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Disentangling astroglial physiology with a realistic cell model in silico

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Electrically non-excitable astroglia take up neurotransmitters, buffer extracellular K+ and generate Ca2+ signals that release molecular regulators of neural circuitry. The underlying machinery remains enigmatic, mainly because the sponge-like astrocyte morphology has been difficult to access experimentally or explore theoretically. Here, we systematically incorporate multi-scale, tri-dimensional astroglial architecture into a realistic multi-compartmental cell model, which we constrain by empirical tests and integrate into the NEURON computational biophysical environment. This approach is implemented as a flexible astrocyte-model builder ASTRO. As a proof-of-concept, we explore an in silico astrocyte to evaluate basic cell physiology features inaccessible experimentally. Our simulations suggest that currents generated by glutamate transporters or K+ channels have negligible distant effects on membrane voltage and that individual astrocytes can successfully handle extracellular K+ hotspots. We show how intracellular Ca2+ buffers affect Ca2+ waves and why the classical Ca2+ sparks-and-puffs mechanism is theoretically compatible with common readouts of astroglial Ca2+ imaging.
Astroglia have emerged as an essential contributor to neural circuit signalling in the brain. In addition to the well-established mechanisms of neurotransmitter uptake and extracellular K⁺ buffering, electrically passive astrocytes appear competent in handling physiological signals using intracellular Ca²⁺ signals⁴–⁶ that display a variety of dynamic ranges and time scales (reviewed in refs. ⁴,⁵). Tri-dimensional (3D) reconstructions of astroglia using electron microscopy (EM) have long revealed a system of nanoscopic processes⁷–⁹ that pervade the entire cell expanse⁸,⁹. Deciphering cellular mechanisms that shape Ca²⁺-dependent signalling and physiological membrane currents in this sponge-like system has been a challenge.

In contrast, cellular machineries underpinning neuronal physiology have been understood in great detail. This is partly because it has been possible to interpret electrophysiological and imaging observations in neurons using realistic biophysical cell models, such as those developed in the NEURON environment¹⁰,¹¹. There have also been numerous attempts to simulate astroglial function, mainly from a reductionist standpoint (reviewed in refs. ¹²,¹³). Aimed at a specific question, such models would normally focus on kinetic reactions inside astroglia¹⁴,¹⁵; between astroglial and neuronal compartments¹⁶,¹⁷ or on astroglial influences in neuronal networks¹⁸,¹⁹. These studies have provided some important insights into the biophysical basis of astroglial physiology. However, their scope would normally exclude complex cell morphology, intracellular heterogeneities or the impact of Ca²⁺ buffering mechanisms on Ca²⁺ signal readout. Thus, integrating cellular functions of an astrocyte on multiple levels, in one realistic entity in silico, remains to be achieved.

Our aim was therefore three-fold. Firstly, to develop a modelling approach that would recapitulate fine astroglial morphology while retaining full capabilities of biophysical simulations enabled by NEURON. We have therefore generated (MATLAB- and NEURON-based) algorithms and software that (a) use experimental data to recreate the space-filling architecture of astroglia, and (b) make this cell architecture NEURON-compatible. Our case study focused on the common type of hippocampal protoplasmic astroglia in area CA1, which has been amongst the main subjects of studies into synaptic plasticity and neuron-glia interactions²⁰–²². We have combined patch-clamp electrophysiology, two-photon excitation (2PE) imaging and 2PE spot-uncaging, fluorescence recovery from photobleaching (FRAP), astroglia-targeted viral transduction Ca²⁺ indicators in vivo, and quantitative correlational 3D EM to systematically document the multi-scale morphology and key physiological traits of these cells. Based on these empirical constraints, we have built a multi-compartmental 3D cell model fully integrated into the NEURON environment. The latter was equipped with additional functionalities relevant to astroglia, such as control of tissue volume filling and surface-to-volume ratios, options for extracellular glutamate application and K⁺ rises, endfoot and gap junctions menus, choice of fluorescence imaging conditions, etc.

Our second objective was to implement this approach as a flexible simulation instrument—cell model builder—capable of recreating and probing various types of astroglia in silico. Thus, we have integrated our algorithms and software as a modelling tool ASTRO, which enables an investigator to generate morphological and functional astroglial features at various scales.

Finally, as a proof of concept, we explore our test-case astrocyte models (that are partly constrained by empirical data) to reveal some important aspects of astroglial physiology that are inaccessible in experiments. We therefore evaluate key electrodynamic features of the astroglial membrane, basic aspects of intracellular K⁺ dynamics, the range of intracellular Ca²⁺ buffering capacity, and how the classical molecular machinery of Ca²⁺ ‘puffs’ and ‘sparks’ could explain some Ca²⁺ imaging observations in astrocytes. Our findings suggest that ASTRO could be a valuable tool for physiological testing and causal interpretation of experimental observations pertinent to astroglia.

Results

Stem tree reconstruction of live astroglia. The gross morphology of hippocampal area CA1 astrocytes points to the cell tree radius of 30–50 µm, somatic diameter of 7–15 µm, and 4–9 primary processes⁹,²³–²⁵. To elucidate this structure further, we used acute hippocampal slices, loaded individual astroglia in whole cell with the morphological tracer Alexa Fluor 594 (Methods), and imaged the cell expanse using two-photon excitation (2PE; Fig. 1a, b). This procedure has been shown to faithfully reveal fine astroglial morphology²⁴.

Our modelling strategy was to start with the principal branch structure (‘stem tree’) which could be resolved in optical images (branch diameter above the diffraction limit, 0.3–0.5 µm; Fig. 1a, b) and then 3D-reconstructed using computer tools previously validated in neuronal studies. Focusing on one cell, we imaged it in a z-stack (Fig. 1a, b), corrected brightness of individual recorded sections for the depth-dependent signal drop, and 3D-reconstructed identifiable cell branches semi-automatically using Simple Neurite Tracer (Fiji-ImageJ, NIH; Fig. 1c; Methods). To store the recorded structure in a NEURON format (Fig. 1d) we used Vaa3D (Allen Institute). Alternatively, the entire 3D-reconstruction procedure could be carried out using commercially available Neurolucida (MBI).

Our complementary approach was to build the ‘typical’ stem tree representing the astrocyte pool under study. First, we used a library neurogliaform cell (P32-DEV136, [http://neuromorpho.org/neuron_info.jsp?neuron_name=P32-DEV136]) as a stem tree skeletal (Fig. 1e, diagram). Second, the numbers and diameters of its branches were adjusted to match measurements from an experimental sample of CA1 astroglia (13 cells, 98 branches; Fig. 1e).

The endfoot. The endfoot surrounding blood vessels is a key feature of most astrocytes. Because its morphology varies enormously, it would seem appropriate to use experimental 3D reconstructions (as in Fig. 1a–d) to incorporate it into the cell architecture. ASTRO provides a separate NEURON menu for constructing the endfoot and connecting it to the main arbour (Supplementary Note 1, ASTRO User Guide, pp 20–21). All nanoscopic process structures and biophysical mechanisms available in the present model builder (sections below) could be incorporated into the endfoot. However, as it represents a highly specialized cell compartment, it will require a separate study to develop its biophysical machinery in accord with experimental observations. Simulation tests in the present study will focus therefore on the ‘main’ astrocyte arbour (parts of which may include processes that surround small blood vessels).

Experimental measurements of nanoscopic astroglial processes. The next model-building step was stochastic generation of nanoscopic processes. The bulk of astroglial morphology comprises irregularly shaped ultrathin branchlets that appear as a blur in optical images (Fig. 1a, b). To quantify such structures, we used correlational 3D EM²⁶. Individual astrocytes were filled with biocytin whole-cell, and after DAB conversion were traced and reconstructed using serial sections (Methods; Supplementary Fig. 1). It has recently transpired that chemical fixation via heart perfusion might shrink tissue by 30–35% also causing aberrations in astroglial morphology²⁷. To minimise such effects, we rapidly fixed thin acute slices by submersion, which causes only ~5%
linear tissue shrinkage\textsuperscript{28} preserving the extracellular volume fraction of \~0.12\textsuperscript{29}, close to \~0.15 under cryofixation\textsuperscript{27}.

Reconstructing the entire astrocyte with 3D EM is difficult and may not necessarily represent the ‘typical’ cell. We instead focused on small fragments sampled from multiple CA1 astrocytes\textsuperscript{26} (Fig. 2a), aiming to extract key statistical features of their nanoscopic processes. Specific ASTRO routines were developed to sample and store branchlets from 3D-reconstructed cell fragments (Fig. 2a, b; Supplementary Note 1, ASTRO User Guide p. 12). Each sampled process comprised varied-length stacks of 60 nm thick sections, with individual sections being represented by 3D point co-ordinates scattered on their ‘polygonal’ perimeters (Fig. 2c; Supplementary Note 1, ASTRO User Guide pp. 12–13). Thus, a representative sample of astrocyte nanoscopic processes was obtained.

**NEURON-compatible transformation of nanoscopic processes.**

NEURON-built cell models use cylindrical compartments that follow the shape of neuronal dendrites or axons. Because astroglial processes have irregular shapes (Fig. 2a–c), we carried out a separate investigation to establish how their geometry and biophysical properties could be recapitulated using cylindrical compartments.

We therefore transformed ‘polygonal’ z-stacks representing 3D-reconstructed processes, into z-stacks of cylindrical slabs (Fig. 2d). Here, the adjacent polygonal slabs, with cross-section areas \( S_i \) and \( S_{i+1} \) and an intersection area of \( T_j \) (Fig. 2d, left and middle), were represented by two ‘main’ cylinder slabs, with base areas \( S_i \) and \( S_{i+1} \) (termed ‘leaves’), plus a ‘transitional slab’ (‘stalk’), with base area \( T_j \) (Fig. 2d, right). This transformation largely preserved the diffusion bottleneck and the surface-volume relationships of the original shape. By applying this rule, we transformed all stored 3D-processes into NEURON-compatible shapes (Fig. 2e).

Next, we employed Monte Carlo simulations to systematically compare original and NEURON-compatible shapes with respect to the two key biophysical traits, diffusion transfer rate and dynamic electrical impedance. This involved ‘injecting’ 3000 Brownian particles into one end and monitoring them at the other end of the shape, with or without an electric field applied (Fig. 2f; example in Supplementary Movie 1). The algorithms involved were tested and validated by us against experimental data previously\textsuperscript{30,31}. In most instances, there was a remarkable similarity between the two shape types in their properties (Fig. 2g); in the remaining cases, a minor adjustment of the cylindrical compartment diameters achieved a similar match.

Importantly, the biophysical match between the original and the cylinder-based shapes held when cylindrical compartments were shuffled randomly (Supplementary Fig. 3). Thus, the frequency distribution of cylinder diameters was sufficient to obtain nanoscopic structures biophysically compatible with real cell processes. The transformation procedure described above has been integrated in ASTRO (Supplementary Note 1, ASTRO User Guide, pp. 11–18).

The next step for ASTRO was therefore to populate the cell stem tree (Fig. 1d, e) with nanoscopic processes using their experimental statistics and further empirical constrains described below.
Transformation of 3D EM reconstructed processes (top) into NEURON-compatible cylinder section stacks (bottom). Individual sections (blue, top) are transformed into cylinders (bottom), as in d. Transformation of the adjacent 3D EM sections (left; grey polygonal slabs with base areas $S_i$ and $S_{i+1}$) with intersection area $T_i$ (middle; green polygons) into NEURON-compatible two main (grey, ‘leaves’) and one transitional (green, ‘stalk’) cylindrical slabs with the corresponding base areas (right).

Transformation of 3D EM reconstructed processes (top) into NEURON-compatible cylinder section stacks (bottom). Individual sections (blue, top) are transformed into ‘main’ cylinders (blue, bottom), and green segments (top) depict adjacent surfaces between sections represented by green ‘transition’ cylinders (bottom), as in d. A characteristic example of a 3D EM reconstructed astroglial process made up by its serial polygonal sections (top) and its representation by serial cylindrical compartments (bottom). Scattered dots illustrate a snapshot of the Monte Carlo simulation test (monitored live in ASTRO: Supplementary Movie 1) in which Brownian particles are injected in the bottom of the 3D structure, and their arrival time at the top is registered, to compare molecular diffusivity (no electric field) and electrodynamic properties ($2.5 \times 10^3$ V m$^{-1}$ electric field in the $z$-direction, one electron charge $e = 1.6 \times 10^{-19}$ C per particle applied) between the two shapes. The outcome of Monte Carlo tests comparing two shapes shown in f, for the molecular diffusion flux (top) and ion current (bottom), measured at the top exit of the shapes (as in f), upon injection of the Brownian particles into the bottom entry (as in f); blue and green, 3D EM reconstructed and NEURON-compatible shapes, respectively. See ref. 31 for electrodiffusion simulation detail.

Fig. 2 Nanoscopic astroglial protrusions: from 3D EM to in silico. a 3D EM serial-section reconstruction of an astrocytic fragment (green) and adjacent dendritic spines (grey) with postsynaptic densities (red) in area CA1; surface rendering applied; dotted square, selected nano-process; Scale cube side, 1 µm. b Fragment in a shown using surface point scatter; false colour scale, $z$-depth as indicated; dotted square, selected nano-process, as in a. c Selected process (highlighted in a and b) as a stack of polygonal sections (60 nm thick, to follow EM sectioning) delimited by surface points. Scale bar, 500 nm.
f Transformation of the adjacent 3D EM sections (left; grey polygonal slabs with base areas $S_i$ and $S_{i+1}$) with intersection area $T_i$ (middle; green polygons) into NEURON-compatible two main (grey, ‘leaves’) and one transitional (green, ‘stalk’) cylindrical slabs with the corresponding base areas (right).

**Tissue volume fraction and surface-to-volume ratios.** The tissue volume fraction (VF) occupied by astroglial processes in the hippocampal neuropil ranges between 5 and 10% 26,28,29,32,33. The VF distribution provides a key descriptor of astroglial morphology because individual astrocytes occupy adjacent tissue domains with little overlap while their processes fill the volume in a sponge-like manner 23,34. 2PE microscopy enables the direct monitoring of VF in live astroglia in situ because it collects emission within a thin focal layer only (Supplementary Fig. 4a). Thus, fluorescence intensity of the dye-filled astroglial scales with VF occupied by local astroglial processes (Supplementary Fig. 4b) whereas somatic cytosol imaged in the same focal plane corresponds to ~100% VF (Supplementary Fig. 4c) 26,35. Thus, the local-to-somatic emission ratio can provide direct readout of astroglial VF (Fig. 3a). We thus obtained the VF distribution within individual CA1 astrocytes (Fig. 3b) and used it to constrain stochastic generation of nanoscopic processes in the model.

The latter is achieved by adjusting two parameters in the ASTRO-NEURON menu: the average size of nanoscopic processes (number of leaves per process, <100; Supplementary Note 1, ASTRO User Guide, Fig. 16, p. 19), and their ‘seed density’ (normally between 1 and 3 per branch; ASTRO SeedNumber parameter; Supplementary Note 1, ASTRO User Guide, p. 20). Another important feature of cell processes is their surface-to-volume ratio (SVR); it determines how transmembrane fluxes are converted into intracellular concentration dynamics. Stereological analyses of hippocampal astroglial processes using 3D EM estimates SVRs in the 15–25 µm$^{-1}$.
The astrocyte fragments sampled in our experiments (Fig. 2a–c) had SVRs within this range, which was faithfully reproduced by the cylinder-based shapes (Fig. 2f bottom, Supplementary Fig. 3b). ASTRO could further fine-tune the average SVR, by adjusting the distribution of ‘leaves’ and ‘stalks’ in stochastically simulated nanoscopic processes (Supplementary Note 1, ASTRO User Guide, pp. 21–23). With these steps completed, we arrived at realistic, NEURON-compatible CA1 astrocyte geometry comprising 35,000–45,000 individual compartments (Fig. 3c; Supplementary Table 1).

Astrogial internal connectivity in experiment versus model. Our final check was to see if the model has intracellular diffusion connectivity similar to that in live astroglia. To test that, we used the fluorescence-recovery-after-photobleaching (FRAP) approach:

Fig. 3 NEURON-based astrocyte model: determining volumetric quantities. a Image panel, a characteristic astrocyte in area CA1 (Alexa Fluor 594, λex = 800 nm) seen in a single 2PE optical section (~1 μm thick) crossing the cell soma; dotted lines, sampling fluorescence intensity profiles reporting the astrocyte tissue volume fraction (VF); scale bar, 15 μm. Graph, VF profiles (fluorescence local/soma ratio) obtained along the dotted lines i and ii in the image, as indicated; scale bar, 10 μm. b Matching modelled (red) and experimental (blue; mean ± SEM; n = 13 astrocytes) VF values (ordinate, dimensionless) sampled at different distances from the soma (abscissa). c A complete NEURON-generated astrocyte model (z-projection), with main branches depicted in orange (partly obscured by smaller processes) and nanoscopic protrusions (schematic depiction) in purple. Note that tortuous processes of real-life astroglia are represented here by biophysically equivalent ‘straightened’ processes. Scale bar, 10 μm. d An example of astroglia as in a; dotted line, line-scan position to measure internal diffusion connectivity (using Alexa Fluor 594 photobleaching); patch pipette tip is seen. Scale bar, 10 μm. e A snapshot of a photobleaching experiment in silico showing the intracellular Alexa concentration dynamics in a modelled astrocyte; arrows, photobleaching line positioning; false colour scale, intracellular Alexa concentration, as indicated (Supplementary Movie 2). f Matching the modelled (red) and the experimental (blue) time course of intracellular Alexa Fluor fluorescence during a photobleaching experiment as shown in d and e, one-cell example (CA1 area, stratum radiatum astrocyte). Grey segments indicate laser shutter-on when fluorescence recovery occurs (red). g Statistical summary of photobleaching experiments (n = 10 astrocytes) and related simulations, as depicted in d and e, comparing experimental (blue) and simulated (red) data.

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comparing the modelled outcome with experimental observations. By adjusting one free model parameter (photobleaching rate), we were able to match experimental and simulated data in individual cells (Fig. 3f) and across the sample (n = 10; Fig. 3g). Thus, the modelled cell faithfully represented inner connectivity of CA1 astrocytes.

Passive electrical properties of astrocytes. In whole-cell configuration, input resistance $R_i$ of CA1 astrocytes in our sample ($2.66 \pm 0.31 \, \text{M} \Omega$, mean $\pm$ SEM, n = 15; Fig. 4a) was consistent with the previous reports. To assess specific membrane conductance $G_m$, we measured resistance of outside-out patches and estimated the patch area using the classical voltage-step method (Methods), which gave $G_m = 0.69 \pm 0.18 \, \text{mS cm}^{-2}$ (mean $\pm$ SEM; Fig. 4b). Next we asked if the model could actually predict experimental $G_m$ (Fig. 4b) when two other model parameters, $R_i$ and total membrane area $S_{\text{mem}}$, were empirically constrained. We have therefore produced a small representative sample of same-type astrocyte models, by repeating stats-constrained stochastic generation of nanoscopic processes on the same stem tree (Fig. 4c). In the sample, the cell volume varied well within the range of 3000–4800 $\mu$m$^3$ characteristic of CA1 astroglia. In each sampled cell model, $G_m$...
was a free parameter which was adjusted until model \( R \) matched its empirical value (Fig. 4a). This test produced the average \( G_m \) value of 0.78 ± 0.04 mS cm\(^{-2}\) (Fig. 4c), which was indistinguishable from the experimental \( G_m \) value (Fig. 4b) or its earlier measurements. This results indicates that stochastic generation of nanoscopic processes produces realistic membrane properties of astrocytes.

We next simulated a voltage-clamp experiment, showing that large currents in these leaky cells induce only small somatic depolarisation while propagating with a space constant of 30–60 µm depending on the signal frequency (Fig. 4d, left). Voltage signals generated at the cell periphery have, due to the 'cable-end' proximity, a shorter constant of 25–30 µm (Fig. 4d, right) suggesting that similar signals at different astrocyte loci could have different membrane effects.

Astrocyte membranes are enriched in potassium channels, in particular Kir4.1 type. Their typical unit conductance is either suggesting that similar signals at different astrocyte loci could have different membrane effects.

Astrocyte membranes are enriched in potassium channels, in particular Kir4.1 type. Their typical unit conductance is either compatible with or lower than the membrane current leak due to the other conductances (channels, exchanges, gap junctions) which were represented, for the sake of simplicity, by a non-specific channel current maintaining resting \( V_m \) near −83...–85 mV. Thus, Kir4.1 should have little effect on the voltage spread profile, even though these channels largely control the cell resting membrane potential. Indeed, when we added evenly distributed Kir4.1, their effect on voltage was detected only when their overall conductance exceeded its expected physiological range (Supplementary Fig. 6a). In physiological circumstances, however, this scenario could be affected by changes in extracellular K\(^+\), and by the poorly understood contribution of other ion channels and exchangers (see below).

**Voltage–current landscape generated by glutamate uptake.** To understand how the current generated by glial glutamate transporters (GLT-1) affects membrane potential across the cell, we first recorded from a CA1 astrocyte the response to two-photon spot-uncaging of glutamate (20 ms) at variable distances from the soma (Fig. 4e, traces). Second, we replicated this test in the model, by implementing the GLT-1 kinetics, scattering the transporters uniformly on the cell surface (at ~10^4 µm\(^{-2}\)), and applying extracellular glutamate within small spherical areas (radius ~3 µm, duration ~20 ms) at quasi-random distances from the soma.

An excellent match between the modelled somatic current and whole-cell recording data could be obtained (Fig. 4e, plot) by adjusting one free model parameter, the amount (peak concentration) of released glutamate. The model unveiled the dynamic landscape of astrocyte membrane voltage, which varied only within ~0.2 mV across the entire cell (Fig. 4f; Supplementary Movie 3).

These simulations illustrate quantitatively that a local current hotspot in the electrically leaky astroglial membrane stays localized, with little effect on the voltage landscape (Fig. 4f). Again, this scenario was only weakly affected by adding Kir4.1 (Supplementary Fig. 6b-d) although in real cells the effect could be more complex. In any case, these tests suggest that GLT-1 currents on their own cannot significantly depolarise astrocyte membrane away from the region of active transport.

**Potassium uptake and redistribution inside astroglia.** Rapid uptake and intracellular redistribution of potassium are essential functions of brain astroglia. Several models have dealt with this mechanism on a cell or tissue level, and here we aimed to understand its complex dynamics inside astroglia. As a proof of concept, we simulated a scenario of intense local activity in which extracellular K\(^+\) concentration \([K^+]_{out}\) was elevated for two seconds, from 3 to 10 mM, inside a 20 µm spherical tissue area (Fig. 5a). The model was populated with Kir4.1 channels with unit conductance of 0.1 mS cm\(^{-2}\). The \([K^+]_{out}\) elevation activated Kir4.1 homogeneously inside the 20 µm area, prompting K\(^+\) entry (peak current density ~0.01 mA cm\(^{-2}\)). The ensuing local increase in intracellular K\(^+\) concentration \([K^+]_{in}\) (from 110 to ~113 mM) dissipated over several seconds after \([K^+]_{out}\) returned to 3 mM (Fig. 4b). The period of elevated \([K^+]_{out}\) also featured very slight depolarisation generated by Kir4.1 (Fig. 5c), not dissimilar to that arising from glutamate uptake (Fig. 4f). These data suggest that extracellular K\(^+\) buffering and its intracellular redistribution could be controlled by local K\(^+\) efflux, in particular through Kir4.1. However, further experimental constraints are required to understand the possible contribution of other membrane mechanisms.

To dissect theoretically a possible role of active K\(^+\) extrusion mechanisms, such as pumps and ion exchangers, we carried out similar tests but with the active removal of intracellular K\(^+\) by a first-order pump (which may also reflect gap-junction escape) and no contribution from Kir4.1 channels (Supplementary Fig. 7). The dynamic spatial landscape of \([K^+]_{in}\) captured by the model confirmed that local K\(^+\) efflux could efficiently limit the spatial spread of \([K^+]_{in}\) elevations (Supplementary Fig. 7).

**Gap junctions and hemicannels.** Neighbouring astroglia are connected via gap junctions (made up by adjoined connexin proteins), enabling current leak and diffusional flow of molecules across the astroglial syncytiu. Astroglia are also enriched in connixin hemicannels permitting molecular transfer to and from the extracellular medium. In our tests, blocking these channels with carbenoxol (CBX, 50 µM) increased \( R \) by ~30% (Supplementary Fig. 8), consistent with previous reports.

We have incorporated basic gap junction options in the ASTRO menu, both as electric conductance and as a diffusion channel (Methods; Supplementary Note 1, ASTRO User Guide, pp. 18, 26–28). For the sake of clarity, however, simulations here consider gap junctions as a constituent contributor to membrane conductance. Dissecting their precise roles will require further experimental detail pertinent to their biophysics and their intercellular distribution.

**Probing the impact of calcium buffering on calcium waves.**

Astrogial Ca\(^{2+}\) waves are thought to rely on Ca\(^{2+}\) stores, channels, and pumps involving endoplasmic reticulum and mitochondria. The underlying molecular machinery appears to engage Ca\(^{2+}\)-dependent Ca\(^{2+}\) release controlled by inositol 1,4,5-trisphosphate (IP\(_3\)) and possibly ryanodine receptor-channels displaying a highly non-linear (sometimes bell-shaped) dependence between channel activity and Ca\(^{2+}\) concentration.

Recently, local Ca\(^{2+}\) transients (but not global waves) have been documented in astroglia lacking IP\(_3\) receptors. In any such cases, intracellular Ca\(^{2+}\) signal propagation must be tightly controlled by the local Ca\(^{2+}\) buffering capacity, the feature comprehensively explored in nerve cells.

The NEURON environment by default incorporates biophysical mechanisms of Ca\(^{2+}\) dynamics including diffusion, buffering, and IP\(_3\) action (Methods), to which we have added two further IP\(_3\)-dependent mechanisms described in the literature. In the default configuration, four parameters control intracellular Ca\(^{2+}\) waves: resting IP\(_3\) concentration \( C_{IP3} \), resting Ca\(^{2+}\) concentration \([Ca^{2+}]_{rest}\), endogenous Ca\(^{2+}\) buffer concentration \([B]\) and its affinity (dissociation constant) \( K_D \). Most studies constrain \( C_{IP3} \) within 0.5–3 µM whereas earlier we were able to measure \([Ca^{2+}]_{rest}\) using time-resolved fluorescence microscopy (range 50–100 nM). In contrast, \([B]\) and \( K_D \) can vary widely across cell types and thus remain unknown.
As expected, the model could generate Ca\(^{2+}\) waves over a wide (physiologically plausible) range of the above parameters. To dissect the basic effect of Ca\(^{2+}\) buffering on Ca\(^{2+}\) wave propagation, we compared wave dynamics with and without a small amount (10 \(\mu\)M) of mobile Ca\(^{2+}\) buffer added (Fig. 6a; Supplementary Movie 4). Adding the buffer appeared to significantly reduce the wave speed and amplitude (Fig. 6a), suggesting that increased Ca\(^{2+}\) buffering may actually prolong periods of elevated [Ca\(^{2+}\)]. However, accurate interpretation of such results requires further empirical constrains.

Assessing calcium-buffering capacity in vivo. Because there is an ongoing debate on whether astroglial Ca\(^{2+}\) waves seen in acute slices are fully physiological, we sought to document such waves in live animals. However, imaging hippocampal astrocytes in vivo involves mechanical invasion of the impact of which on astroglial function is not fully understood. We therefore imaged somatosensory cortex astroglia (accessible with intact brain surface), which appears remarkably similar in their basic morphological features and territorial volumes to hippocampal astrocytes.

We asked whether \([B]\) and \(K_D\) could be assessed by matching the empirical Ca\(^{2+}\) wave dynamics to the modelled outcome. Spontaneous activity of astrocytes in vivo was recorded using a virus-transduced Ca\(^{2+}\) indicator expressed under a GFAP promoter: to minimize filtering effects of free-diffusing Ca\(^{2+}\) indicators, we used the plasma-membrane-tethered GCaMP6f (~50 ms fluorescence response time). The animals were anaesthetized, to limit bursts of sensory input-evoked prominent Ca\(^{2+}\) rises that could be mistaken for self-propagating Ca\(^{2+}\) waves. Gross morphology of astrocytes was monitored in the red channel using bolus-loading of sulforhodamine 101.

In baseline conditions, spontaneous Ca\(^{2+}\) waves engulfing individual cells (spread over 10–20 µm) appeared on average ~5.4 times a minute over a 160 µm x160 µm ROI (Fig. 6c, top; Supplementary Movie 5), in good correspondence with previous observations. The time first derivative of the fluorescence transients revealed their wave-front dynamics (Fig. 6c, bottom) helping to identify centrifugally spreading events and thus to distinguish single-cell regenerative waves from synchronous signals evoked by external influences.

We found that such waves propagated with an average radial speed of 3.94 ± 0.16 µm s\(^{-1}\) (mean ± SEM, n = 54 events). Intriguingly, this speed appears significantly lower than that of the stimulus-induced astroglial Ca\(^{2+}\) waves in brain slices or in culture (15–25 µm s\(^{-1}\); reviewed in ref. 61). One possible explanation is that an exogenous stimulus in situ, such as agonist application, is likely to trigger a synchronous receptor response over the entire cell expanse. Similarly, in awake animals, we detected single-cell Ca\(^{2+}\) waves resembling those under anaesthesia, albeit at lower frequencies and magnitudes, in between prominent, region-wide Ca\(^{2+}\) elevations (Supplementary Movie 6). Possible contamination with such events made the anaesthetized animals a preferred choice in assessing Ca\(^{2+}\) buffering properties of astroglia.

In the astrocyte model, experimentally observed Ca\(^{2+}\) waves could be readily reproduced using an instantaneous local Ca\(^{2+}\) rise (5 \(\mu\)M for 0.1 ms) near the soma (Fig. 6d). Although the model could generate waves over a wide range of Ca\(^{2+}\) buffering parameters, their experimental speed required only certain combinations of \([B]\) and \(K_D\) (Fig. 6e) reflected in an almost perfectly linear relationship (in \(\mu\)M): \([B] = 170(1 + K_D)\). This
simple formula captures the buffering properties of cortical astrocytes, also suggesting the lower-limit Ca²⁺ buffer concentration of ~170 µM (ignoring the residual effect of GCaMP6f).

Decoding fluorescent Ca²⁺ signals recorded in astroglia. Historically, global and slow Ca²⁺ elevations had been the key indicator of astroglial activity. Recent advances in Ca²⁺ imaging...
revealed faster and more local Ca$^{2+}$ signals prevalent in small astrocyte processes (reviewed in refs. 43, 62–64). Any such observations rely however on high-affinity Ca$^{2+}$ indicators, which provide only a crude reference to the underlying Ca$^{2+}$ signals35. To translate recorded fluorescence into Ca$^{2+}$ dynamics, one has to model Ca$^{2+}$ entry, diffusion, and buffering (by endogenous proteins as well as Ca$^{2+}$ indicators), as demonstrated in numerous studies of nerve and muscle cells. We therefore used ASTRO to apply a similar modelling strategy to CA1 astrocytes loaded with Fluo-4, which show robust, multi-scale spontaneous Ca$^{2+}$ activity in acute slices (Fig. 7a, b; Supplementary Movie 7). While the precise mechanism underlying astroglial Ca$^{2+}$ signalling is still poorly understood, there has been a large body of work exploring the biophysical machinery of Ca$^{2+}$ oscillations in other cell types (reviewed in ref. 65). The key features emerging from these studies are the occurrence of Ca$^{2+}$ micro-domains

Fig. 7 Ca$^{2+}$ dynamics decoded from fluorescence Ca$^{2+}$ imaging in situ. a Example, astrocyte (CA1 area, acute hippocampal slice; Fluo 4 channel, $\lambda_{ex}^{2P} = 800$ nm) held in whole cell, with regions of interest for Ca$^{2+}$ monitoring (circles, ROIs 1–7; Supplementary Movie 7). Scale bar, 5 µm. b Time course of Ca$^{2+}$ sensitive fluorescence (Fluo-4 channel) recorded in ROIs 1–7 as in a, over 100 s; same colours correspond to ROIs on the same branch (ROIs 2–3 and 4–5). Scale bars (v, h): 200% ΔF/F, 20 s. c An astrocyte model with localized Ca$^{2+}$-puff sources (orange dots) and four recording points (arrows, 1–4); dotted oval, region for analyses: cell area outside has a negligible effect of the Ca$^{2+}$ sources as shown (Supplementary Movie 8). The model is ‘filled’ with free-diffusing Fluo-4 (150 µM; $k_{on} = 600$ mM$^{-1}$ ms$^{-1}$, $k_{off} = 21$ ms$^{-1}$) and the endogenous buffer as estimated (Fig. 6d; 200 µM, $K_D = 0.2$ µM; other combinations produced similar results). d Example of channel-like local Ca$^{2+}$ entry activity generated by a single localized Ca$^{2+}$ source, in accord with the known biophysical properties of cellular Ca$^{2+}$ sparks and hotspots. Scale bar, 1 s. e Time course of simulated Fluo-4 fluorescence (150 µM ‘added’) in ROIs 1–4 shown in c; it has statistical properties similar to those recorded in situ (b); shaded area, time window for higher temporal resolution (see f); same line colours correspond to ROIs on the same cell branch. Scale bars (v, h): 100% ΔF/F, 20 s. f, Right, simulated intracellular [Ca$^{2+}$] dynamics underlying Fluo-4 fluorescence shown in e. Left, trace fragments on the expanded time scale (shaded area in e), as indicated; the fragments correspond to the period of relatively high [Ca$^{2+}$].
(0.5–5 µm apart) represented by clusters of stochastically activated Ca2+ receptor channels (including IP3 or ryanodine receptors), with the agonist-dependent mean opening time of 2–20 ms, peak amplitude of ~5 pA, and an inter-opening interval varying between 0 and 50 ms. Stochastic activation of this system produces local Ca2+ ‘sparks’ (reflecting ryanodine-receptor clusters) or ‘puffs’ (reflecting IP3-receptor clusters), typically with an event frequency of 0.1–2 Hz, sometimes leading to global Ca2+ rises. The kinetic properties of other potential Ca2+ sources in astroglia, such as mitochondria and other Ca2+ channels, remain to be established.

In keeping with the basic paradigm, we have scattered local clusters of Ca2+ channels (IP3 receptor type) along several branches of the modelled astrocyte, 1–5 µm apart (Fig. 7c), within a ~20 µm area of interest: Ca2+ activity outside the area was of little consequence because of rapid diffusional dissipation in the absence of global Ca2+ events. Next, we 'filled' the model with Fluo-4 (details in Fig. 7c) and explored simulated channel openings, within and among Ca2+ channel clusters, over the plausible range of their characteristic frequencies (Fig. 7d). Thus, Ca2+-dependent fluorescence (represented by the concentration of Ca-bound Fluo-4) was recorded at four arbitrarily selected points in the area of interest (Fig. 7c; Supplementary Movie 8).

We found that at an average interval between channel openings of ~3 ms within clusters, and ~7 s among clusters, the simulated fluorescence signals (Fig. 7e) were similar to the experimental recordings (Fig. 7b). The intracellular Ca2+ dynamics underlying these signals was readily revealed by the model (Fig. 7f). Constraining this dynamics further would require experimental probing involving varied Ca2+ buffering conditions and molecular dissection of the Ca2+ cascades involved. Nonetheless, this example indicates that the classical molecular machinery of Ca2+ signalling long explored in other cell types could explain Ca2+ imaging data collected in astrocytes. Our further exploration of the IP3 arrangement within astrocyte branches revealed a complex relationship between inter-cluster distances, spontaneous Ca2+ activity and its fluorescent-indicator readout (see below).

Probing impact of variable astroglial features on function. One advantage of cell modelling is the possibility to predict theoretically the impact of a specific cellular or environmental feature on cell’s behaviour. This approach could also reveal whether certain feature combinations can make the modelled cell biophysically unstable. We therefore carried tests in which some key functional traits of modelled cells were monitored against changes in model parameters.

First, to see how strongly gross cell morphology could influence its biophysics, we compared two different modelled cells, one with the stem tree reconstructed in an experiment (Fig. 1a–d), and the ‘typical CA1 astrocyte’, with the stem tree adjusted to match the average features of CA1 astrocytes (Fig. 1e). The two cells thus featured different stem trees but were populated with nanoscopic processes based on the same statistical constraints (as in Figs. 2 and 3). The test revealed only subtle differences between the cells in their membrane voltage spread, input resistance, or Ca2+ wave generation (Supplementary Fig. 9). In a similar context, simulating astrocyte swelling by ~20% (by evenly increasing the cell process width throughout) had only moderate consequences (Supplementary Fig. 10). These examples suggest a relatively narrow range of effects arising from morphological variations per se, when all other features remain unchanged.

We next asked how intracellular diffusivity would affect the molecule equilibration time across the astrocyte (e.g., during whole-cell dialysis). Simulations mimicking somatic dialysis showed how reducing diffusivity (from 0.6 to 0.05 µm2 ms−1, reflecting diffusion of small ions and 2–3 kDa molecules, respectively) slows down dye equilibration (Supplementary Fig. 11). These examples, however, should be further constrained by experimental data, mainly because large molecules tend to undergo significant additional steric and viscous hindrance inside small cell compartments19. In contrast, smaller molecules can escape via gap junctions: this feature could be explored with ASTRO (Supplementary Note 1, ASTRO User Guide, pp. 27–28) once the gap junction diffusion sink rate has been experimentally constrained.

Finally, we explored simulation settings shown in Fig. 7 to ask how the clustering of IP3-dependent Ca2+ sources affects local Ca2+ activity. It appears that spreading this signalling mechanism into individual (equally spaced) clusters, with the same total amount of the IP3 activity, prompts de novo Ca2+ events which could feature prominently in fluorescent recordings (Supplementary Fig. 12).

Discussion

The present study sought to create a simulation tool ASTRO that would allow exploration and testing of mechanistic hypotheses pertinent to astroglial physiology, on the scale from nanoscopic processes to the entire cell expanse. Biophysical cell models replicating cell morphology have significantly influenced our understanding of neural function yet there have hitherto been no similar tools available to study astroglia. We therefore aimed at filling this knowledge gap.

The task of recreating astroglia in silico included three steps. The first step was to construct a ‘stem tree’ based on experimental data on main astrocyte branches that are readily identifiable in the optical microscope. This procedure is similar to the common 3D reconstructions of nerve cells using z-stacks of their optical sections. The second step, which was the key methodological challenge here, was to recreate the complex morphology of numerous nanoscopic astroglial processes pervading the synaptic neuropil. We therefore developed algorithms and computational tools (a) to quantify such processes using an empirical 3D EM sample and (b) to transform them into NEURON-compatible (cylinder-compartment based) shapes with matching biophysical properties. The latter was to be verified using dedicated Monte Carlo simulation tests (for diffusion and electrodiffusion) incorporated in ASTRO. This procedure provided all the key statistics characterising nanoscopic astroglial processes in the model.

Thus, the third step was to populate the modelled stem tree with stochastically generated nanoscopic processes, in accord with their morphometric stats obtained as outlined above. The main experimental constraint here was the VF occupied by astroglial processes, which we and others could measure directly using either 2PE microscopy or 3D EM. Because neighbouring astrocytes do not overlap in tissue, local VF faithfully reflects the space-filling properties of individual astroglial cells. We could therefore stochastically generate individual nanoscopic processes on dendritic branches until their bulk matched the empirical VF distribution. This procedure was to complete the modelled cell architecture: the model could now be explored using NEURON simulation environment which we equipped with several additional functions specific to astroglia.

In our case study, we obtained detailed morphological data on hippocampal astroglia in area CA1 using 3D EM and 2PE imaging, recreated the ‘typical’ cell in silico, and partly constrained its functional features by further experimental tests. Exploring the model has shed light on some traits of astroglial physiology that have not been attainable in experiments. Our simulations predict that local glutamate uptake or K+ intake via K+4.1 generates only...
very small membrane depolarisation across the astrocyte. It appears that transient rises of extracellular K$^+$ concentration prompt relatively small changes of intra-astroglial K$^+$, which dissipate relatively quickly, within one cell, due to efficient K$^+$ efflux through K$_a$4.1 channels. Our tests illustrated that relatively small changes in Ca$^{2+}$ buffering properties might significantly influence the spread of regenerative intra-glial Ca$^{2+}$ waves. The model also showed that the classical mechanisms of rapid Ca$^{2+}$ spikes and hotspots described in other cell types could be consistent with the slow Ca$^{2+}$ signals reported by common Ca$^{2+}$-sensitive fluorescent indicators.

Thus, the modelling approach presented here serves several general purposes. Firstly, to recapitulate complex astrocyte morphology at multiple scales, paving the way to interrogating the form-function relationship in astroglia. Secondly, to understand the physiological and biophysical characteristics of individual cellular features (morphology, Ca$^{2+}$ buffering, channel current density, molecular transport, etc.) and the associated Ca$^{2+}$ signaling. Finally, to predict the relationships between biophysical and biophysical simulations of astroglial membranes. The steady-state current response to the whole-cell patch voltage step was used to calculate the patch conductance, which was then normalized to the membrane area to obtain $G_{m}$.

**Experimental methods: 2P imaging, uncaging, and FRAP ex vivo.** Astrocytes were loaded with 100 µM Alexa Fluor 594 and 100 µM Alexa Fluor 546 (Invitrogen) and were imaged using an Olympus FLX-100MPE microscope (Chameleon, Coherent, tuned to 720 nm) with a 100×/1.45 NA oil objective. Fluorescence recordings were normally carried out in line-scan mode (500 Hz). In FRAP experiments, laser power was increased to 15–20 mW during photobleaching epochs. To record spontaneous Ca$^{2+}$ transients in free scan mode, 200 µM Fluo-4 (Invitrogen) and 100 µM Alexa Fluor 594 (Invitrogen) were added to the intracellular solution. 350–500 µm² fields of view where imaged within the astrocyte arbour and the fluorescence emitted by Alexa Fluor 594 (λ$_{ex}$ 540 nm; λ$_{em}$ 594 nm) was collected at a rate of 3–5 Hz for 2–3 min. Time-dependent fluorescence transients were expressed as A/GR where G corresponds to the background-subtracted Fluor-4 fluorescence and R to the background-subtracted Alexa Fluor 594 fluorescence. Further details of the imaging methods were reported previously.

For MINI-glutamate uncaging, astrocytes were loaded with 100 µM Alexa Fluor 594 as a morphological marker. Astrocytes were held in voltage-clamp mode at their resting membrane potential (typically between 80 and 90 mV). The MINI-glutamate (12.5 mM) was either puffed within the tissue from a glass pipette placed through the astrocyte arbour and across the soma) at the end of the voltage step is measured, giving electric charge $Q$, and (c) membrane patch capacitance is estimated as $c = Q/AV$. Thus the patch area is evaluated from the ratio $c / c_{m}$, where specific membrane capacitance $c_{m} = 1 \mu$F cm$^{-2}$ is a common characteristic of astroglial membranes. Finally, we stress that our aim was not to present a comprehensive model of astrocyte morphology at multiple scales, paving the way to interrogating the form-function relationship in astroglia.
CA, USA) and kept warm on a heating blanket. The scalp was shaved and disinfected using three washes of topical chlorhexidine. Upon loss of pedal withdrawal reflex, the incision was made to expose the skull. A given injection bolus contained between 0.25 and 0.5 × 10¹⁰ genomic copies, in a volume not exceeding 500 nL. After injection, pipettes were left in place for 5 min before retraction. The scalp was sutured with absorbable 7–0 sutures (Ethicon Endo-Surgery GmbH, Norderstedt, Germany) and the animal was left to recover in a heated chamber. Meloxicam (subcutaneous, 1 mg kg⁻¹) was administered once daily for up to two days following surgery. After a 4–6-week AAV incubation period, animals were prepared for multiphoton imaging through a cranial window implantation as described below.

**Experimental methods: two-photon excitation imaging of astrogia in vivo.** Following viral transduction of LkGCaMP6f as above, male C57BL/6 mice (7–9 weeks of age) were prepared for cranial window implantation and 2P microscopy. Animals were anaesthetized using fentanyl, midazolam and medetomidine (i.p., 0.05, 5 and 0.5 mg kg⁻¹, respectively). Adequate anaesthesia was ensured by continuously checking for the loss of pedal withdrawal reflex and anaesthesia was supplemented appropriately throughout the procedure (typically 10–20 % of the original dose per hour). Body temperature was maintained at 37.0 ± 0.5 °C using a feedback rectal thermometer and heating blanket. The animal was secured in a stereotactic frame and a craniotomy of ~2.5 mm diameter was carried out over right parietal cortex, at a depth of ~1.2 mm lateral to the midline (i.p., 0.05, 5 and 0.5 mg kg⁻¹) was administered once daily for up to two days following surgery. After a 4–6-week AAV incubation period, animals were prepared for multiphoton imaging through a cranial window implantation as described below.

In these experiments, two-photon excitation was carried out using a Newport-SpectroPhysics Ti:sapphire MaiTai laser pulsing at 90 MHz, and an Olympus Water FV1000 with XLPlan N ×25 water immersion objective (NA 1.05). Acquisitions were carried out using a wavelength of 920 nm and the mean laser power under the objective was kept at 20–35 mW. Cortical astrocytes were readily identified through SR101 labelling and verified for GCaMP6f expression by frame-scanning for 5 min to eliminate blood background, washed with 0.1 M TBS (tris buffer saline, pH 7.4) to eliminate any SR101 labelling and verified for GCaMP6f expression by frame-scanning for 5 min to eliminate blood background, washed with 0.1 M TBS (tris buffer saline, pH 7.4) to eliminate any SR101 labelling and verified for GCaMP6f expression by frame-scanning for 5 min to eliminate blood background, washed with 0.1 M TBS (tris buffer saline, pH 7.4) to eliminate any SR101 labelling. The cortical surface was covered with 1% agarose and a glass coverslip was placed on top. Using tissue adhesive (DermaFuse, Vet-Tech Solutions, UK), the coverslip was partially secured and a custom-built headplate fixed to the skull. A single cranial-mounted screw was inserted over the contralateral hemisphere and the entire assembly was then secured using dental cement. During imaging, the headplate was used to secure the animal under the objective on a custom-built stage.

In a subset of experiments we loaded an astrocyte with biocytin, and after the experiment the slices were rapidly fixed (by submersion) with 1.25% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M PB (phosphate buffer, pH 7.4), to be kept overnight, submerged in 10% sucrose in PB for 10 min and then in 20% sucrose in PB for 12 h, before being cryoprotected in liquid nitrogen for 3 s each to gently crack intracellular membranes and embedded in 1% low gelling temperature agarose in PB (Sigma-Aldrich, USA). Sections (50 μm) were incubated in 1% H₂O₂ in PB for 20 min to eliminate blood background, washed with 0.1 M TBS (tris buffer saline, pH 7.4) and incubated with ABC solution (VECTASTAIN ABC, Vector laboratories, USA) for 30 min at room temperature. Next sections were washed with 0.1 M TB (tris buffer, pH 7.4), pre-incubated with DAB (3.3'-Diaminobenzidine tablets—Sigma-Aldrich, USA) solution (10 mg DAB tablet + 40 ml TB) for 30 min at room temperature, followed by three washes in DAB (5 μL of 33% H₂O₂ + 25 ml of DAB solution) for 10–20 min at room temperature in the dark. The DAB stained sections was washed in PB, post-fixed in 2% osmium tetroxide and further processing and embedding protocols were essentially similar to those reported previously. Briefly, the tissue was dehydrated in graded aqueous solutions of ethanol (40% v/v from 37% v/v at 70–80 °C), followed as modified as 3 times in 100% acetone, embedded into a mixture of 50% epoxy resin (Epox 812/Aradite M) and 50% acetone for 30 min at room temperature, embedded in pure epoxy resin, and polymerized overnight at 80 °C. Sections in blocks were coded and all further analyses were carried out blind as to the experimental status of the tissue.

**Experimental methods: 3D electron microscopy.** Serial sections (60–70 nm thick) were cut with a Diatome diamond knife and systematically collected using Pioloform-coated slat copper grids (each series consist of up to 100 serial sections). Sections were counterstained with 4% uranyl acetate, followed by lead citrate. Finally sections were imaged in stratum radiatum area of CA1 (hippocampus) using a Zeiss 980 12 megapixel camera in JOEL1400 electron microscopy. Serial sections were aligned as JPEG images using SEM align 1.26b (software available from http://synapses.clm.utexas.edu/). 3D reconstructions of DAB-stained astrocyte processes the adjoined to stained astrocytes dendritic spines (that clearly identifiable excitatory synapses) were performed in Trace 1.6b software (http://synapses.clm.utexas.edu/). 3D reconstructions of selected astrocytic segments and dendritic spines were rendered to 3D-Studies for rendering the reconstructed structures. However, ASTRO can upload other standard text-formatted files with 3D coordinates representing the cell-membrane 'mesh'.

**Astrocyte model: generating ‘invisible’ nanoscopic morphology.** Nanoscopic processes of the astrocyte model were generated in a probablistic manner based on the sample statistics from 3D EM reconstructions (Fig. 2). The total cell surface area Smain represented by the cylinder-based shape approximations (Fig. 2d, e) were divided to the surface area of the main cylinder bases Sb (blue in Fig. 2d, bottom) minus the surface areas of ‘transitional’ cylinder bases Strans (green in Fig. 2d, bottom). In our case, computations indicated that Sb = 1.02Strans throughout modelling; thus, the formula Strans = Sb – 0.5Strans was applied. In the generated cell models, the S/V ratios were ranging from ~7 μm⁻¹ near the soma to an average of ~22 μm⁻¹ in the bulk of the cell arbor, in accord with the empirical observations.

**Astrocyte model: transporter/channel kinetics and diffusion-reaction mechanisms.** Models built with ASTRO can incorporate many dozens of NEURON-enabled channel and transporter kinetic mechanisms that have been tested and validated in numerous studies combining experiments and simulations. The formal description and the respective algorithms are included in an extensive NEURON database at https://senselab.med.yale.edu/modeldb/ which also contains references and links to the original studies and the mathematical formulation involved. Upon ASTRO installation on the host computer, these mechanisms could also be inspected in the respective .mod files in the ‘neurons’ directory or, alternatively, online here https://github.com/LeonidSavtchenko/Astro/tree/master/neuronSims.

Several channel current and diffusion-reaction mechanisms have been written specifically for the present model. The kinetics of glutamate transporter GLT-1 involving glutamate and ion fluxes has been incorporated in accordance with 46 (description in the GluTrans.mod file). The K<sub>Ca</sub>1.1 potassium current has been incorporated in accordance with 47 (Kir1.1.mod; Fig. 5 legend), intracellular K<sup>+</sup> diffusion was incorporated as longitudinal diffusion (no radial rings) using a built-in Ca²⁺ diffusion algorithm described in the next section (potassium.mod), the FRAP mechanism incorporated the same algorithm plus a reaction-diffusion step (FRAP.mod), and K<sup>+</sup> extrusion was modelled as a first-order pump (Kpump.mod; Supplementary Fig. 7 legend). Gap junction mechanisms were enabled either as (zero-order) current leak (gap.mod) or as a diffuse escape (gapCa.mod).

Throughout these mechanisms, the respective kinetic parameters can be set using the relevant NEURON-enabled ASTRO menus, as described in the User Guide (https://github.com/LeonidSavtchenko/Astro/blob/master/ASTRO_User_Guide.pdf).

**Astrocyte model: Ca²⁺ homeostasis and diffusion.** Astrophoretic algorithms enabling intracellular Ca²⁺ homeostasis and diffusion (simulation including that among adjacent compartments of unequal size) are detailed in Chapter 9 of the NEURON Book 20 (also here https://www.neuron.yale.edu/neuron/docs), and can be accessed to the moddb at neuronDB. In brief, Ca²⁺ diffusion is modeled freely whereas buffer-bound Ca²⁺ (which has much lower diffusivity) is considered stationary, for the sake of simplicity. In individual cylindrical cell...
compartments, radial diffusion occurs through four concentric shells surrounding a cylindrical central core, and longitudinal diffusion is calculated using fluxes between the corresponding concentric compartments adjusted for the cross-section areas. The longitudinal and radial diffusion coefficient for Ca^{2+} was set to 0.3 μm² ms⁻¹, the basal level was set to 50 nM, and IP₃ concentration at 0 unless specified otherwise.

In addition to free diffusion, Ca^{2+} homoeostasis mechanisms included the SERCA pump, SERCA channel and SERCA leak, the endogenous (stationary) and exogenous (Ca^{2+} indicator) mobile buffers, and a plasma membrane Ca^{2+} pump with the threshold mechanism (cadimus.mod). The kinetics of buffers can be modified using NEURON-enabled menus. The mechanistic details of Ca^{2+} SERCA pump were as described earlier. The current model implementation assumes that IP₃ is distributed uniformly across cell compartments, i.e. that diffusion equilibration of IP₃ is fast compared to Ca^{2+} concentration transients in space or time.

Modelling with ASTRO: on-line access and installation. Detailed information on the installation and running of ASTRO can be found in the User’s Manual (Supplementary Material; online download at https://github.com/LeonidSavtchenko/Astro/blob/master/Manual). The current version of ASTRO can also be downloaded directly from https://github.com/LeonidSavtchenko/Astro. The (regularly updated) User Guide can be downloaded from the same location or found (current version) in the Supplementary Note.

In brief, running ASTRO with de novo 3D EM reconstructions and model building, but without full-scale simulations of intracellular Ca^{2+} dynamics, requires the host computer to have MATLAB (2012 version or later, https://uk.mathworks.com/products/matlab.html), or at least free-download MATLAB Runtime package (https://www.mathworks.com/products/compiler/incre), and NEURON 7.2 or later, https://neuron.yale.edu/neuron/download] installed under Windows 7 or Windows 10. Simulations using ready-made astrocyte models require NEURON installation only.

Simulating full intracellular Ca^{2+} dynamics is highly resource-consuming and normally requires an additional Worker computer/cluster operating under Linux, with preinstalled NEURON (https://neuron.yale.edu/neuron/download/compile_linux) and MPI whereas the Host computer will require MATLAB (2013 version or later), NEURON (7.0 or later), and access to the Internet. In house, the Linux version with the parallel computations including intracellular diffusion simulation was routinely run using a 12-node in-house computer cluster, taking advantage of the computational optimization routines developed by us earlier for compartmental models and Monte Carlo simulations.

Data availability
The current version of ASTRO, with open code access, can also be downloaded directly from https://github.com/LeonidSavtchenko/Astro, with the User’s Manual available at https://github.com/LeonidSavtchenko/Astro/blob/master/Manual. See above for further detail. All experimental recording data are available from the authors upon request.

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Author contributions

D.A.R., L.P.S. and C.H. conceived the study; L.P.S. developed and implemented the modelling approach and its computing environment; C.H. designed and carried out morphometric studies and analyses ex vivo; C.H. and L.P. carried out patch-clamp and imaging experiments and analyses ex vivo; T.J. carried out some imaging experiments ex vivo; I.K., M.M. and M.G.S. designed and carried out quantitative 3D EM studies; D.A.R. narrated the study, carried out 3D stem-tree reconstructions, some data analyses and wrote the paper which was subsequently contributed to by all the authors. Data availability: The current version of ASTRO, with open code access, can also be downloaded directly from https://github.com/LeonidSavtchenko/Astro, with the User’s Manual available at https://github.com/LeonidSavtchenko/Astro/blob/master/Manual). See above for further detail. All experimental recording data are available from the authors upon request.

Additional information

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