top glial layer of an astrocyte-rich culture. (b) Synapses on dentritic spines (arrows) deep within the neuropil layer of an astrocyte-rich culture. (c, d) Synapses on dendritic shafts (arrows) in the neuropil layer of astrocyte-rich cultures. Scale bar in a also applies to b; scale bar in c also applies to d.

no intervening astrocytic processes were counted and the percentage of culture interface covered by glia was determined. In addition, the number of synapses occurring beneath neuronal cell bodies, but without overlying astrocytic processes, were counted as these are more likely to be shielded from the medium than synapses directly in the medium.

A subset of 12 micrographs was selected from the photomontages of each culture condition for a more detailed analysis of neurite and synapse locations. In the astrocyte-rich condition, eight micrographs were randomly selected and four micrographs that also had synapses in the medium were evaluated to obtain four analyses per coverslip. For the astrocyte-poor condition, four-electron micrographs were randomly selected from each of the three coverslips for a total of 12. Each process encountered along a line drawn through the depth of the culture in the middle of each of the selected micrographs was identified as glial or neuronal. Then the depths of the top glial layer, middle neuropil, and bottom glial layers were computed. In addition, all synapses and vesicle-containing processes occurring under the top glial layer in the 12 micrographs from the astrocyte-rich condition were counted to estimate their relative frequencies along the culture length.

Statistical analyses

The RS1 statistical package (Bolt, Beranek and Newman, Cambridge, MA) was used to obtain correlations and comparisons described in the Results.

RESULTS

In phase-contrast light microscopy, astrocyte-rich cultures have a dense layer of astrocytes apparently covering the dish beneath round, phase-bright neurons (Fig. 2a). Astrocyte-poor cultures have somewhat fewer neurons and lack the dense plexus of astrocytes (Fig. 2b). In both culture conditions, neurites are dark processes (arrows) extending from the phase-bright cells.

Ultrastructural components of astrocyte-rich cortical cultures

The astrocyte-rich cultures were characterized by three rather distinct layers: the top glial layer, the middle neuropil layer, and the bottom glial layer (Fig. 3a). The top glial layer contained astrocytes and their processes; the middle neuropil layer contained neurons, dendrites, axons, synapses, and some glia; and the bottom glial layer contained only astrocytes and astrocytic processes. Where the top glial layer was discontinuous, the underlying neuropil tended to be thinner than in portions of the culture having a continuous top glial layer (Fig. 3b). Astrocytes were characterized by bundles of fibrils and glycogen

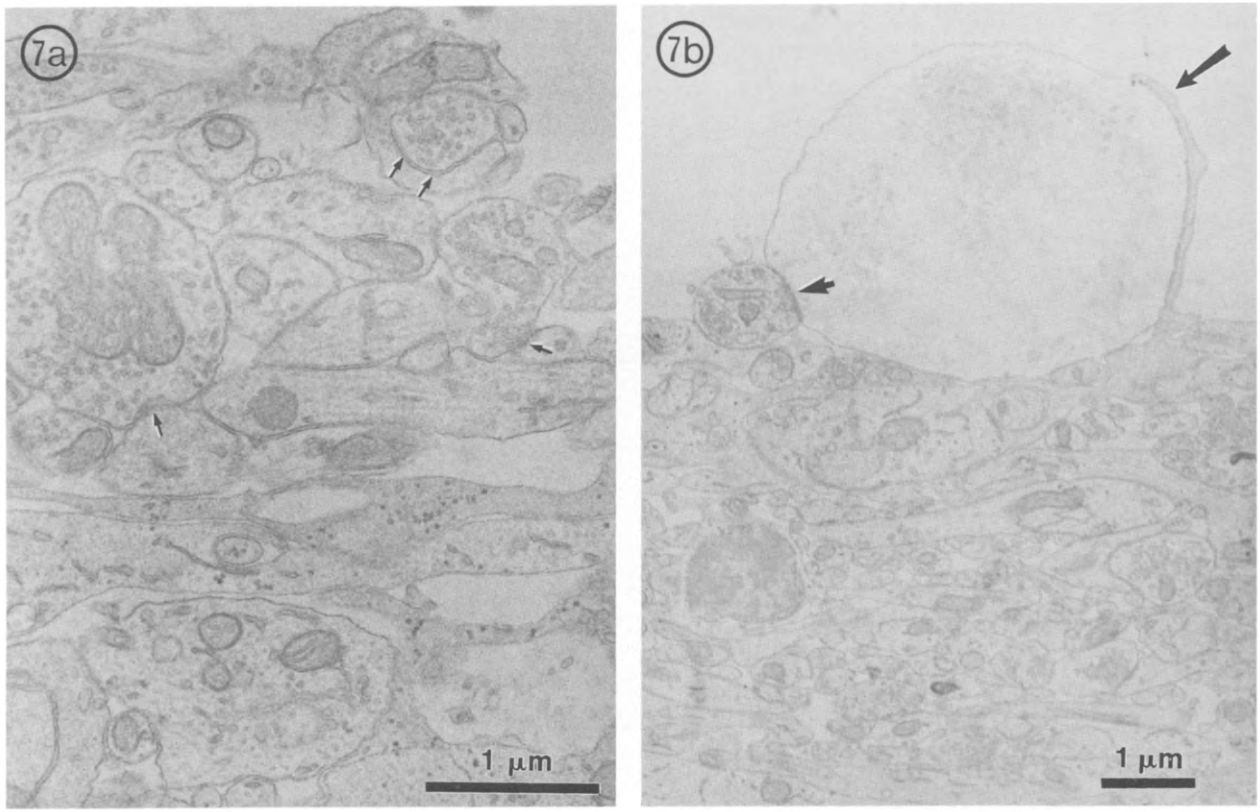

Fig. 7. Synapses above the top glial layer of astroctye-rich cultures. (a) Synapses or parts of synaptic complexes (arrows) at the edge of a neurite cluster at the culture–medium interface of an astrocytic-rich culture. (b) Presynaptic bouton and postsynaptic density (short arrow) on a large swollen process adjacent to an astrocytic finger (long arrow) which projects up from the top glial layer at the culture–medium interface of an astrocytic-rich culture.

granules in the cytoplasm (Fig. 4a). At the medium surface, overlapping astrocytic processes segregated the underlying neuropil from the medium (Fig. 4b). Dark staining material obscured the intermembrane zones of adjacent astrocytic processes (Fig. 4c).

Neuron-like cells lacked these astrocytic components and neurons were unambiguously identified by the presence of axosomal synapses (Fig. 5a). Clusters of neurites located outside the top glial layer were identified by the presence of dendrites, axons, synapses, and vesicle-containing processes within the clusters (Fig. 5b). Small protrusions from the top astrocytic processes referred to as "astrocytic fingers" penetrated and often surrounded parts of these neurite clusters.

Synapses were identified by the presence of a presynaptic axon containing small vesicles, and a postsynaptic element with an asymmetric postsynaptic density or a symmetric pre- and postsynaptic density. Synapses were found beneath the top glial layer on dendritic spines (Fig. 6a, b) and dendritic shafts (Fig. 6c, d). Some synapses occurred between morphologically normal axons and dendrites within neurite clusters or adjacent to the clusters and in the medium (Fig. 7a). Presynaptic axons were also found to synapse with empty swollen processes above the glial layer that were presumed to have been dendritic (Fig. 7b).

Ultrastructural components of astrocyte-poor cortical cultures.

In contrast with the astrocyte-rich cultures most of the astrocyte-poor cultures had only the neuropil layer. This layer was thinner in astrocyte-poor than in astrocyte-rich cultures, and contained few if any glia (Fig. 8). Synapses located on dendritic spines (Fig. 8a,b) and dendritic shafts (Fig. 8c) were freely

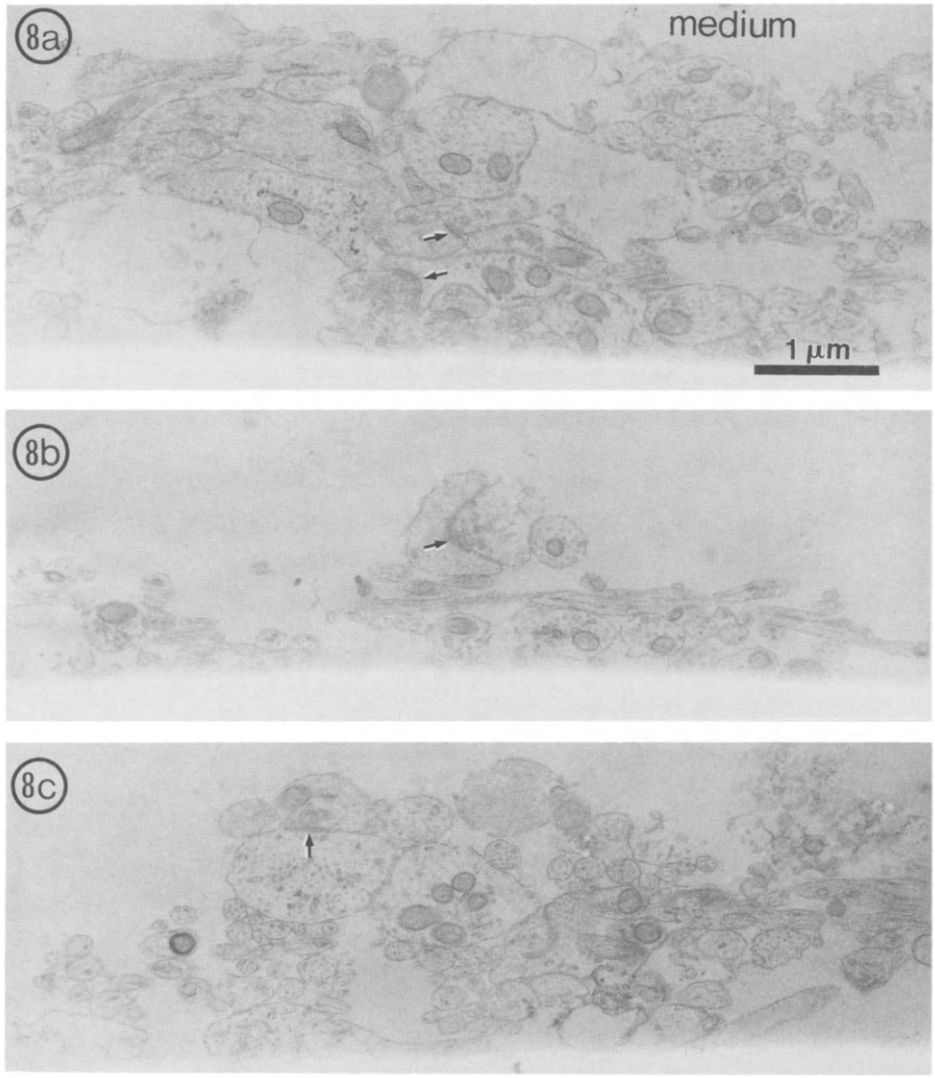

Fig. 8. Full depths of astrocyte-poor cultures contain primarily neuropil that is "freely exposed" to the surrounding medium. (a) Synapses on dendritic spines (arrows) located within this neuropil and (b) the culture–medium interface. (c) Synapse on a dendritic shaft (arrow) at the culture–medium interface. Scale bar in a also applies to b and c.

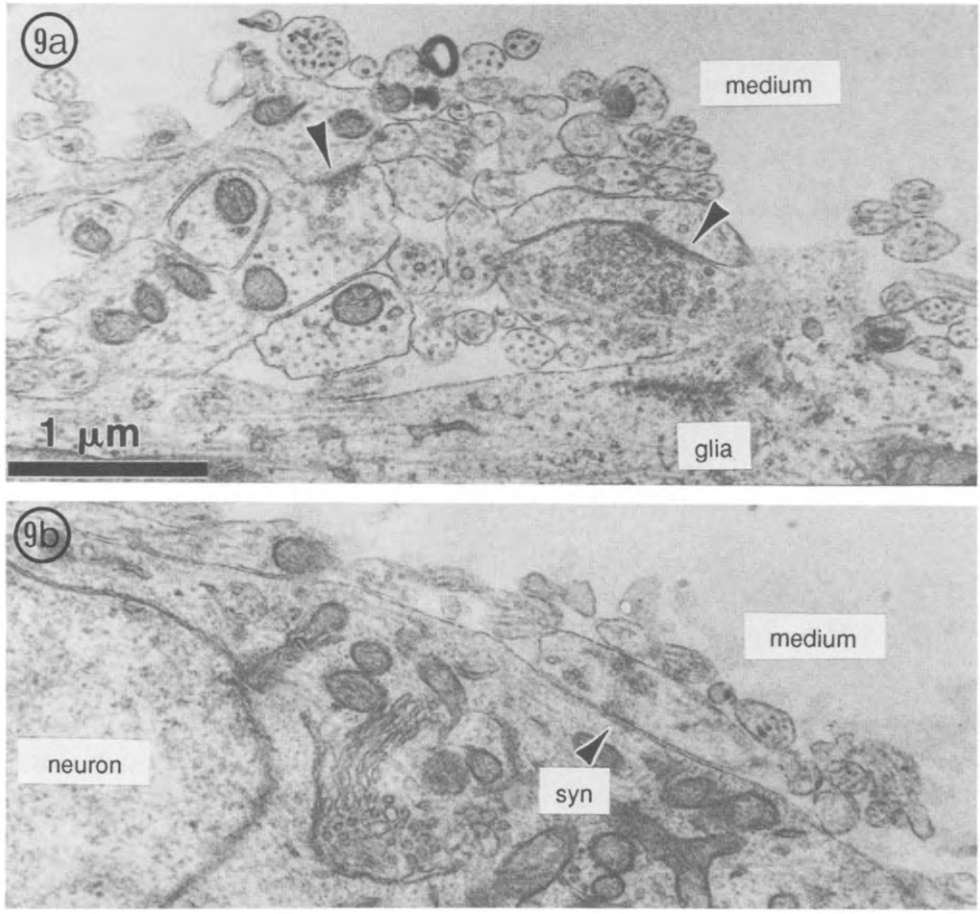

Fig. 9. Other synapses in astrocyte-poor cultures. (a) Synapses (arrows) occur in a neurite cluster on top of a glia-like process. (b) Axosomal synapses were found at the culture–medium interface. Scale bar in a also applies to b.

exposed to the surrounding medium. Some synapses were found in neuron clusters on top of glia-like processes (Fig. 9a). Neuron-like cell bodies were somewhat more flattened in astrocyte-poor than in astrocyte-rich cultures, and lacked the fibrils and glycogen granules of astrocytes. Neurons were unambiguously identified by the presence of axo-somatic synapses which were located on the top surface of the cell, freely exposed to the medium (Fig. 9b).

Quantification of the relative thickness of the top glial, middle neuropil, and bottom glial layers in astrocyte-rich and astrocyte-poor cultures

There was considerable variability in the total thickness of the astrocyte-rich cultures which was also reflected in the variability of the thicknesses of the three layers (Fig. 10a). Nevertheless, the thickness of the neuropil layer was found to be proportional to the thickness of the top glial layer, but not the bottom glial layer, in the astrocyte-rich cultures ($r = 0.74$, $P < 0.005$). In contrast, the astrocyte-poor cultures had a significantly thinner layer of neuropil when compared to the astrocyte-rich cultures ($P < 0.002$), with no correlation between the neuropil thickness and the glial components (Fig. 10b).

Quantification of the relative distribution of astrocytes, neuropil, and synapses at the culture–medium interface of astrocyte-rich and astrocyte-poor cultures and synapse density in the depth of the astrocyte-rich cultures (*Table* 1)

The astrocyte-rich cultures were continuous across the entire length of the photomontages while the more sparsely plated astrocyte-poor cultures had empty space for 10% of the culture–medium interface. Nearly all of the astrocyte-rich culture surface had a continuous top glial layer, while less than 1% of the astrocyte-poor culture surface had overlying glia. Neuronal cell bodies were freely exposed to the medium in both culture conditions and only four neurite clusters were encountered outside the top glial layer in the astrocyte-rich condition. All of the synapses and vesicle-containing processes observed in the astrocyte-poor condition were located such that they were freely exposed to the medium, while most of the synapses in the astrocyte-rich culture were

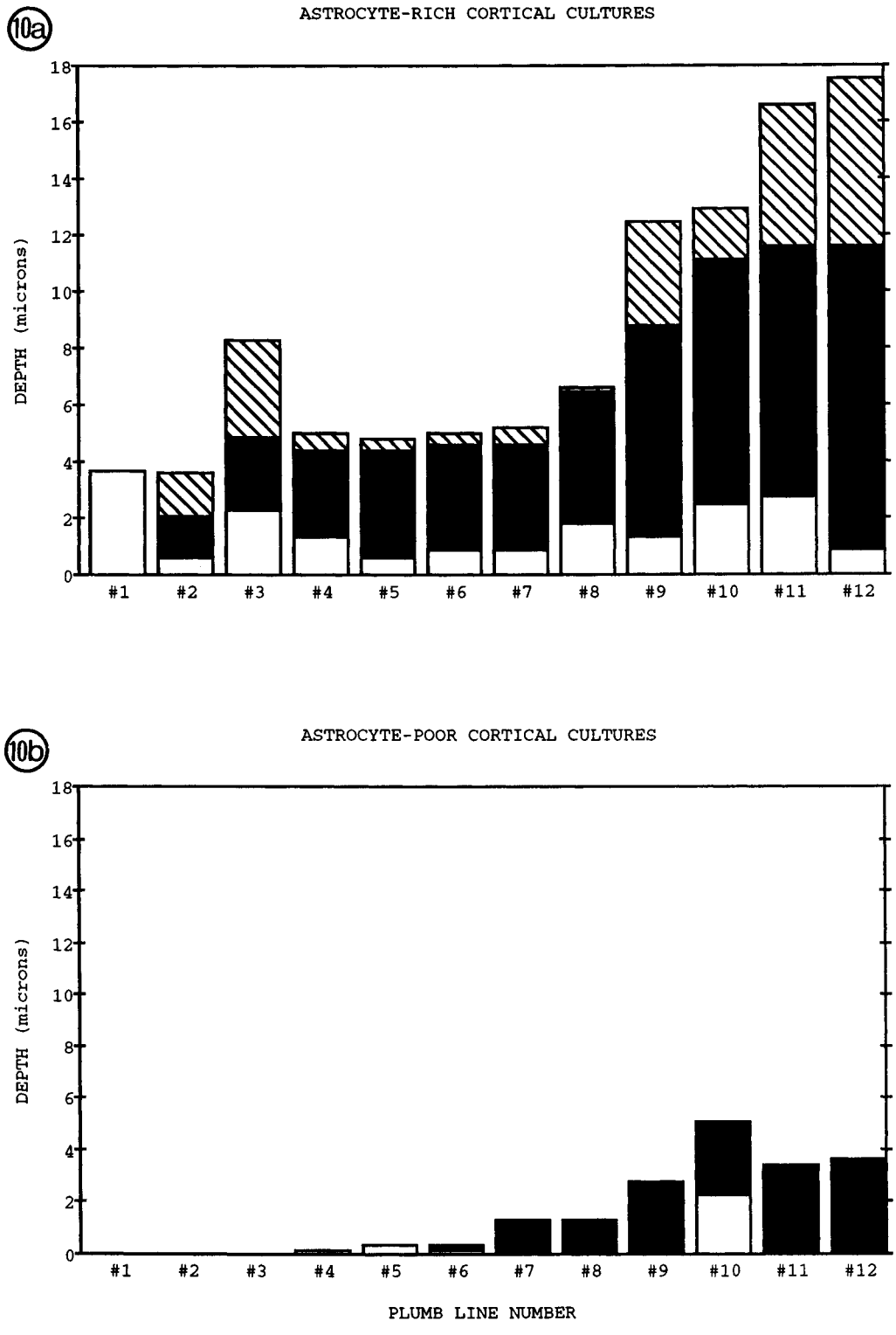

Fig. 10. Depth of top glial (hatched), neuropil (filled), and bottom glial (open) layers at randomly selected positions (plumb line number) in the astrocyte-rich (a) and astrocyte-poor (b) cultures.

Table 1. Structural components of astrocyte-rich and astrocyte-poor cultures

Component	Astrocyte-rich $n = 3$	Astrocyte-poor $n = 3$
Total length of CMI examined (μm)	1850	1980
Length of CMI per coverslip (μm)	616 ± 116	660 ± 211
Per cent length with no tissue culture present	0%	$10 \pm 8\%$
Per cent length with a TGL	$99 \pm 1\%$	$0.8 \pm 1\%$
No. of neuronal somas encountered at CMI	8 ± 3	1.3 ± 1.5
No. of neurite clusters encountered above the TGL	1.3 ± 1.5	n/a
No. of synapses encountered under neuronal somas	1 ± 2	0
Frequency of synapses at the CMI (no. per 100 μm)	0.5 ± 0.8	4 ± 3
Frequency of synapses beneath TGL (no. per 100 μm)	45 ± 40	n/a
Frequency of vesicle-containing profiles at the CMI (no. per 100 μm)	1 ± 0.9	3 ± 0.6
Frequency of vesicle-containing profiles beneath TGL (no. per 100 μm)	156 ± 111	n/a

Frequencies are normalized per 100 μm of length along the photomontages, synapses had both pre- and postsynaptic features visible, vesicle-containing profiles contained the small synaptic-like vesicles typically found in presynaptic axonal boutons but no postsynaptic element was seen in the plane of section; n is the number of coverslips on which continuous montages were photographed. The frequency of synapses and vesicle containing profiles located under the top glial layer (TGL) in the astrocyte-rich cultures were estimated from the 12 sample photomicrographs used for the plumb line analyses in Fig. 10a. The estimates were obtained by counting the number of synapses (mean observed = 6 ± 5) or vesicle-containing profiles (mean observed = 21 ± 14) observed in the depth of the culture and dividing by the length of the culture over which the counts were made (mean = 13 ± 2 μm) and normalizing for 100 μm of culture length to compare with the frequencies of synapses observed at the culture–medium interface (CMI).

located beneath the top glial layer. In addition, the frequency of synapses along the culture length was nearly 10-fold higher in the astrocyte-rich than in the astrocyte-poor cultures.

DISCUSSION

Cortical cultures have been used extensively to examine mechanisms of glutamate toxicity *in vitro*. This discussion examines the role of anatomical relationships between the astrocytes and neurons in the differential sensitivity to glutamate toxicity observed in astrocyte-rich and astrocyte-poor cultures. An obvious candidate conferring resistance to neurons *in vivo* against glutamate toxicity is glutamate uptake. However, uptake has been less plausible as the only protective mechanism *in vitro* because the thin layer of tissue is confronted with a relatively large reservoir of medium containing the substance of interest. Especially for short exposure periods, uptake mechanisms would not be expected to deplete significantly the glutamate in the medium. Neuronal somas and axosomal synapses are not covered by glia in either astrocyte-rich or astrocyte-poor cortical cultures. Since only a 5-min exposure to glutamate can be toxic,[8] the occurrence of even avid uptake would appear to be irrelevant, if the somas were the site of the toxic injury.

Our ultrastructural observations suggest that astrocytes form a physical buffer between the medium and most dendrites, axons and synapses in the astrocyte-rich cultures. If dendrites and axo-dendritic synapses were the primary target for glutamate agonists, then a combination of the physical buffer and glutamate uptake could play an important role in protecting neurons from glutamate toxicity in the astrocyte-rich cultures. The permeability of the astrocytic physical buffer has not yet been determined; if it were impervious glutamate uptake would not be required at all. Tight junctions were not frequently observed, suggesting that the physical buffer of the astrocytes may be relatively loose. In addition, since the transport properties of glutamate agonists have been shown to play an important role in determining their relative potencies, it is likely that astrocytic uptake mechanisms do play an important role in protecting neurons in culture against glutamate toxicity.[28] If the physical buffer were to reduce diffusion of substances from the extracellular medium, then the uptake process might be able to maintain glutamate concentrations low enough within the interstitium of the astrocyte-rich cultures to protect the neurons.

In this discussion, glutamate uptake has been assumed to be astrocytic in location; however, neuronal glutamate uptake could also play an important role in clearing glutamate and maintaining extracellular levels below the toxic threshold, especially in regions of the cultures where the neuropil is well developed. Neurites and synapses occurring within neuropil clusters above the top glial layer of astrocyte-rich cultures were structurally similar to neurites located deep within the cultures. In contrast, isolated processes located above the top glial layer, that had synapses and therefore were presumed to be dendrites, were grossly swollen, a characteristic of neurites suffering from glutamate toxicity *in vivo*.[30] The concentration of glutamate normally found in the medium of astrocyte-rich cultures is within a range (2–15 μM) that is toxic to neurons in the astrocyte-poor cultures.[26] The concentration of glutamate in the astrocyte-poor cultures must be kept at less than 1.6 μM by using conditioned medium[26] in order for the neurons to survive, and for the normal neuronal and synaptic morphology described here. Perhaps synapses in the neurite clusters of astrocyte-rich cultures are protected from the relatively high glutamate concentrations

normally found in the medium of astrocyte-rich cultures by a combination of local neuronal and astrocytic glutamate uptake. The glia underlying the neurite clusters and the isolated dendrites had finger-like projections positioned such that they may have been in the process of "surrounding" the neurites at the time of fixation. Substantial neuropil occurred under the top glial layer in astrocyte-rich, but not astrocyte-poor cultures, and the width of this neuropiwas related to the amount of glia between it and the extracellular medium. The explanation for the apparent increase in neuropil in the astrocyte-rich cultures is unclear, but could be due to a greater number of neurons, decreased glutamate receptor stimulation favoring neurite outgrowth, or a trophic effect of the astrocytes. In contrast, neurite outgrowth may be inhibited by excessive glutamate stimulation in the astrocyte-poor cultures or in regions of the astrocyte-rich cultures where the protective layer of astrocytes is thin.[17]

CONCLUSIONS

Extracellular glutamate concentrations in hippocampus have been measured at 2 μM.[6] This presumably is the extracellular concentration of glutamate in the presence of astrocytes *in vivo*. Since the EC_{50} for the neurotoxic effects of glutamate on cortical neurons in astrocyte-poor cultures is 2–5 μM, there are likely to be mechanisms in place for protecting neurons from the toxic effects of the extracellular concentration of glutamate normally present *in vivo*. One possibility is that astrocytes protect neurites *in vivo* much as they do *in vitro*, by physically isolating sensitive regions of the neuronal membrane from ambient glutamate.[23,31] Here too, the physical buffer may allow glutamate uptake by the astrocytes to maintain a lower local concentration thereby protecting the neurons. In any case, there does not seem to be a need to postulate a large rise in extracellular glutamate as the primary event in glutamate toxicity. Rather, glutamate toxicity may be caused by some compromise of the mechanisms normally at work to protect neurons and their processes from exposure to concentrations of glutamate that are close to those normally present in the extracellular fluid of the brain.

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