The Excitatory Neurotransmitter Glutamate Causes Filopodia Formation in Cultured Hippocampal Astrocytes

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ABSTRACT Can neurons induce surrounding glia to provide a more favorable microenvironment? Synapses and nerve growth cones have been shown to release neurotransmitters (Hume et al. Nature 1983;305:632–634; Kater et al. Trends Neurosci. 1988;11:315–321; Young and Poo. Nature 1983;305:634–637) providing a possible mechanism for this type of control. The excitatory neurotransmitter glutamate induces an increase in the number of filopodia on the surface of astrocytes cultured from the neonatal rat hippocampus. This seems to be associated with a receptor-mediated event that is activated to a lesser degree by the quisqualate and kainate, but not NMDA receptors. In addition, time-lapse video recordings have revealed a rapid extension of filopodia from the apical margins of cells treated with glutamate. The apical margins of glutamate-treated cells studied with electron microscopy contained dense cortical actin networks that are devoid of microtubules. Coated pits are often seen to invaginate from the apical membrane in the vicinity of filopodia. A receptor-binding step may be followed by a rapid reorganization of cortical actin resulting in actin-containing filopodia. This process may be mediated by inositol lipid hydrolysis. Pyramidal neurons settled on glial cultures induced filopodia to form around the entire margin of growth cones and neurite tips suggesting that these events might occur in situ.

INTRODUCTION

In the central nervous system neurotransmitters play an elemental role in developing nervous circuitry (Lipton and Kater, 1989; Mattson, 1988; Mattson et al., 1988), in guiding neuroplasticity (Bear and Singer, 1986; Cohan and Kater, 1986; Haydon et al., 1985; Mattson et al., 1988), and in modulating synaptogenesis (Chang and Greenough, 1984; Sutula et al., 1988). They also may be involved in neurite elongation and branching (Brewer and Cotman, 1989; Lipton, 1988; Mattson and Kater, 1987; Mattson et al., 1988; McCobb and Kater, 1986; Pearce et al., 1987). Recent studies suggest that at low concentrations excitatory neurotransmitters stimulate neural sprouting (Cornell-Bell et al., 1988; Patterson, 1988). However, at intermediate levels they halt neurite elongation, and at somewhat higher concentrations they dramatically prune dendrites though not axons (Lipton and Kater, 1989; Mattson et al., 1988). At high levels glutamate and related excitatory amino acids will cause striking neurodegeneration (Choi, 1990; Wiley-Liss, Inc.)
ASTROCYTES FORM FILOPODIA AFTER GLUTAMATE

Fig. 1

E  NEURONS: MEAN FILOPODIAL PROJECTIONS

F  GLIA: MEAN FILOPODIAL PROJECTIONS

PROJECTIONS/µM MEMBRANE

CONTROL  GLUTAMATE

0.0  0.2  0.4  0.6

PROJECTIONS/SQUARE

CONTROL  GLUTAMATE

0  1  2

Fig. 1.
We have found that glutamate also induces major morphological changes in cultured hippocampal astrocytes, namely an increase in the number of filopodia on the cell surface. This seems to be associated with a receptor-mediated event involving activation of the quisqualate and kainate, but not NMDA receptors.

Interaction between neurons and glia also plays an important part in the normal development and function of the nervous system. Glia are not only crucial for the guidance of neurons and axons to their appropriate sites (Bastiani and Goodman, 1986; Edmonson and Hatten, 1987; Hatten and Liem, 1981; Hatten et al., 1984; Rakic, 1971; Trenkner and Sidman, 1977), but also influence neuronal morphogenesis in the peripheral (Mudge, 1984) and central nervous systems (Banker, 1980). For example, when mesencephalic neurons are plated with glia from striatum, the shapes of their neuritic arbors change (Barbin et al., 1988; Denis-Donini et al., 1984). Astroglia also regulate the interstitial fluid environment of neurons in the adult brain by maintaining favorable ionic and neurotransmitter levels (Cserr and Bundgaard, 1986). Moreover, neuronal behavior can be related to astroglial morphology: neurons migrate along Bergman-like glia, but not along the stellate form in cerebellar cultures (Hatten and Mason, 1986).

An interesting question is whether glial qualities that influence neuronal behavior are intrinsic to particular glial subtypes (see Raff et al., 1983), or whether neurons influence the glia around them to provide a favorable environment. The neuronal microenvironment has been shown to influence lineage decisions of multipotential glial progenitor cells from optic nerve and cerebellum (Levine, 1989). Both synapses and nerve growth cones have been shown to release neurotransmitter (Hume et al., 1983; Kater et al., 1988; Young and Poo, 1983) and neurons could influence surrounding astrocytes by this mechanism (Haydon et al., 1985). We have found that nerve growth cones from hippocampal neurons are capable of stimulating underlying astrocytes to sprout filopodia. Filopodia form around the entire margin of growth cones and neurite tips, but not in the vicinity of the nucleus or radiating neurites.

Recently, different motile activities in neuronal growth cones have been related to interactions of the cytoskeleton (Tosney and Wessells, 1983). Filopodia that protrude from the growth cone’s cell surface are filled with actin, which presumably provides both the force and the structural elements for protrusion (Johnston and Wessells, 1980). Interestingly, cytochalasin-B treatment, which disrupts the actin network of growth cones, results in impairment of their pathfinding ability (Bentley and Toroaian-Raymond, 1986; Burmeister and Goldberg, 1988; Marsh and Letourneau, 1984). The tips of filopodia play an important role in the formation of adhesive connections (Bray, 1987; Bunge, 1986; Letourneau, 1981; Tsui et al., 1985, 1988), and there is some evidence (Tsui et al., 1985) that adhesive filopodia have the potential for developing into synapse-forming branches of mature axons. Although the examples above are of neuronal filopodia, one could postulate that filopodia formed on the astrocyte surface may have an adhesive role that could aid growth cones in pathfinding. It would be interesting to find out if anchoring and cell adhesion molecules (i.e., N-CAMS, extracellular matrix proteins, or adherons) found to be involved in adhesive junctions formed by neuronal filopodia (Tsui et al., 1988) are involved in contacts between neurons and astroglial filopodia.

**MATERIALS AND METHODS**

**Cell Culture and Solutions**

Mixed hippocampal cultures (Finkbeiner and Stevens, 1988) isolated from the CA1 and CA3 region of neonatal rat were plated at low density (1,000 cells/cm²) on glass coverslips coated with poly-d-lysine. Cells were isolated in Earl’s Minimal Essential Medium (MEM) without phenol red (GIBCO, Grand Island, NY). Isolated cells were settled in a complete medium containing Earl’s MEM, Penn/Strep, and fetal bovine serum (GIBCO). Following 4–7 days maturation in culture, cells were used for microscopy. These cultures stained positively (> 98% of cells) with anti-glial fibrillary acidic protein antibody (Amersham, Arlington Heights, IL, and Sigma Chemical, St. Louis, MO) and negatively with A2B5 antibody (gift from Dr. Beth Friedman).

Normal saline containing 137 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 25 mM sorbitol (pH 7.2, 295 mOsm) was used to make the experimental solutions. Glutamate (100 μM), and the glutamate agonists quisqualate (100 μM), kainate (100 μM), and NMDA (100 μM) were all purchased from Sigma Chemical. Ca²⁺-free Ringer’s contained 108 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 3 mM EGTA, and 3 mM HEPES (pH 7.2, 292 mOsm).

**Electron Microscopy**

**Transmission electron microscopy**

After treating with glutamate for 30 sec (Fig. 1) or 5 min (Figs. 5, 6) cells were fixed for 5 min with 3% glutaraldehyde (EMS, Ft. Washington, PA) in normal saline containing HEPES buffer (pH 7.2). Following fixation for 15 min at room temperature, cells were rinsed in normal saline. Cultures were post-fixed in 0.5% osmium with 0.8% potassium ferricyanide (McDonald, 1984). Coverslips were inverted into a mold containing EMBED 812 (EMS) according to Coulter (1967). An aclar mask (Allied Chemical, Morristown, NJ) was cut to fit around the periphery of the coverslip before embedding (Buchanan et al., 1989). Following polymerization, the aclar mask is removed exposing the glass coverslip to hydrofluoric acid (Moore, 1975). Sections stained with 2% uranyl acetate and lead citrate (Venable and Coggeshall, 1965) were examined at 60 KV in a JEOL 100 CXII electron microscope.
Scanning electron microscopy and morphometry

Cultures of glial cells were grown on coverslips and were fixed as previously described for TEM. Following the osmium ferricyanide step the coverslips were broken into smaller pieces, and the cells were dehydrated using an ETOH series (50, 70, 80, 95, 100%). They were transferred to a freon 113 series (25, 50, 75, 100%) and critically point dried in a Polaron E3000 critical point dryer. Coverslip pieces were mounted on copper specimen holders, transferred to a Denton vacuum evaporator (bench top turbo), and coated with 50–75 nm (as determined by an Inficon XTC thin-film thickness and rate monitor) of platinum using a Denton DSM-3000 cold sputter module. Scanning micrographs were taken using an ASID scanning attachment on the JEOL 100 CXII electron microscope and Polaroid Type 55 positive/negative film.

A minimum of 25 scanning micrographs were taken for each experimental situation. For all comparisons cells were fixed, critically point dried, and coated at the same time. A coherent point grid (d = 15 mm) was dropped on a micrograph in a random fashion. All the projections in every 15 mm square were counted using the following guidelines: 1) the square was completely filled with astrocyte surface; and 2) projections were counted if they fell into the range of > 0.5 mm and < 5 mm in length. The total number of projections in a micrograph was then divided by the total number of squares counted to get the average number of projections per square for each micrograph.

Video Microscopy

Cells were continuously perfused and time-lapse Nomarski video recordings were made using a SIT...
camera (Hamamatsu Corp., Middlesex, NJ) mounted on a Zeiss IM 35 inverted microscope with a Zeiss 63X/1.25 NA Neofluar objective. An Imaging Technology (Woodburn, MA) Series 151 image processor controlled by a host IBM-AT computer, was used for boxcar (eight video frames) or rolling frame averaging (0.5 sec time constant). Video images were recorded on an optical memory disk recorder (Panasonic OMDR model TQ-2026F). An Imaging Technology PFG plus640 personal frame grabber controlled by a Compaq 386 personal computer with Image-Pro software (Media Cybernetics, Bethesda, MD) was used to acquire single images from the OMDR for digital image processing. A slide-stretch contrast enhancement and an unsharp mask non-convolution filter (Pratt, 1978) were used. Photographs were taken directly from a high-resolution (1,024 by 680 pixel array) Hitachi monitor (model HM-4115-D-BA-0, Woodbury, NY) using a 35 mm camera with a macro lens and Kodak T-Max ASA 100 film.

Immunofluorescence and cytochemical staining

Cells were exposed to 2 min glutamate (100 μM) and were fixed for 5 min with 2% formaldehyde and 0.05% glutaraldehyde in the Ca²⁺-free saline solution described above. Cells were then extracted for 2 min with 1% Triton X-100 (Polysciences, Warrington, PA) in Ca²⁺-free saline to permeabilize cell membranes. The following procedure was used for anti-GFAP and A2B5 antibody staining. Five percent normal goat serum (NGS) was applied as a block for 5 min. A primary antibody was diluted 1:200 from a normal stock of 50 pg/ml and cells were incubated for 2 hr in this solution. After 3 washes with calcium-free saline a biotinylated secondary antibody (horse anti-mouse, from Vector Stains, Burlingame, CA) in Ca²⁺-free saline with 1% NGS was applied for 15 min. Following 3 washes with Ca²⁺-free saline, FITC or rhodamine-labelled avidin (50 μg/ml, Vector Stains, Burlingame, CA) was applied for 15 min. Actin was stained with 8 IU/ml rhodamine-labelled phalloidin (Molecular Probes, Eugene, OR) for 5 min.

RESULTS

In recent experiments in our lab, (Smith and Jahr, unpublished observations; Cornell-Bell et al., 1988) glutamate (1mM) was applied onto the surface of pyramidal neurons in a continuous perfusion chamber and was followed using computer enhanced video microscopy. Following glutamate application, filopodia erupted from the surface of the apical dendrites at a velocity of 1–2 μm/sec. We have completed a morphometric study that confirmed the increased presence of filopodia on glutamate-treated vs. control pyramidal neurons; in the process it became apparent that the surface membranes of the underlying astrocytes were at least as responsive to glutamate as the neurons. A second morphometric study was done comparing the surfaces of astrocytes treated with a bath application of 100 μM glutamate to control astrocytes exposed to normal saline solution.

Filopodial eruption from the surface membrane of hippocampal pyramidal neurons and astrocytes is illustrated in the scanning electron micrographs of Fig. 1. Following glutamate application (30 sec), the number of filopodia on the cell nucleus and peripheral neurites (Fig. 1A) is greatly increased over the number seen on control neurons (Fig. 1B). Small numbers of filopodia are present on the apical dendrites of untreated cells (see arrowheads, Fig. 1B). Astrocytes sprout innumerable filopodia in response to glutamate (see bold arrowheads in Fig. 1C), whereas the surface of control astrocytes remains smooth (Fig. 1D). Also, the margins of the glutamate-treated cells (see arrows in 1C) are elaborated into well-defined filopodia not seen along margins of control cells.

The results from morphometric analysis of glutamate-treated neurons (315 measurements from 52 scanning electron micrographs) compared to control neurons (303 measurements from 41 micrographs) is shown in Fig. 1E. A t-test was run on the data, which had been transformed to give a parametric distribution. There is a significant increase (P<.001) in the number of filopodia/μm membrane for glutamate-treated cells (mean = 0.64 ± 0.02 filopodia/μm membrane) when compared to control cells (mean = 0.46 ± 0.03 filopodia/μm membrane). A similar comparison was made on glutamate-treated astrocytes (810 measurements from 27 micrographs) vs. control astrocytes (755 measurements from 25 micrographs). A significant increase (P<.001, Mann Whitney U-test) in the number of filopodia/15 mm grid square was counted for astrocytes exposed to glutamate (mean = 1.9 ± 0.1 filopodia/15 mm square) when compared to control astrocytes (mean = 0.9 ± 0.1 filopodia/15 mm square) (Fig. 1F).

Time-lapse videos using Nomarski optics were made of filopodial protrusion from the margins of astrocytes plated on glass coverslips. The panels in Fig. 2A-D illustrate the rapid extension of filopodia at 6, 33, and 60 sec after glutamate addition to the perfusion system. Very long filopodia develop at sites marked by the arrows in this figure. In addition, lamellipodia develop (bold arrow at 6 sec), which further elaborate into numerous shorter filopodia (arrowheads at 33 sec). In the culture studied the glutamate effects were seen in roughly 38% of the cells. The maximum number of filopodia were counted on cells 4 days after plating, and older cultures were unresponsive to glutamate application at the cell margins. A more complete study involving the developmental time course of filopodial eruption would be valuable.

Filopodial characteristics were analyzed from the video time-lapse studies. The velocity of filopodial outgrowth from astrocyte margins (0.092 ± 0.006 μm/sec) was slower than the velocity of filopodia emerging from the dendrites of pyramidal neurons (1–2 μm/sec) reported by Cornell-Bell et al. (1988). Astrocyte filopodia attained lengths (3.2 ± 0.2 μm) comparable to those measured from pyramidal neurons (2.8 ± 0.3 μm).
Fig. 3. A: Filopodia (f) are evident in transmission electron micrographs. The apical astrocyte margin contains a network of cortical actin (a), which excludes microtubules (mt, arrows). Microtubules are evident beneath this zone (arrows) often associated with organelles. Intermediate filaments (i) are commonly seen throughout the cytoplasm. Coated pits (arrowhead) are often seen in the cytoplasm beneath the filopodia. B: Actin extends into the filopodia whereas microtubules (mt, arrows) are more commonly found parallel to the apical surface. Intermediate filaments (i) are extensive in the cytoplasm but do not appear to extend into the filopodia; however, colloidal gold labelling of anti-GFAP is needed to confirm this observation. C: Coated pits (bold arrows) are formed from invaginations of the apical membrane near filopodia (f) and may be an indication of receptor-mediated endocytosis.
An electron microscopic study revealed numerous filopodia (Fig. 3). The apical margins of cells contain dense cortical actin networks that are devoid of microtubules. Microtubules are more commonly a component of the deeper cytoplasm (Fig. 3B) and are generally oriented parallel to the apical margin. Coated pits are often seen in the vicinity of filopodia (see arrowhead in Fig. 3A) and are seen to invaginate from the apical membrane (see bold arrows, Fig. 3C). In order to confirm the presence of actin in filopodia astrocytes were exposed to glutamate (100 μM for 2 min) and then stained with rhodamine-phalloidin (Fig. 4A). Even the smallest filopodia protruding from the cell margins stained brightly for actin. In contrast, microtubules (Fig. 4B) and intermediate filaments (Fig. 4C) were generally localized to the perinuclear regions of cells.

Once morphometry confirmed the relationship between glutamate and astrocyte filopodial formation, the basis for this activity was investigated in more detail. Astrocytes were exposed for 5 min to a control media containing fetal bovine serum (Fig. 5A) and also to media without serum (Fig. 5B). One possibility investigated was that filopodia are produced simply as a result of glutamate’s depolarizing effect. To test this, astrocytes were exposed for 5 min to a depolarizing concentration of 55 mM K+ (Fig. 5C). As a check for the filopodial reactivity of astrocytes exposed to these agents, the cells were also exposed to glutamate (Fig. 5D). An analysis of variance run on the data, which had been transformed to give a parametric distribution, showed strong evidence (P < .001) of a difference between the four means. There was no significant difference in the number of filopodia between cells exposed to control (mean = 0.80 ± 0.09 filopodia/15 mm grid square, 1,247 measurements from 36 micrographs), serum free (mean = 0.44 ± 0.06 filopodia/15 mm grid square, 735 measurements from 25 micrographs), and potassium (mean = 0.8 ± 0.1 filopodia/15 mm grid square, 717 measurements from 25 micrographs) solutions. There was a significant increase (P < .001) in filopodia on the surface of cells exposed to glutamate (mean = 2.3 ± 0.2, 802 measurements from 25 micrographs). This information is represented graphically in Fig. 5E.

Another possibility, that astrocyte filopodia formation is somehow associated with a receptor-mediated event was also explored using morphometry (Fig. 6). Astrocytes were exposed to six different treatments for 5 min: control and serum free media, glutamate, and the glutamate agonists quisqualate, kainate, and NMDA. The data were transformed to give a parametric distribution, and an analysis of variance gave strong evidence (P < .001) of a difference between the means of the six treatments. There was no significant difference in number of filopodia between control (mean = 0.8 ± 0.1 filopodia/15 mm grid square, 1,111 measurements from 36 micrographs) and NMDA (mean = 0.77 ± 0.08 filopodia/15 mm grid square, 1,056 measurements from 34 micrographs) treatments; however, there was evi-

Fig. 4. Immunohistochemical labelling of the cytoskeleton of the astrocyte exposed to glutamate. A: Astrocytes stained with rhodamine-phalloidin show actin extending into the cell periphery. Filopodia protruding from cell margins (arrows) stain brightly for actin. B: Astrocytes stained with a monoclonal to β-tubulin on the other hand, show greater staining in the perinuclear region with little microtubule staining at extreme cell margins. C: Likewise, more intermediate filaments are localized in the perinuclear region in astrocytes stained with anti-GFAP. Few if any of the small processes stain brightly for intermediate filaments.
Astrocytes form filopodia after glutamate, but not depolarizing K⁺ or serum-free media. The astrocyte surface in control (A) or serum-free (B) media is characteristically smooth with few filopodia evident. Following exposure to 55 mM K⁺ (C), the numbers of filopodia are not significantly greater than those counted for controls.

On the other hand, the surface of the astrocyte following glutamate (D) has a significantly greater number of filopodia when compared to the other treatments (see graph (E)). CTRL, control; SF, serum-free; K, 55 mM K⁺; Glut, 100 μM glutamate.
ence ($P < .01$) of a difference between these treatments and the serum free treatment (mean = 0.9 ± 0.1 filopodia/15 mm grid square, 969 measurements from 31 micrographs). There was a significant increase ($P < .01$) in the number of filopodia on cells exposed to quisqualate (mean = 2.0 ± 0.3 filopodia/15 mm grid square, 869 observations from 30 micrographs) when compared to the control, serum free, and NMDA treatments. Kainate also showed a significant increase ($P < .001$) in number of filopodia (mean = 2.6 ± 0.4 filopodia/15 mm grid square, 798 measurements from 26 micrographs) when compared to the control, serum free, and NMDA treatments. Although quisqualate had a significantly lower filopodial response than glutamate, there was no significant difference between the kainate and glutamate (mean = 4.4 ± 0.5 filopodia/15 mm grid square, 879 measurements from 29 micrographs) response. These results are graphically illustrated in Fig. 6A. Micrographs of the control and serum free treatments (Fig. 6B, C) show few filopodia. Likewise, there is no obvious sprouting from the surface of the NMDA-treated cells (Fig. 6D). Statistically, the number of filopodia following quisqualate (Fig. 6E) and kainate treatment (Fig. 6F) are similar. The surfaces of cells exposed to quisqualate, however, sprout filopodia that are shorter and smaller than the filopodia on the surface of a kainate-treated cell. The expected filopodial response following glutamate is shown (Fig. 6G) for comparison.

We were interested in whether neuronal cues play a part in filopodial formation on the astrocyte. Hippocampal pyramidal neurons were allowed to settle on astrocytes that were grown in control media containing serum. When neurons settled on top, they had a profound effect on the surface of the astrocyte (Fig. 7). Numerous glial filopodia develop upon contact with the pyramidal cell growth cone (Fig. 7A, D) and neurite endings (Fig. 7B, C). Very few filopodia sprout on the astrocyte membrane under the long stretches of neurites connecting the nuclear region and the periphery (Fig. 7B). Astrocytes that have no neuronal connections have the characteristically smooth surface seen in control situations (Fig. 7D).

**DISCUSSION**

Neurotransmitters, which have been shown to induce rearrangements of the morphology in neurons (Lipton and Kater, 1989; Mattson et al., 1987), apparently have a similar effect on astrocytes. Astrocytes exposed for short periods (30 sec to 5 min) to the excitatory neurotransmitter glutamate sprouted numerous filopodia from the cell surface. Filopodial extension does not seem to be due to the depolarization caused by the glutamate (100 µM), since glia exposed to a depolarizing 55 mM $K^+$ showed no filopodial response. Thus, a receptor-mediated mechanism seems to be at work. EM observations (cf. Fig. 3) showed coated pits on the apical membrane just beneath the filopodia. Uptake of ligand–receptor complexes is mediated by coated pits, and receptosomes are formed from these structures (Pastan and Willingham, 1985). Only the quisqualate and kainate receptors seem to be involved in filopodia formation; application of NMDA produced no filopodia. This is not surprising since recent findings indicate that astrocytes do not possess functional NMDA receptors (Bowman and Kimelberg, 1984; Cornell-Bell et al., 1990; Kettenmann and Schachner, 1985; Usowicz et al., 1989).

If filopodial protrusion is a receptor-mediated event, phosphoinositid turnover may play a part. Cells respond to external signals by utilizing a limited number of internal signal pathways. One such pathway is driven by inositol lipid hydrolysis, which initiates an intracellular calcium signalling cascade responsible for regulating a wide range of cellular processes (Berridge, 1988). Phosphoinositidases have been implicated in cell motility through modulation of profilactin (Lassing and Lindberg, 1988) and gelsolin (Janney et al., 1987), which regulate actin polymerization. When cells are stimulated there is a marked realignment of the actin network with phosphatidyl inositol [PtdIns(4,5)P$_2$] functioning either as a catalyst to alter activity of proteins that modulate actin polymerization or as a substrate used by the receptor to generate second messengers (Berridge, 1988; Forscher, 1989). Since filopodia produced by astrocytes following glutamate exposure primarily contain actin (phalloidin staining and EM evidence), the mechanism described above is a plausible explanation for their protrusion. Few if any filopodia are seen on astrocyte surfaces prior to glutamate exposure suggesting that filopodia form only after the receptor-mediated event.

There appeared to be a more robust filopodial response following 30 sec exposure to glutamate (cf. Fig. 1), i.e., filopodia were longer and thicker compared to 5 min exposure to glutamate (Figs. 5, 6). Others have shown that glutamate (100 µM) causes a graded reduction in the length of dendrites of pyramidal neurons of the hippocampus (Mattson et al., 1988). Possibly the decrease in astrocyte filopodial length following longer exposures to glutamate may proceed by a mechanism similar to the one responsible for the decrease in dendritic length.

Two separate filopodial sprouting events were seen on astrocytes: 1) filopodial production along the cell margin; and 2) filopodial production by the apical cell surface. Sprouting from astrocyte cell margins studied with video microscopy was seen as early as 2 days in culture and the response was maximal after 4 days in culture. Hippocampal pyramidal neurons exhibited maximal sensitivity to glutamate after 4 days in culture as well (Mattson et al., 1987). Focal application of glutamate to individual dendritic growth cones caused a retraction of filopodia and a reduction in dendritic length (Mattson et al., 1987). Neurotransmitter-releasing axonal growth cones were suggested to be involved in controlling the geometry of the dendritic arbor by acting on receptors that inhibited dendritic outgrowth and initiated synapse formation (Mattson
Fig. 6. A statistical comparison of the ability of glutamate and its agonists to induce filopodial formation on the astrocyte surface was made. In the graph (A) it becomes evident that the ability to form filopodia follows the sequence of glutamate > kainate or quisqualate, with NMDA having no ability to induce filopodial formation. CTRL, control; SF, serum-free; QU, 100 μM quisqualate; KA, 100 μM kainate; NM, 100 μM NMDA; GL, 100 μM glutamate. The surface of astrocytes exposed to NMDA (D) is as smooth as cells exposed to control media (B) or media with serum removed (C). Although the number of filopodia following quisqualate is statistically similar to kainate, the filopodia following quisqualate (E) are shorter and less robust than those seen following kainate (F). An example of the astrocyte surface following glutamate (G) is included for comparison.
Fig. 7. Pyramidal neurons induce filopodial formation on the surface of astrocytes. A: Pyramidal neurons were allowed to settle onto the surface of cultured astrocytes. Filopodia (f) formed on the astrocyte in the vicinity of the growth cone (g), whereas regions of the astrocyte membrane underlying the neurite (n) were smooth (s). B: Filopodia (f) on the surface of the astrocyte were also localized to a region around neurite endings and were never seen in the smooth areas (s) surrounding the nucleus (N). C: The area outlined in B is enlarged to clearly show filopodia (f) that develop in the vicinity of neurite endings. D: A montage illustrating that astrocyte filopodia are localized to peripheral regions of the neurons, which have settled on top. Astrocytes without neurons have characteristic smooth surfaces (s).

et al., 1987). Interestingly, at around 4 days in mixed hippocampal cultures pyramidal neurons form large numbers of synapses (Cornell-Bell, unpublished EM observations). It would be interesting to pursue whether the formation of filopodia along the astrocyte margin at day 4 was in any way related to junction formation between astrocytes. We have recently described a long-range astrocyte signalling system in which calcium is released from intracellular stores following glutamate stimulation (Cornell-Bell et al., 1990). A dynamic wave of calcium or second messenger is spread intercellularly through gap junctions between syncytial hippocampal astrocytes. Formation of junctions between astrocytes would be essential in the establishment of astrocyte communication. Recent work in our laboratory (Cooper and Smith, 1988) has further studied contact formation in mixed hippocampal cultures that have not been exposed to excitatory amino acids. Time-lapse studies have shown that the margin of the target cell, be it a neuron or glial cell, ruffles and often sends out filopodia prior to contact formation. Excitatory amino acids released by cells in these cultures may bind via a receptor and initiate the ruffling of the target cell membrane. It appears that these membrane phenomena are prelimi-
nary steps to contact formation so it is not unreasonable to think there may be a correlate at the astrocyte margin following glutamate stimulation in the present studies.

Filopodial production by the apical surface of the astrocyte in response to glutamate is a separate and distinct event that can be induced on cells kept 3–4 weeks in culture. We were able to induce filopodial formation on the surface of astrocytes by settling pyramidal neurons on top of glia. Filopodia formed in the region of the neuronal growth cones and neurite tips. Perhaps anchoring and cell adhesion molecules similar to those described for neurons, including extracellular matrix proteins and adherons molecules, may also be located on astrocyte filopodia (Tsui et al., 1988). Many studies have looked at adhesive filopodia on neurons (Bray, 1987; Tsui et al., 1988) and have suggested they may play a role in establishing synapse-forming branches of mature axons. If filopodia induced to form on the surface of the cultured astrocytes by the neuronal growth cone are responsible for anchoring the neuron to the glial surface, there may be some interesting implications in which astrocytes stabilize neurons during pathfinding and development of neuronal circuitry in embryogenesis. Alternatively, filopodia may grow from the glial surface in the direction of glutamate, a chemotactic signal released by the neuron. Extension of glial filopodia in areas surrounding growth cones or synaptic contacts may be a component of extracellular glutamate regulation (Schouboe et al., 1988).

We have reported that filopodia are elaborated from the surface of cultured hippocampal astrocytes following a bath application of the excitatory neurotransmitter glutamate. The glutamate agonists quisqualate and kainate, but not NMDA induce a similar, but less robust filopodial response to that following glutamate exposure. Our current thinking suggests that a ligand–receptor binding step may be followed by a rapid reorganization of cortical actin, which results in the formation of actin-containing filopodia. This process may be mediated by inositol lipid hydrolysis. The two separate filopodial sprouting events we have described (1) at the cell margin and (2) on the apical surface), may be important in junction formation and in stabilizing and anchoring neurons during development. Ideally, studies concerning the interaction between neurons and glia, including junction formation and the development of the glial syncytium and neuronal circuitry need to be done in intact tissue. Preliminary studies (Cooper et al., 1989) looking at intact developing neocortex and hippocampus with the confocal scanning laser microscope have shown that the technology to do these studies in living tissue is rapidly approaching.

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