

The endoplasmic reticulum in perisynaptic astrocytic processes: shape, distribution and effect on calcium activity

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1 **Astrocytes recently emerged as key regulators of information processing in the**
2 **brain. Ca^{2+} signals in perisynaptic astrocytic processes (PAPs) notably allow**
3 **astrocytes to fine-tune neurotransmission at so-called tripartite synapses. As**
4 **most PAPs are below the diffraction limit, their content in Ca^{2+} stores and**
5 **the contribution of the latter to astrocytic Ca^{2+} activity is unclear. Here, we**
6 **reconstruct tripartite synapses in 3D from electron microscopy and find that**
7 **75% of PAPs contain some endoplasmic reticulum (ER), a major astrocytic**
8 **Ca^{2+} store, displaying strikingly diverse geometrical properties. To investigate**
9 **the role of such spatial properties, we implemented an algorithm that creates**
10 **3D PAP meshes of various ER distributions and constant shape. Reaction-**
11 **diffusion simulations in those meshes reveal that astrocyte activity is shaped by**
12 **a complex interplay between the location of Ca^{2+} channels, Ca^{2+} buffering, ER**
13 **shape and distribution. Overall, this study sheds new light into mechanisms**
14 **regulating signal transmission in the brain.**

15 Introduction

16 Astrocytes, the most abundant glial cells of the central nervous system, are essential to nu-
17 merous brain functions [74]. Notably, astrocytes are key modulators of neurotransmission at
18 so-called tripartite synapses [4, 60]. A single astrocyte in the CA1 region of the mouse hip-
19 pocampus is in contact with hundreds of thousands of synapses simultaneously, at perisynaptic
20 astrocytic processes (PAPs) [14]. Around 75 % of cortical and 65 % of hippocampal synapses
21 are contacted by an astrocytic process [78, 46]. This close contact between astrocytes and neu-
22 rons allows astrocytes to control various synaptic functions, from glutamate uptake [37], and
23 spillover [34, 9], to synapse homeostasis [57], stability [11], synaptogenesis [73], and neu-
24 rotransmission [3, 60]. Those synaptic functions are associated with specific local molecular
25 expression in PAPs [49, 28], which changes upon fear conditioning [49]. Importantly, the al-
26 teration of the proximity of PAPs to hippocampal synapses of the CA1 region *in vivo* affects
27 neuronal activity and cognitive performance [9]. Conversely, neuronal activity has been shown
28 to induce the remodeling of synaptic coverage by PAPs in various brain regions, both *in vivo*
29 and in acute slices [34, 51, 54, 11, 46, 56, 29, 76]. Together, those results illustrate that PAPs
30 are preferential sites of neuron-astrocyte communication. Although the recent emergence of
31 super-resolution techniques has provided key insights into the properties and functions of PAPs
32 [33, 5], our understanding of PAP physiology and function in live tissue is hindered by their
33 nanoscopic size [59, 1].

34
35 Ca^{2+} signals are commonly interpreted as a measure of astrocyte activity, notably in re-
36 sponse to neurotransmitter release at synapses [75, 59, 61]. The recent advances in Ca^{2+} imag-
37 ing approaches have improved the spatio-temporal resolution of Ca^{2+} signals in astrocytes [64,
38 61]. Strikingly, it revealed that astrocytes in acute slices and *in vivo* exhibit spatially-restricted
39 Ca^{2+} signals, also referred to as hotspots or microdomains, stable over time and which activity
40 varies under physiological conditions such as locomotion or sensory stimulation [44, 30, 2, 12,
41 8, 66, 70, 69, 68, 63, 53, 25, 45]. Growing evidence supports that PAPs are preferential sites dis-
42 playing spatially-restricted Ca^{2+} microdomains in response to neurotransmission [53, 25, 52, 7,
43 44]. As a single astrocyte can contact hundreds of thousands of synapses simultaneously [14],
44 such spatially-restricted Ca^{2+} microdomains might enable the astrocyte to finely tune synaptic
45 transmission at the single synapse level.

46
47 mGluR activation on the astrocytic membrane following neurotransmission at glutamater-
48 gic synapses results in Ca^{2+} transients mediated by G_q proteins and Ca^{2+} stores such as the
49 endoplasmic reticulum (ER) [64], which can trigger the release of molecules that modulate neu-
50 rotransmission, referred to as gliotransmitters[15, 48, 3, 60]. Most astrocytic Ca^{2+} signals are
51 mediated by the Inositol 3-Phosphate (IP_3) receptors on the membrane of the endoplasmic retic-
52 ulum (ER) [62]. Because of their nanoscopic size, the Ca^{2+} pathways involved in microdomain
53 Ca^{2+} signals in PAPs are still unclear. Notably, the presence of ER in PAPs and its involvement
54 in microdomain Ca^{2+} signals at synapses is highly debated. During the last decade, PAPs have

55 notably been regarded as devoid of ER, with a minimum distance between the synapse and the
56 closest astrocytic ER $> 0.5 \mu\text{m}$ [55, 59]. In contrast, inhibiting ER-mediated Ca^{2+} signaling in
57 fine processes results in a decreased number of Ca^{2+} domains [2] and a decreased Ca^{2+} peak
58 frequency [2, 7, 63]. Furthermore, some astrocytic ER has been detected near synapses in other
59 EM studies [1, 10]. Yet, the geometrical properties of the ER in PAPs and its distribution re-
60 main poorly characterized, but could have a strong impact on neuron-astrocyte communication
61 at tripartite synapses.

62

63 Here, we use a $220 \mu\text{m}^3$ hippocampal astrocytic volume from the CA1 stratum radiatum
64 region (6 nm voxel resolution) [17], reconstructed from electron microscopy (EM), to create 46
65 three dimensional meshes of tripartite synapses. Strikingly, we find that 75 % of PAPs contain
66 some ER, which can be as close as 72 nm to the post-synaptic density (PSD). Analysis of the
67 geometrical features of those meshes reveal the vast diversity of ER shapes and distributions
68 within PAPs from a single cell. We then used a detailed stochastic reaction-diffusion model of
69 Ca^{2+} signals in PAPs to investigate the mechanistic link between the spatial features of the ER
70 measured in the 3D meshes and the spatio-temporal properties of Ca^{2+} microdomain activity
71 in PAPs. To be able to decipher the effect of ER distribution within the PAP independently
72 from the effect of its shape, we developed an algorithm that automatically creates realistic 3D
73 tetrahedral PAP meshes with various ER distributions from the realistic meshes reconstructed
74 from EM. *In silico* experiments in those meshes reveal that the spatio-temporal properties of
75 Ca^{2+} signals in PAPs are tightly regulated by a complex interplay between the clustering of
76 Ca^{2+} channels, the ratio between ER surface area and PAP volume, Ca^{2+} buffering and ER
77 spatial distribution. Together, this study provides new insights into the geometrical properties
78 of hippocampal tripartite synapses and predicts mechanistic links between those features and
79 Ca^{2+} microdomain activity at tripartite synapses.

80 **Results**

81 **Quantification of the main geometrical properties of hippocampal tripar-
82 tite synapses**

83 To characterize the presence, shape and distribution of the endoplasmic reticulum (ER) in
84 perisynaptic astrocytic processes (PAPs), we used a $220 \mu\text{m}^3$ ($7.07 \mu\text{m} \times 6.75 \mu\text{m} \times 4.75 \mu\text{m}$)
85 hippocampal astrocytic volume from the CA1 stratum radiatum region reconstructed from a
86 perfectly isotropic EM stack (6 nm voxel resolution) [17]. Elements from the neuropil, i.e. bou-
87 tons, dendritic spines and post-synaptic densities (PSDs), were also reconstructed. Following
88 the workflow presented in Fig. 1A, forty four excitatory and two inhibitory tripartite synapse
89 meshes were created, containing all elements belonging to the astrocyte and to the neuropil
90 within a cube of $1.5 \mu\text{m}$ edge length ($3.375 \mu\text{m}^3$) centered at the center of mass of the PSD
91 (Supplementary Movie 1). Five of those tripartite synapse meshes are displayed in Fig. 1B.

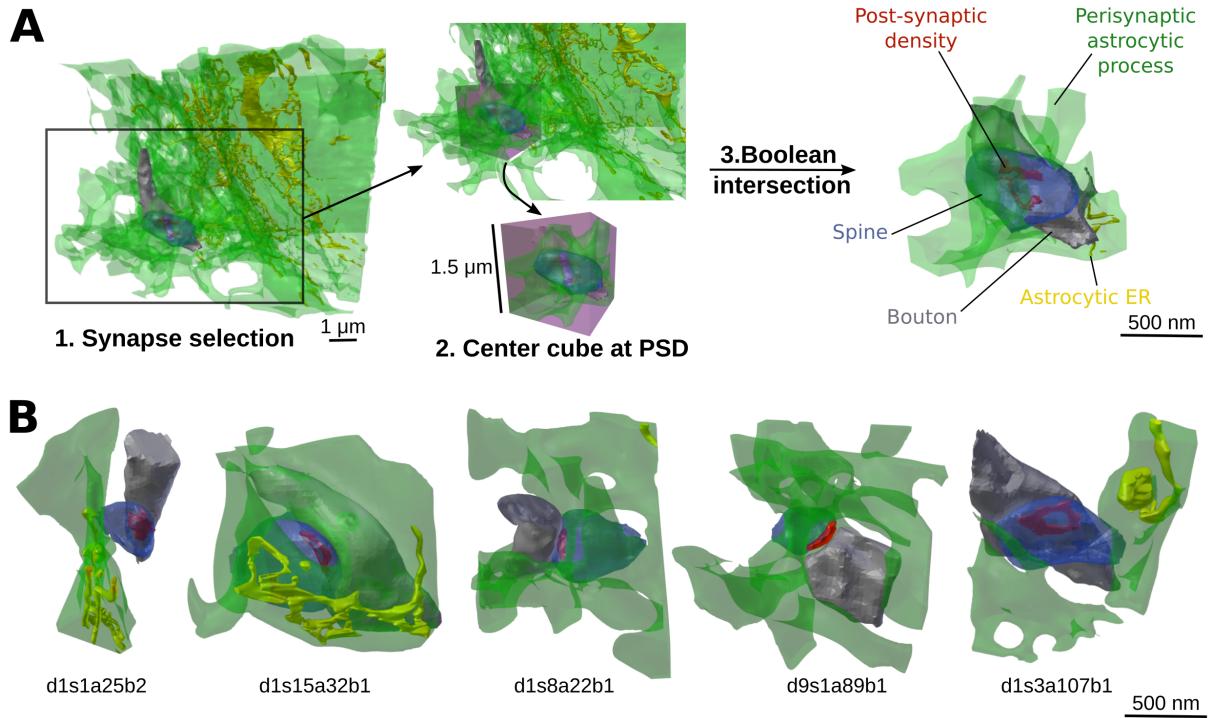


Fig 1: Reconstruction of 46 tripartite synapse meshes from electron microscopy. (A) Schematic presenting our tripartite synapse mesh creation workflow, here performed on synapse d10s1a2b1. 1. Synapses in contact with the $220 \mu\text{m}^3$ astrocytic volume were selected one by one. 2. A cube of $1.5 \mu\text{m}$ edge length ($3.375 \mu\text{m}^3$) was created and centered at the center of mass of the post-synaptic density (PSD, red). 3. Boolean intersection between the neuronal and astrocytic objects and the cube resulted in the isolation of the elements of the tripartite synapse mesh: the perisynaptic astrocytic process (PAP, green), the astrocytic endoplasmic reticulum (ER, yellow), the bouton (grey) and the spine (blue). This workflow resulted in the creation of 44 excitatory and 2 inhibitory tripartite synapse meshes. (B) Images of five of the 3D tripartite synapse meshes created, d1s1a25b2, d1s15a32b1, d1s8a22b1, d9s1a89b1, d1s3a107b1, revealing their diverse geometrical properties.

92 Among those meshes, seventeen were located at the borders of the $220 \mu\text{m}^3$ astrocytic volume.
93 They were thus omitted from data analysis as synaptic elements in those meshes could not be
94 fully reconstructed. The volume, surface area and surface-volume ratio (SVR) of each synaptic
95 element, i.e the PAP, astrocytic ER, spine and bouton, of the remaining twenty seven fully re-
96 constructed excitatory tripartite synapses are presented in Fig. 2C-E and Supplementary Table
97 S1. The minimum distance between each vertex on the membrane of the PAP and the center
98 of mass of the PSD was measured in each of the twenty seven meshes (Fig. 2B), providing
99 a quantification of the distribution of the astrocyte around the synapse. Our results highlight
100 the diverse distances between PSDs and PAPs belonging to a single cell. In accordance with
101 previous studies [46, 50, 55], PAP membrane vertices could be as close as 5 nm to the PSD,
102 with an average distance between the PSD and the closest PAP vertex of 65 nm. Importantly,
103 we found that PM-PSD distance is the shortest, i.e PAPs are the closest to the synapse, when
104 bouton surface area is low (Fig. 2F, $p=0.013$). PAP-PSD distance was not correlated to the
105 surface area of the PAP (Fig. 2G, $p=0.14$) or spine (Fig. 2H, $p=0.24$).

106 **Presence and geometrical properties of the endoplasmic reticulum in perisy-
107 naptic astrocytic processes**

108 Because of the small size of most PAPs, the Ca^{2+} pathways that regulate astrocytic Ca^{2+} mi-
109 crodomain activity at tripartite synapses remain to be uncovered. Notably, the presence of ER
110 in PAPs is controversial [55, 1, 10, 41]. We have thus analyzed the presence and shape of the
111 ER in the PAPs from the twenty seven fully reconstructed excitatory tripartite synapse meshes
112 presented in Fig. 2.

113 75% of PAPs contained some ER (Fig. 3C), which challenges the widespread belief that tri-
114 partite synapses are devoid of astrocytic ER. ER surface area, volume and SVR were measured
115 in ER-containing PAPs and highlight that ER shape is highly variable between PAPs from the
116 same cell (Fig. 3B). Note that there was no significant difference between bouton, spine and
117 PAP surface area, volume and SVR between synapses with vs without astrocytic ER (Supple-
118 mentary Fig. S1). We further characterized the vicinity of the astrocytic ER to the synapse. To
119 do so, we measured the distance between each vertex on the ER membrane to the center of mass
120 of the PSD ($n=20$). We found that ER-PSD distance varies drastically from synapse to synapse
121 (Fig. 3E) and can be as little as 72 nm, far below the $> 0.5 \mu\text{m}$ ER-PSD distance reported pre-
122 viously [55, 59]. The closest ER vertex was on average 432 nm away from the center of mass
123 of the PSD. Interestingly, the larger the surface area of the ER, the closer it was to the PSD (Fig.
124 3F, $p=0.013$). Astrocytic ER was closer to the PSD in PAPs with higher surface area (Fig. 3G,
125 $p=0.024$). The minimum ER-PSD distance was not correlated to the surface area of the spine
126 (Fig. 3H, $p=0.54$) or bouton (Fig. 3I, $p=0.29$). Overall, our results highlight that most PAPs
127 contain some ER and that its shape is highly variable, which could have strong implications on
128 ER-dependent Ca^{2+} signaling in PAPs resulting from synaptic transmission.

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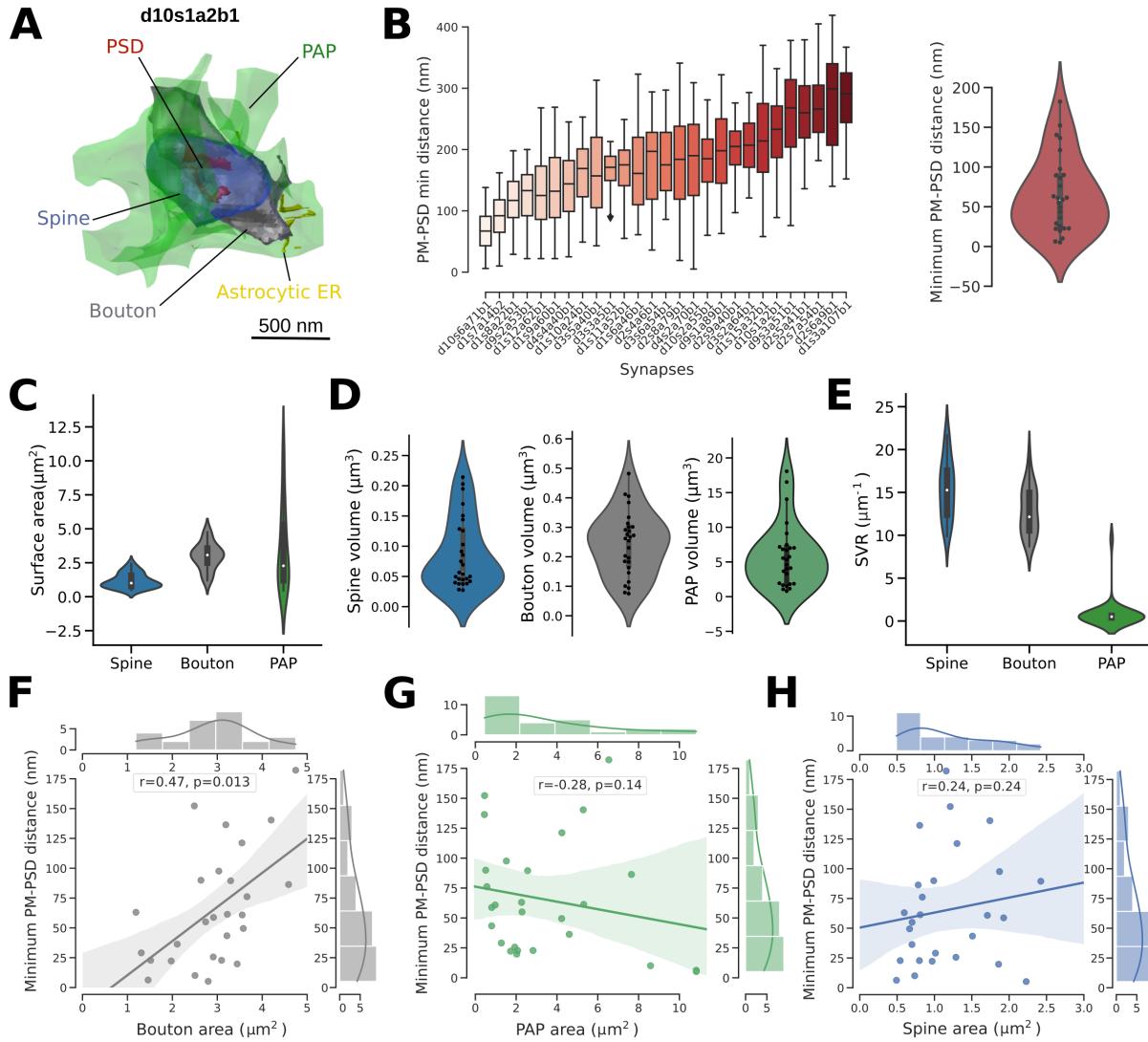


Fig 2: Characterization of the geometrical properties of hippocampal tripartite synapses.

(A) Image of a tripartite synapse mesh, d10s1a2b1, containing a bouton (grey), spine (blue), post-synaptic density (PSD, red), perisynaptic astrocytic process (PAP, green) and the astrocytic endoplasmic reticulum (ER, yellow). (B) Left: Boxplots presenting the distribution of the minimum distance between each vertex on the PAP membrane and the center of mass of the PSD, measured in the twenty seven excitatory tripartite synapse meshes fully reconstructed in this study. Right: Distribution of the minimum distance between the PAP and the PSD (n=27). (C-E) Violin plots presenting the distribution of spine, bouton and PAP surface area (C), volume (D) and surface-volume ratio (E). (F-H) Scatterplots presenting the variation of the minimum PAP-PSD distance as a function of bouton surface area (left), PAP surface area (middle) and spine surface area (right). Plots are presented with univariate kernel density estimation curves and a linear regression fit. Spearman correlation coefficients, r, and p-values, p, are displayed onto each regression plot, n=27.

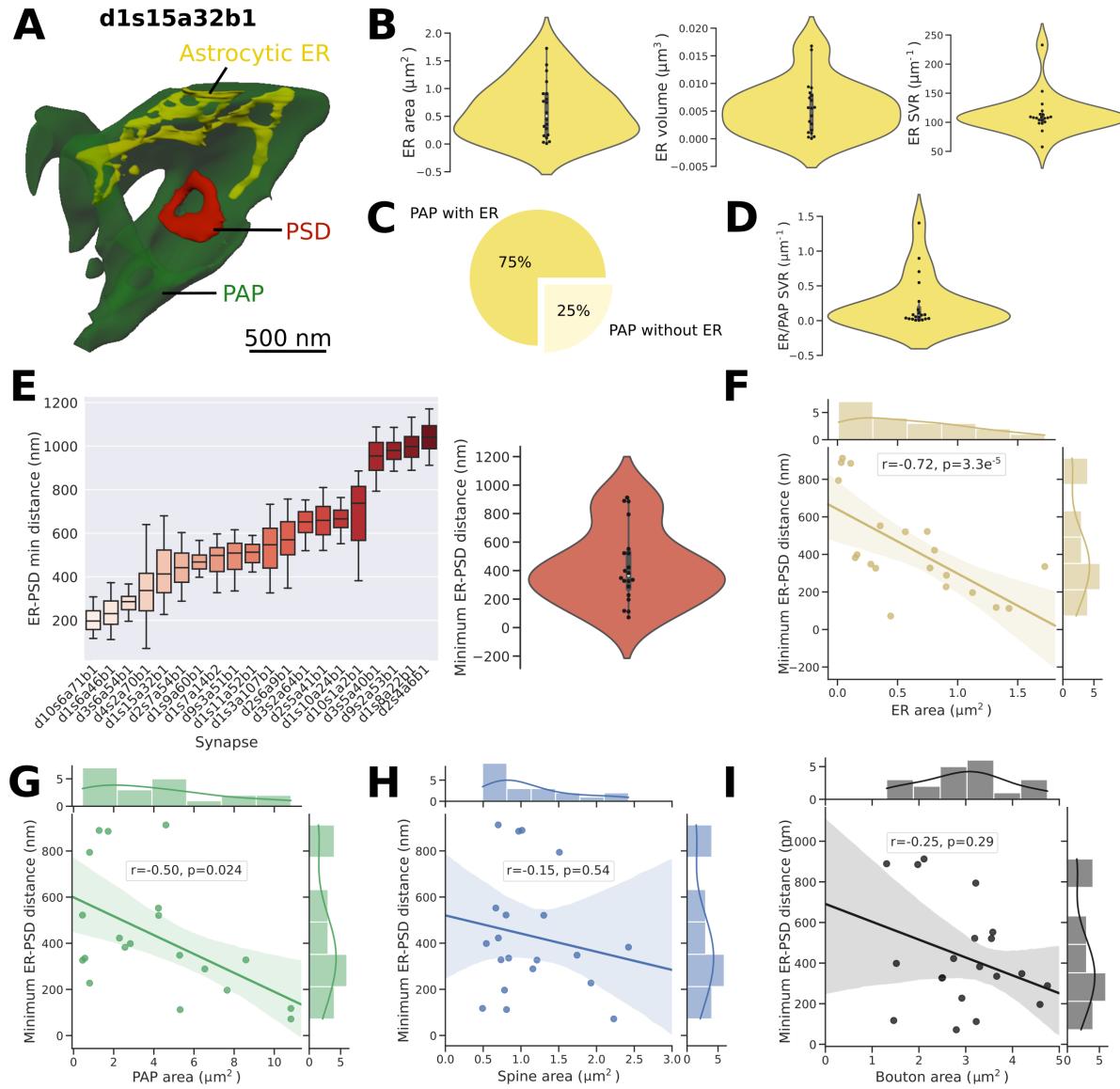


Fig 3: Presence and geometrical properties of the endoplasmic reticulum in perisynaptic astrocytic processes. (A) Image of the d1s15a32b1 PSD (red) and the neighboring PAP (green), that contains ER (yellow). (B) Violin plots representing the distribution of ER surface area (left), volume (middle) and surface volume ratio (right) within PAPs, n=20. (C) Among the twenty seven fully reconstructed PAP meshes extracted, 75 % contained some ER. (D) Distribution of the ratio between the ER surface area and PAP volume (n=20). (E) Quantitative analysis of the distance between the astrocytic ER and the neighboring PSD, n=20. (Left) Boxplots presenting the distribution of the distance of ER membrane vertices to the center of mass of the PSD in each PAP. (Right) Distribution of the minimum ER-PSD distance in PAPs, n=20. The lowest ER-PSD distance measured was 70nm (synapse d4s2a70b1). (F-I) Scatter plots showing the correlation between the minimum ER-PSD distance and the area of the PAP (F), spines (H) or boutons (I) containing the PSD.

Fig 3: (F-I) Scatterplots presenting the variation of the minimum ER-PSD distance as a function of ER surface area (F), PAP surface area (G), spine surface area (H) and bouton surface area (I), n=20. Plots are presented with univariate kernel density estimation curves and a linear regression fit. Spearman correlation coefficient, r, and p-value, p, are displayed onto each regression plot.

130 **Reaction-diffusion simulations reveal different spatio-temporal properties 131 of Ca^{2+} signals in PAPs of the same cell**

132 PAPs are characterized by highly diverse sizes and shapes of the ER (Fig. 3), which could have
133 strong implications on ER-mediated Ca^{2+} signals in PAPs. Because of their nanometric size,
134 measuring Ca^{2+} activity and deciphering the involvement of ER-mediated signals in individual
135 PAPs in live tissue is extremely challenging [61]. A better understanding of the mechanistic
136 link between the geometrical properties of the ER and the spatio-temporal properties of Ca^{2+}
137 microdomain signals in PAPs is crucial, yet hard to test experimentally. Here, we use the PAP
138 meshes presented in Fig. 3 together with a spatial stochastic model of Ca^{2+} signaling adapted
139 from the model of Denizot and collaborators [20] to investigate the mechanistic link between
140 ER shape and Ca^{2+} microdomain activity in PAPs. Ca^{2+} influx in the PAP cytosol in the model
141 is mediated by Inositol 3-Phosphate (IP_3) receptors on the membrane of the ER and by Ca^{2+}
142 channels at the plasma membrane, Ch_{PM} . The reactions modeled are presented in Fig. 4A and
143 in the Methods section. Neuronal activity was simulated at t=1s by infusing 50 IP_3 molecules
144 at the PM of the PAP. The implementation of this model with STEPS software [35] allows to
145 perform simulations in tetrahedral meshes in 3 spatial dimensions, such as the ones created in
146 this study. Representative Ca-GCaMP traces in a cylindrical mesh, corresponding to the con-
147 centration of Ca^{2+} bound to Ca^{2+} indicators added to the cytosol of the model, display similar
148 spatio-temporal characteristics to Ca^{2+} signals measured in organotypic hippocampal astrocytic
149 cultures [21] (Fig. 4A, right).

150
151 We performed simulations in six PAP meshes reconstructed from electron microscopy, char-
152 acterized by various geometrical properties of the ER: d1s3a107b1, d1s8a22b1, d1s10a24b1,
153 d2s6a9b1, d9s4a34b1 and d10s1a2b1 (Fig. 4B, Table 1). To do so, meshes were pre-processed
154 to allow their use in reaction-diffusion simulations. The pre-processing workflow is described in
155 Fig. 4C and in the Methods section, and produced 3D tetrahedral meshes from the 2D triangular
156 meshes reconstructed from EM. Screenshots of simulations performed in two realistic tetrahe-
157 dral PAP meshes are presented in Fig. 4B. Ca-GCaMP and free Ca^{2+} signals, in simulations
158 with and without Ca^{2+} indicators in the cytosol, respectively, were measured in d1s3a107b1,
159 d1s8a22b1, d1s10a24b1, d2s6a9b1, d9s4a34b1 and d10s1a2b1 PAP meshes. A simulation in
160 PAP d9s4a34b1 is presented in Supplementary movie 2. Representative traces are displayed
161 in Fig. 4E. Signals varied greatly depending on the mesh (Fig. 4F). Note that, in accordance
162 with previous studies [20, 81], Ca-GCaMP and free Ca^{2+} signals displayed different spatio-

Table 1: Characteristics of the 3D PAP meshes used in the reaction-diffusion simulations.

V_{cyt} is the cytosolic volume, S_{PM} is the plasma membrane surface area, S_{ER} is the ER surface area, $SVR_{ER/PAP}$ is the ratio between the ER surface area and the cytosolic volume. ER_c is the number of ER vertices at ER-PM contact sites, i.e. ≤ 20 nm from the closest PM vertex. $d1s15a32b1_{f0}$, $d1s15a32b1_{f21}$, $d1s15a32b1_{f64}$ and $d1s15a32b1_{f250}$ refer to meshes from frames 0, 21, 64 and 250 of the $d1s15a32b1$ PAP mesh presented in Fig. 8-9.

Geom	$V_{cyt} (\mu m^3)$	$S_{PM} (\mu m^2)$	$S_{ER} (\mu m^2)$	$SVR_{ER/PAP} (\mu m^{-1})$	ER_c
$d1s3a107b1$	0.112	2.00	0.315	2.81	183
$d1s8a22b1$	0.397	8.60	0.031	0.078	31
$d1s10a24b1$	0.331	5.80	0.344	1.04	0
$d2s6a9b1$	0.505	10.0	0.273	0.54	158
$d9s4a34b1$	0.410	7.05	0.807	1.97	20958
$d10s1a2b1$	0.531	10.0	0.136	0.26	3771
$d1s15a32b1_{f0}$	0.426	6.91	0.85	2.00	295
$d1s15a32b1_{f21}$	0.426	6.91	0.85	2.00	2337
$d1s15a32b1_{f64}$	0.426	6.91	0.85	2.00	1683
$d1s15a32b1_{f250}$	0.426	6.91	0.85	2.00	2408
$PAP1_v$	0.434	3.55	0.088	0.21	0
$PAP1_w$	0.432	3.55	0.428	0.99	0
$PAP1_x$	0.428	3.55	0.834	1.95	125
$PAP1_y$	0.423	3.55	1.27	3.00	0
$PAP1_z$	0.418	3.55	1.62	3.88	555

163 temporal properties (Fig. 4F). Those results suggest that the diverse geometrical features of
 164 PAPs and ER reported in this study (Fig. 2-3) strongly influence Ca^{2+} microdomain activity at
 165 tripartite synapses.

166 The effect of IP₃R clustering differs from PAP to PAP

167 IP₃R channels are not randomly distributed on the surface of the ER and form stable clusters of
 168 various sizes [77, 72, 71, 67]. Numerous computational studies performed in 2 spatial dimen-
 169 sions have predicted that IP₃R cluster size shapes Ca^{2+} activity (see [58] for a review). Whether
 170 this effect still holds in 3D, notably in complex shapes such as that of the PAPs reconstructed
 171 in this study, remains to be uncovered. We thus next simulated Ca^{2+} signaling in the 6 realistic
 172 PAP meshes presented in Fig. 4, reconstructed from EM, $d1s3a197b1$, $d1s8a22b1$, $d1s10a24b1$,
 173 $d2s6a9b1$, $d9s4a34b1$ and $d10s1a2b1$, with various distributions of IP₃Rs on the membrane of
 174 the ER.

175 Simulations of the model were performed with different IP₃R cluster sizes η . Ca^{2+} chan-
 176 nels were either randomly placed on the membrane of the PAP (cocl=0) or clustered onto the

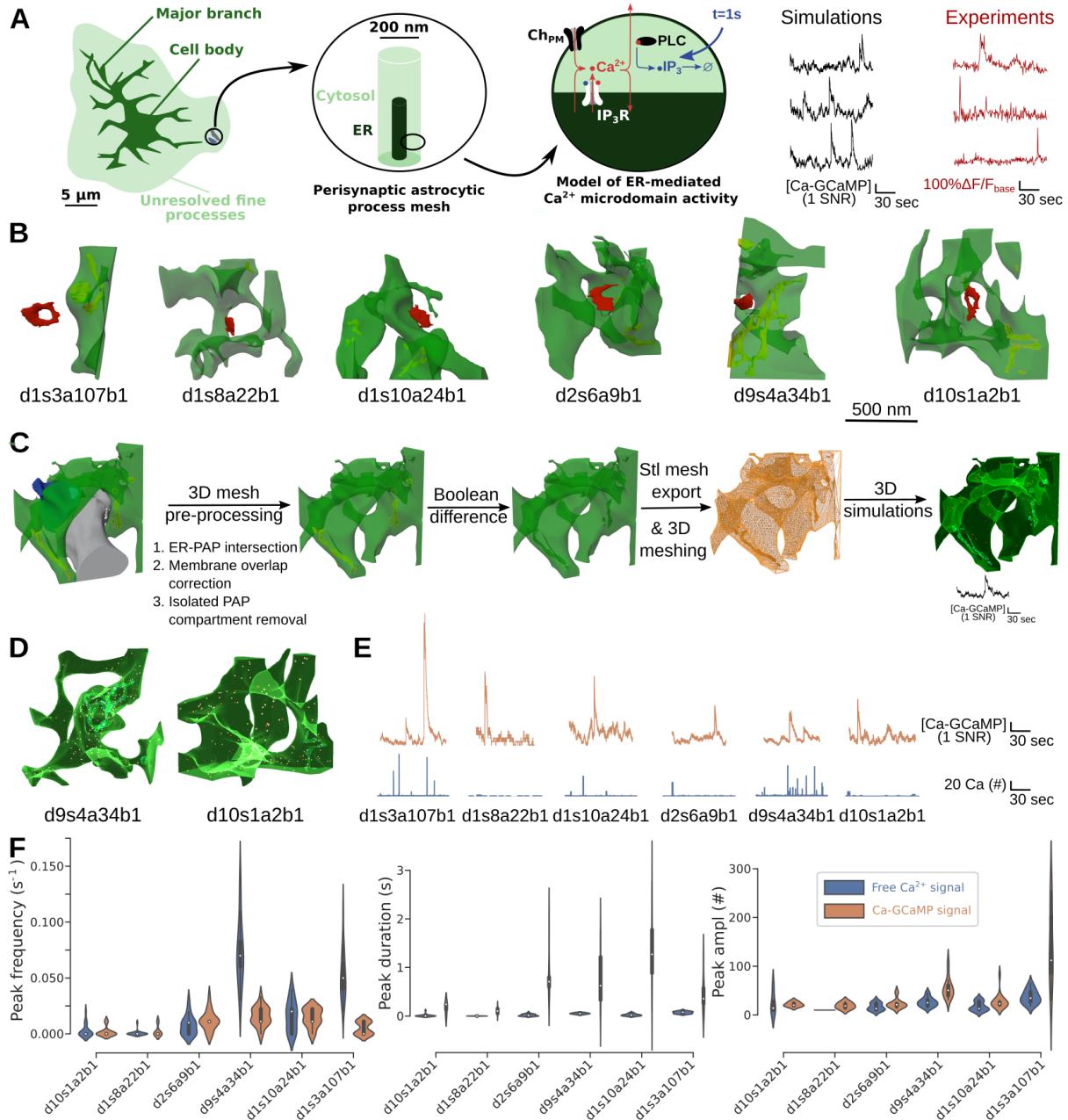


Fig 4: Reaction-diffusion simulations reveal different spatio-temporal properties of Ca^{2+} signals between PAPs of the same cell. (Left) Schematic representation of the model of Ca^{2+} signaling in PAPs used in this study. The model is stochastic, spatially-extended and simulations can be performed in 3D meshes. Ca^{2+} influx into the cytosol results from Ca^{2+} channels on the plasma membrane and from IP_3R channels on the ER. At $t=1\text{s}$, 50 IP_3 molecules were injected at the plasma membrane of the PAP, simulating neuronal activity. (Right) Representative Ca-GCaMP traces from simulations in a cylindrical mesh, 200 nm in diameter, 1 μm long (left, black) and experiments (right, red) [21].

Fig 4: (B) Images representing the 6 PAP meshes in which simulations were performed: d1s3a197b1, d1s8a22b1, d1s10a24b1, d2s6a9b1, d9s4a34b1 and d10s1a2b1. (C) Workflow to prepare the PAP meshes for 3D simulations illustrated on d2s6a9b1 mesh (see Methods section). The geometrical features of the resulting PAP meshes are presented in Table 1. (D) Screenshots of simulations in PAP meshes d9s4a34b1 and d10s1a2b1. Note that the darker and lighter greens result from 3D shading and rendering of the meshes. (E) Representative Ca-GCaMP (top, orange) and free Ca^{2+} (bottom, blue). Ca^{2+} traces were measured in separate simulations, where no GCaMP was added into the cytosol of the PAP. IP₃R channels and Ca^{2+} channels at the plasma membrane, Ch_{PM}, were randomly distributed onto the ER membrane and plasma membrane, respectively. (F) Quantification of peak frequency (left), duration (middle) and amplitude (right) of free Ca^{2+} (left, blue, n=20) and Ca-GCaMP (right, orange, n=20) signals measured *in silico* in 3D meshes of the PAPs presented in panel B.

177 PM triangles that were the closest to the ER triangles containing an IP₃R cluster (cocl=1), em-
178 ulating co-localization of Ca^{2+} channels, reported in neurons and astrocytes [43]. As IP₃R
179 density was kept constant across simulations, $3.5e^{-3}/\mu\text{m}^2$ [20], the total number of IP₃Rs,
180 N_{IP3R} , varied depending on the mesh: 90, 230, 78, 8, 40 and 96 in PAP meshes from synapses
181 d1s3a107b1, d9s4a34b1, d2s6a9b1, d1s8a22b1, d10s1a2b1 and d1s10a24b1, respectively. As
182 IP₃R cluster size was a divider of N_{IP3R} , cluster sizes tested varied slightly depending on the
183 mesh. The range of IP₃R cluster size tested varied from $\eta=1$ -26. Representative free Ca^{2+}
184 traces measured in d1s3a197b1, d1s8a22b1, d1s10a24b1, d2s6a9b1, d9s4a34b1 and d10s1a2b1
185 PAP meshes with various IP₃R cluster sizes η are displayed in Fig. 5B. Strikingly, IP₃R clustering
186 only affected Ca^{2+} activity in a subset of the PAP meshes studied (Fig. 5C). Indeed, Ca^{2+}
187 peak duration and amplitude increased with IP₃R cluster size in PAP meshes from synapses
188 d1s3a107b1 (ANOVA, p= $5.1e^{-5}$ and $1.9e^{-7}$), d2s6a9b1 (ANOVA, p= $3.16e^{-3}$ and 0.026) and
189 d9s4a34b1 (ANOVA, p=0.018 and 0.028) but not in d1s10a24b1 (ANOVA, p=0.44 and 0.32)
190 and d10s1a2b1 (ANOVA, p=0.69 and 0.83). This effect was associated with an increased fre-
191 quency of IP₃R opening with cluster size in d1s3a107b1, d2s6a9b1 and d9s4a34b1 meshes
192 (ANOVA, p= $3.4e^{-4}$, 0.007 and 0.037, respectively). Conversely, cluster size had no effect on
193 IP₃R opening frequency in d1s10a24b1 (ANOVA, p=0.050) and d10s1a2b1 (ANOVA, p=0.15).
194 Interestingly, such differences in IP₃R clustering effects on Ca^{2+} activity were still observed
195 in the absence of co-localization of Ca^{2+} channels at the plasma membrane with IP₃R clusters
196 (Supplementary Fig. S2). Those results highlight that different PAP and ER shapes are associ-
197 ated with different IP₃R clustering effects.
198

199 Interestingly, increased neuronal stimulation, simulated as an increased amount of IP₃ in-
200 fused, i, in the PAP at t=1s, triggered clustering effects in a PAP in which no clustering effect
201 was observed after a milder neuronal stimulation (Fig. 5D). This effect was characterized by an
202 increase of Ca^{2+} peak amplitude (ANOVA, p= $8.2e^{-5}$ for i=150 and p= $4.66e^{-7}$ for i=200), fre-
203 quency (ANOVA, p=0.005 for i=150 and p=0.006 for i=200) and duration (ANOVA, p= $3.4e^{-5}$

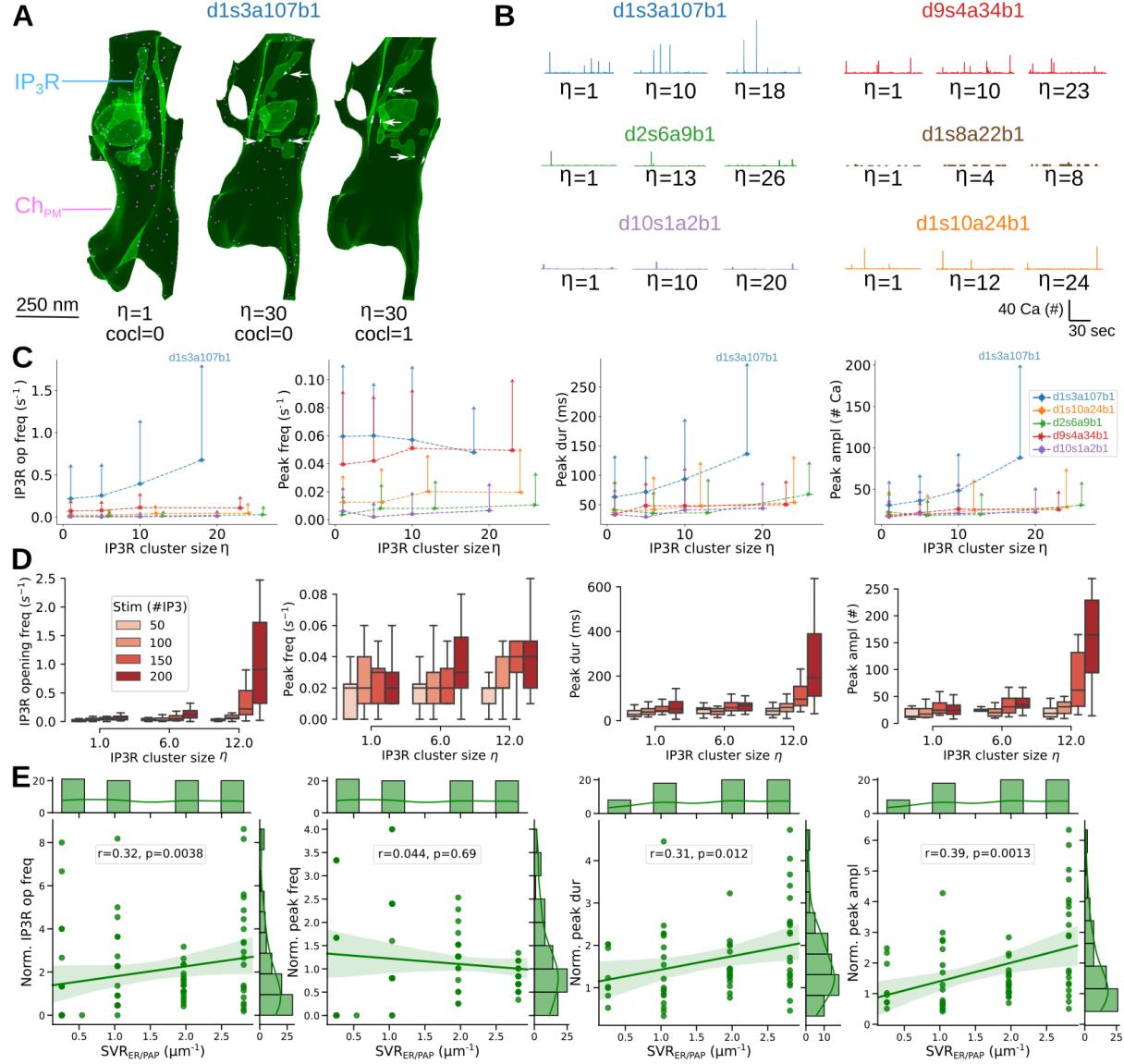


Fig 5: The effect of IP₃R clustering differs from PAP to PAP. (A) Screenshots of simulations in d1s3a107b1, with IP₃R cluster size $\eta=1$ and $\eta=30$. Simulations were performed with (cocl=1) and without (cocl=0) co-clustering of Ca²⁺ channels at the plasma membrane (purple) with IP₃Rs on the ER (blue). IP₃R clusters are indicated with a white arrow. (B) Representative free Ca²⁺ traces measured *in silico* in d1s3a107b1 (blue), d9s4a34b1 (red), d2s6a9b1 (green), d1s8a22b1 (brown), d10s1a2b1 (purple) and d1s10a24b1 (orange) meshes. (C) Quantification of IP₃R opening frequency (left), Ca²⁺ peak frequency (middle left), duration (middle right) and amplitude (right) as a function of η , in d1s3a107b1 (blue circle), d1s10a24b1 (orange diamond), d2s6a9b1 (green triangle), d9s4a34b1 (red triangle) and d10s1a2b1 (purple hexagon), cocl=1. Data are represented as mean \pm STD, n=20 for each mesh and cluster size tested.

Fig 5: Lines are guides for the eyes. Note that no peaks were detected in simulations in d1s8a22b1. (D) Quantification of IP₃R opening frequency (left), Ca²⁺ peak frequency (middle left), duration (middle right) and amplitude (right), in d1s10a24b1, for $\eta=1, 6$ and 12 and various levels of neuronal stimulation: IP₃ infused $i=50, 100, 150$ and 200 (from left to right, light red to dark red), $n=20$ for each parameter set tested. (E) Scatterplots presenting the variation of IP₃R opening frequency (left), Ca²⁺ peak frequency (middle left), duration (middle right) and amplitude (right) for $\eta=18-24$, normalized with Ca²⁺ peak characteristics measured for $\eta=1$, as a function of SVR_{ER/PAP}. Plots are accompanied by univariate kernel density estimation curves and a linear regression fit. Spearman correlation coefficient, r , and p-value, p , are displayed onto each regression plot.

for $i=150$, and $p=0.026$ for $i=200$) with IP₃R cluster size for $i=150$ and 200 , while cluster size did not affect peak amplitude (ANOVA, $p=0.27$ for $i=50$ and $p=0.08$ for $i=100$), frequency (ANOVA, $p=0.86$ for $i=50$ and $p=0.72$ for $i=100$) and duration ($p=0.13$ for $i=50$ and $p=0.15$ for $i=100$) for $i=50$ and 100 . This effect was mediated by an increased IP₃R opening frequency with cluster size when neuronal stimulation was larger (ANOVA, $p=0.024, 5.23e^{-7}$ and $7.8e^{-5}$, for $i=100, 150$ and 200 , respectively), while clustering had no effect on IP₃R opening frequency for milder neuronal stimulation (ANOVA, $p=0.21$, $i=50$). This suggests that IP₃R clustering in PAPs could act as an amplifier of neuronal stimulation.

212

Unexpectedly, the PAPs in which an IP₃R clustering effect was observed were not the PAPs with the highest ER surface area S_{ER} , i.e with the highest number of IP₃R channels. Indeed, although the normalized IP₃R opening frequency and Ca²⁺ peak frequency at high cluster size were positively correlated with S_{ER} , normalized Ca²⁺ peak amplitude and duration were not correlated with S_{ER} (Supplementary Fig. S3). Rather, normalized IP₃R opening frequency, Ca²⁺ peak amplitude and duration were positively correlated to the ratio between ER surface area and PAP volume SVR_{ER/PAP} (Fig. 5E, $p=0.0038, 0.012$ and 0.0013 , respectively). Ca²⁺ peak frequency however did not vary with SVR_{ER/PAP} (Fig. 5E, $p=0.69$). This probably results from our peak definition. Indeed, as a peak is considered terminated when the Ca²⁺ trace decreases below peak threshold, a higher frequency of IP₃R opening events can result in successive opening events occurring before peak termination, resulting in a similar peak frequency but larger peak duration.

225

Overall, our simulation results nuance the effect of the clustering of Ca²⁺ channels on Ca²⁺ signals in small sub-cellular compartments like PAPs. Strikingly, in contrast with reports from models in 2 spatial dimensions [23, 58], Ca²⁺ activity increased with cluster size in only a subset of the realistic 3D PAP meshes tested, highlighting the complex interplay between the ER surface to PAP volume ratio, the intensity of neuronal stimulation and IP₃R clustering on Ca²⁺ microdomain activity. This highlights the importance of cautious interpretation of simulation results on geometrical effects depending on the geometry used.

233 **The surface-volume ratio of the ER conditions the amplification of Ca^{2+} 234 activity by IP_3R clustering in 3D**

235 Our simulation results revealed that the increase of Ca^{2+} activity resulting from IP_3R clustering increased with the ratio between the ER surface area and the PAP volume ($\text{SVR}_{\text{ER}/\text{PAP}}$, Fig
236 5E). In the PAP meshes studied in Fig. 5, $\text{SVR}_{\text{ER}/\text{PAP}}$ varied together with PAP shape and ER
237 shape. To discern the effect of ER and PAP shape from $\text{SVR}_{\text{ER}/\text{PAP}}$ on Ca^{2+} activity in PAPs,
238 we created meshes with various ER size and constant ER and PAP shapes. The original mesh
239 was extracted from the $220 \mu\text{m}^3$ astrocytic volume, located at the vicinity of the d9s3a51b1
240 PSD and referred to as PAP1. The location of PAP1 in the $220 \mu\text{m}^3$ reconstructed hippocampal
241 astrocytic volume is presented in Supplementary Fig. S4. Meshes with various $\text{SVR}_{\text{ER}/\text{PAP}}$
242 were created from PAP1 by rescaling the ER using Blender software. Meshes were then created
243 following the mesh pre-processing workflow described in Fig. 4C, resulting in the creation of
244 PAP1_v, PAP1_w, PAP1_x, PAP1_y and PAP1_z meshes (Fig. 6A). The geometrical properties of
245 those meshes are presented in Table 1.
246

247
248 IP₃R opening frequency, Ca^{2+} peak frequency, duration and amplitude increased with
249 $\text{SVR}_{\text{ER}/\text{PAP}}$ (Fig. 6B-F). This is not surprising as ER surface area increases with $\text{SVR}_{\text{ER}/\text{PAP}}$
250 in those meshes, thus resulting in an increase of the amount of IP₃R channels with $\text{SVR}_{\text{ER}/\text{PAP}}$.
251 The total number of IP₃R channels, $N_{\text{IP}_3\text{R}}$, thus was 24, 120, 240, 360 and 460, in PAP1_v,
252 PAP1_w, PAP1_x, PAP1_y and PAP1_z meshes, respectively. Importantly, Ca^{2+} peak frequency
253 (Fig. 6D, ANOVA, $p=2.39e^{-8}$), duration (Fig. 6E, ANOVA, $p=7.52e^{-17}$) and amplitude (Fig.
254 6F, ANOVA, $p=1.29e^{-14}$) increased with IP₃R cluster size in PAP1_z mesh. This resulted
255 from an increase of IP₃R opening frequency with cluster size in PAP1_z (Fig. 6C, ANOVA,
256 $p=5.93e^{-24}$). No clustering effect was observed in PAP1_{w-y} meshes, characterized by a lower
257 $\text{SVR}_{\text{ER}/\text{PAP}}$, confirming the mechanistic link between $\text{SVR}_{\text{ER}/\text{PAP}}$ and the amplification of
258 Ca^{2+} activity mediated by IP₃R clustering suggested in Fig. 5. Note that no Ca^{2+} signals were
259 detected in PAP1_v mesh. Simulations in PAP1_z meshes with constant IP₃R channel number
260 further highlight that this effect results both from the increased number of IP₃R channels in
261 PAP1_z and from ER shape (Supplementary Fig. S5). Supplementary Fig. S6 reveals that IP₃R
262 opening frequency and Ca^{2+} peak frequency increased with $\text{SVR}_{\text{ER}/\text{PAP}}$ even when IP₃R chan-
263 nels were not clustered ($\eta=1$).

264 Increasing ER surface area in PAP1 however also resulted in a decreased distance between the
265 ER and the plasma membrane (PM) in the PAP (Fig. 6G). Importantly, Ca^{2+} peak frequency
266 (Fig. 6H, $p=2.56e^{-11}$), duration (Fig. 6I, $p=2.18e^{-6}$) and amplitude (Fig. 6J, $p=1.32e^{-6}$) in-
267 creased with the amount of ER vertices at ER-PM contact sites ($\leq 20 \text{ nm}$ to the closest PM
268 vertex [80, 79]). This suggests that the increased IP₃R clustering effect on Ca^{2+} microdomain
269 activity in PAP1_z could result either from its increased $\text{SVR}_{\text{ER}/\text{PAP}}$ or to the decreased ER-PM
270 distances in this mesh compared to PAP1_{w-y} meshes. Overall, our simulation results suggest
271 that ER shape, notably the ratio between its surface area and PAP volume, shape astrocytic
272 Ca^{2+} microdomain activity at synapses.

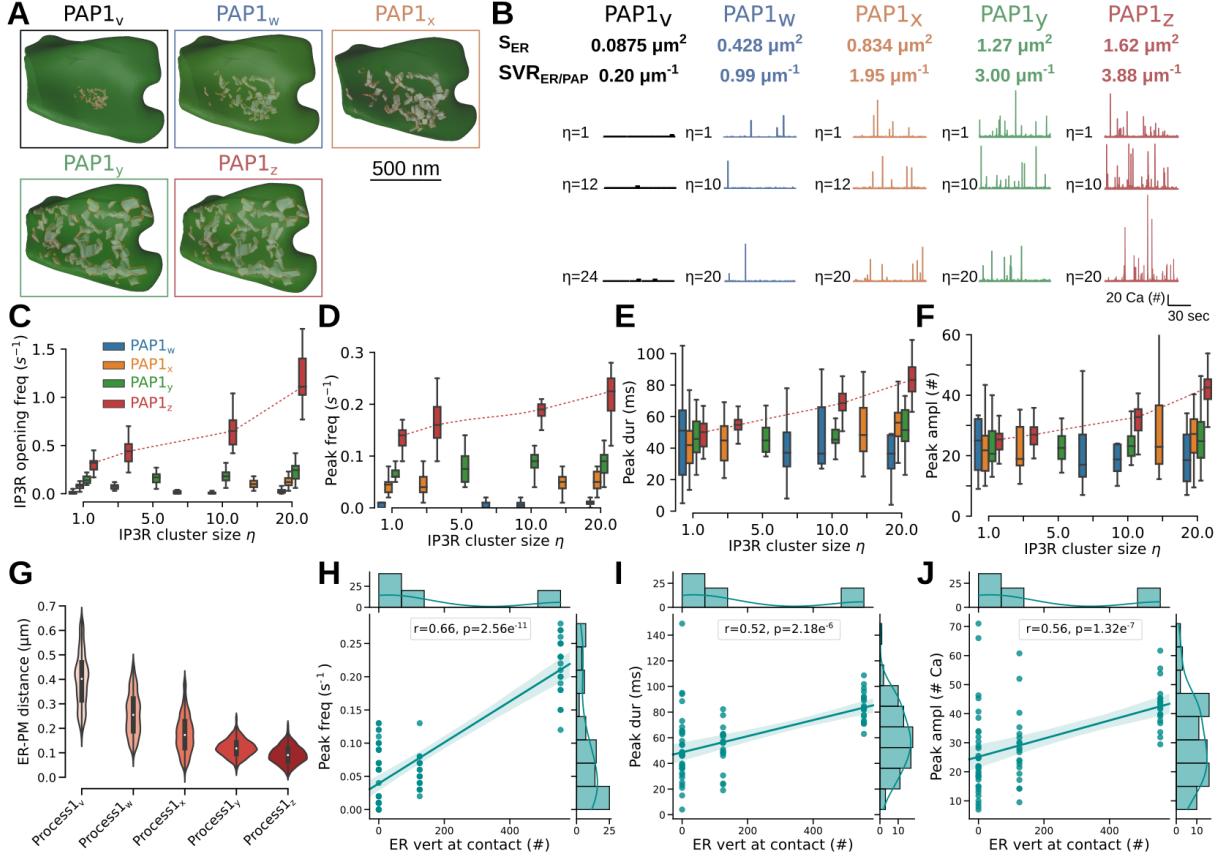


Fig 6: The surface-volume ratio of the ER in PAPs conditions the amplification of Ca^{2+} activity by IP_3R clustering in 3D. (A) Images of the different PAP meshes created to investigate the effect of the ratio between ER surface area and PAP volume, $SVR_{ER/PAP}$, on Ca^{2+} microdomain activity: PAP1_{v-z}. Meshes were obtained by rescaling the ER object in PAP1, located at the vicinity of the d9s3a51b1 PSD (Supplementary Fig. S4). Geometrical features of the meshes are presented in Table 1. (B) Representative free Ca^{2+} traces measured in PAP1_v (black), PAP1_w (blue), PAP1_x (orange), PAP1_y (green) and PAP1_z (red), for IP_3R cluster size $\eta=1$ (top), $\eta=12$ (middle) and $\eta=24$ (bottom). (C-F) Quantification of IP_3R opening frequency (C), Ca^{2+} peak frequency (D), duration (E) and amplitude (F), in PAP1_w (blue), PAP1_x (orange), PAP1_y (green) and PAP1_z (red), for $\eta=1-23$. Note that η varies depending on the mesh (see Methods). Lines were added to visualize the effect of η on Ca^{2+} peak characteristics in PAP1_z mesh. (G) Quantification of the variation of the distance between each ER vertex and the closest plasma membrane (PM) vertex in PAP1_{v-z} meshes. (H-J) Scatterplots presenting Ca^{2+} peak frequency (H), duration (I) and amplitude (J) in PAP1_{v-z} meshes for $\eta=20$, as a function of the number of ER vertices ≤ 20 nm to the closest PM vertex.

Fig 6: Plots are presented with univariate kernel density estimation curves and a linear regression fit. Spearman correlation coefficient, r , and p-value, p , are displayed onto each regression plot.

273 Quantification of ER-PM distance distribution within PAPs

274 As simulation results suggested that Ca^{2+} activity in $\text{PAP1}_{\text{v}-\text{z}}$ meshes varies depending on
275 the distribution of the ER within the PAP, we next aimed at quantifying ER distribution in the
276 twenty ER-containing PAP meshes reconstructed from EM and presented in Fig. 3. To do so,
277 we measured the distance between each vertex on the plasma membrane (PM) and the closest
278 vertex on the ER. We found that ER-PM distance is highly variable in PAPs from a single cell,
279 with an average ER-PM distance within a single PAP from around 200 nm to 1200 nm (Fig.
280 7B-C). Not surprisingly, mean ER-PM distance decreases as ER (Fig. 7D, $p=5.01e^{-10}$) and
281 PAP (Fig. 7E, $p=0.055$) surface area increase. Interestingly, ER-PM distance was lower in
282 PAPs contacting boutons with higher surface area (Fig. 7F, $p=0.022$). Note that there was no
283 correlation between ER-PM distance and spine surface area (Fig. 7G, $p=0.73$). Importantly,
284 we found that PAPs closer to the synapse are characterized by lower mean ER-PM distance
285 (Fig. 7H, $p=2.2e^{-5}$), which, according to simulation results presented in Fig. 6, could result in
286 enhanced Ca^{2+} activity in those PAPs.

287 Effect of ER-PM distance in PAPs on Ca^{2+} microdomain activity

288 To discern the effect of $\text{SVR}_{\text{ER}/\text{PAP}}$ from the effect of ER-PM distance on Ca^{2+} microdomain
289 activity in PAPs reported in Fig. 6, we implemented an algorithm that creates realistic tetra-
290 hedral 3D meshes of PAPs characterized by various distributions of the ER within the same
291 PAP. The workflow is presented in Fig. 8. Briefly, the ER is split into small portions of sim-
292 ilar size, then resized to match the total ER surface area of the original mesh. Simulations in
293 meshes with the original ER and with split ER confirmed that this ER splitting algorithm does
294 not alter Ca^{2+} activity in the PAP (Supplementary Fig. S7). A simulation of n frames is then
295 generated in Blender, which alters the location of the ER objects within the PAP. Each frame is
296 thus characterized by a unique distribution of the ER objects within the PAP, while ER and PAP
297 shape, surface area, volume and SVR are constant across frames (Supplementary movie 3). The
298 mesh processing workflow presented in Fig. 4C is then automatically applied to each frame
299 of interest. This workflow allows the creation of numerous realistic 3D PAP meshes, that can
300 be used for reaction-diffusion simulations in 3D. Fig. 7B-D displays the quantification of ER
301 distribution in the PAP meshes created with this workflow on PAP d1s15a32b1. The workflow
302 successfully produced realistic tetrahedral PAP meshes characterized by various ER-PM dis-
303 tances (Fig. 7B). Note that the distribution of ER-PM distance at ER-PM contact sites (ER-PM
304 distance $\leq 20 \text{ nm}$) did not vary, while the number of ER vertices belonging to contact sites, and
305 thus the size of the contact sites, increased with frame number (Fig. 7C).

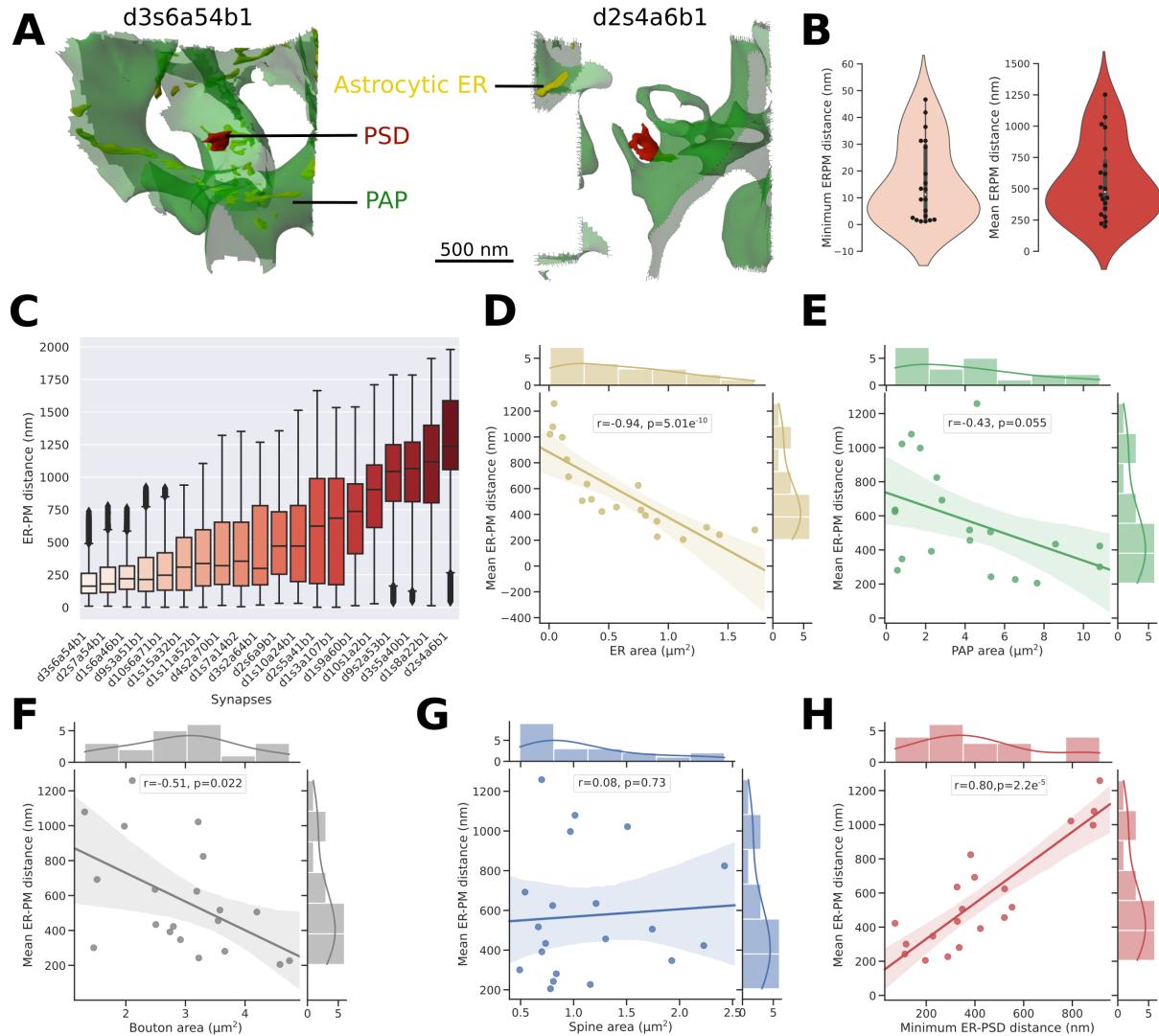


Fig 7: ER-PM distance in PAPs is highly variable and is decreased in PAPs close to the synapse. (A) Images of 2 PAP meshes (green), d3s6a54b1 (left) and d2s4a6b1 (right) with the neighboring PSD (red), displaying the diverse shapes and distributions of the ER (yellow) in PAPs from the same cell. (B) Distribution of the minimum (left) and mean (right) distance between each vertex on the plasma membrane (PM) and the closest ER vertex, measured in PAP meshes reconstructed from EM, n=20 (Fig. 3). (C) Quantification of the distance between each PM vertex and the closest ER vertex in each PAP mesh. (D-F) Scatterplots presenting the variation of the mean distance between each PM vertex and the closest ER vertex as a function of the surface area of the ER (D), PAP (E), bouton (F), and spine (G), and as a function of the minimum ER-PSD distance (H), n=20. Plots are presented with univariate kernel density estimation curves and a linear regression fit. Spearman correlation coefficient, r, and p-value, p, are displayed onto each regression plot.

306

307 To test the effect of ER distribution in PAPs, simulations were performed in meshes from
308 frames 0, 21, 64 and 250 of PAP d1s15a32b1 (Fig. 9A) with different IP₃R cluster sizes, η .
309 Free Ca²⁺ signals did not vary depending on ER distribution within the PAP (Fig. 9C). Inter-
310 estingly, ER distribution did not affect the amplification of IP₃R opening frequency (ANOVA,
311 p=0.059), Ca²⁺ peak duration (ANOVA, p=0.55) and amplitude (ANOVA, p=0.15) with IP₃R
312 cluster size when IP₃R clusters were randomly distributed on the membrane of the ER (Fig.
313 9D). However, the increase of peak frequency with cluster size was larger in meshes in which
314 the ER was closer to the plasma membrane (Fig 9D, ANOVA, p=0.0048). This suggests that
315 a distribution of the ER closer to the plasma membrane might allow increased Ca²⁺ peak fre-
316 quency with IP₃R cluster size.

317

318 As IP₃R clusters are believed to be preferentially located at ER-PM contact sites [72], we
319 performed simulations in the same meshes while positioning IP₃R clusters at ER-PM contact
320 sites. Screenshots of simulations with different locations of IP₃R clusters are presented in Fig.
321 9B. Interestingly, locating IP₃R clusters at ER-PM contact sites resulted in larger increases of
322 IP₃R opening frequency (ANOVA, p=0.0019) and Ca²⁺ peak frequency (ANOVA, p=0.0043)
323 with cluster size in meshes in which the ER was closer to the PM (Fig. 9D). However, the loca-
324 tion of the ER did not impact the effect of IP₃R cluster size on Ca²⁺ peak duration (ANOVA,
325 p=0.45) and amplitude (ANOVA, p=0.069). Together, those results suggest that a distribution
326 of the ER closer to the plasma membrane, coupled with a location of IP₃R clusters at ER-PM
327 contact sites, favors an increase of Ca²⁺ peak frequency with cluster size. ER-PM contact sites
328 could act as diffusional barriers. Locating IP₃R channels at ER-PM contact sites would thus
329 increase the residency time of Ca²⁺ ions and IP₃ molecules at the vicinity of the channels, thus
330 increasing the probability of Ca²⁺ and IP₃ binding to IP₃Rs and resulting in an increased IP₃R
331 opening frequency in meshes with larger ER-PM contact sites. To further test the interplay
332 between ER distribution, IP₃R clustering and local diffusional properties, Ca²⁺ buffers, here
333 Ca²⁺ indicators GCaMP6s, were added to the model. Strikingly, ER distribution had a greater
334 effect on buffered Ca²⁺ signals compared to free Ca²⁺ signals (Fig. 9E). More precisely, the in-
335 crease of IP₃R opening frequency (ANOVA, p=2.37e⁻⁷), Ca-GCaMP peak frequency (ANOVA,
336 p=1.14e⁻⁴), duration (ANOVA, p=7.78e⁻³) and amplitude (ANOVA, p=0.023) with IP₃R clus-
337 ter size was significantly larger in meshes in which the ER was located closer to the plasma
338 membrane. Those results highlight that the effect of ER distribution within the PAP on Ca²⁺
339 microdomain activity is conditioned by the location of IP₃R channels at ER-PM contact sites
340 and by local Ca²⁺ buffering.

341 Discussion

342 Here, we extracted 3D meshes of tripartite synapses from a 220 μm^3 hippocampal astrocytic
343 volume from the CA1 stratum radiatum region, reconstructed from EM [17]. Quantification

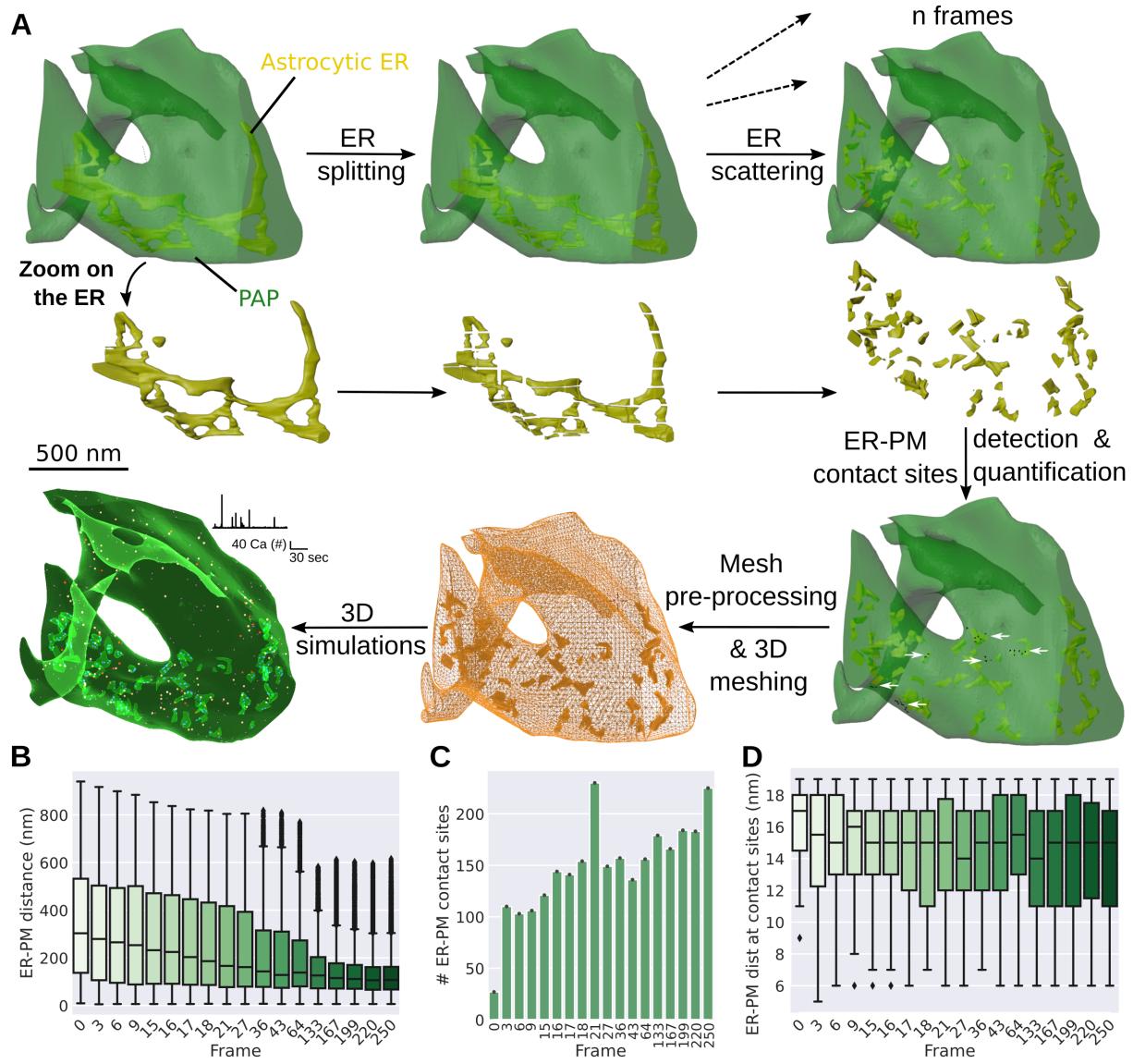


Fig 8: Automated realistic 3D PAP mesh generation with diverse ER distributions. (A) Schematic representing the workflow developed in this study to create realistic PAP meshes in 3 spatial dimensions with various ER distributions and constant shape, volume and surface area of PAP and ER, used on the PAP mesh d1s15a32b1. The ER is split and a simulation with n frames is generated in Blender, in which ER objects are subject to physical forces that alter their spatial distribution. The n frames are thus characterized by different locations of the ER elements within the PAP, with constant ER and PAP shapes. The pipeline detects, quantifies and exports in a text file the distance between each vertex at the plasma membrane (PM) and the closest vertex at the membrane of the ER. A point cloud can be created to visualize the ER vertices at ER-PM contact sites (ER-PM distance ≤ 20 nm, white arrows). The mesh pre-processing workflow presented in Fig. 4C is then applied to the mesh of each desired frame. The resulting 3D tetrahedral meshes can then be used for 3D reaction-diffusion simulations.

Fig 8: (B) Quantification of the distance between each PM vertex and the closest ER vertex in the meshes generated by the workflow presented in panel A, applied to the d1s15a32b1 PAP mesh. (C) Quantification of the number of ER vertices located at ER-PM contact sites, i.e. ≤ 20 nm to the closest PM vertex, in each frame from mesh d1s15a32b1. (D) Quantification of the distance between each PM vertex and the closest ER vertex at ER-PM contact sites, in each frame from mesh d1s15a32b1.

344 of the geometrical features of those meshes highlighted the diverse geometrical properties of
345 PAPs from a single astrocyte and revealed, contrary to a widespread belief that PAPs are devoid
346 of ER [55, 59], that 75 % of PAPs contained some ER. Interestingly, we found that PAPs are
347 the closest to the synapse when bouton surface area is low, which could result from the spa-
348 tial constraints imposed by larger boutons, preventing the PAP from getting in close contact to
349 the PSD. Reaction-diffusion simulations in the realistic PAP 3D meshes reconstructed in this
350 study provided key insights into the effect of the diverse shapes and distributions of the ER in
351 PAPs on microdomain Ca^{2+} activity. As reactive astrocytes, hallmark of brain diseases [26],
352 are characterized by a remodelling of ER volume and shape [39], our results suggest that such
353 geometrical alterations of the ER could be one of the factors responsible for the altered astro-
354 cytic Ca^{2+} activity reported in pathological conditions [65].

355

356 Fine-tuning the spatial distribution of Ca^{2+} channels, monitoring channel opening events at
357 each channel, while independently manipulating ER shape and distribution, such as performed
358 in this study, is not feasible experimentally. It is yet essential to understand the mechanistic link
359 between the spatial features of the astrocyte and its Ca^{2+} microdomain activity. Combining our
360 detailed biophysical model of Ca^{2+} signals in PAPs, the PAP meshes that we extracted from
361 EM and the realistic PAP meshes with various ER distributions generated by our automated
362 mesh generator allowed us to provide key insights into Ca^{2+} signaling in PAPs. Notably, we
363 predict how the complex interplay between the clustering of Ca^{2+} channels, the ratio between
364 ER surface surface area and PAP volume, Ca^{2+} buffering and the size and location of ER-PM
365 contact sites shapes Ca^{2+} microdomain signals at tripartite synapses. This study is the first to
366 our knowledge to model Ca^{2+} activity in astrocytes with realistic shapes in 3D at the nanoscale
367 that accounts for the complex and diverse spatial characteristics of Ca^{2+} stores in PAPs. Fur-
368 thermore, our results highlight the impact of the modeling choices on simulation results, notably
369 when investigating spatial effects. Importantly, our results nuance the effect of the clustering of
370 Ca^{2+} channels, which is stronger in 2D or simple 3D shapes than in more realistic 3D meshes.
371 This is crucial as, until now, modeling studies on PAPs were conducted in 1D, 2D or in simple
372 3D shapes, notably cylinders [47, 24, 13, 20, 22, 32]. The 3D meshes provided by this study,
373 together with our realistic 3D PAP mesh generator, pave the way for future modeling studies in
374 realistic 3D meshes to investigate the mechanisms governing neuron-astrocyte communication
375 at tripartite synapses.

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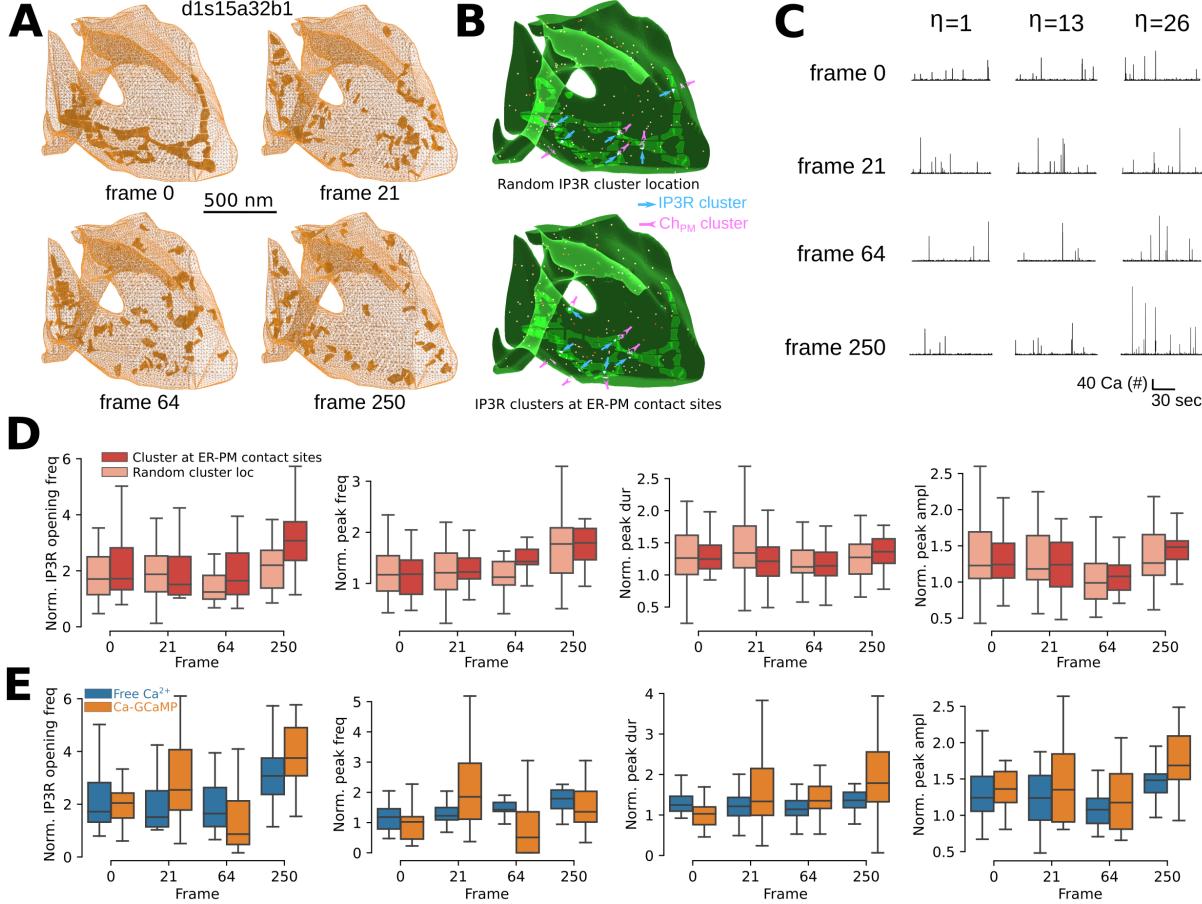


Fig 9: Ca²⁺ buffering and IP₃R channel location mediate the effect of ER-PM distance on Ca²⁺ microdomain activity in PAPs. (A) Images presenting different meshes created from PAP d1s15a32b1 using the automated workflow presented in Fig. 8: frames 0, 21, 64 and 250, characterized by diverse ER distributions within the PAP with constant PAP and ER shape, volume and surface area. Characteristics of ER-PM distance in those meshes are displayed in Fig. 8B-D. (B) Screenshots of simulations performed in d1s15a32b1_{f0} mesh (frame 0), with IP₃R clusters (blue, arrows) distributed randomly on the ER membrane (top) or at ER-PM contact sites (bottom). IP₃R clusters were co-localized with Ch_{PM} clusters at the plasma membrane (purple, inverse arrows): colcl=1. (C) Representative free Ca²⁺ traces in frames 0, 21, 64 and 250, with IP₃R cluster size η=1, 13 and 26 and random distribution of IP₃R clusters on the ER. (D) Quantification of IP₃R opening frequency (left), Ca²⁺ peak frequency (middle left), duration (middle right) and amplitude (right), in frames 0, 21, 64 and 250, for η=26, normalized by Ca²⁺ peak characteristics for η=1 in each mesh, with IP₃R clusters at random locations on the ER (left, light red) or at ER-PM contact sites (right, dark red).

Fig 9: (E) Quantification of IP₃R opening frequency (left), peak frequency (middle left), duration (middle right) and amplitude (right) of free Ca²⁺ signals (blue) and Ca-GCaMP signals (orange), in frames 0, 21, 64 and 250, for $\eta=26$, normalized by Ca²⁺ peak characteristics for $\eta=1$. IP₃R clusters were located at ER-PM contact sites. n=20 for each parameter set tested.

377 The geometrical data used here were extracted from electron microscopy, which is the only
378 tool that can resolve PAP and ER shape at a high spatial resolution (6 nm here), yet results
379 in potential alterations of the ultrastructure of the extracellular space [42] and cannot be used
380 to study live cells. Furthermore, the model used in this study, focusing on the effect of the ER
381 shape and distribution on Ca²⁺ activity, describes with great details the kinetics of ER-mediated
382 Ca²⁺ signals while simplifying other Ca²⁺ sources. Other Ca²⁺ sources and channels however
383 contribute to Ca²⁺ microdomain activity in PAPs, including mitochondria, the Na⁺/Ca²⁺ ex-
384 changer, transient receptor potential ankyrin 1 channels, L-type voltage gated channels and
385 other pathways [64, 62]. According to our model's predictions, the spatial distribution of
386 Ca²⁺ channels can alter the spatio-temporal properties of Ca²⁺ microdomain signals in PAPs
387 as well as their amplification upon neuronal stimulation. Further quantification of the Ca²⁺
388 channels expressed in PAPs, their density, location and remodeling in live tissue under (patho-
389)physiological conditions is thus essential to better understand astrocyte activity at synapses.
390 The recent advances in super-resolution techniques, notably single particle tracking methods,
391 provide a promising avenue to overcome current limitations in obtaining such data [33, 6].

392
393 Recent super-resolution studies in live neurons revealed dynamical remodeling of ER-PM
394 contact sites [27] and diffusional trapping of molecules resulting from the ER remodeling [18]
395 in neurons. Those observations, together with our model predictions, highlight the need for fur-
396 ther quantification of the dynamical shape and distribution of the ER in astrocytes in live tissue
397 to fully grasp its influence on Ca²⁺ microdomain activity in astrocytes. According to our model
398 predictions, preferential location of IP₃Rs at ER-PM contact sites might be essential to allow
399 signal amplification with IP₃R cluster size and could thus strongly alter the spatio-temporal
400 properties of astrocytic Ca²⁺ signals evoked by neurotransmitters, potentially affecting the sub-
401 sequent modulation of neuronal activity by astrocytes. Our results, in accordance with previous
402 computational studies in other cell types [81], highlight that Ca²⁺ buffering plays a crucial role
403 in shaping Ca²⁺ activity at ER-PM contact sites. The Ca²⁺ buffering effect described here was
404 mediated by Ca²⁺ indicators. Future experimental and computational studies will be essential
405 to assess Ca²⁺ buffering mechanisms in astrocytes and PAPs, which are still poorly understood,
406 yet, according to our simulation results, play crucial roles in shaping Ca²⁺ microdomain activ-
407 ity in astrocytes.

408
409 Overall, this study provides new insights into astrocytic activity at tripartite synapses by
410 characterizing the presence, shape and distribution of the ER in PAPs and by shedding light
411 to the mechanistic link between those features and microdomain Ca²⁺ activity at tripartite

412 synapses. The realistic 3D meshes of tripartite synapses created in this study pave the way for
413 new modeling studies of neuron-astrocyte communication in the synaptic micro-environment,
414 allowing the study of various processes, such as glutamate spillover or gliotransmission. Such
415 studies will be crucial to decipher whether the various nano-architectures displayed by tripartite
416 synapses reflect distinct functional identities.

417 Methods

418 3D reconstruction from electron microscopy

419 Sample preparation and imaging

420 The original dataset used in this work (EM stack and 3D reconstructions) was previously pub-
421 lished in [17]. The block was a gift from Graham Knott (BioEM imaging facility at EPFL,
422 Lausanne, Switzerland). All procedures were performed according to the Swiss Federal Laws.
423 One P90 Sprague-Dawley rat was deeply anesthetized with isoflurane and transcardially per-
424 fused using 2% paraformaldehyde and 2.5% glutaraldehyde in PBS 0.1M. Coronal sections
425 (100 μm) were obtained and washed in cacodylate buffer, followed by a post-fixation using
426 osmium tetroxide and uranyl acetate. Finally, the sections were embedded in Durcupan. Re-
427 gions of the hippocampus were dissected under a stereoscopic microscope, mounted onto a
428 blank resin slab, and trimmed using an ultramicrotome (Leica Ultracut UC-7). Imaging was
429 performed using an NVision 40 FIB-SEM (Carl Zeiss) with an acceleration voltage of 1.5 kV, a
430 current of 350 pA, and a dwell time of 10 $\mu\text{s}/\text{pixel}$. Serial images were obtained using backscat-
431 tered electrons and collected at a 6 nm/pixel magnification and 5 nm of milling depth between
432 images.

433 3D reconstruction and rendering

434 The serial micrographs were first registered using Multistackreg, a freely available plug-in
435 for Fiji [17]. Then, using those micrographs, we proceeded to the image segmentation and
436 3D model reconstructions by using TrackEM2 (a plug-in for Fiji) for manual segmentation,
437 and iLastik, for a semi-automated segmentation. The extracted models were then imported to
438 Blender software for visualization and rendering purposes [16].

439 Extraction of tripartite synapse meshes

440 For each synapse in contact with the $220 \mu\text{m}^3$ astrocytic volume, a cube of edge length $1.5 \mu\text{m}$
441 ($3.375 \mu\text{m}^3$) was created and centered at the center of mass of the PSD. All the elements of
442 the mesh (astrocyte, astrocytic ER, spine and bouton) that were within the cubic volume were
443 isolated using a boolean intersection operator available in Blender, forming what we refer to as
444 a tripartite synapse mesh. The size of the cube was chosen to be large enough to contain the

445 whole spine and bouton elements while containing a single synapse, taking into consideration
446 that the neuropil is believed to contain around one synapse per micrometer cube. This workflow
447 resulted in the creation of 44 excitatory and 2 inhibitory synapse meshes.

448 **3D mesh manipulation**

449 All 3D mesh manipulations were performed with open-access, open-source software. All 3D
450 PAP meshes used in this study will be available online upon paper acceptance.

451 **3D PAP mesh processing for reaction-diffusion simulations**

452 PAP meshes from tripartite synapse meshes were pre-processed using Blender software so that
453 they could be used for reaction-diffusion simulations. The workflow is illustrated in Fig. 4C.
454 Intersection between ER and PAP membranes was prevented by using a boolean intersection
455 operator. ER was relocated a few nanometers away from the plasma membrane. PAP compart-
456 ments that did not belong to the main PAP volume were deleted. Boolean difference operation
457 between PAP and ER elements was performed. Non-manifold vertices were repaired. The
458 resulting PAP mesh was exported in .stl format, which was then converted into a .msh 3D tetra-
459 hedral mesh using TetWild software [38]. Lastly, the mesh was imported into Gmsh software
460 to be converted into 2.2 ASCII format, format supported by the STEPS mesh importer.

461 **Automated 3D PAP mesh generation**

462 We have implemented a workflow to generate realistic 3D tetrahedral PAP meshes characterized
463 by various ER locations and constant ER shape. The algorithm is written in python, can be
464 imported in Blender and is available at <https://bit.ly/3Nc2Qin>. The workflow is presented in Fig.
465 8. First, all elements of the mesh, i.e the PAP and the ER, are relocated so that their center of
466 mass is centered at the origin. Then, the ER is split into smaller ER objects using a custom-made
467 function. Briefly, n cubes of a given size are placed along the ER object. Intersection boolean
468 operation is then performed between the ER and each cube, resulting in the creation of n ER
469 objects. ER objects smaller than 30 nm^3 are deleted. The remaining ER objects are rescaled
470 so that the sum of their surface areas matches the area of the original ER element, measured
471 with the Blender 3D Print add-on. The number and size of cubes can be altered depending on
472 the size of the original ER and on the mesh characteristics desired. Using Blender's physics
473 engine, a simulation with n frames is generated, in which ER objects are subject to physical
474 forces that alter their location between each frame. Inputs of the 'RunPhysics' function include
475 parameters that affect how close objects can get, which can be altered to prevent membrane
476 intersection. Note that successful scattering of the ER depends on the geometrical properties of
477 each mesh so that adjusting the parameters of physics simulation might be necessary depending
478 on the mesh used. Details are provided in comments of the code to allow the user to adjust
479 the code to the mesh under study. Examples of frames generated by this workflow applied to

480 d1s15a32b1 PAP mesh are presented in Supplementary movie 3. For each selected frame, the
481 mesh pre-processing steps presented in Fig. 4C are performed automatically, resulting in the
482 export of a .stl triangular mesh. 3D meshing and format conversion can then be performed using
483 TetWild and Gmsh software, as described above. The resulting meshes can be used to perform
484 reaction-diffusion simulations.

485 **Analysis of the geometrical properties of 3D meshes**

486 The volume and surface area of each synaptic element, i.e the PAP, astrocytic ER, spine and
487 bouton, were measured using the Blender add-on Neuromorph [40]. We implemented a python
488 script that can be imported in Blender software that measures distances between mesh elements
489 of interest. The distance between each vertex of the plasma membrane of the PAP and the
490 center of mass of the neighboring PSD was computed in Blender and stored in a list. Similarly,
491 ER-PSD distance was quantified by measuring the distance between each vertex of the ER
492 membrane and the center of mass of the PSD. To characterize ER-PM distance, for each vertex
493 on the PM, the closest ER vertex was detected and its distance to the PM vertex was stored in
494 a list. PM-PSD, ER-PSD and ER-PM distance lists were exported to a text file for analysis and
495 visualisation. The analysis code, implemented in python and imported in Blender, is available
496 at <https://bit.ly/3Nc2Qin>.

497 **Computational modeling**

498 **Modeled reactions and computational approach**

499 Astrocytic Ca^{2+} signals in PAPs were simulated using the reaction-diffusion voxel-based model
500 of ER-dependent Ca^{2+} signaling from Denizot and colleagues ([20] Table 2, Fig. 6-7). Briefly,
501 the model describes Ca^{2+} fluxes in and out of the astrocytic cytosol. The opening of IP_3R
502 channels on the ER membrane triggers Ca^{2+} influx in the cytosol. IP_3 can be synthesized
503 by the Ca^{2+} -dependent activity of Phospholipase C (PLC) δ . IP_3 removal from the cytosol is
504 described by a decay rate. IP_3R dynamics is derived from the De Young & Keizer's model
505 [19]. Each IP_3R has 3 binding sites: one to IP_3 and two to Ca^{2+} (activating and inhibiting).
506 The channel can thus be in 8 different states. The open state is $\{110\}$: IP_3 and Ca^{2+} bound
507 to the activating sites and the Ca^{2+} inactivating site is unbound. In a subset of simulations,
508 GCaMPs6s, genetically-encoded Ca^{2+} indicators [64], were added to the cytosol and variations
509 of $[\text{Ca-GCaMP}]$ concentration, mimicking experimental Ca^{2+} imaging, were measured. For
510 further details on the kinetic scheme, parameter values and model assumptions, please refer to
511 the original paper presenting the model [20]. We slightly altered this model to better describe
512 and control IP_3R -independent Ca^{2+} fluxes. To do so, IP_3R -independent Ca^{2+} influx was mod-
513 eled as an influx through Ca^{2+} channels at the plasma membrane, Ch_{PM} . For simplicity, the
514 amount of Ch_{PM} channels equals the total number of IP_3R channels, N_{IP3R} . Ca^{2+} influx rate
515 at Ch_{PM} channels, $\gamma_{\text{ch}_{\text{PM}}}$, is $15 \times 10^{-8} \text{s}^{-1}$. The reactions modeled here are illustrated in Fig. 4A.
516

517 The model was implemented using the STochastic Engine for Pathway Simulation (STEPS)
518 python package (<http://steps.sourceforge.net/>) [36]. This software uses a spatialized version of
519 Gillespie's SSA algorithm [31] to perform exact stochastic simulations of reaction-diffusion
520 systems. Simulations in STEPS allow the diffusion of molecules in 3D tetrahedral meshes and
521 onto the surfaces of the mesh, such as the ER and plasma membrane. STEPS allows volume
522 and surface reactions. Reactions can only occur between molecules within the same tetrahe-
523 dron (volume reactions) or in adjacent triangle and tetrahedron (surface reactions). Boundary
524 conditions were reflective. Simulation time was 100s. The states and amounts of all molecular
525 species were measured at each time step (1 ms).

526 **Neuronal stimulation simulation**

527 Unless specified otherwise, glutamatergic transmission at the synapse was modeled and oc-
528 curred at simulation time t=1s. To do so, IP₃ molecules were injected in tetrahedra below
529 the plasma membrane of the PAP, emulating IP₃ synthesis resulting from the activation of
530 metabotropic glutamatergic receptors at the membrane of the PAP. Supplementary movie 4
531 presents a visualization of a simulation at neuronal stimulation time, in the d2s6a9b1 PAP mesh.

532 **Ca²⁺ channel clustering algorithm**

533 Surfaces correspond to triangular meshes. To simulate IP₃R clustering, N_{IP3R}/η IP₃R clusters
534 were randomly positioned onto the membrane of the ER, where N_{IP3R} is the total number of
535 IP₃Rs and η is the number of channels per cluster. As η is an integer, it must be a divider
536 of N_{IP3R} . As IP₃R density was kept constant across simulations, $3.5e^{-3}/\mu m^2$ [20], the total
537 number of IP₃Rs, N_{IP3R} , and IP₃R cluster size η varied depending on the mesh. Each IP₃R
538 cluster was located within a region of interest, as defined in STEPS, consisting in 4 triangles.
539 Clusters could not overlap. In a subset of simulations, IP₃R clusters were located at ER-PM
540 contact sites. To do so, ER triangles were sorted depending on the distance between their center
541 of mass and the closest PM triangle. Cluster center was then located at the ER triangle in which
542 no cluster was already located characterized by the lowest ER-PM distance in the mesh. The
543 cluster ROI consisted in this cluster center triangle and the neighboring triangles. Similarly,
544 clusters could not overlap. The number of IP₃R opening events at each cluster ROI was mea-
545 sured at each time step.

546
547 IP₃R channels were co-clustered with Ch_{PM} Ca²⁺ channels at the plasma membrane (cocl=1),
548 unless specified otherwise. If cocl=0, Ch_{PM} channels were randomly distributed onto the
549 plasma membrane. If cocl=1, Ch_{PM} channels were co-clustered with IP₃Rs. To do so, Ch_{PM}
550 cluster center was defined as the triangle on the plasma membrane that was the closest to the
551 IP₃R cluster center triangle on the ER. The cluster ROI then consisted in this Ch_{PM} cluster
552 center and the neighboring triangles. Similarly to IP₃R cluster ROIs, Ch_{PM} clusters could not
553 overlap. For simplicity, Ch_{PM} cluster size was identical to IP₃R cluster size: η .

554 **Simulation code**

555 Simulations were performed using the model of Ca^{2+} signals in fine processes from Denizot
556 and collaborators [20], available at <http://modeldb.yale.edu/247694>. The simulation code used
557 in this study is available at <https://bit.ly/3Nc2Qin>.

558 **Ca^{2+} peak detection and characterization**

559 Ca^{2+} peaks were considered initiated and terminated when Ca^{2+} concentration increased above
560 and decreased below peak threshold, respectively. Peak threshold was $[Ca]_b + n\sigma_{\text{Ca}}$, where
561 $[Ca]_b$ is the basal Ca^{2+} concentration and σ_{Ca} is the standard deviation of the $[\text{Ca}^{2+}]$ histogram
562 in the absence of neuronal stimulation. n varied depending on signal/noise ratio of the simula-
563 tion of interest, notably when measuring Ca-GCaMP signals, noisier than free Ca^{2+} signals (see
564 (e.g.) Fig 4E). Ca^{2+} peak frequency, duration and amplitude were measured in each simulation.
565 Ca^{2+} peak duration corresponds to the time between peak initiation and termination, Ca^{2+} peak
566 amplitude corresponds to the maximum number of Ca^{2+} ions in the cytosol measured within
567 peak duration time and Ca^{2+} peak frequency corresponds to the amount of peaks detected dur-
568 ing simulation time. The number of IP_3R peak opening events was recorded at each time step,
569 in the whole cell as well as at each IP_3R cluster ROI.

570 **Statistical analysis**

571 Data analysis and statistics were performed using open-access and open-source software: the
572 SciPy and pandas python libraries. Data visualization was performed using Seaborn and Mat-
573plotlib python libraries. Sample size for each analysis, n , is described in the figure legend.
574 Prior to statistical analysis, normality of data distribution was inferred using the Shapiro-Wilk
575 test. Relationship between Ca^{2+} peak characteristics and parameter values was inferred using
576 one-way ANOVA if values followed a Gaussian distribution, Kruskal-Wallis one-way ANOVA
577 otherwise. Note that the effect of IP_3R clustering was quantified by measuring the ratio be-
578 tween the Ca^{2+} peak characteristic of interest measured at a given IP_3R cluster size, $\eta > 1$
579 and its mean value for $\eta=1$. The linear relationship between two datasets was evaluated using
580 Spearman's correlation coefficient. The test and p-value, p , associated with each analysis is
581 described in the figure legend or in the main text.

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