GLIA 55:13–23 (2007)

Plasticity of Perisynaptic Astroglia During Synaptogenesis in the Mature Rat Hippocampus

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KEY WORDS
astrocyte; three-dimensional reconstruction; dendritic spine; ultrastructure; synaptogenesis; tripartite

ABSTRACT
Astroglia are integral components of synapse formation and maturation during development. Less is known about how astroglia might influence synaptogenesis in the mature brain. Preparation of mature hippocampal slices results in synapse loss followed by recuperative synaptogenesis during subsequent maintenance in vitro. Hence, this model system was used to discern whether perisynaptic astroglial processes are similarly plastic, associating more or less with recently formed synapses in mature brain slices. Perisynaptic astroglia was quantified through serial section electron microscopy in perfusion-fixed or sliced hippocampus from adult male Long-Evans rats that were 65–75 days old. Fewer synapses had perisynaptic astroglia in the recovered hippocampal slices (42.4% ± 3.4%) than in the intact hippocampus (62.2% ± 2.6%), yet synapses were larger when perisynaptic astroglia was present (0.055 ± 0.003 μm^2) than when it was absent (0.036 ± 0.004 μm^2) in both conditions. Importantly, the length of the synaptic perimeter surrounded by perisynaptic astroglia and the distance between neighboring synapses was not proportional to synapse size. Instead, larger synapses had longer astroglia-free perimeters where substances could escape from or enter into the synaptic clefts. Thus, smaller presumably newer synapses as well as established larger synapses have equal access to extracellular glutamate and secreted astroglial factors, which may facilitate recuperative synaptogenesis. These findings suggest that as synapses enlarge and release more neurotransmitter, they attract astroglial processes to a discrete portion of their perimeters, further enhancing synaptic efficacy without limiting the potential for cross talk with neighboring synapses in the mature rat hippocampus.

INTRODUCTION

Historically, astroglia were thought to provide only metabolic and physical support for neurons. It is now clear that astroglia are directly involved in neuronal signaling, even locally at synapses (Allen and Barres, 2005; Barres, 1991; Bergles et al., 1997; Hatton and Parpura, 2004; Lin and Bergles, 2004; Volterra et al., 2002; Volterra and Meldolesi, 2005). They are the primary source of energy for neurons (Brown et al., 2004) and serve to control ionic homeostasis and neuronal excitability by buffering potassium (Kofuji and Newman, 2004). Astroglia synthesize and recycle glutamate (Hertz and Zielke, 2004) and respond to synaptic release of neurotransmitters with both calcium waves and release of gliotransmitters that can further influence synaptic activity (Cornell-Bell et al., 1990a; Grosche et al., 1999; Pascual et al., 2005; Perea and Araque, 2005; Schipke and Kettenmann, 2004). Perisynaptic astroglial processes may detect spill out of glutamate and other substances from active synapses (Diamond, 2005; Rusakov and Kullmann, 1998), and respond structurally by extending and modifying their processes (Cornell-Bell et al., 1990b; Hirrlinger et al., 2004). Variation in synapse strength and the degree to which substances escape the perimeter might determine whether astroglial processes grow towards and ensheath parts of some synapses and avoid or retract from others (Cornell-Bell et al., 1990b; Hatton and Parpura, 2004). Recent studies show that astroglia secrete substances, such as thrombospondins and cholesterol, that are critical to the formation and function of synapses during development (Christopherson et al., 2005; Goritz et al., 2005; Mauch et al., 2001; Ullian et al., 2001; Ullian et al., 2004). Astroglial membranes also contain contact-mediated factors that influence synapse maturation (Hama et al., 2004; Mazzanti and Haydon, 2003; Murai et al., 2003). Thus, astroglia may influence where synapses form and how large they become.

Less is known about whether astroglia are similarly plastic or critical for synaptogenesis in the mature brain. In the mature brain, access to astroglial-secreted factors will be relatively limited because their levels decrease globally (Christopherson et al., 2005) and intervening structures fill the extracellular space (ECS), which was substantially greater during development (Fiala et al., 1998). The positioning of perisynaptic astroglia is diverse among mature brain regions (Chao et al.,

Grant sponsor: American Academy of Neurology; Grant numbers: NIH T32 NRSA NS045543, NIH Grants K010MH02000, NS21184, NS 33574 and EB002170.

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Received 19 June 2006; Revised 31 July 2006; Accepted 2 August 2006

DOI 10.1002/glia.20415

Published online 25 September 2006 in Wiley InterScience (www.interscience.wiley.com).

2002; Spacek, 1985); for example about 90% of cerebellar synapses have some perisynaptic astroglia, but less than 50% of hippocampal and cortical synapses having any (Grosche et al., 1999; Palay and Chan-Palay, 1974; Spacek, 1985; Ventura and Harris, 1999; Xu-Friedman et al., 2001). Here we compared perisynaptic astroglial processes in perfusion-fixed hippocampus and in hippocampal slices that had undergone substantial synapse loss and recuperative synaptogenesis (Kirov et al., 1999, 2004) to test whether astroglial processes were more likely to associate with smaller, presumably newer, or larger more established synapses during synaptogenesis in the mature brain. We use the phrase "recuperative synaptogenesis" in this mature hippocampus to distinguish it from developmental synaptogenesis, although similar processes may be involved.

MATERIALS AND METHODS

Tissue Preparation

Four mature male rats of the Long-Evans strain ranging in age from postnatal days 65–75 were used, two each for perfusion fixation and preparation of acute hippocampal slices (Kirov et al., 1999; Sorra and Harris, 1998). Intracardiac perfusions with fixative were done under pentobarbital anesthesia (80 mg/kg) and started within 1 min after opening the chest cavity to minimize hypoxia-ischemia. The fixative contained 2% paraformaldehyde and 6% glutaraldehyde in 0.1 M cacodylate buffer with 2 mM $CaCl_2$ and 4 mM $MgCl_2$ at pH 7.4. Fixative was delivered to the body at 37°C and 4 psi backing pressure of 95% O_2 and 5% CO_2.

Briefly, the acute hippocampal slices were prepared in accordance with National Institutes of Health guidelines and approved animal care protocols (Kirov et al., 1999; Sorra and Harris, 1998). One rat was first anesthetized with 80 mg/kg pentobarbital to mimic the "pre-fixation" conditions of the perfusion protocol; the second rat was rapidly decapitated without prior anesthesia to avoid potential artifacts of anesthesia. The hippocampus was cut at 400 μm thickness and slices were received into ice-cold artificial cerebral spinal fluid (ACSF, containing 117 mM NaCl, 5.3 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM glucose at pH 7.4 and bubbled with 95% O_2–5% CO_2). Slices were equilibrated at 30–32°C at the interface of the same ACSF and humidified O_2 (95%) and CO_2 (5%) for 9 or 4.5 h, respectively (Kirov et al., 1999; Sorra and Harris, 1998). The slice from the first rat received control stimulation for 30 min prior to fixation (Kirov et al., 1999). The sampled region of the slice from the second rat received three sets of tetanic stimulation at 100 Hz for 1 s at half-maximal stimulation followed by recording 2 h of stable potentiation (Sorra and Harris, 1998). Both slices were then rapidly fixed during 8 s of microwave irradiation in mixed aldehydes (2% paraformaldehyde and 6% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and containing 2 mM $CaCl_2$ and 4 mM $MgCl_2$) and stored overnight in fixative at room temperature.

Perfusion-fixed hippocampus and hippocampal slices were both subsequently fixed in reduced osmium (1% OsO_4 with 1.5% $K_4Fe(CN)_6$) followed by 1% OsO_4, to enhance membranes, and then exposed to 1% uranyl acetate, dehydrated, and embedded in Epoxy Resins and hardened for 48 h prior to collecting serial thin sections. The serial thin sections were stained with ethanolic uranyl acetate followed by Reynolds' lead citrate. Serial sections were photographed in the middle of stratum radiatum at a location about 150–200 μm from the CA1 pyramidal cell body layer at an optimal depth of 100–180 μm from the cut surfaces. These protocols produced well-stained and readily identifiable astroglial processes (Figs. 1 and 5).

Quantification Through Serial Section Electron Microscopy

Series were photographed at the JEOL (Peabody, MA) 1200EX or 1230 electron microscopes and negatives were scanned or images were digitally captured (Gatan, Pleasanton, CA). The series were coded and analyzed blind as to condition. Images were digitally optimized for brightness and contrast to visualize structures of interest, and reconstructions were colorized for clarity of presentation. Three-dimensional reconstructions and analyses were performed using the software entitled RECONSTRUCT (Fiala, 2005), and freely available from (http://synapses.bu.edu). Pixel size was calibrated relative to a diffraction grating replica (Ernest F. Fullam, Latham, NY) and section thickness was computed by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned (Fiala and Harris, 2001).

Surface areas of cross-sectioned synapses were computed by measuring their lengths on individual sections, multiplying by section thickness, and summing over sections. Synapses cut enface were outlined and the enclosed area calculated. A total of 201 synapses were evaluated along eight lateral dendritic segments (0.39–0.92 μm diameters) in the intact perfusion-fixed hippocampus from two animals. Similarly, 210 synapses were evaluated along eight lateral dendritic segments (0.45–0.97 μm diameters) in the hippocampal slices from two animals. EM and statistical analysis of synaptic and astroglial ultrastructural features revealed no significant differences between the two acute slices, hence they were grouped for comparison in subsequent analyses.

Statistics

Excel software (Microsoft, Redwood, CA) was used to organize the data, and Statistica (StatSoft, Tulsa, OK) was used to obtain means and standard deviations, to graph the data and perform regressions and statistical analyses of nested ANOVAs and ANCOVAs by dendrite, animal, and condition. Criterion P was <0.05.

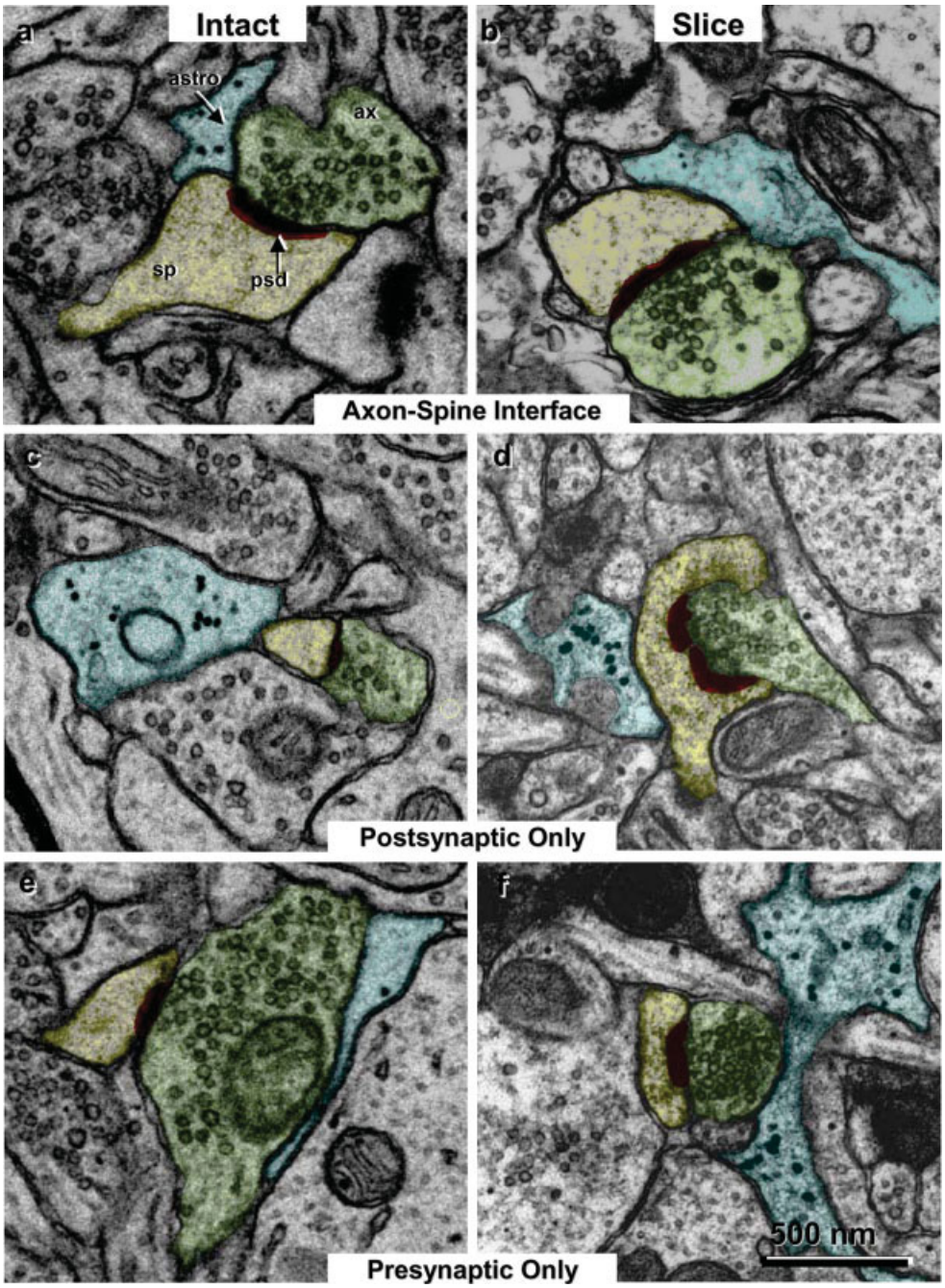

Fig. 1. Locations of perisynaptic astrocyte in area CA1 of intact, perfusion-fixed hippocampus (Intact) or hippocampal slices (Slice) from mature rats. (**a**,**b**) Astroglial processes at the axon-spine interface (ASI, astro, astroglial process (blue); psd, postsynaptic density (red); sp, dendritic spine head (yellow); ax, axonal bouton (green). (**c**,**d**) Astroglial processes apposed to the postsynaptic dendritic spine only. (**e**,**f**) Astroglial processes apposed to the back surface of a presynaptic axon only. Scale bar in f is for a–f. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

Locations of Perisynaptic Astroglia

Astroglial processes were identified by their irregular shapes interdigitating among neuronal processes and by the presence of glycogen granules, intermediate filament bundles, and a relatively clear cytoplasm (Fig. 1; see also Ventura and Harris, 1999). Thin astroglial processes were traced through serial sections to larger processes to confirm their identities. The perisynaptic location of astroglia in relationship to the axon–spine interface (ASI), and the presynaptic and postsynaptic partners were defined as illustrated in the intact perfusion-fixed hippocampus (Figs. 1a,c,d "intact") and in acutely recovered hippocampal slices (Figs. 1b,d,f). Astrocytic distribution varied between apposition at the ASI (Figs. 1a,b), the postsynaptic dendritic spine only (Figs. 1c,d), or the presynaptic axonal bouton only (Figs. 1e,f). Other synapses had no perisynaptic astroglia. These images also reflect accurately the equally low extracellular volume in the intact and acutely sliced mature hippocampus.

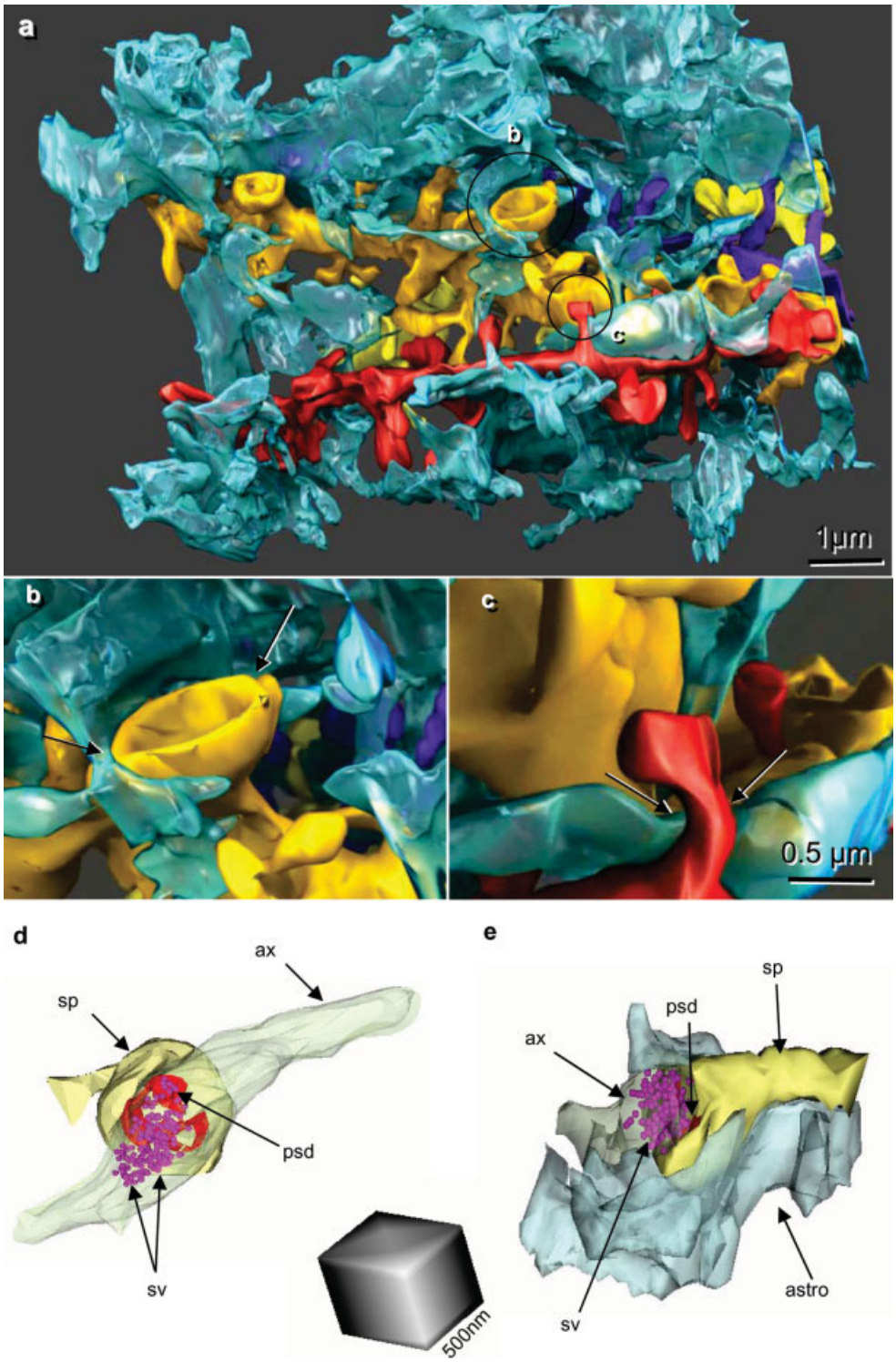

Fig. 2. Reconstructed dendrites, synapses, and associated astroglial processes. (**a**) Three-dimensional reconstruction of a single astroglial process (blue) interdigitating among many dendrites, four of which are reconstructed here (gold, yellow, red, purple). Axonal boutons are not displayed. (**b**) Approximately 50% of the ASI of a mushroom spine was apposed by astroglia (arrows). (**c**) Only the neck of this thin dendritic spine was apposed by astroglia (arrows). Scale bar in c is for b and c. Synapses on mushroom dendritic spines without (**d**) and with (**e**) perisynaptic astroglia (sv, synaptic vesicles). Scale cube shows 500 nm for d and e. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Complete three-dimensional reconstructions illustrated the complex relationships between a single astroglial process (light blue) and synapses along different dendritic segments (red, yellow, gold, and purple); presynaptic axons and other dendrites are excluded for clarity (Fig. 2a). For example, this astroglial process surrounded part of the ASI of a large mushroom spine (Fig. 2b) but encountered only the neck of another spine on a different dendrite (Fig. 2c). Other reconstructions show that spines of similar sizes can be devoid of perisynaptic astroglial processes (Fig. 2d) or richly ensheathed (Fig. 2e). These findings illustrate the nonuniform distribution of astroglial processes among dendritic spine synapses.

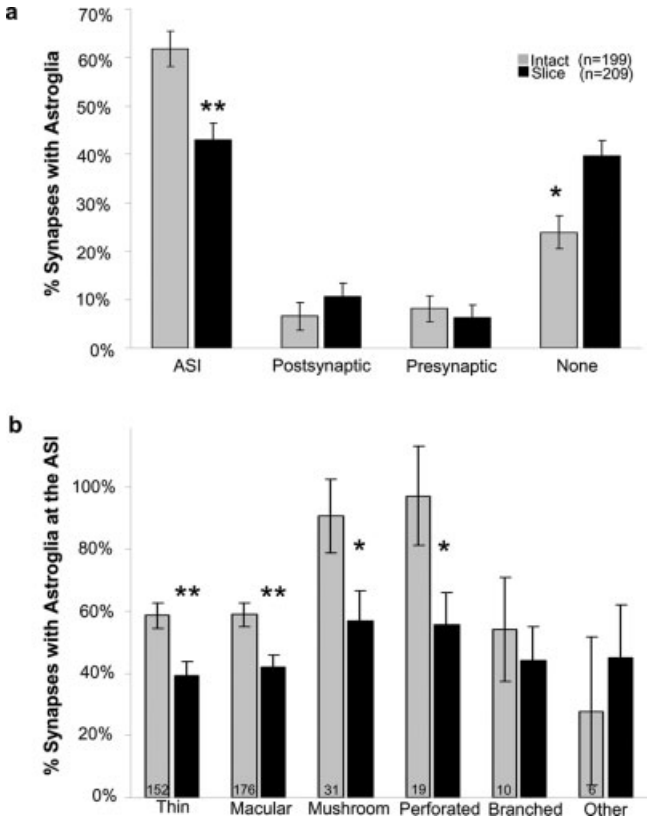

Fig. 3. Nonuniform distribution of perisynaptic astroglia among synapses. (**a**) The percentage of synapses with perisynaptic astrocyte at the ASI was greater in the intact hippocampus (61.9% ± 3.7%) than in slices (43.0% ± 3.5% $^{**}P < 0.0005$). Astroglial processes were located only at the postsynaptic dendritic spines for 6.6% ± 2.9% and 10.6% ± 2.8% of the synapses and the presynaptic boutons for 8.1% ± 2.7% and 6.3% ± 2.7% of the synapses for intact hippocampus and slices, respectively. Fewer synapses had no perisynaptic astroglia ("none") in intact hippocampus (23.9% ± 3.4%) than in the slices (39.6% ± 3.3%, $^{*}P < 0.005$). (**b**) Spines of both thin or mushroom shapes with macular or perforated synapses were more likely to have astroglia at the perimeter of their ASIs in the intact hippocampus than slices ($^{*}P < 0.05$; $^{**}P < 0.005$). There were no significant differences among the other spine types (branched, multisynaptic, or stubby) regarding perisynaptic astroglia. n at the base of each bar is the total number of synapses in each category by condition.

Quantification of Perisynaptic Astroglia at the Perimeter of the ASI

A quantitative analysis was performed to determine whether astroglial processes are preferentially located at specific synapses in the intact or sliced hippocampus. A higher percentage of synapses had perisynaptic astroglia at their ASIs in the intact than the sliced hippocampus (Fig. 3a). In both conditions, relatively few synapses had perisynaptic astroglia at the presynaptic or postsynaptic side only (Fig. 3a). Conversely, a lower percentage of synapses had no perisynaptic astroglia in the intact than in the sliced hippocampus (Fig. 3a).

Synapses with astroglia at the ASI were also categorized by spine and synapse shape (Fig. 3b). Only 40–60% of the thin spines and thin or mushroom spines with macular synapses had astroglia at the ASI. In contrast, 60–99% of the larger mushroom spines and spines with perforated synapses had perisynaptic astroglia. The presence of perisynaptic astroglia was greater in intact hippocampus for all spine and synapse shapes than in the acutely recovered slices (Fig. 3b). Branched and other spine shapes (stubby and multisynaptic) did not differ significantly between conditions with respect to perisynaptic astroglia (Fig. 3b). These observations show that perisynaptic astroglial processes are not restricted to particular spine or synapse shapes.

Larger Synapses Were More Likely to Have Perisynaptic Astroglia

The next question was whether differences in synapse size were related to the presence or absence of perisynaptic astroglia. The surface area of the PSD was measured across serial sections for each synapse. PSDs were larger when perisynaptic astroglial processes were present (0.055 ± 0.003 μm^2) than when they were absent (0.036 ± 0.004 μm^2) at the perimeter of the ASI, or along the dendritic spine head; but not when astroglia was present along the presynaptic axonal bouton only (Fig. 4a). Synapses were larger when astroglial processes were present at the ASI in both the sliced and intact hippocampus (Fig. 4b).

Partial Ensheathment of Synapses by Perisynaptic Astroglia

Membrane-associated molecules interact at the neuron-glial interface and can have both supportive and detrimental effects on synapses. Here we measured whether the amount of perisynaptic astroglial ensheathment was related to spine type or synapse size, and if it was altered in the acutely recovered slices that had undergone recuperative synaptogenesis. We determined the length and fraction of the ASI perimeter that was surrounded by perisynaptic astroglia as described in Figures 5a–j. The average length of the ASI apposed by perisynaptic astroglia did not differ between intact and sliced hippocampus (Fig. 5k). Similarly, the fraction of the ASI perimeter ensheathed by astroglia did not differ significantly between intact (0.38 ± 0.02) and sliced hippocampus (0.35 ± 0.02, $P = 0.75$, data not shown), nor between different spine shapes ($P = 0.4$; data not shown). Although a strong correlation exists between PSD area and ASI area (Ventura and Harris, 1999); there is only a weak correlation between PSD area and the length of ASI that was apposed to perisynaptic astroglia (Fig. 5l, $r = 0.26$; $P < 0.05$).

Conversely, the degree to which glutamate and other substances can escape or enter the perimeter of the synapse will depend upon the length of the astroglia-free interface. The vast majority of synapses (99.5%) had some portion of their ASI perimeter that was free from astroglia. The length of the astroglia-free ASI perimeter was computed as described above, measuring the astroglia-free length instead (red dots and lines in Figs. 5a–j). There was a strong correlation between the astroglia-

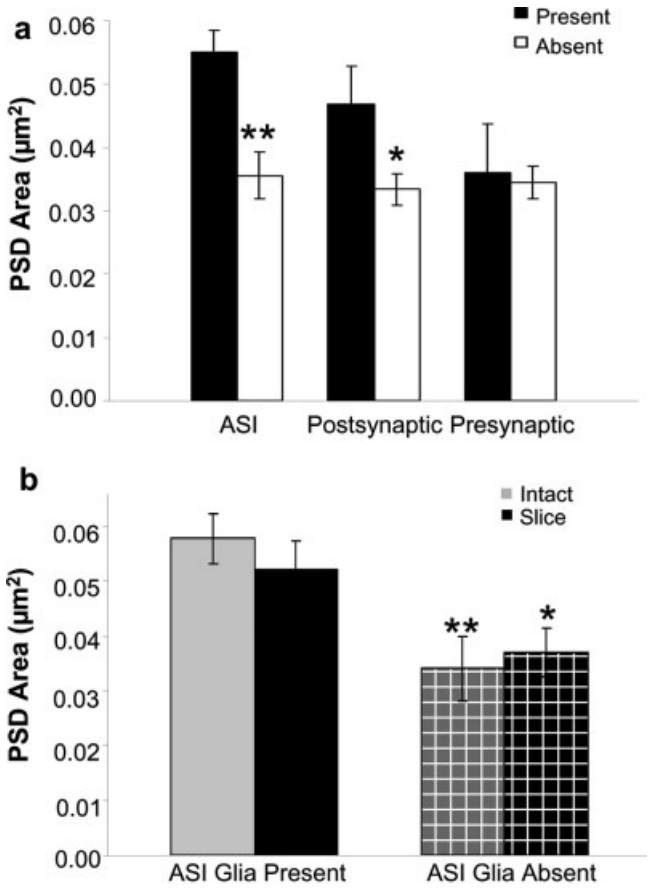

Fig. 4. Mean synapse size was larger for synapses with perisynaptic astroglia apposed to the ASI or postsynaptic spine. (**a**) Mean synapse size was larger when astroglia was present at the ASI (0.055 ± 0.003 μm^2) than when it was absent (0.036 ± 0.004 μm^2; $^{**}P < 0.0005$). Mean synapse size was also greater when astroglia was present at the postsynaptic dendritic spine only (0.047 ± 0.006 μm^2 vs. 0.033 ± 0.002 μm^2; $^{*}P < 0.05$). (**b**) Mean synapse size was larger when perisynaptic astrocyte was present at the ASI in both the intact ($^{**}P < 0.005$) and sliced ($^{*}P < 0.05$) hippocampus.

free length of the ASI perimeter and PSD area whether some portion ($r = 0.74$, $P < 0.0001$) or no portion ($r = 0.68$; $P < 0.0001$) of the ASI perimeter was in direct apposition to perisynaptic astroglia. Synapses without astroglia at the ASI had longer mean unapposed lengths (0.79 ± 0.02 μm) than synapses with astroglia at the ASI (0.58 ± 0.02 μm; $P < 0.0001$, Fig. 5m). These findings suggest that larger synapses with their greater capacity for neurotransmitter release (Harris and Stevens, 1989; Harris and Sultan, 1995; Lisman and Harris, 1993; Schikorski and Stevens, 2001) also have a longer interface from which glutamate and other substances can escape from or enter into the synapse.

Distance Along Astroglia-Free Path to Nearest Neighboring Synapse

One important role that astroglial processes are thought to perform is controlling glutamate spillover and crosstalk between neighboring synapses. Whether glutamate or other substances escaping the synapse can influence neighboring synapses depends on the extracellular distance and tortuosity in the path between neighboring synapses. Here we determined these lengths based on astroglia-free paths because it is known that astroglial membranes contain a high density of glutamate transporters, which remove glutamate from the ECS. A subset of 64 synapses was selected at equal intervals across the range of PSD size for synapses with and without astroglia at the ASI. For each synapse, the shortest astroglia-free route was measured from the perimeter through ECS to a neighboring synapse. The nearest neighboring synapse was determined by viewing serial sections and measuring the shortest three-dimensional linear distance within (Fig. 6a) or across (Fig. 6b) serial sections. The curvilinear distance was measured through ECS between neighboring synapses. Curvilinear distances did not differ between intact and slice conditions, nor did they correlate with synapse size (Fig. 6c). These measurements showed that most synapses had neighbors located within 1 μm and all of the synapses had neighbors located within 2 μm along an astroglia-free path through the ECS. Tortuosity was computed based on the ratio of the curvilinear to the straight linear paths and was less than three for all distances, and did not correlate with PSD area (Fig. 6d). Similarly, whether or not astroglia occurred along some part of the ASI did not influence the curvilinear path length or tortuosity (data not shown). These findings indicate that once substances leave the astroglia-free perimeter of a synapse there is a relatively short diffusion path to a neighboring synapse for all synapse sizes under both conditions.

DISCUSSION

More synapses had perisynaptic astroglia in the perfusion fixed intact hippocampus than acutely recovered hippocampal slices, suggesting that recuperative synaptogenesis occurred without sustained contact by astroglia in mature hippocampus. Perisynaptic astroglial processes were more likely to be present at larger synapses; thus, eventual contact with astroglia permitted or enhanced synapse growth. Alternatively, as a synapse enlarged and released more neurotransmitter, it may become attractive to astroglial processes as glutamate escapes its perimeter (Cornell-Bell et al., 1990b). Most of the perimeter was not ensheathed by perisynaptic astroglia, so that substances in the ECS could escape from or enter into both new and established synapses in mature hippocampus. The distances along astroglia-free paths were usually less than a micron so that spillover could be detected among neighboring synapses in mature hippocampus (Diamond, 2005). Thus, sharing of neurotransmitter such as glutamate, could facilitate the integration of smaller "silent" synapses into active networks during recuperative synaptogenesis in mature hippocampus, although glutamate spillover must be controlled to avoid seizures (Tanaka et al., 1997). Our structural analyses suggest that astroglial glutamate transport may control

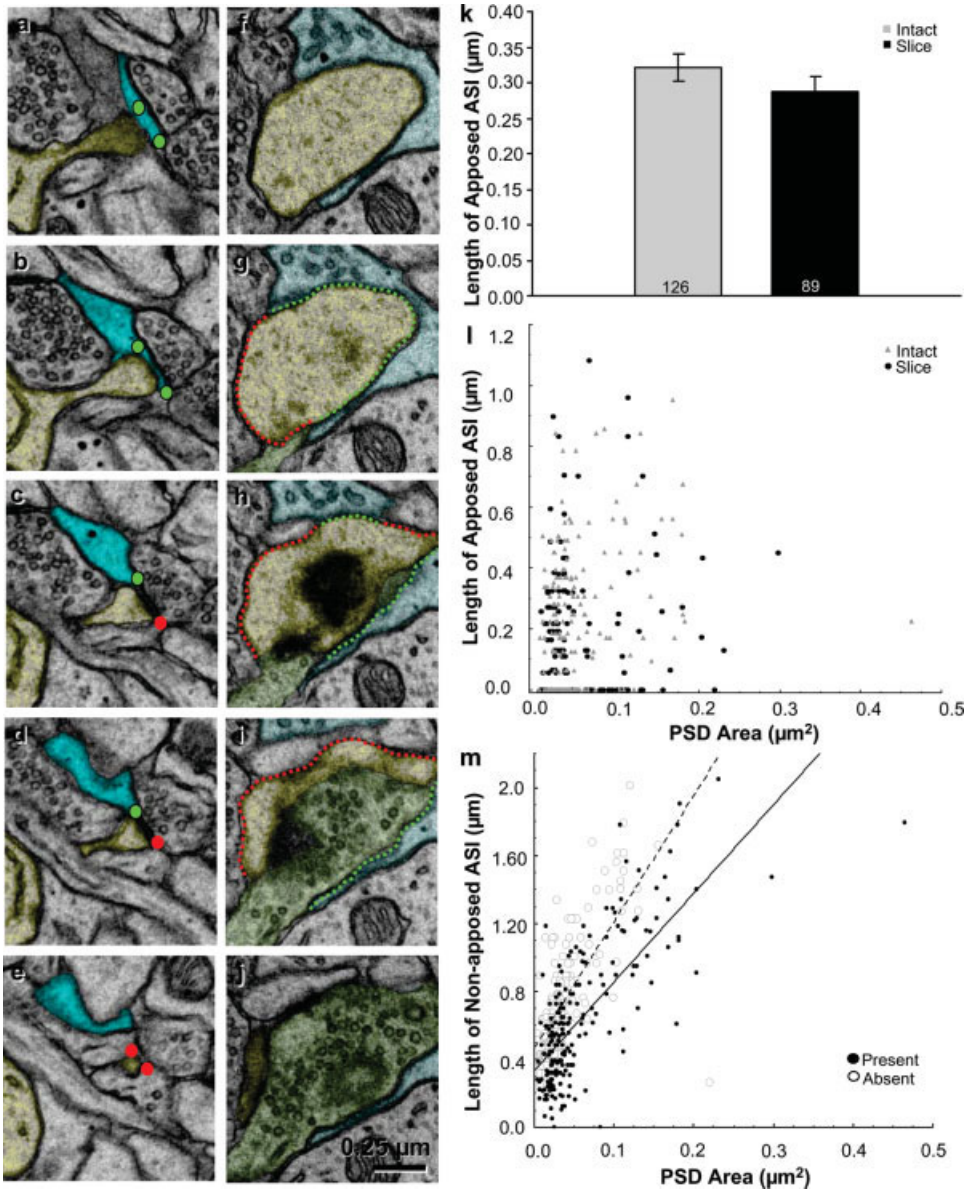

Fig. 5. Reconstruction and quantification of perisynaptic astroglia. (**a–e**) For cross-sectioned synapses, the length of the ASI perimeter associated with (green dots) or without (red dots) astroglia was calculated by counting the number of dots and multiplying by the number of sections spanned and section thickness. (**f–j**) For obliquely-sectioned and enface synapses, the fraction of ASI apposition was measured as the fractional length of entire perimeter with (dotted green line) or without (dotted red line) astroglia. (**k**) The length of the ASI ensheathed by astroglia was similar in the intact (0.32 ± 0.019 µm) and sliced hippocampus (0.29 ± 0.02 µm; $P = 0.23$). Synapses without astroglia were excluded. (**l**) Synapse size was only weakly correlated with the length of the ASI apposed to astroglia ($r = 0.26$; $P < 0.05$). (**m**) The length of ASI perimeter that was unapposed by astroglial process was strongly correlated with PSD area whether astroglia was present at the ASI ($r = 0.74$; $P < 0.0001$) or absent from the ASI ($r = 0.68$; $P < 0.0001$). Synapses without astroglia at the ASI had a longer length open for the flow of glutamate and other substances to and from the synapse (0.79 ± 0.02 µm and 0.58 ± 0.02 µm, respectively; $P < 0.0001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

spillover across relatively large domains of neuropil, while neuronal glutamate transport may be required to provide local control near individual hippocampal synapses (Chen et al., 2004; Diamond, 2001; He et al., 2000).

Role of Delayed Contact Between Astroglial Processes and Synapses

Astroglial processes were absent from the perimeter of more synapses in the hippocampal slices where there had been a recent loss, recovery, and proliferation of synapses (Fiala et al., 2003; Kirov et al., 1999, 2004; Petrak et al., 2005). This finding suggests that, in the mature system, new synapses are more likely to form where astroglial processes are not in direct contact with them. Astroglia respond during slice preparation with a complete loss of glycogen that requires 1–3 h to recover in vitro (Fiala et al., 2003). During this reactive period, astroglia may also release soluble substances that promote synaptogenesis in the slices. Preliminary strength-

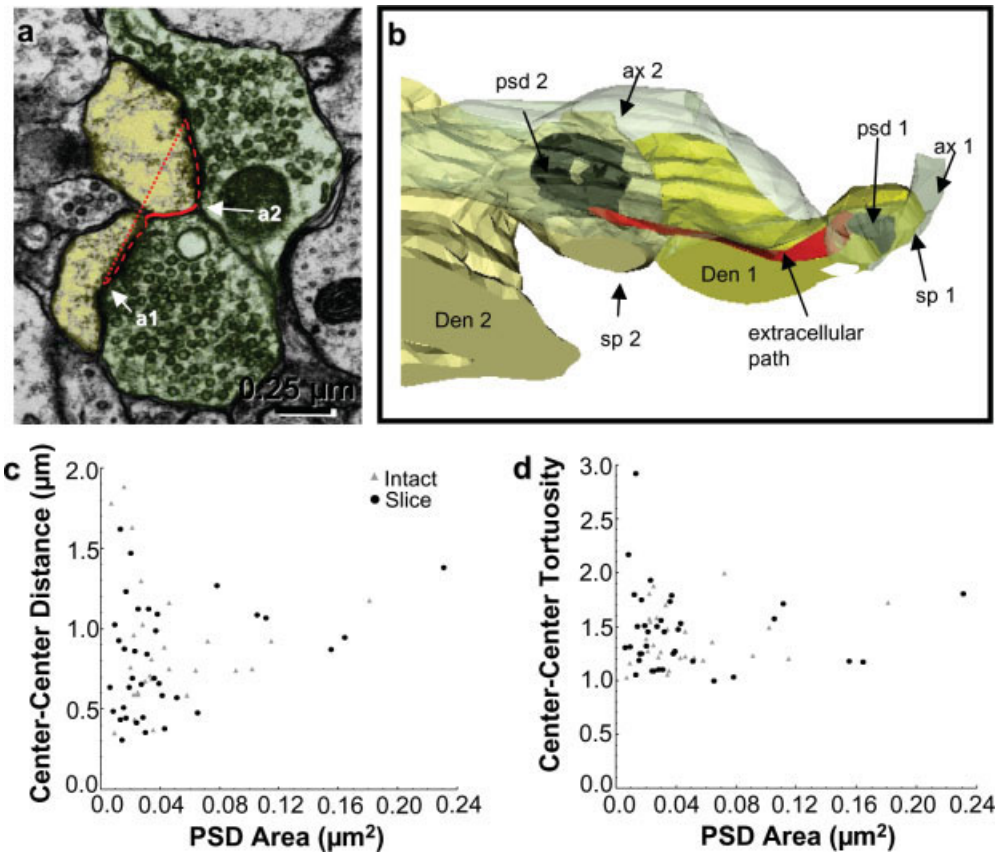

Fig. 6. Distances and tortuosity of the glia-free path between nearest neighboring synapses. (**a**) The curvilinear distances were measured between the edges (solid red line), centers (dashed red line) and center to edge (distance along dashed and solid lines from a1 to a2) of nearest neighboring synapses in both conditions (n = 64). The tortuosity in these paths was computed as the curvilinear distance through ECS divided by the straight linear distance between the points (e.g. dotted red line for center to center on this synapse). (**b**) Sometimes the extracellular path distance between nearest neighboring synapses had to be measured through 3D reconstruction (red = extracellular path; black = psd 1 and 2; gold = dendrite1 [Den 1] and spine1 [sp 1]; yellow = dendrite2 [Den 2] and spine2 [sp 2]; green = axon1 [ax 1] and axon2 [ax 2]. Distances to nearest neighboring synapses were similar in intact and sliced hippocampus when measured (**c**) center to center (0.87 ± 0.07 μm and 0.82 ± 0.07 μm, respectively; P = 0.97) or edge to edge (0.41 ± 0.08 μm and 0.40 ± 0.05 μm, respectively; P = 0.95, data not shown), or center to edge (0.63 ± 0.07 μm and 0.59 ± 0.05 μm, respectively; P = 0.90, data not shown). (**d**) Tortuosity of extracellular paths between nearest neighboring synapses also did not differ between intact and sliced hippocampus when computed center to center (1.39 ± 0.05 μm and 1.47 ± 0.06 μm, respectively; P = 0.70) edge to edge (1.16 ± 0.04 μm and 1.34 ± 0.06 μm, respectively; P = 0.17, data not shown), or center to edge (1.34 ± 0.07 μm and 1.36 ± 0.05 μm, respectively; P = 0.74, data not shown). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ening of synapses during the 4–9 h that these slices remained in vitro could have been supported by cholesterol and other substances released from astroglia (Goritz et al., 2005; Mauch et al., 2001). Consistent with this hypothesis there are more synapses in astrocyte-rich than in astrocyte-poor cortical cultures (Harris and Rosenberg, 1993). Furthermore, soluble substances in astrocyte-conditioned medium also promote synaptogenesis on developing retinal ganglion cells isolated on postnatal day 5 and maintained in culture (Christopherson et al., 2005).

In contrast, direct contact with astroglial processes might be detrimental during the early phases of synaptogenesis. Contact with astroglial processes augments calcium influx through N-type calcium channels on cultured hippocampal neurons (Mazzanti and Haydon, 2003), which in turn can cause depolymerization of actin filaments and retraction of filopodia or loss of unstable new spines (Oertner and Matus, 2005). Contact with astroglia may also cause ephrin-mediated remodeling or collapse of nascent dendritic spines (Murai et al., 2003). These observations suggest that synaptogenesis in mature hippocampus might be restricted to paths through the neuropil where new filopodia do not physically contact astroglial processes, but still have access to soluble, synapse-promoting factors released from distant astroglial processes.

Why and how might the larger synapses attract astroglial processes to their perimeters? Previous studies have shown that the number of presynaptic vesicles increases proportionately with the area of the PSD on dendritic spines (Harris and Sultan, 1995; Lisman and Harris, 1993; Schikorski and Stevens, 2001). The astroglial processes could have been attracted to the greater amount of glutamate or other substances escaping the ASI of larger synapses (Cornell-Bell et al., 1990b; Hirrlinger et al., 2004). Subsequent contact with astroglia attracted to these larger synapses could facilitate growth and maturation of the synapse. For example, puncta adherens

junctions between astroglial processes and dendritic spines (Spacek and Harris, 1998) contain a variety of cell adhesion molecules such as integrins, NCAM, cadherins, and others (Schuster et al., 2001). Integrins can activate the protein kinase C signaling cascades in astroglia, which serve to enhance synaptic efficacy (Hama et al., 2004). GAP43, another contact-mediated growth associated protein, activates the MAP kinase pathway and facilitates elaboration of dendrites via astroglial contact (Piontek et al., 2002). Thus, contact with astroglial processes could enhance synaptic efficacy as the spine cytoskeleton stabilizes (Smart and Halpain, 2000).

How Might Glutamate Spillover Awaken Newly Recovered Synapses?

A persistent question has been whether astroglial processes serve as barriers to glutamate spillover and crosstalk between neighboring synapses. It is likely that at least some glutamate escapes from hippocampal synapses, because synaptically driven transporter currents can be recorded from hippocampal astroglia in acute slices (Bergles and Jahr, 1997; Diamond et al., 1998). Furthermore, we show here that nearly all hippocampal synapses had regions along their ASI perimeters where substances could escape or enter the synaptic cleft without encountering astroglia. The rate of glutamate clearance from the ECS increases proportionately with expression of glial glutamate transporters during hippocampal maturation (Diamond, 2005). In the absence of glutamate transporters, synapses separated by distances less than a micron would likely be affected by glutamate spillover (Diamond, 2005). The distance along the shortest glia-free extracellular route between neighboring synapses was usually less than 1 μm. In addition, the apparent low volume of the ECS suggests that most synapses in both the intact and sliced hippocampus would experience a similar amount of glutamate spillover from neighboring synapses. More release of glutamate means more transporters will be occupied such that subsequently-released glutamate could diffuse farther (Diamond, 2005). Glutamate transporters located in neuronal membranes can also remove glutamate from the ECS (Chaudhry et al., 1995; Diamond, 2001; He et al., 2000). If neuronal glutamate transporters were concentrated at the perimeter of synapses without astroglia (Chen et al., 2004), then they might prevent significant spillover of glutamate during recuperative synaptogenesis in mature hippocampus.

Smaller synapses in the mature hippocampal slices are reminiscent of early development (Fiala et al., 1998) where small synapses are relatively silent and contain only NMDA glutamate receptors (Isaac et al., 1997; Liao et al., 1999; Takumi et al., 1999). The small developing synapses might also be presynaptically silent under normal conditions, increasing neurotransmitter release during synaptic plasticity (Ma et al., 1999). The absence of perisynaptic astroglia from small synapses might allow sufficient glutamate to enter the cleft from the ECS to activate NMDA receptors (Asztely et al., 1997; Kullmann et al., 1996), while other synapses on the neuron are firing. This coactivation would facilitate insertion of AMPA receptors and incorporation of the synapses into a functional network.

Astroglia-Free microdomains for Synaptogenesis

Astroglia form multiple domains within hippocampal CA1 neuropil. Individual astroglia serve as one large domain with little overlap among processes of neighboring astroglia (Bushong et al., 2002). The whole astrocyte spans the dendritic arbors of many neurons. An individual astroglial process can coordinate neuronal excitability among a few neurons by amplifying calcium signaling (Fellin et al., 2004; Pascual et al., 2005). Here we demonstrate astroglia-free microdomains through which new filopodia might emerge to form synapses without contacting astroglial processes (Fiala et al., 1998; Harris et al., 1992). Regulation of the composition of the extracellular matrix, through secretion of proteases or reduced secretion of protease inhibitors from astroglia, might also be important to provide an unobstructed path for new spine outgrowth (Giau et al., 2005; Pavlov et al., 2004). Ongoing maintenance of these astroglia-free microdomains could be important for synaptic plasticity in the mature hippocampus.

Plasticity of Perisynaptic Astroglia

The degree to which astroglial processes surround synapses depends on many factors including age, brain region, local neuropil, and experience. For example, astroglial ensheathment of parallel fiber synapses increases with maturation (Grosche et al., 1999), and the astroglial volume per Purkinje neuron increases with acrobatic learning but not exercise alone (Anderson et al., 1994). Astroglial volume also increases in visual and motor cortex proportionately with synaptogenesis and expansion of the dendritic arbor during environmental enrichment and motor learning (Jones and Greenough, 2002). In hippocampal neuropil, astroglial volume fluctuates with synaptogenesis during the oestrus cycle (Klintsova et al., 1995) and increases during long-term potentiation (Wenzel et al., 1991) or following kindling (Hawrylak et al., 1993). These findings show that perisynaptic astroglial processes undergo dynamic reorganization during synaptic plasticity. Whether these and other forms of synaptic plasticity result in astroglial processes preferentially ensheathing portions of recently stabilized synapses remains to be determined.

ACKNOWLEDGMENTS

We thank Marcia Feinberg, Elizabeth Perry, and Robert Smith for technical assistance; Dr. John Fiala for the RECONSTRUCT software; and Cameron Slayden (Cosmocyte, Inc.) for illustrating Figure 2. We thank Paul Rosenberg and Mark Ellisman for their insightful

comments leading to the idea about how proteases may clear a path for spine outgrowth. Paul Rosenberg is further thanked for many years of astute conversation about perisynaptic astroglia as well as the role of neuronal glutamate transporters in controlling spillover at shorter distances.

REFERENCES

Allen NJ, Barres BA. 2005. Signaling between glia and neurons: Focus on synaptic plasticity. Curr Opin Neurobiol 15:542–548.

Anderson BJ, Li X, Alcantara AA, Isaacs KR, Black JE, Greenough WT. 1994. Glial hypertrophy is associated with synaptogenesis following motor-skill learning, but not with angiogenesis following exercise. Glia 11:73–80.

Asztely F, Erdemli G, Kullmann DM. 1997. Extrasynaptic glutamate spillover in the hippocampus: Dependence on temperature and the role of active glutamate uptake. Neuron 18:281–293.

Barres BA. 1991. New roles for glia. J Neurosci 11:3685–3694.

Bergles DE, Dzubay JA, Jahr CE. 1997. Glutamate transporter currents in bergmann glial cells follow the time course of extrasynaptic glutamate. Proc Natl Acad Sci USA 94:14821–14825.

Bergles DE, Jahr CE. 1997. Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19:1297–1308.

Brown AM, Tekkok SB, Ransom BR. 2004. Glial-neuronal interactions and brain energy metabolism. In: Hatton GI, Parpura V, editors. Glial-neuronal signaling. Boston, MA: Kluwer Academic. pp 279–296.

Bushong EA, Martone ME, Jones YZ, Ellisman MH. 2002. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183–192.

Chao TI, Rickmann M, Wolff JR. 2002. The synapse-astrocyte boundary: An anatomical basis for an integrative role of glia in synaptic transmission. In: Volterra A, Magistretti PJ, Haydon PG, editors. The tripartite synapse. Oxford: Oxford University Press. pp 3–23.

Chaudhry FA, Lehre KP, van Lookeren C, Ottersen OP, Danbolt NC, Storm-Mathisen J. 1995. Glutamate transporters in glial plasma membranes: Highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15:711–720.

Chen W, Mahadomrongkul V, Berger UV, Bassan M, DeSilva T, Tanaka K, Irwin N, Aoki C, Rosenberg PA. 2004. The glutamate transporter GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons. J Neurosci 24:1136–1148.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421–433.

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. 1990a. Glutamate induces calcium waves in cultured astrocytes: Long-range glial signaling. Science 247:470–473.

Cornell-Bell AH, Thomas PG, Smith SJ. 1990b. The excitatory neurotransmitter glutamate causes filopodia formation in cultured hippocampal astrocytes. Glia 3:322–334.

Diamond JS. 2001. Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. J Neurosci 21:8328–8338.

Diamond JS. 2005. Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: Transmitter uptake gets faster during development. J Neurosci 25:2906–2916.

Diamond JS, Bergles DE, Jahr CE. 1998. Glutamate release monitored with astrocyte transporter currents during LTP. Neuron 21:425–433.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. 2004. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729–743.

Fiala JC. 2005. Reconstruct: A free editor for serial section microscopy. J Microsc 218:52–61.

Fiala JC, Feinberg M, Popov V, Harris KM. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. J Neurosci 18:8900–8911.

Fiala JC, Harris KM. 2001. Cylindrical diameters method for calibrating section thickness in serial electron microscopy. J Microsc 202: 468–472.

Fiala JC, Kirov SA, Feinberg MD, Petrak LJ, George P, Goddard CA, Harris KM. 2003. Timing of neuronal and glial ultrastructure disruption during brain slice preparation and recovery in vitro. J Comp Neurol 465:90–103.

Giau R, Carrette J, Bockaert J, Homburger V. 2005. Constitutive secretion of protease nexin-1 by glial cells and its regulation by G-protein-coupled receptors. J Neurosci 25:8995–9004.

Goritz C, Mauch DH, Pfrieger FW. 2005. Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. Mol Cell Neurosci 29:190–201.

Grosche J, Matyash V, Moller T, Verkhratsky A, Reichenbach A, Kettenmann H. 1999. Microdomains for neuron-glia interactions: Parallel fiber signaling to Bergmann glial cells. Nat Neurosci 2:139–143.

Hama H, Hara C, Yamaguchi K, Miyawaki A. 2004. PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. Neuron 41:405–415.

Harris KM, Jensen FE, Tsao B. 1992. Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: Implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12:2685–2705.

Harris KM, Rosenberg PA. 1993. Localization of synapses in rat cortical cultures. Neuroscience 53:495–508.

Harris KM, Stevens JK. 1989. Dendritic spines of CA1 pyramidal cells in the rat hippocampus: Serial electron microscopy with reference to their biophysical characteristics. J Neurosci 9:2982–2997.

Harris KM, Sultan P. 1995. Variation in number, location, and size of synaptic vesicles provides an anatomical basis for the non-uniform probability of release at hippocampal CA1 synapses. J Neuropharmacol 34:1387–1395.

Hatton GI, Parpura V. 2004. Glial neuronal signaling. Boston: Kluwer Academic.

Hawrylak N, Chang FL, Greenough WT. 1993. Astrocytic and synaptic response to kindling in hippocampal subfield CA1. II. Synaptogenesis and astrocytic process increases to in vivo kindling. Brain Res 603: 309–316.

He Y, Janssen WG, Rothstein JD, Morrison JH. 2000. Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. J Comp Neurol 418:255–269.

Hertz L, Zielke HR. 2004. Astrocytic control of glutamatergic activity: Astrocytes as stars of the show. Trends Neurosci 27:735–743.

Hirrlinger J, Hulsmann S, Kirchhoff F. 2004. Astroglial processes show spontaneous motility at active synaptic terminals in situ. Eur J Neurosci 20:2235–2239.

Isaac JT, Crair MC, Nicoll RA, Malenka RC. 1997. Silent synapses during development of thalamocortical inputs. Neuron 18:269–280.

Jones TA, Greenough WT. 2002. Behavioral experience-dependent plasticity of glial-neuronal interactions. In: Volterra A, Magistretti PJ, Haydon PG, editors. The tripartite synapse: Glia in synaptic transmission. Oxford: Oxford University Press. pp 248–265.

Kirov SA, Petrak LJ, Fiala JC, Harris KM. 2004. Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippocampus. Neuroscience 127:69–80.

Kirov SA, Sorra KE, Harris KM. 1999. Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats. J Neurosci 19:2876–2886.

Klintsova A, Levy WB, Desmond NL. 1995. Astrocytic volume fluctuates in the hippocampal CA1 region across the estrous cycle. Brain Res 690:269–274.

Kofuji P, Newman EA. 2004. Potassium buffering in the central nervous system. Neuroscience 129:1045–1056.

Kullmann DM, Erdemli G, Asztely F. 1996. LTP of AMPA and NMDA receptor-mediated signals: Evidence for presynaptic expression and extrasynaptic glutamate spill-over. Neuron 17:461–474.

Liao D, Zhang X, O'Brien R, Ehlers MD, Huganir RL. 1999. Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. Nat Neurosci 2:37–43.

Lin SC, Bergles DE. 2004. Synaptic signaling between neurons and glia. Glia 47:290–298.

Lisman J, Harris KM. 1993. Quantal analysis and synaptic anatomy—Integrating two views of hippocampal plasticity. Trends Neurosci 16: 141–147.

Ma L, Zablow L, Kandel ER, Siegelbaum SA. 1999. Cyclic AMP induces functional presynaptic boutons in hippocampal CA3-CA1 neuronal cultures. Nat Neurosci 2:24–30.

Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW. 2001. CNS synaptogenesis promoted by glia-derived cholesterol. Science 294:1354–1357.

Mazzanti M, Haydon PG. 2003. Astrocytes selectively enhance N-type calcium current in hippocampal neurons. Glia 41:128–136.

Murai KK, Nguyen LN, Irie F, Yamaguchi Y, Pasquale EB. 2003. Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. Nat Neurosci 6:153–160.

Oertner TG, Matus A. 2005. Calcium regulation of actin dynamics in dendritic spines. Cell Calcium 37:477–482.

Palay SL, Chan-Palay V. 1974. Cerebellar cortex: cytology and organization. New York: Springer-Verlag.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG. 2005. Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113–116.

Pavlov I, Lauri S, Taira T, Rauvala H. 2004. The role of ECM molecules in activity-dependent synaptic development and plasticity. Birth Defects Res C Embryo Today 72:12–24.
Perea G, Araque A. 2005. Synaptic regulation of the astrocyte calcium signal. J Neural Transm 112:127–135.
Petrak LJ, Harris KM, Kirov SA. 2005. Synaptogenesis on mature hippocampal dendrites occurs via filopodia and immature spines during blocked synaptic transmission. J Comp Neurol 484:183–190.
Piontek J, Regnier-Vigouroux A, Brandt R. 2002. Contact with astroglial membranes induces axonal and dendritic growth of human CNS model neurons and affects the distribution of the growth-associated proteins MAP1B and GAP43. J Neurosci Res 67:471–483.
Rusakov DA, Kullmann DM. 1998. Extrasynaptic glutamate diffusion in the hippocampus: Ultrastructural constraints, uptake, and receptor activation. J Neurosci 18:3158–3170.
Schikorski T, Stevens CF. 2001. Morphological correlates of functionally defined synaptic vesicle populations. Nat Neurosci 4:391–395.
Schipke CG, Kettenmann H. 2004. Astrocyte responses to neuronal activity. Glia 47:226–232.
Schuster T, Krug M, Stalder M, Hackel N, Gerardy-Schahn R, Schachner M. 2001. Immunoelectron microscopic localization of the neural recognition molecules L1, NCAM, and its isoform NCAM180, the NCAM-associated polysialic acid, β1 integrin and the extracellular matrix molecule tenascin-R in synapses of the adult rat hippocampus. J Neurobiol 49:142–158.
Smart FM, Halpain S. 2000. Regulation of dendritic spine stability. Hippocampus 10:542–554.
Sorra KE, Harris KM. 1998. Stability in synapse number and size at 2 hr after long-term potentiation in hippocampal area CA1. J Neurosci 18:658–671.
Spacek J. 1985. Three-dimensional analysis of dendritic spines. III. Glial sheath Anat Embryol (Berl) 171:245–252.
Spacek J, Harris KM. 1998. Three-dimensional organization of cell adhesion junctions at synapses and dendritic spines in area CA1 of the rat hippocampus. J Comp Neurol 393:58–68.
Takumi Y, Ramirez-Leon V, Laake P, Rinvik E, Ottersen OP. 1999. Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. Nat Neurosci 2:618–624.
Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276:1699–1702.
Ullian EM, Christopherson KS, Barres BA. 2004. Role for glia in synaptogenesis. Glia 47:209–216.
Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. 2001. Control of synapse number by glia. Science 291:657–661.
Ventura R, Harris KM. 1999. Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897–6906.
Volterra A, Magistretti PJ, Haydon PG. 2002. The tripartite synapse. Oxford: Oxford University Press.
Volterra A, Meldolesi J. 2005. Astrocytes, from brain glue to communication elements: The revolution continues. Nat Rev Neurosci 6:626–640.
Wenzel J, Lammert G, Meyer U, Krug M. 1991. The influence of long-term potentiation on the spatial relationship between astrocyte processes and potentiated synapses in the dentate gyrus neuropil of rat brain. Brain Res 560:122–131.
Xu-Friedman MA, Harris KM, Regehr WG. 2001. Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar Purkinje cells. J Neurosci 21:6666–6672.