OPINION

Astrocyte Ca^{2+} signalling: an unexpected complexity

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Abstract | Astrocyte Ca^{2+} signalling has been proposed to link neuronal information in different spatial–temporal dimensions to achieve a higher level of brain integration. However, some discrepancies in the results of recent studies challenge this view and highlight key insufficiencies in our current understanding. In parallel, new experimental approaches that enable the study of astrocyte physiology at higher spatial–temporal resolution in intact brain preparations are beginning to reveal an unexpected level of compartmentalization and sophistication in astrocytic Ca^{2+} dynamics. This newly revealed complexity needs to be attentively considered in order to understand how astrocytes may contribute to brain information processing.

In the early 1990s, application of Ca^{2+} imaging techniques to the study of astrocytes in brain slices led to the remarkable discovery that these cells respond to synaptic activity by increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[1–3]. Research in the following years established that similar increases in astrocytic $[Ca^{2+}]_i$ occur in living animals in response to sensory stimuli[4–6], during locomotor activity[7] and during autonomic function[8], and further elucidated details of the nature of these Ca^{2+} fluxes and their physiological roles. The picture that emerged was one of diverse and wide-ranging astrocytic activation in response to neuronal function: astrocytic $[Ca^{2+}]_i$ changes occur in most brain areas and in response to the release of numerous neurotransmitters and factors (for reviews, see REFS 9–12). According to current views, neurons transfer information to astrocytes mainly through spillover of synaptic transmitters and factors, which bind to high-affinity astrocytic G protein-coupled receptors (GPCRs)[13–16] that trigger inositol-1,4,5-trisphosphate (IP3) production and Ca^{2+} release from the endoplasmic reticulum (ER). Activation of this signalling system can then generate a wide range of oscillatory Ca^{2+} signals[3,17,18]. In addition, synaptic activity can induce a rise in astrocytic $[Ca^{2+}]_i$ through the stimulation of Ca^{2+}-permeant ionotropic receptors, but only in some regions (for example, the cerebral cortex by NMDA receptor stimulation[19]) and via specialized mechanisms (such as 'ectopic' release onto glial AMPA receptors in the cerebellum[20]). Astrocytic $[Ca^{2+}]_i$ increases can also be induced via reversal of Na^+/Ca^{2+} exchangers following neurotransmitter uptake or other pumping activities[21,22] that lead to an increase in astrocytic Na^+ levels. Astrocytes also show transient $[Ca^{2+}]_i$ rises that occur independently of synaptic activity, particularly during development[23–26], and Ca^{2+} fluctuations due to transient receptor potential A1 (TRPA1) channel activity that contribute to resting Ca^{2+} levels[27,28].

A specific consequence of $[Ca^{2+}]_i$ increases in astrocytes is release (within tens of milliseconds to seconds[29–32]) of chemical mediators called gliotransmitters[33–35], which are capable of modulatory actions on other glial, neuronal or vascular cells. There are various types of gliotransmitter (for example, glutamate, D-serine and adenosine triphosphate; the latter is often rapidly converted to adenosine), and their synaptic actions result in several, often opposite, types of effects, including stimulation or inhibition of synaptic transmission and participation in long-term potentiation (LTP) or long-term depression (LTD), heterosynaptic facilitation or depression and homeostatic plasticity (reviewed in REFS 10–12). How astrocytic Ca^{2+} signalling can generate such an impressive diversity of synaptic effects is currently not understood. Available data suggest that several factors might be important, including specificities in the properties of different receptors that trigger an increase in $[Ca^{2+}]_i$ in astrocytes[36], in the Ca^{2+}-dependent mechanisms that govern the production or release of different mediators[30,32,37–39] and in the gliotransmitter targets in effector cells[40–47].

Although the above studies hint at a complexity of the relations linking astrocytic $[Ca^{2+}]_i$ changes to synaptic changes, a more problematic scenario has emerged from an important set of recent studies. By manipulating astrocytic $[Ca^{2+}]_i$ in several ways, these studies failed to produce any effect on hippocampal synaptic transmission and plasticity[48,49]. Moreover, the negative results were in direct contradiction with other results[28,50,51] obtained in the same region (see a detailed comparative analysis in BOX 1). These discrepancies have been difficult to reconcile in the context of current models of astrocyte function, suggesting that such models may be simplistic. In parallel, new data obtained owing to methodological advances have started to reveal a previously unanticipated level of diversification in astrocyte Ca^{2+} dynamics (FIG. 1). In this Opinion article, we call for more attention to be paid to the emerging complexity in the modes of astrocyte activation and to the potential specificities of different types of astrocytic $[Ca^{2+}]_i$ changes. We also argue that the experimental approaches to study astrocytic Ca^{2+} phenomena should be rethought to better match this new perspective, bearing in mind the importance of testing under physiologically relevant conditions and according to a specific biological question. This refined strategy will hopefully produce the more detailed and robust understanding of astrocyte Ca^{2+} signalling that is needed to reconcile apparently conflicting views of astrocyte physiology.

A challenge to the current models

A series of studies addressing the role of astrocyte Ca^{2+} signalling in synaptic transmission and plasticity at CA3–CA1

Box 1 | **Role of astrocytic Ca^{2+} signalling in hippocampal LTP: different approaches give different outcomes***

Four studies have been reported that examined the role of astrocytic Ca^{2+} signalling in long-term potentiation (LTP) in the CA3–CA1 hippocampal circuit (see the table). They reached different conclusions, possibly because of important differences in experimental settings. First, they used preparations of different age, and the coupling between neuronal activity and astrocytic Ca^{2+} signalling is probably different in the mature and immature circuitry. Second, they used different LTP induction protocols (studies A, B and C used tetanic stimulation, whereas study D used alveus theta-burst stimulation of acetylcholine (ACh) fibres), resulting in the recruitment of different forms of LTP, which rely on different astrocytic mechanisms (FIG. 3). Thus, high-frequency stimulation of CA3 Schaffer collaterals (SCs) (in studies A, B and C) induces NMDA receptor (NMDAR)-dependent postsynaptic LTP (nLTP), which possibly requires release of astrocyte D-serine as a co-agonist; theta-burst stimulation of alveus cholinergic afferents (in study D) induces cholinergic LTP (cLTP), possibly through a presynaptic mechanism[51] requiring astrocytic muscarinic ACh receptor (mAChR) activation and glutamate release (see main text). Third, different strategies for blocking astrocyte Ca^{2+} signalling were used in studies A, B and C): astrocytic Ca^{2+} chelation (study A) interferes with all Ca^{2+} activity regardless of the source, whereas each individual gene knockout targets a single and distinct source of Ca^{2+} (studies B and C). Fourth, different protocols were used to test the effect of an artificial increase in astrocytic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on LTP (study B versus study D). In study B, astrocytic $[Ca^{2+}]_i$ was increased through transgenic G protein-coupled receptor (GPCR) stimulation prior to neuronal stimulation and remained steadily raised during neuronal stimulation: this protocol did not enhance LTP. However, native astrocytic Ca^{2+} signals produced during LTP induction were not blocked in parallel. Moreover, the evoked $[Ca^{2+}]_i$ increases had a longer duration (~5 min) than the native signals (~30 s, see main text). In study D, astrocytic $[Ca^{2+}]_i$ was increased by pulsatile Ca^{2+} uncaging[47] coincidentally with the start of subthreshold neuronal stimulation and after blocking native GPCR-dependent Ca^{2+} signalling: this protocol induced LTP.

	Study A	Study B	Study C	Study D
Key experimental observations				
Astrocytic Ca^{2+} involved in LTP?	Yes	No	Yes	Yes
Identified astrocytic Ca^{2+} source	Not defined	Not applicable	External, via TRPA1	ER, via IP3R2 (mAChR)
Downstream gliotransmitter	D-serine	Not applicable	D-serine	Glutamate
Experimental settings				
Species and age	Adult rats (4–8-week-old)	Young mice (P16–P17)	Adult mice (8–18-week-old)	Young mice (P13–P18) Young rats (P12–P17)
Method of LTP induction	SC tetanic stimulation, 100 Hz, 1 s×3	SC tetanic stimulation, 100 Hz, 1 s×2	SC tetanic stimulation, 100 Hz, 1 s×2	Alveus theta-burst stimulation (ACh fibres)
Endogenous Ca^{2+} response of astrocytes during LTP induction				
Astrocyte Ca^{2+} response (soma)	~54% of astrocytes	~19% of astrocytes	Not tested	~50% of astrocytes
Duration of response	~30 s (estimated)	<30 s (estimated)	Not tested	~30 s (estimated)
Effect of interfering with an astrocytic Ca^{2+} source				
Astrocytic Ca^{2+} chelation (all sources)	LTP disrupted	Not tested	Not tested	LTP disrupted
IP3R2-knockout mice	Not tested	LTP not affected	LTP not affected	LTP disrupted
TRPA1-knockout mice	Not tested	Not tested	LTP disrupted	Not tested
Effect of artificially induced Ca^{2+} increase in the astrocytes				
Transgenic GPCR stimulation	Not tested	LTP not affected	Not tested	Not tested
UV uncaging	Not tested	Not tested	Not tested	LTP induced in specific conditions

ER, endoplasmic reticulum; IP3R2, inositol-1,4,5-triphosphate receptor 2; P, postnatal day; TRPA1, transient receptor potential A1. *Study A refers to REF. 50, study B refers to REF. 48, study C refers to REF. 28 and study D refers to REF. 51.

hippocampal synapses led to discrepant results. These apparently contradictory responses are difficult to explain, but given the emerging subtleties of astrocytic Ca^{2+} signalling, the core of the problem could reside in the use of different experimental paradigms that interfere with astrocytic Ca^{2+} (details in BOX 1). Notably, a genetic mouse model created ad hoc to study the effect of $[Ca^{2+}]_i$ increases selectively in astrocytes[48,49] provided fully unexpected results. In this model, a GPCR linked to IP3 production (but which is not endogenously present in brain), MRGA1, was conditionally expressed in astrocytes and activated on demand by applying its natural agonists, arginine and amidated-phenylalanine motif-containing peptides. Robust MRGA1 activation led to $[Ca^{2+}]_i$ increases that lasted for minutes and occurred ubiquitously in astrocytes. Surprisingly, such persistent $[Ca^{2+}]_i$ increases neither detectably modified synaptic function[49] nor contributed to LTP induction[48]. Furthermore, similar $[Ca^{2+}]_i$ increases induced by stimulation of endogenous GPCRs were also ineffective[48,49], and even the opposite approach (testing synaptic responses in IP3 receptor 2 (IP3R2)-null mice lacking Ca^{2+} responses to astrocyte GPCR stimulation[52]) failed to produce any effect on synaptic transmission and plasticity (specifically, LTP)[48,52]. These data would seem to suggest that astrocyte Ca^{2+} signalling does not have a crucial role in these phenomena. However, other studies reached the opposite conclusion by using different strategies to interfere with astrocyte Ca^{2+}. Some infused Ca^{2+} chelators into

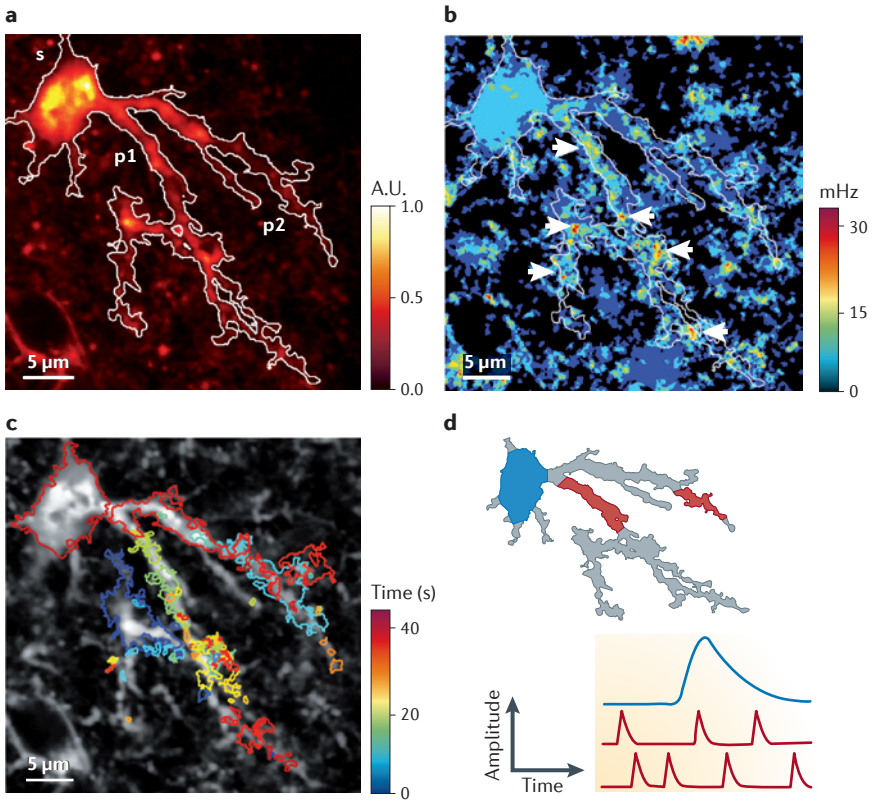


Figure 1 | **Diversity of endogenous Ca^{2+} activity in a mature hippocampal astrocyte *in situ*: Ca^{2+} signals in cell body and processes are different.** **a** | Cumulative Ca^{2+} activity recorded in an astrocyte over a 165 second period revealed by the Ca^{2+} indicator Fluo-4-AM. The visible boundaries of the astrocyte are shown in white. Note the different intensities of spatially confined local activity in the astrocyte soma (s), primary process (p1) stemming from the soma and secondary processes (p2) branching from a primary process. The intensity of the normalized cumulative activity is expressed in arbitrary units (A.U.) and is shown in pseudocolour, from dark (lowest activity) to white (highest activity). **b** | A frequency map of the Ca^{2+} activity in the astrocyte during the 165 second period as in part **a**. Activity is measured in individual pixels, expressed in mHz and colour-coded from black (never active) to dark red (frequently active). Most of the activity is within the white boundaries, and the most frequently active pixels are in defined small regions (arrowheads) of the primary and secondary processes (30 mHz), whereas pixels in the somatic region are less active (~10 mHz). This indicates that most of the astrocytic Ca^{2+} activity — as well as the activity with the highest frequency — occurs in the processes, not in the soma. **c** | A spatiotemporal map showing that the Ca^{2+} activity occurs asynchronously in different processes and in different portions of an individual process, and that Ca^{2+} events in the cell body and processes are temporally dissociated. The maximal spatial spread of each Ca^{2+} event is shown in colour, and the temporal sequence of the events is colour-coded from dark blue, which outlines events occurring in the first seconds, to dark red (around 40 seconds). **d** | A cartoon summary of the diversity of astrocytic Ca^{2+} signalling in the cell body and processes. The Ca^{2+} events typically observed in the soma (blue) are less frequent but longer and larger in amplitude compared with those occurring in the processes (red). Data presented in parts **a**–**c** are based on two-photon imaging at 2 Hz performed in the outer molecular layer of the dentate gyrus in an acute hippocampal slice of a mouse at postnatal day 35 at a depth of >50 µm (A.V., N.L. and C. Dürst, unpublished observations). Images used in parts **a**–**c** courtesy of C. Dürst, University of Lausanne, Switzerland.

astrocytes, preventing any endogenous $[Ca^{2+}]_i$ rise, whereas others targeted an IP3R-independent Ca^{2+} source[28], and each observed changes in synaptic transmission[27,44,46,50,51,53–57] and/or suppression of synaptic plasticity[28,51,58–60]. Even more puzzlingly, one of these studies found disrupted LTP in IP3R2-null mice[51].

Although these inconsistent results highlight deficiencies in our understanding of astrocytic Ca^{2+} phenomena, mounting evidence that astrocytic Ca^{2+} signals are richly diverse suggests that 'details' such as how (the mechanism), where (in the complex astrocytic arbor or in the cell soma) and when Ca^{2+} fluxes are naturally generated or experimentally produced can fundamentally change the functional consequences.

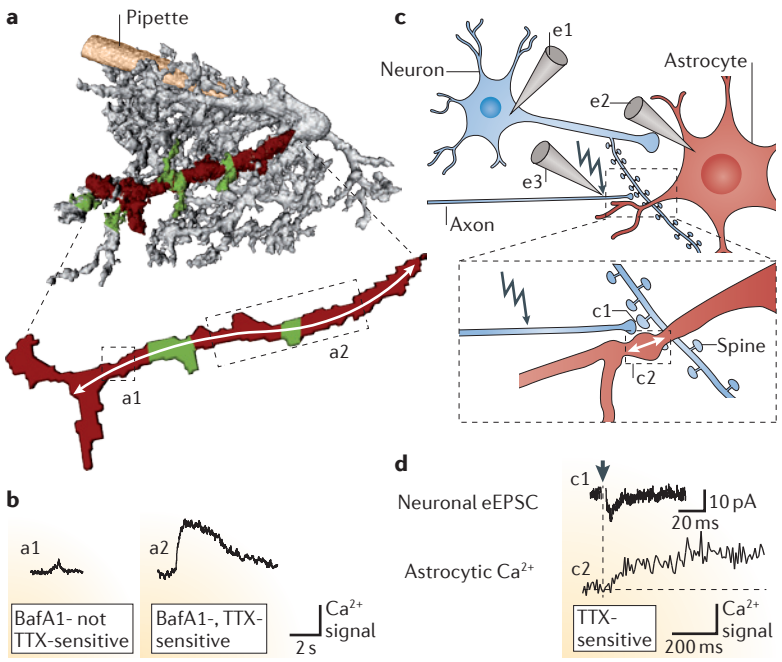
Ca^{2+} dynamics in astrocytic processes

Initial research in astrocytes monitored Ca^{2+} responses mainly in the soma because it was a methodologically accessible, first approximation readout of astrocyte activity. However, work in cerebellar Bergmann glia[61] subsequently revealed the existence of Ca^{2+} responses generated in cell processes that do not propagate to the soma, thereby identifying autonomous functional domains (microdomains). Use of large Bergmann glial cells facilitated measurement of Ca^{2+} in domains (20–50 μm^2), but determining whether similar microdomains existed in astrocytic processes (cross-section of ≤1–2 µm) proved to be much more difficult[24]. Only recently, technical obstacles were surmounted (BOX 2), leading to the first description of local astrocytic Ca^{2+} dynamics[46,55] (FIGS 1,2). This methodological progress revealed that astrocyte–synapse communications operate on previously unrecognized spatial–temporal scales and was an important advance in the conceptual understanding of astrocytic Ca^{2+} signalling. For example, early work that monitored mainly somatic $[Ca^{2+}]_i$ increases concluded that astrocytes respond only to intense neuronal firing patterns[2], but the work in astrocytic processes revealed that they also respond (locally) to low levels of synaptic activity[46,55], suggesting that the profile of astrocytic Ca^{2+} activity encompasses the whole spectrum of neuronal communication. This advance is important because it highlights different levels of synaptic activation of astrocytes and focuses attention on the relationship between patterns of the neuronal activation and the types of astrocytic Ca^{2+} responses[3,62–64]. Moreover, it introduces the issue of how astrocytes integrate, via $[Ca^{2+}]_i$ changes, neuronal activities of different intensities, which occur at different times, in different astrocytic locations and via different mediators[64,65].

Different Ca^{2+} signals in astrocyte processes and the cell body. Direct monitoring of Ca^{2+} dynamics in the processes of adult mouse hippocampal astrocytes[46,55] has revealed intense and previously unappreciated local activity that is dissociated from activity in the cell body (see also REF. 65). This activity is much more frequent than the somatic activity and occurs asynchronously in various processes as if they were functionally independent (FIG. 1). Even within an individual process, Ca^{2+}

Box 2 | **The methodological challenge of studying Ca^{2+} dynamics in astrocytic processes**

Monitoring fast local Ca^{2+} dynamics in astrocytic processes *in situ* is methodologically challenging. High-resolution two-photon techniques that are suitable for studies in dendritic spines[110] cannot easily be adapted for studies in astrocytes because astrocytic processes are more complex and their appendages are often not compartmentally isolated nor entirely contained within the imaged focal volume (~ 1 μm^3), and the sites of communication with synapses (for example, where G protein-coupled receptors are located) are not yet clear (FIG. 2). Nevertheless, by adopting different strategies, two studies[46,55] succeeded in recording fast Ca^{2+} dynamics in astrocytic processes. Part **a** of the figure depicts the approach used in REF. 55. A single astrocyte was patched (orange pipette) and loaded with two dyes, one for monitoring Ca^{2+} dynamics and the other for obtaining a three-dimensional map of the astrocyte morphology (grey). Ca^{2+} dynamics were recorded in a process lying favourably in the focal plane using the two-photon 'line-scan' approach, which sequentially samples activity at ultra-fast speed along a single line rather than in a volume. Dark red shading represents unbranched subregions of the astrocytic process and green represents branched subregions; also shown magnified below. In this experiment, the Ca^{2+} activity evoked by the synaptic activity from surrounding neurons was recorded by line scan along the process length (white arrow in magnified image) and is shown as black traces in part **b**. The three-dimensional morphological map was then used to distinguish truly local Ca^{2+} signals that were confined to single subregions of the astrocytic process (boxed area a1) from larger and more extensive Ca^{2+} signals involving contiguous subregions and also branches that exited the focal plane (boxed area a2). This approach led to the identification of two types of Ca^{2+} signals in the processes, dubbed 'focal' (a1, part **b** of the figure) and 'expanded' (a2, part **b** of the figure). Focal events (a1) are spatially restricted (~4 µm), small and fast events, whereas expanded events (a2) are spatially enlarged (~13 µm), bigger and longer-lasting events. Focal and expanded events are pharmacologically distinguishable: only expanded events are sensitive to tetrodotoxin (TTX), which blocks neuronal firing and thereby abolishes evoked synaptic release, whereas both types of events are sensitive to bafilomycin A1 (BafA1), which depletes synaptic vesicles and thereby abolishes any type of synaptic release. Part **c** of the figure depicts the alternative approach used in REF. 46. Here, researchers patch-clamped a neuron (azure) using electrode e1 and a neighbouring astrocyte (dark red) using electrode e2 while using a third electrode (e3) to stimulate nearby axons. They minimally stimulated axons (using electrode e3) and in parallel scanned a small region of the proximal astrocytic process, looking for temporally correlated Ca^{2+} events (lower image). They often observed (see the figure, part **d**) a single-synapse evoked excitatory postsynaptic current (eEPSC) in the contiguous synapse (c1) and, simultaneously, a local Ca^{2+} response in the monitored astrocytic process region (c2). Notice that the astrocytic Ca^{2+} signal resembles the expanded event in part **b** in its prolonged time course and, likewise, is TTX-sensitive. The dual indicator approach[110] and selection of the appropriate dyes[46,55] contributed to the success of both studies. Whole-cell patch-clamp dye infusion in an astrocyte enabled resolution of even very tiny Ca^{2+} events as far as 35 µm from the soma during periods of several minutes. In parallel, it enabled constant control of the cell's membrane electrical properties and loading of pharmacological agents. However, a disadvantage of this method is the possible dialysis of intracellular components. The advent of dye loading by cell electroporation[111,112], which is potentially less intrusive, and the use of genetically encoded Ca^{2+} indicators[65] (see main text) provide plausible alternatives for the study of astrocytic Ca^{2+} dynamics. Parts **a** and **b** are modified, with permission, from REF. 55 © (2011) Macmillan Publishers Ltd. All rights reserved. Part **d** is modified, with permission, from REF. 46 © (2011) Elsevier.



activity is complex and may be comprised of different types of events (see tentative classification of 'focal' and 'expanded' events in BOX 2)[55] and depend on the specific properties and organization of the underlying signalling components (FIG. 2B,C).

The fastest and most local events (focal events) that have been detected so far in hippocampal astrocyte processes[55] are events that are sensitive to pharmacological blockade or genetic deletion of IP3Rs, thus possibly representing 'Ca^{2+} puffs', the elementary IP3R-mediated transients that have been described in many cell types[17,66]. The pharmacological properties of focal events in astrocytes (BOX 2) suggest that these events are independent of neuronal firing but could originate from spontaneous neurotransmitter release at neighbouring synapses[55]. Their functional significance is unknown, but it could be speculated that they act in register with miniature synaptic events to stabilize 'tripartite' connections and/or coordinate plastic adaptations of these connections[55,67]. This hypothesis is supported by the recent observation that genetic suppression of astrocytic IP3-dependent Ca^{2+} signalling reduces astrocytic coverage of hippocampal synapses[68]. A second, distinct type of local Ca^{2+} event, possibly generated by Ca^{2+} influx via TRPA1 channels, was recently described in adult hippocampal astrocytes[28,65].

A different class of Ca^{2+} events in astrocytic processes (expanded events), which are sensitive to tetrodotoxin (TTX), are most probably generated by the firing of neighbouring axons. Compared with focal events, these transients are much larger (in amplitude, duration and spatial extent) and occupy substantial portions of a process (BOX 2). They arise in regions otherwise displaying asynchronous focal Ca^{2+} activity, are IP3R-dependent and, peculiarly, can display multiple initiation points and peaks[55]. This might mean that they result from the synchronization of several autonomous microdomains and represent a first level of local spatial–temporal integration, producing

a stronger and more temporally coordinated astrocytic Ca^{2+} response. Interestingly, expanded Ca^{2+} signals were shown to increase transmitter release probability at local hippocampal synapses, suggesting that they trigger gliotransmission[46,55]. Consistent with this, in the hypothalamus, Ca^{2+} transients that were evoked in astrocytic processes by stimulated neuronal firing induced an ATP-dependent increase in synaptic currents that was restricted to the neuron directly apposed to the active astrocytic process[69].

$[Ca^{2+}]_i$ changes observed in the astrocytic cell body are significantly slower and less frequent than any of the transients in the processes (FIG. 1d). They might, therefore, occur only above a certain threshold of cell activation and trigger biological processes that are not activated by the transients confined to the processes — for example, more global and/or persistent cell or network responses. For instance, slow, large and concerted somatic Ca^{2+} responses (but not faster responses in processes) were recently associated with prolonged blood-oxygen-level-dependent (BOLD) signals, suggesting that they contribute to persistent vascular responses[70].

Overall, these recent observations provide a new and more comprehensive (albeit far from exhaustive) view of astrocyte Ca^{2+} dynamics. More studies are needed to understand the functional specificities of the different types of Ca^{2+} phenomena that have been observed (FIG. 2). A higher level of experimental sophistication will also be needed to keep abreast of the emerging biological complexity. What is clear is that experimental manipulation of astrocytic $[Ca^{2+}]_i$ is not a straightforward practice and can produce different results depending on approach and context. Although this is almost a natural side effect of our deficient understanding of astrocytic encoding rules, several experimental issues need attention, and these are discussed below.

A deeper understanding of astrocytes

Physiological relevance of the experimental approach. Currently, the main limitation in selecting an experimental approach to evoke or suppress astrocytic $[Ca^{2+}]_i$ increases is the lack of a clear-cut, physiologically based rationale. Different approaches to increase astrocytic $[Ca^{2+}]_i$ (such as transgenic receptor expression, caged Ca^{2+} photolysis and pharmacological or optogenetic stimulation) do not have equivalent biological effects, and thus their results are not directly comparable. Moreover, and importantly, these artificial methods may not mimic physiological

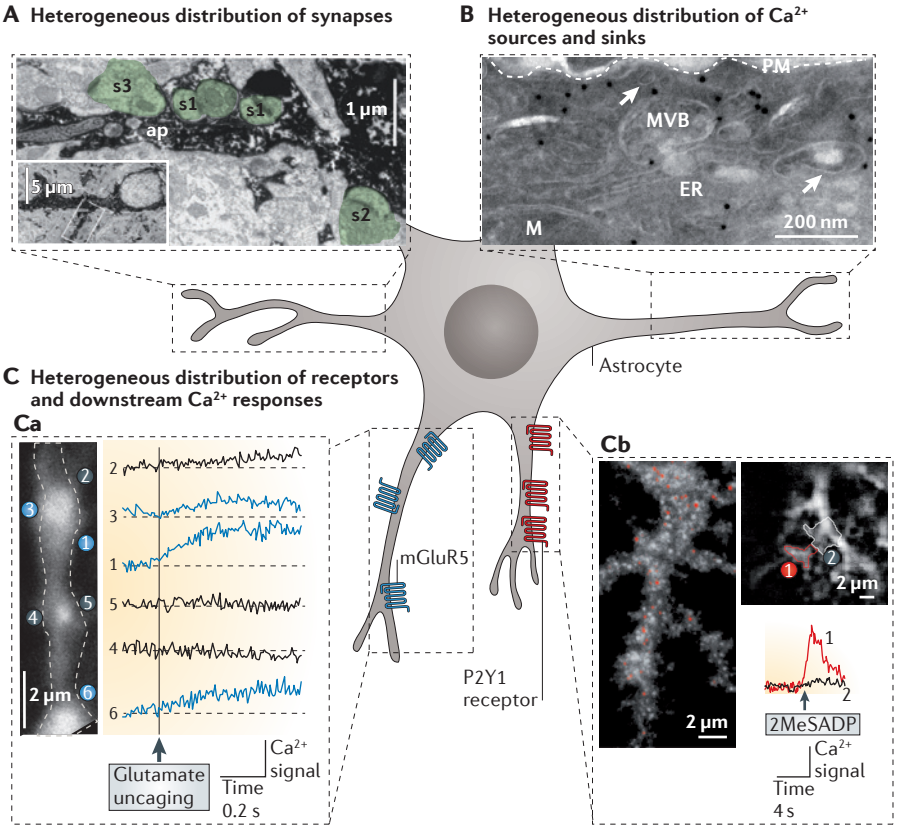


Figure 2 | **An emerging view: astrocytes display structural–functional heterogeneity that shapes the diversity of Ca^{2+} responses.** The figure presents experimental data exemplifying some types of heterogeneity, drawn arbitrarily along the processes of an astrocyte. **A** | An electron micrograph showing diversity in the ultrastructural relations between synapses (s, green) and an astrocytic process (ap, black). Some synapses are enclosed completely by the astrocytic process (s1), whereas others are contacted only on one side of the synaptic cleft (s2) or completely lack astrocytic contact (s3). The image represents a magnification of the secondary astrocytic process branching out of the primary process shown in the inset. The astrocyte was visualized by S100-immunoreaction in the cerebral cortex of an adult rat. **B** | An electron micrograph showing heterogeneous distribution of organelles, including Ca^{2+} sources and sinks, along an astrocytic process from the hippocampus of an adult mouse. Recognizable organelles include: an endoplasmic reticulum (ER), a multivesicular body (MVB), a mitochondrion (M) and tubular vesicular structures (arrows). The plasma membrane (PM) is delineated with a dashed white line. These astrocytic structures are present in tissue sections (70–90 nm) from a transgenic mouse expressing enhanced green fluorescent protein (GFP) from the glial fibrillary acidic protein promoter[113] prepared with the Tokuyasu method[114] and were identified by immunolabelling with GFP-specific antibodies and using 15 nm gold particles for detection (A.V. and D. Bouvier, unpublished observations). **Ca**,**b** | Localization and functional data showing heterogeneity in the expression of endogenous G protein-coupled receptors (GPCRs) and related Ca^{2+} responses along an astrocytic process. Two examples are presented concerning metabotropic glutamate receptor 5 (mGluR5) and purinergic P2Y1 receptor signalling. Part **Ca** shows that targeted two-photon uncaging of glutamate along an astrocytic process (highlighted with a dashed line) elicits a local Ca^{2+} response in some regions (blue bullets and traces) but not in other neighbouring regions (black bullets and traces) of the process (caged photo-releasable glutamate, 10 mM). Part **Cb** (left panel) shows a super-resolution image obtained using stimulated emission depletion (STED) in which P2Y1 receptors appear to be unevenly distributed along a multi-branched astrocytic process in the adult mouse hippocampus (A.V., N.L. and D. A. Sahlender, unpublished observations). The maximum z-projection image shows the three-dimensional contour of the process (grey scale) according to glutamine synthase labelling, and P2Y1 receptor labelling (red) within the process. In the right panel of part **Cb**, two adjacent regions of an astrocytic process show either a Ca^{2+} response (bullet and trace 1) or no Ca^{2+} response (bullet and trace 2) to a brief focal puff application of the P2Y1 receptor agonist 2MeSADP. Part **A** is modified, with permission, from REF. 115 © (2002) Oxford University Press. Image used in part **B** courtesy of D. Bouvier, C. Loussert and B. Humbel, University of Lausanne, Switzerland. Part **Ca** is modified, with permission, from REF. 46 © (2011) Elsevier. Image used in the left panel of part **Cb** courtesy of D. A. Sahlender, University of Lausanne, Switzerland. The right panel of part **Cb** is modified, with permission, from REF. 39 © (2011) Elsevier.

astrocytic responses, leading to results that must be interpreted cautiously. Indeed, the prolonged (minutes), ubiquitous and steady increase in $[Ca^{2+}]_i$ reported upon MRGA1 activation or robust pharmacological stimulation of other receptors[48,49,71,72] does not correspond with the Ca^{2+} signals seen in astrocytes under physiological conditions (BOX 2; FIGS 1,2), including the relatively long-lasting (tens of seconds) somatic $[Ca^{2+}]_i$ increases evoked by high-frequency neuronal stimulations that induce LTP (BOX 1). At first sight, it seems counterintuitive that a greater increase in $[Ca^{2+}]_i$ does not affect synaptic function[48,49], whereas smaller increases do[28,46,50,55]. However, in the case of IP3-dependent Ca^{2+} dynamics, the importance of frequency encoding is well known. This signalling system reaches maximal efficacy in triggering downstream biological responses (for example, enzyme activation or gene expression) at specific Ca^{2+} oscillation frequencies, whereas it is poorly effective when $[Ca^{2+}]_i$ increases are prolonged[73–75]. Further support for this notion has been obtained in cultured astrocytes, in which a long-lasting $[Ca^{2+}]_i$ increase that was induced by strong GPCR stimulation triggered just one solitary episode of glutamate release at the onset of the $[Ca^{2+}]_i$ change, whereas oscillatory short-lasting Ca^{2+} transients resulted in multiple glutamate-release episodes and in repeated and more substantial activation of neuronal receptors[29]. In keeping with this, induction of spike timing-dependent LTD in the cerebral cortex, which requires astrocyte glutamate release, is typically associated with an increased frequency of astrocytic Ca^{2+} oscillations[57]. In conclusion, defining the range of Ca^{2+} events generated in an astrocyte by genuine physiological activity and teasing those events apart from unnatural signals is likely to be crucial for correctly addressing the functional role of astrocyte–synapse crosstalk.

Selection of the experimental parameters for Ca^{2+} imaging and analysis. Results of astrocytic studies (and their interpretation) also depend on the methods used for measuring and analysing Ca^{2+} signals, including the regions of interest in which Ca^{2+} activity is monitored and the image acquisition parameters that are set. Although these should match the physiological question that is being addressed, in astrocytes they are often selected arbitrarily. Consequently, Ca^{2+} dynamics may happen to be sampled inadequately with respect to the biological phenomenon under study. Many studies, as mentioned earlier, have used the astrocytic soma as a region of interest that is representative of the entire astrocytic activity, because of its large size and the good visibility of Ca^{2+} signals. Accordingly, images are acquired under conditions calibrated on the properties of somatic Ca^{2+} signals — notably, at low spatial–temporal resolution[4,76,77]. This approach is still convenient for studying neuron–glia population dynamics[56,78], but as discussed above, it is unlikely to produce data that can be reliably used to infer aspects of communication between neurons and astrocytic processes (often referred to as tripartite communication)[79], such as the speed of astrocyte–synapse reciprocal signalling and the chemical mediators and receptors involved. In this case, communication should be studied directly in astrocytic processes and under conditions that maximize the signal-to-noise ratio and can capture the small and fast local signals (details in BOX 2).

Selection of Ca^{2+} indicators. The recently recognized diversity in amplitude, kinetics and cell location of Ca^{2+} events in astrocytes raises another relevant methodological issue: that is, that none of the available Ca^{2+} indicators can faithfully report the full spectrum of astrocytic activities. All indicators, by buffering free Ca^{2+}, interfere with the endogenous Ca^{2+} dynamics and act as filters that could misrepresent or even negate a given class of Ca^{2+} events. Each indicator produces a type of interference related to its specific properties (for example, affinity, concentration, dynamic range and mobility)[80–83]. Different indicators may therefore reveal different spectra of Ca^{2+} phenomena or report an individual phenomenon differently. Ultimately, selection of the indicator will depend on the class (or classes) of events and/or cell location (or locations) of interest in relation to the physiological question being studied, and will involve a trade-off or compromise of some sort. Large somatic Ca^{2+} events are well detected by most indicators. A suitable choice is Oregon Green BAPTA1 which, in its cell-permeant form, loads neurons and astrocytes equally well, enabling a relatively faithful study of population Ca^{2+} dynamics in neuron–glia networks *in vivo*[77]. Another good indicator for network studies is Rhod-2, which loads astrocytes selectively[70]. If the aim is to measure Ca^{2+} signals with different characteristics simultaneously — for example, in the cell body and processes — at present Fluo-4 probably offers the best compromise, given its superior dynamic fluorescence range and signal-to-noise ratio[84,85] (BOX 2; FIG. 1). Of increasing interest are the so-called genetically encoded Ca^{2+} indicators (GECIs)[84,86–88], a class of Ca^{2+}-sensing proteins. Indeed, more and more variants of these proteins with constantly improved performance are becoming available. Owing to transgenic or fast viral expression, GECIs can be selectively targeted to astrocytes and to astrocytic subcompartments (for example, the plasma membrane), providing (in principle) more accurate information about compartmental Ca^{2+} dynamics than the above mobile cytosolic indicators. Moreover, in contrast to synthetic dyes, GECIs are suitable for chronic imaging studies *in vivo*[89]. Despite these indubitable advantages, use of GECIs is not free of drawbacks and requires important controls. Long-term expression in cells may alter physiology (GECIs are Ca^{2+} buffers), which makes it hard to predict (and correct) the effects, because GECI levels cannot be fully controlled experimentally[90–92]. In this respect, transgenic GECI expression in astrocytic populations can be variable, as it depends on the type of astrocytic promoter being used and the brain area being investigated[93]. When GECIs are expressed via viral infection, injection of the viral vectors in the brain can be an additional problem, because astrocytes are highly sensitive to this invasive approach. Finally, how GECIs report astrocyte Ca^{2+} dynamics *in situ* remains an issue that needs to be addressed. Recently, a test using membrane-bound GCaMP3 (REF. 65) showed promise in revealing Ca^{2+} dynamics quite uniformly along the astrocytic arborization but did not perform as well as Fluo-4 in terms of sensitivity and dynamic range[84], suggesting that GCaMP3 may not adequately resolve fast and small focal events that can be detected using Fluo-4 (REF. 55) (BOX 2). Interestingly, newer GECI generations[94–96] already outperform GCaMP3. From the properties exhibited in neurons, the latest GCaMP6 variants[96] may provide a real alternative to Fluo-4 and other 'top' synthetic dyes.

Circuit and synaptic phenomena under study. It is not just the design, execution and analysis of studies focusing on astrocyte Ca^{2+} dynamics that require attention to 'details'; interpretation of their results is also crucial. A paradigmatic example of the importance of interpretation comes from several recent, apparently conflicting, studies of LTP induction at hippocampal CA1–CA3 synapses, the results of which are summarized in BOX 1. These studies have fuelled debate as to whether astrocyte Ca^{2+} signalling is relevant for hippocampal synaptic plasticity[97]. However, when the experimental settings of each of those studies are attentively considered, the results may not in fact be contradictory — just unexpected.

For example, the opposite observations that LTP is either unaffected[48] or abolished[51,58,60] in mice lacking IP3R2 selectively in astrocytes could be reconciled by the fact that the studies examined different forms of LTP that were generated by different circuit interactions and sustained by different signalling mechanisms (FIG. 3). Thus, in the study in the hippocampus[51] and in two analogous studies in the cerebral cortex[58,60] in which abolishing astrocyte IP3R2-dependent signalling affected LTP, the generation of LTP was found to require cholinergic fibre stimulation and astrocytic muscarinic receptor activation. By contrast, in the studies in which LTP was unaffected by disabling astrocyte IP3R2 signalling[28,48], the LTP was independent of cholinergic input but required Ca^{2+}-dependent D-serine (NMDA co-agonist) release from astrocytes[28,50]. Crucially — and unexpectedly — the release of D-serine from astrocytes seems to be triggered primarily by influx of external Ca^{2+}, not by the activation of the IP3-dependent pathway. If this is the case, it might explain why disabling IP3R2 signalling would not affect this type of LTP. Three lines of evidence support the idea that astrocytic D-serine release is dependent on extracellular Ca^{2+}: studies in cultured astrocytes testing the dependence of this amino acid's release on different Ca^{2+} sources[38]; recent work *in situ* showing that both D-serine release and LTP are attenuated by pharmacological or genetic interference with TRPA1 channels, which are a part of an external Ca^{2+} permeation pathway of astrocytes[28]; and data obtained from 'IP3 sponge' mice, a model that is different from IP3R2-null mice but similarly deficient in astrocyte IP3-dependent signalling[68]. In CA1 astrocytes of these mice, GPCR-evoked $[Ca^{2+}]_i$ increases are abolished, but not baseline Ca^{2+} activity, which might rely on a different Ca^{2+} source[27]. Moreover, if D-serine-dependent LTP relies mainly on Ca^{2+} influx rather than on ER-dependent Ca^{2+} release, this could explain why CA3–CA1 LTP was blocked in the studies that used Ca^{2+} chelators[50], which abolish all astrocytic $[Ca^{2+}]_i$ increases independently of the Ca^{2+} source, whereas it was not blocked in the studies that used IP3R2-null mice, which lack only the IP3-dependent pathway[48]. In conclusion, the different forms of LTP that are elicited and/or the different modes of interfering with astrocytic $[Ca^{2+}]_i$ seem to account for the discrepant results of the above studies. However, additional experimental differences between the studies — for example, in the species or age of the animals used (BOX 1) — could also play a part.

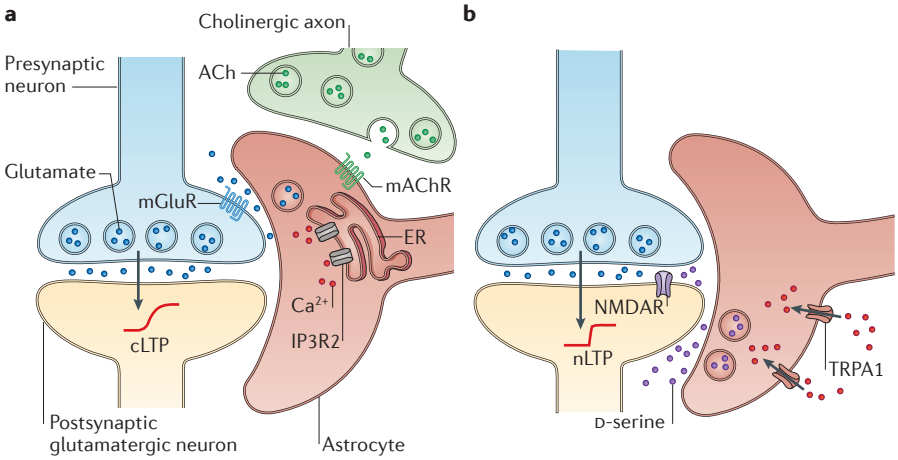


Figure 3 | **Different astrocytic Ca^{2+} signalling pathways may contribute to different forms of hippocampal LTP.** The figure presents a schematic summary of the current hypothesis concerning the astrocytic involvement in hippocampal long-term potentiation (LTP); this hypothesis integrates the results from REFS 28,48,50,51 (see main text and BOX 1 for details). **a** | Astrocytes would mediate cholinergic LTP (cLTP) at CA3–CA1 synapses as follows: acetylcholine (ACh) released from afferent fibres coming from the alveus activates astrocytic muscarinic ACh receptors (mAChR), and this activation is coupled to inositol-1,4,5-trisphosphate receptor 2 (IP3R2)-mediated Ca^{2+} release from the endoplasmic reticulum (ER) stores. This increase in Ca^{2+} levels would lead to the release of glutamate from the astrocyte, which then activates presynaptic metabotropic glutamate receptors (mGluRs), potentiating synaptic release of glutamate (see main text). **b** | By contrast, astrocytes would contribute to classical NMDA receptor (NMDAR)-dependent LTP (nLTP) at the same CA3–CA1 synapses through a different mechanism. Their activation during LTP induction would cause an influx of external Ca^{2+}, possibly through plasma membrane transient receptor potential A1 (TRPA1) channels (whose exact activation mechanism is unknown). This increase in Ca^{2+} (which apparently is not dependent on IP3R2) would result in D-serine release from the astrocyte, which would act as a co-agonist to potentiate activation of postsynaptic NMDARs.

Age of the animal from which the preparation is taken. Most work on astrocyte Ca^{2+} dynamics to date has been carried out in acute slices of the developing brain (2–3 postnatal weeks) to facilitate dye loading, but these results cannot be directly translated to the functioning of the adult brain: properties and roles of astrocytic Ca^{2+} activity may change with maturation of the synaptic circuitry[55,79]. Indeed, the endogenous activity observed in astrocytes from the immature brain seems to arise independently of synaptic activity (that is, activity that is insensitive to TTX and the synaptic release blocker bafilomycin A1 (REFS 23,24)), but the activity in slices from 5–6-week-old animals is largely blocked by these agents[55] (BOX 2). Moreover, when the astrocyte GPCR metabotropic glutamate receptor 5 (GluR5) is pharmacologically stimulated, somatic $[Ca^{2+}]_i$ increases are observed in astrocytes from the immature but not mature brain[79,98]. Indeed, after the third postnatal week, astrocytic mGluR5 expression declines[79] and the receptor may segregate to domains in cell processes[99,100]. These observations suggest caution in comparing data from preparations of different ages.

Conclusions and perspectives

Decoding astrocytic Ca^{2+} signalling is one of the keys to understanding the roles of these cells in brain function. The anatomical complexity of astrocytes does not facilitate the task, but recent high-resolution studies in astrocytic processes[46,55,65] have successfully enabled new levels of analysis. Further substantial progress may come from adaptation of super-resolution techniques such as stimulated emission depletion (STED) to Ca^{2+} dynamics studies[101], from their combination with fluorescence lifetime imaging microscopy (FLIM), a technique that can report free $[Ca^{2+}]_i$ levels with high sensitivity[102], and from the availability of further-improved GECIs[84,103,104]. These developments may enable Ca^{2+} analysis at the level of the fine astrocytic arborization (<500 nm) and their highly complex interactions with synapses, which are currently beyond conventional optical resolution. This will help to decisively advance our understanding of tripartite astrocyte–synapse interactions[105]. Improved techniques are likely to be complemented by advances in rapid scanning of brain tissue, not just in individual planes but in volumes[106], which promises to offer an integrated view of astrocytic Ca^{2+} phenomena.

These ambitious methodological advances will help to answer the many pending questions about Ca^{2+} signalling in astrocytes. For example, astrocytes are known to release several gliotransmitters in a Ca^{2+}-dependent manner, but whether a single astrocyte releases more than one gliotransmitter, and, if so, what determines the release of transmitter A or B is unknown. The link between the input of information to an astrocyte to its output responses is likely to be encoded in the properties of Ca^{2+} signals, and the identity and spatial location of input–output signalling components (including GPCRs, IP3Rs, Ca^{2+} permeation pathways, transmitter release determinants, and so on; see FIG. 2) probably define the type of astrocytic response. However, the underlying mechanisms are not yet known; the structure–function relations and molecular machinery that make each synapse–astrocyte interaction specific remain unidentified. Astrocytes can participate in the modulation of synaptic transmission and plasticity, but how this occurs in time and space is unclear. Moreover, via Ca^{2+} dynamics, astrocytes most probably perform genuine processing, but how this is accomplished is, again, mysterious. Astrocytic processing could be amazingly elaborate given that, in principle, any astrocyte can receive inputs from many thousands of synapses belonging to different circuits and neurons[107,108] and treat them on different spatial–temporal scales. Indeed, an astrocyte can potentially bridge the disconnected neuronal circuits that are present in its territory, and do so on very local or more integrated spatial scales, in relatively fast or much slower timescales, thereby offering numerous new possibilities of signal integration. Moreover, during processing, astrocytes could not just integrate neuronal information into Ca^{2+} signals but also multiplex it with other signals reporting the cell's or environment's state[8,109] to eventually produce uniquely informative integrated output responses.

Addressing all of the above issues is a formidable challenge. However, in the years to come, methodological advances, refined experimental strategies and a progressively improved comprehension of the basic properties of astrocytes promise to guide scientists towards deciphering the language of these fascinating cells and their roles in brain function.

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Competing interests statement
The authors declare no competing interests.