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Glia selectively approach synapses on thin dendritic spines

Nikolai Medvedev1, Victor Popov1,2,†, Christian Henneberger3,4, Igor Kraev1, Dmitri A. Rusakov3 and Michael G. Stewart1

1Department of Life and Health Sciences, The Open University, Milton Keynes MK7 6AA, UK
2Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino 142290, Russia
3Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK
4Institute of Cellular Neuroscience, University of Bonn Medical School, Bonn, Germany

This paper examines the relationship between the morphological modality of 189 dendritic spines and the surrounding astroglia using full three-dimensional reconstructions of neuropil fragments. An integrative measure of three-dimensional glial coverage confirms that thin spine PSDs are more tightly surrounded by glia. This distinction suggests that diffusion-dependent synapse–glia communication near ‘learning’ synapses (associated with thin spines) could be stronger than that near ‘memory’ synapses (associated with larger spines).

1. Introduction

In the hippocampus, high-affinity transporters populating astroglia membranes rapidly buffer the excitatory neurotransmitter glutamate released by synaptic discharges [1–4]. This powerful uptake system maintains low ambient extracellular glutamate (20–30 nM), providing a ‘silent’ background for transient excitatory signals [5]. However, astrocytes occupy less than 10% of tissue volume in area CA1 of the hippocampus [6], and synchronous synaptic releases may give rise to extra- or inter-synaptic actions of glutamate [7–9]. Indeed, astrocyte protrusions occur unevenly in the hippocampal neuropil, closely approaching only 20–30% of excitatory synapses [10], with no apparent relationship to the morphology of the host dendritic spines [11]. However, glial coverage of synapses varies depending on the organism’s physiological state [12,13], which raises the question as to whether there is an adaptive significance for the uneven distribution of astroglia near hippocampal synapses?

To investigate this, we first documented the architectonics and the tissue volume fraction occupied by live astrocytes in dentate neuropil and, second, reconstructed in three dimensions the ultrastructure of contiguous astrocyte fragments together with the adjacent excitatory synapses. To quantify juxtaposition of synapses and astroglia, we first calculated the shortest distances between astrocyte membranes and the nearest edges of postsynaptic densities (PSDs) at 136 ‘thin’ and 53 ‘mushroom’ dendritic spines [14,15] of dentate granule cells. Second, we applied an integrative weighted-distance measure providing cumulative data about spatial glial coverage of PSDs. We found that glial membranes occur substantially closer to the PSDs of thin dendritic spines compared with mushroom spines. Because mushroom and thin dendritic spines have been associated with the different dominant synaptic receptors [16–18] and different stages of synaptic plasticity [19–21] (although see [22]), the results suggest that glia may selectively approach synapses that are in the process of physiological ‘learning’.

2. Material and methods

(a) Two-photon microscopy and morphometry

Acute 350 μm slices from male Wistar rats (approx. eight-week-old) were transferred to the recording submersion chamber (Scientific Systems Design, NJ, USA) in an
Olympus microscope integrated with a BioRad Radiance 2100 imaging system and coupled with a femtosecond-infrared laser (MaTiAi, SpectraPhysics) and patch-clamp electrophysiology [23].

The superfusion solution contained (mM): 124 NaCl, 2 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, bubbled with 95%/5% of O2/CO2. Astrocytes in dentate gyrus were held in whole-cell mode; the internal solution contained (mM): 135 K methanesulfonate, 10 HEPES, 10 a2Phosphocreatine, 4 MgCl2, 4 NaATP, 0.4 NaGTP.

Astrocytes in dentate gyrus were held in whole-cell mode; patch pipettes are seen. Image on far right, an astrocyte representing a sub-group with prominent dendritic trunks [24].

(b) Input resistance measurement in a typical ‘passive’ astrocyte; whole-cell current clamp mode, a voltage response to a 200 pA current injection pulse is shown (upper trace; also text and electronic supplementary material, figure S1). (c) In each astrocyte, distribution of the tissue volume fraction \( G^v \) occupied by thin, indicator-filled astrocyte processes (excluding areas containing large dendritic trunks) is measured in a \( Z \)-series of thin \( X-Y \) optical sections provided by two-photon excitation. Left panel, an image stack average (as in (d)). Inset panels, examples of individual \( X-Y \) sections (section number reference is indicated; the effective optical width, approx. 1 \( \mu m \)).

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Figure 1. Morphology of live astrocytes in dentate gyrus. (a) Characteristic morphologies of individual astrocytes held in whole-cell mode and filled with Alexa Fluor 594 (\( \lambda_c = 800 \) nm); each fluorescence image represents an averaged \( Z \)-stack of 70 – 110 consecutive \( X-Y \) two-photon excitation sections taken with 0.5 \( \mu m \) \( Z \)-axis steps; patch pipettes are seen. Image on far right, an astrocyte representing a sub-group with prominent dendritic trunks [24].

(b) Inset panels, examples of individual \( X-Y \) sections (section number reference is indicated; the effective optical width, approx. 1 \( \mu m \)) containing the soma; dotted line, a sampling segment (example) for the fluorescence brightness profile. Such samples were taken systematically in each \( X-Y \) section, normally by rotating the sampling segment in approximately 20 \(^\circ\) increments around the soma, throughout the \( Z \)-stack containing the soma. Plot, an example of \( G^v \) measurements in eight different \( X-Y \) sections of the same astrocyte (shown on the left): brightness profiles are normalized with respect to the brightness level inside the soma (see text) within each \( X-Y \) section; grey, individual profiles; black, average.

(b) Tissue preparation and processing for electron microscopy

Animals were anaesthetized (urethane 1.5 g kg \(^{-1}\)), perfused transcardially with 100 ml saline, followed by 100 ml 3.5% paraformaldehyde and 0.5% glutaraldehyde (0.1 M Na-cacodylate buffer, pH 7.4) at room temperature (RT-22 \(^\circ\) C). After perfusion, the brains were removed and coronal serial 50-\( \mu m \) sections were cut from each brain containing the whole dorsal portion of the hippocampus. Slices were immersed in 2.5% glutaraldehyde (0.1 M Na...
cycadylate buffer) for 24 h. The tissue was post-fixed with 1% OsO₄
and 0.01% potassium dichromate (same buffer, 1–2 h, RT) dehy-
drated in graded solutions of ethanol (10 min each) and then
100% acetone (three changes 10 min each). Specimens were
embedded in epoxy resin (Epon 812/AralditeM) as detailed earlier
[25]. A total of 60–70 nm thick serial sections were cut (2–5% etha-
ol in water), allowed to form a ribbon on the bath surface and
collected with Pioloform-coated slab copper grids. Sections were
counterstained with saturated ethanolic uranyl acetate, followed
by lead citrate, and were then placed in a rotating grid holder to
allow uniform orientation of sections on adjacent grids in the
electron microscope. Electron micrographs (6000 ×) were obtained
in a JEOL 1010 electron microscope from the medial molecular
layer of dentate gyrus, at a distance of 80–100 μm from the layer
of neuronal cell bodies. Up to 100 serial sections per series were
photographed. A cross-sectioned myelinated axon, mitochond-
dria and dendrites spanning each section provided a fiduciary
reference for initial alignment of serial sections.

(c) Three-dimensional reconstruction and morphometry

Digital electron micrographs (1200 dpi) were aligned using SEM
Align 1.26 and contours of individual dendritic spines, PSDs and
astrocytic processes were traced digitally using IGL Trace 1.26
(http://www.synapses.clm.utexas.edu/). Section thickness was
determined as described earlier [26] and was normally 60–70 nm
(grey/white colour). Volumes and surface areas of individual
structures were computed and three-dimensional (3D) objects
were generated using the IGL Trace. Dendritic spines adjacent to
identified astrocytic processes were reconstructed and analysed
(see Results). Additional criteria for ‘mushroom’ spines were the
presence of a spine apparatus and a complex PSD (perforated,
U-shaped or segmented) whereas ‘thin’ spines had only macular
PSDs and no spine apparatus [27,28].

For distance measurements, we used reconstructed surfaces
represented by quasi-regular triangular lattices: lattice vertices
provided a set of surface coordinates. Where required, centroids
were calculated directly from such sets. Running through and
measuring all distances between PSDs (all surface points) and the
astrocyte membrane (all surface points with an upper distance
limit) was carried out in 3D using the IGL Trace; the distance
metric was Euclidean throughout. We exported 3D reconstructions
of dendritic spines adjacent to the electron microscopy level.

3. Results

(a) Astrocytes in dentate gyrus: neuropil volume
fraction in live tissue

To understand architectonics and space-filling features of indi-
vidual astrocytes in dentate gyrus, we carried out single-cell
experiments in acute hippocampal slices. We applied visual
patch-clamp routines to identify individual astrocytes (occur-
ing preferentially in the medial perforant path layer) filling
them, in whole-cell mode, with a fluorescent tracer Alexa
Fluor 594. To restrict indicator diffusion to individual cells,
gap junctions were blocked by carbocyanine (Materials and
methods). Astrocytes were visualized in serial stacks of
fluorescence images obtained with two-photon (2P) excita-
tion of Alexa (figure 1a). Stable fluorescence was usually
achieved within 5–10 min (access resistance typically approx.
10 MΩm). Pictures were taken about 20 min after entering
whole-cell. All recorded cells were electrically passive: the
average resting $V_m$ and the input resistance were, respectively,
$-88.1 \pm 0.7$ mV and $5.9 \pm 1.1$ MΩm (mean ± s.e.m., $n = 10$;
figure 1b; electronic supplementary material, figure S1), reflect-
ing the properties of regular ‘passive’ astrocytes [29]. Cell
morphology was consistent with that reported in the same
area using Lucifer Yellow staining [24], including a proportion of
astrocytes with long dendrite-like trunks (figure 1a). The average
cross-sectional area occupied by an individual astro-
ocyte arbour was $2631 \pm 268 \mu m^2$ ($n = 10$); this corresponded
to an average caliper size of $57.8 \pm 18.5 \mu m$, consistent with
previous reports [30].

The tissue volume fraction occupied by glia, $G_V$, should
reflect basic properties of glutamate uptake in the area. $G_V$
has hitherto been measured in fixed preparations using stereo-
ological rules [2,6]. To measure $G_V$ in live tissue, we applied 2P
microscopy: 2P excitation occurs entirely within approximately
1 μm thick optical layer ensuring that no contaminating fluo-
rescence is generated outside the focal plane. Because glial
protrusions are normally much thinner than the translucent
excitation layer, the sampled emission $F(i,j)$ (i and j, pixel co-
ordinates in the X–Y plane) is proportional to the local volume
fraction of indicator-filled glia, or $G_V(i,j)$.

To translate $G_V(i,j)$ into the local volume fraction, we
related it to the fluorescence $F_{max}(i,j)$ inside the dye-filled cell
soma (i.e. 100% volume fraction) in the same focal plane
(figure 1c): $G_V(i,j) = (F(i,j) - F_0)/(F_{max} - F_0)$ where $F_0$
is background fluorescence (outside any stained structures). We
used this approach to obtain two measurements. First, we
excluded the areas occupied by the soma and thick (more
than 1 μm) astrocyte processes. The resulting average value
$G_{min}^V = 4.0 \pm 0.3\%$ ($n = 10$ cells) thus reflected the tissue
volume fraction occupied by the fine glial protrusions. Im-
portantly, local $G_{min}^V(i,j)$ were relatively constant away from
the soma (figure 1c) suggesting homogeneous glial coverage
throughout the astrocyte domain. Second, we measured the
$G_V$ value that included all dendrite-like processes: $G_V =
8.9 \pm 0.7\%$ ($n = 10$). This value should correspond to electron
microscopy observations, in which fragments of astrocyte
processes are sampled arbitrarily. Because hippocampal astrocytes
tend to occupy separate neuropil domains, with little or no
spatial overlap [24,30,31], the value of $G_V$ should therefore pro-
vide control for the completeness of astroglia reconstruction at
the electron microscopy level.

(b) Spatial juxtaposition of synapses and glia

In serial-section electron micrographs of medial molecular
layer (Materials and methods), we identified glial fragments
using the criteria described earlier [3,6,11,32] and further vali-
dated this by tracing contiguous structures across the series
(figure 2a; electronic supplementary material, figure S2). Through-
out the sample, the average tissue volume fraction occupied
by glia was $G_V = 9.18 \pm 0.65\%$ ($n = 16$ astrocyte frag-
ments). This was in excellent agreement with $G_V$ measured in
live tissue (see above), indicating the accuracy of glial identifi-
cation. We next reconstructed fragments of astrocytes together
with the adjacent dendritic spines containing PSDs (figure 2b;
electronic supplementary material, figure S2).

In 3D reconstructions, we could unambiguously dis-
tinguish between thin ($n = 136$) and mushroom ($n = 53$)
dendritic spines (figure 2c–d) which differ more than fivefold
in their average head volume [14,21,25]. Indeed, the average
head volume for mushroom and thin spines was, respectively,
Figure 2. Three-dimensional reconstruction of contiguous astrocyte fragments with adjacent synapses. (a–d) A fragment of a dentate astrocyte reconstructed in three dimensions (blue, (a,b)) together with the adjacent dendritic spines (grey and dark yellow structures, (b)) equipped with PSDs (red). The spines are unambiguously separated into the sub-groups of thin (dark yellow, shown separately in (c)) and mushroom (grey, (d)). See the electronic supplementary material, figure S2, for examples of the original serial sections with identified structures. (Online version in colour.)

0.1998 ± 0.0156 and 0.0323 ± 0.0019 µm^3 (mean ± s.e.m.). An average 3D nearest-neighbour distance between the PSD centroids among all spines was 0.57 ± 0.02 µm (n = 190). This was consistent with the volume density of excitatory synapses in dentate [33,34], and similar to the average nearest-neighbour inter-synaptic distance in area CA1 [10,35]. This consistency further validated the accuracy of reconstruction procedures.

First, we attempted to gauge glial coverage of synapses by calculating the proportion of synaptic circumvent approached by high-affinity glial glutamate transporters [2,4,39], which immediately synapse [7,9]. This ‘spill-in’ signal is controlled by high-affinity synaptic NMDA receptors could provide one possible explanation for tighter and snugger synapses relying on AMPAR-mediated transmission [36,37], turning NMDA receptor-dominated (‘silent’) synapses into AMPAR-enriched large, mushroom-type spines [19]. Furthermore, classical electrophysiological observations propose that LTP may turn NMDA receptor-dominated (‘silent’) synapses into synapses relying on AMPAR-mediated transmission [36,37], evoking a concept of ‘learning spines’ as opposed to ‘memory spines’ [21,28].

This functional distinction between the two spine types may provide one possible explanation for tighter and snugger glial coverage around thin spines. In contrast to low-affinity AMPARs, high-affinity synapt NMDA receptors could in some conditions sense glutamate released outside the immediate synapse [7,9]. This ‘spill-in’ signal is controlled by high-affinity glial glutamate transporters [2,4,39], which therefore developed an integrative measure that would indicate how much glial surface how closely approaches each PSD. Because the closer glial surfaces are to the PSD the stronger impact they must have on any diffusion-mediated glia–synapse communication (figure 3c), we weighted all PSD–glia distances accordingly. The chosen weight function \( w(r) = A\exp(-18r^2) \) approximated the drop of transmitter concentration at a distance \( r \) from the release site shortly post-release [6,9], with the factor \( A \) normalizing the sum of all weights as one: \( A^{-1} = \int_0^{r_{\text{max}}} w(r) \, dr \). For each PSD, therefore, we calculated the average weighted PSD–glia distance \( D_w = \int_0^{r_{\text{max}}} w(r) \, dr \) that incorporates information about all glial surfaces occurring at \( r < r_{\text{max}} \) from the PSD (\( r_{\text{max}} = 0.5 \mu m \) corresponded to the average distance to the next PSD).

The average \( D_w \) for mushroom and thin spines, respectively, was 0.385 ± 0.006 µm and 0.276 ± 0.005 µm (\( p < 0.001 \)) when \( D_w \) was referred to the PSD centroid and 0.324 ± 0.004 µm and 0.264 ± 0.004 µm (\( p < 0.001 \)) when \( D_w \) was referred to all points on the surface of each PSD. This result indicated that astroglia approached PSDs on thin dendritic spines more closely than PSDs on mushroom spines. (Additional calculations indicated that this difference was relatively insensitive to a particular form of \( w(r) \).)

4. Discussion

Here, we have analysed fine 3D architectonics of astrocyte compartments and adjacent excitatory synapses in the dentate neuropil. We have found that on average astroglia are significantly nearer to synapses on thin dendritic spines compared with those on mushroom spines. What could be the adaptive significance of this difference?

2P uncaging of glutamate at sub-synaptic resolution provided a map of AMPA receptor (AMPAR) distribution in dendritic spines visualized in hippocampal pyramidal neuron [16]. The authors showed that AMPARs are abundant (up to 150 per spine) in mushroom spines but are scarcely present in thin spines or filopodia of dendrites. This observation was consistent with the molecular machinery that controls, in a synergistic manner, both dendritic spine elongation and the pruning of local AMPARs in cultured cortical neurons [20]. In agreement with this, dendritic spines with larger, complex PSDs showed greater numbers of immuno-gold labelled AMPARs compared with spines with simple, macular PSDs in hippocampal neurons [17]. Remarkably, induction of long-term potentiation (LTP) by local glutamate uncaging could transform, in real time, thin spines deficient in AMPARs into the AMPAR-enriched large, mushroom-type spines [19]. Furthermore, classical electrophysiological observations propose that LTP may turn NMDA receptor-dominated (‘silent’) synapses into synapses relying on AMPAR-mediated transmission [36,37], evoking a concept of ‘learning spines’ as opposed to ‘memory spines’ [21,28].

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Figure 3. Astrocyte membranes are much closer to the PSDs occurring on thin, compared to mushroom, dendritic spines. (a) A diagram illustrating automatic measurement of the nearest edge-to-edge distances $D_{\text{min}}$ between all individual PSDs (red mesh) and astrocyte membranes (white mesh) in space, at two levels of detail. Blue segments show $D_{\text{min}}$, determined in 3D automatically; smooth surface rendering (as in figure 2) is omitted for clarity. (b) Distribution (cumulative probability plot) of $D_{\text{min}}$ for individual PSDs occurring on thin (red, $n = 136$) and mushroom (black, $n = 53$) dendritic spines. (c) A two-dimensional diagram illustrating a rationale behind the weighting of PSD–glia distances. Distances between PSD (red, either the centroid only or all surface points) and all points on the surface of a neighbouring glial process (green) are measured, with larger distances bearing less weight (indicated by arrow thickness and shades of grey). The weighting reflects a rapid drop of transmitter concentration with distance from the site of release (yellow dot). See Results for details. (d) Grey and white columns, average (± s.e.m.) weighted PSD–astroglia distances $D_p$ for thin and mushroom dendritic spines, respectively, measured with respect to either PSD centroids or all PSD surface points, as indicated (**p < 0.001). (Online version in colour.)

could thus be particularly important for synapses that rely on high-affinity (NMDA) receptors.

The important adaptive role for the extent of synaptic glial coverage could also arise from the findings that hippocampal astrocytes can exchange transmitter signals with the neighbouring neurons [40,41]. Because such signalling relies on rapid extracellular diffusion outside the synaptic cleft, changes in the spatial juxtaposition of synapses and glia should have a direct impact on this mode of neuron–glia communication [12,13].

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