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Journal: Nature reviews. Neuroscience

Year: 2014 May

Volume: 15

Issue: 5

Pages: 327-35

DOI: 10.1038/nrn3725

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

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Preface

Astrocyte calcium signalling has been proposed to link neuronal information in different spatial-temporal dimensions to achieve a higher level of brain integration. Some discrepancies in the results of recent studies challenge this view, however, and highlight key insufficiencies in our current understanding. In parallel, new experimental approaches are rising that allow study of astrocyte physiology at higher spatial-temporal resolution in intact brain preparations, and begin 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 90s, 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 with elevations in intracellular calcium ($[Ca^{2+}]_i$)[1-3]. Research in the following years established that similar astrocytic $[Ca^{2+}]_i$ elevations occur in living animals in response to sensory stimuli[4-6], during locomotor activity[7] and autonomic function[8], and further elucidated details of the nature of these calcium 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 release of numerous neurotransmitters and factors (for reviews see[9-12]). According to current views, neurons transfer information to astrocytes mainly via spillover of synaptic transmitters and factors which bind to high-affinity astrocytic G protein-coupled receptors (GPCR)[13-16] linked to inositol 1,4,5-trisphosphate (IP_3) 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 astrocytic $[Ca^{2+}]_i$ elevations via stimulation of Ca^{2+}-permeant ionotropic receptors, but only in some regions (e.g., cerebral cortex for NMDA receptors[19]), and via specialized mechanisms ("ectopic" release onto glial AMPA receptors in cerebellum[20]). Astrocytic $[Ca^{2+}]_i$ elevations can also be induced via reversal of Na^+/Ca^{2+} exchangers following neurotransmitter uptake or other pumping activities[21,22] leading to astrocytic Na^+ elevation. Astrocytes also show transient $[Ca^{2+}]_i$ rises independent of synaptic activity, particularly during development[23-26], and Ca^{2+} fluctuations due to TRPA1 channel activity that contribute to resting Ca^{2+} levels[27,28].

A specific consequence of $[Ca^{2+}]_i$ elevations 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 (e.g., glutamate, D-serine, ATP, the latter often rapidly converted to adenosine), and their synaptic actions result in several, often opposite, types of effects, including stimulation/inhibition of synaptic transmission, participation to long-term potentiation/depression (LTP/LTD), to heterosynaptic facilitation/depression and to homeostatic plasticity (reviewed in[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: specificities in the properties of different receptors triggering $[Ca^{2+}]_i$ elevation in astrocytes[36], in the Ca^{2+}-dependent mechanisms governing 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 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 (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 thanks to methodological advances start to reveal a previously unanticipated level of diversification in astrocyte Ca^{2+} dynamics (**Fig. 1**). In this Perspective, 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 model of astrocyte calcium signalling

A series of studies addressing the role of astrocyte Ca^{2+} signalling in synaptic transmission and plasticity at CA3-CA1 hippocampal synapses led to discrepant results. These apparently contradictory responses are difficult to explain but given the emerging subtleties of astrocytic calcium signalling, the core of the problem could reside in the use of different experimental paradigms, including in interfering with astrocytic calcium (details in **Box 1**) Notably, a genetic mouse model created *ad hoc* to study the effect of $[Ca^{2+}]_i$ elevations selectively in astrocytes[48,49] provided fully unexpected results. In this model, a GPCR linked to IP_3 production (but 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 (RF amides). Robust MrgA1 activation led to $[Ca^{2+}]_i$ increases lasting minutes and occurring ubiquitously in astrocytes. Surprisingly, such persistent $[Ca^{2+}]_i$ elevations neither detectably modified synaptic function[49] nor contributed to LTP induction[48]. Furthermore, similar $[Ca^{2+}]_i$ elevations induced via stimulation of endogenous GPCRs were also ineffective[48,49], and even the opposite approach (testing synaptic responses in IP_3R2 null

mice lacking Ca^{2+} responses to astrocyte GPCR stimulation[52]), failed to produce any effect on synaptic transmission and plasticity (LTP)[48,52]. These data would seem to suggest that astrocyte Ca^{2+} signalling lacks a crucialrole in these phenomena. However, other studies reached the opposite conclusion by using different strategies to interfere with astrocyte calcium. Some infused Ca^{2+} chelators in astrocytes preventing any endogenous $[Ca^{2+}]_i$ rise, others targeted an IP_3 receptor-*in*dependent 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 IP_3R2 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 (mechanism), where (in the complex astrocytic arbor or in the cell soma) and when calcium 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 mainlyin 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 Ca^{2+} measures in domains (20-50 μm^2), but determining whether similar microdomains existed in in astrocytic processes (cross-section: ≤1-2 μm) proved much more difficult[24]. Only recently technical obstacles were surmounted (see **Box 2**) leading to the first description of local astrocytic Ca^{2+} dynamics[46,55] (**Figs. 1 and 2**). This methodological progress revealed that astrocyte-synapse communications operate on previously unrecognized spatial-temporal scales and constituted an important advancein the conceptual understanding of astrocytic Ca^{2+} signalling. For example, early work that monitored mainly somatic $[Ca^{2+}]_i$ elevations, concluded that astrocytes respond only to intense neuronal firing patterns[2], but the work in astrocytic processes revealed that they respond also (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 type

of astrocytic Ca^{2+} response[3,62-64]. Moreover, it introduces the issue as to how astrocytes integrate, via $[Ca^{2+}]_i$ changes, neuronal activities of different intensities, occurring at different times, different astrocytic locations, and via different mediators[64,65].

Different calcium signals in astrocyte processes and cell body

Direct monitoring of Ca^{2+} dynamics in the processes of adult mouse hippocampal astrocytes[46,55] has revealed an intense and previously unappreciated local activity dissociated from activity in the cell body (see also[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+} activity is complex and may be comprised of different types of event (see tentative classification in "focal" and "expanded" events in **Box2 A**)[55] and depend on the specific properties and organization of the underlying signalling components (**Fig. 2B and C**).

The fastest and most local events ("focal events") detected so far in hippocampal astrocyte processes[55] are events sensitive to pharmacological blockade or genetic deletion of IP_3 receptors, thus possibly representing "Ca^{2+} puffs", the elementary IP_3 receptor-mediated transients described in many cell types[17,66]. Their pharmacological properties in astrocytes (**Box2 A)**, 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 their plastic adaptations[55,67]. This hypothesis is supported by the recent observation that genetic suppression of astrocytic IP_3-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"), sensitive to tetrodotoxin (TTX), are most likely generated by the firing of neighboring axons. Compared to "focal events", these transients are much larger (in amplitude, duration and spatial extent), and occupy substantial portions of a process (**Box2 A** and **B**). They arise in regions otherwise displaying asynchronous focal Ca^{2+} activity, are IP_3 receptor-dependent and, peculiarly, can display multiple initiation points and peaks[55]. This might mean that they result from 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 evoked in astrocytic processes by stimulated neuronal firing induced ATP-dependent increase in synaptic currents 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 not activated by the transients confined to the processes, e.g. more global/persistent cell or network responses. For instance, slow, large, and concerted somatic Ca^{2+} responses (but not faster responses in processes) were recently associated to prolonged 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 in order to understand the functional specificities of the different types of Ca^{2+} phenomena 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 astrocyte physiology

Physiological relevance of the experimental approach

Currently, the main limitation in selecting an experimental approach to evoke or suppress astrocytic $[Ca^{2+}]_i$ elevations is lack of a clear-cut, physiologically-based rationale. Different approaches to elevate astrocytic $[Ca^{2+}]_i$ (transgenic receptor expression, caged Ca^{2+} photolysis, 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 astrocytic responses, leading to results that must be interpreted

cautiously. Indeed, the prolonged (minutes), ubiquitous and steady $[Ca^{2+}]_i$ elevation reported upon MrgA1 activation or robust pharmacological stimulation of other receptors[48,49,71,72] has does not correspond with Ca^{2+} signals seen in astrocytes under physiological conditions (**Figs. 1, 2 and Box 2**), including the relatively long-lasting (tens of seconds) somatic $[Ca^{2+}]_i$ elevations evoked by high-frequency neuronal stimulations inducing LTP (**Box 1**). At first sight it seems counterintuitive that a greater $[Ca^{2+}]_i$ elevation does not affect synaptic function[48,49], whereas smaller ones do[28,46,50,55]. However, in the case of IP_3-dependent Ca^{2+} dynamics, the importance of frequency encoding is well known. This signaling system reaches maximal efficacy in triggering downstream biological responses (e.g., enzyme activation or gene expression) at specific Ca^{2+} oscillation frequencies, whereas it is poorly effective when $[Ca^{2+}]_i$ elevations are prolonged[73-75]. Further support for this notion has been obtained in cultured astrocytes, where a long-lasting $[Ca^{2+}]_i$ increase 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 it apart from unnatural signals is likely to be crucial to 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) depend also on the methods used for measuring and analyzing Ca^{2+} signals, including the regions of interest (ROIs) in which Ca^{2+} activity is monitored and the image acquisition parameters that are set. Although these should match the physiological question, 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 ROI 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 speed of astrocyte-synapse reciprocal signaling, chemical mediators and receptors involved. In this case, communication should be studied directly in astrocytic processes and using under conditions that maximize the signal-to-noise ratio and can capture the small and fast local signals (details in **Box 2**).

Selection of calcium indicators

The recently recognized diversity in amplitude, kinetics, and cell location of Ca^{2+} events in astrocytes raises another relevant methodological issue, i.e., that none of the available Ca^{2+} indicators can faithfully report the full spectrum of astrocytic activities. All indicators, by buffering free calcium, 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 (affinity, concentration, dynamic range, mobility etc.)[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(es) of events and/or cell location(s) of interest in relation to the physiological question 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 (OGB1) which, in its cell-permeant form, loads neurons and astrocytes equally well, allowing a relatively faithful study of population Ca^{2+} dynamics in neuron-glia networks *in vivo*[77]. A good companion for network studies is Rhod-2 which loads astrocytes selectively[70]. If the aim is to measure simultaneously Ca^{2+} signals with different characteristics, e.g. in cell body and processes, today Fluo-4 probably offers the best compromise, given its superior dynamic fluorescence range and signal-to-noise ratio[84,85] (**Fig. 1** and **Box 2**). Of increasing interest are the so-called genetically-encoded Ca^{2+} indicators (GECI)[84,86-88], a class of Ca^{2+}-sensing proteins with constantly improved performance. Thanks to transgenic or fast viral expression, GECI can be selectively targeted to astrocytes and to astrocytic sub-compartments (e.g., plasma membrane), providing in principle more accurate information on the compartmental Ca^{2+} dynamics than the above mobile cytosolic indicators. Moreover, different from synthetic dyes, GECI are suitable for chronic imaging studies *in vivo*[89]. In spite of these indubitable advantages, use of GECI is not free of drawbacks and requires important controls. Long-term expression in cells may alter physiology (GECI are Ca^{2+} buffers!), with effects hard to predict (and correct) because GECI levels cannot be fully controlled experimentally[90-92]. In this respect, transgenic GECI expression in astrocytic populations can be variable, also depending on the type of astrocytic promoter used and the brain area investigated[93]. When GECI 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, it remains the issue as to how GECI report astrocyte Ca^{2+} dynamics *in situ*. Recently, test of membrane-bound GCaMP3[65] showed promise in revealing Ca^{2+} dynamics quite uniformly along the astrocytic arborization, but did not perform as well as Fluo4 in terms of sensitivity and dynamic range[84], suggesting that GCaMP3 may not adequately resolve fast and small "focal" events detected using Fluo4[55] (**Box2 A**). Interestingly, newer GECI generations[94-96] already outperform GCaMP3. From the properties exhibited in neurons, the latest GCaMP6 variants[96] may represent a real alternative to Fluo4 and other "top" synthetic dyes.

Circuit and synaptic phenomenon under study

It is not just the design, execution and analysis of studies focusing on astrocyte Ca^{2+} dynamics that requires attention to "details", but also interpretation of their results. 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 or not 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 instance, the opposite observations that LTP is either unaffected[48] or abolished[51,58,60] in mice lacking IP_3R2 selectively in astrocytes could be reconciled by the fact that the studies looked at different forms of LTP generated by different circuit interactions and sustained by different signalling mechanisms (**Figure 3**). Thus, in the study where abolishing astrocyte IP_3R2-dependent signalling affected LTP, the generation of LTP was found to require cholinergic fibre stimulation and astrocytic muscarinic receptor activation[51,58,60]. By contrast, in the studies where LTP was unaffected by disabling astrocyte IP_3R2 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 activation of the IP_3-dependent pathway, and if this is the case it might explain why disabling IP_3R2 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 the amino acid 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, an external Ca^{2+} permeation pathway of astrocytes[28]; data obtained in

"IP_3 sponge" mice, a model different from IP_3R2-null mice but similarly deficient in astrocyte IP_3-dependent signalling[68]. In CA1 astrocytes of these mice, GPCR-evoked $[Ca^{2+}]_i$ elevations 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 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 $[Ca^{2+}]_i$ elevations independent of the Ca^{2+} source whereas it was not blocked in the studies that used IP_3R2 mice, which lack only the IP_3-dependent pathway[48]. In conclusion, different forms of LTP elicited and/or 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, e.g. in the species or age of the animals used (**Box 1**), could also play a part.

Age of the animal from which the preparation is taken

Most work on astrocyte Ca^{2+} dynamics to date has been performed in acute slices of developing brain (2-3 post-natal 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 immature brain seems to arise independently of synaptic activity (insensitive to TTX and the synaptic release blocker bafilomycin A1[23,24]), but the one in slices from 5-6 weeks-old animals is largely blocked by these agents[55] (**Box2 A**). Moreover, when the astrocyte GPCR, mGluR5, is pharmacologically stimulated, somatic $[Ca^{2+}]_i$ elevations are observed in astrocytes from the immature but not mature brain[79,98]. Indeed, after the third post-natal 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 understand 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 opened 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 availability of further improved GECI[84,103,104]. These developments may enable Ca^{2+} analysis at the level of the fine astrocytic arborisation (<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]. This is likely to be complemented by advances in rapid scanning of brain tissue, not just in individual planes but in volumes[106] and 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 instance, 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 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 (GPCR, IP_3R, Ca^{2+} ion permeation pathways, transmitter release determinants etc., see **Fig. 2**) probably defines the type of astrocytic response. However, the underlying mechanisms are not known yet; the structure-function relations and molecular machinery that make each synapse-astrocyte interaction specific remain unidentified. Astrocytes can participate in modulation of synaptic transmission and plasticity, but how this occurs in time and space, is unclear. Moreover, via Ca^{2+} dynamics, astrocytes most likely 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 synapses belonging to different circuits and neurons[107,108] and treat them on different spatial-temporal scales. Indeed, an astrocyte can potentially bridge disconnected neuronal circuits present in its territory, and do so on very local or more integrated spatial scales, in relatively fast or much slower time scales, thereby offering a myriad of 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 the above issues represents 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.

Acknowledgments

Research in Andrea Volterra's lab is supported by the ERC Advanced grant 340368 "Astromnesis" and by Swiss National Science Foundation grants 31003A 140999, NCCR "Synapsy" and NCCR "Transcure". The authors are grateful to Céline Dürst for the experimental data presented in **Fig. 1**, to Daniela Sahlender for the STED image in **Fig. 2**; and to David Bouvier, Céline Loussert and Bruno Humbel for the EM image of an astrocytic process in **Fig. 2**.

Figure legends

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 s period revealed by the calcium indicator Fluo4-AM. The visible boundaries of the astrocyte are shown in white. Note the different intensities of spatially-confined local activity in the astrocyte cell body (s), primary process (p1) stemming from the soma and secondary processes (p2) branching from a primary process. Intensity of the normalized cumulative activity is expressed in arbitrary units (a.u.) and shown in pseudocolour, from dark (lowest) to white (highest). **(B)** Frequency map of the Ca^{2+} activity in the astrocyte during the 165 s period as in A. Activity is measured in individual pixels, expressed in mHz and color-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 of the soma 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)** Spatio-temporal map showing that the Ca2+ 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 color and the temporal sequence of the events is color-coded, from dark blue, which outlines events occurring in the first seconds, to dark red (around 40 seconds). **(D)** Cartoon summary of the diversity of astrocytic Ca^{2+} signaling 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 as compared to those occurring in the processes (red). Two-photon imaging at 2 Hz performed in the outer molecular layer of the dentate gyrus in an acute hippocampal slice of a P35 mouse at a depth of >50 µm. Scale bar for A, B and C: 5 µm.

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)** Electron micrograph showing diversity in the ultrastractural relations between synapses (green) and an astrocytic process (black). Some synapses are enclosed completely by the astrocytic process (s1), while others are contacted only on one side of the synaptic cleft (s2) or lack completely astrocytic contact (s3). (al) astrocyte lamellae; (af) astrocyte filopodia; (ap), secondary astrocytic process

branching out of the primary process as framed in the inset; cross-section: 0.5-1 µm. Astrocyte visualized by S100-immunoreaction in the cerebral cortex of adult rat. Scale bar: 1 µm. Adapted with permission from[110].**(B)** Electron micrograph showing heterogeneous distribution of organelles, including Ca^{2+} sources, along an astrocytic process from the hippocampus of an adult mouse. Recognizable organelles include: endoplasmic reticulum (ER), multivesicular body (MVB), mitochondrium (M), and tubular vesicular structures (arrows). The plasma membrane (PM) is delineated with a dotted line. Scale bars: 200 nm. Astrocytic structures in tissue sections (70-90 nm) from a GFAP-eGFP mouse[111] prepared with the Tokuyasu method[112] were identified by immunolabeling with anti-GFP and using 15 nm gold particles for detection. **(C)** Localization and functional data showing heterogeneity in the expression of endogenous GPCRs and related Ca^{2+} responses along an astrocytic process. Two examples are presented concerning mGluR5 (left) and P2Y1R signaling (right). Left: 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, Ca^{2+} signal bar: 200% $\Delta F/F$) but not in other neighboring regions (black bullets and traces) of the process (caged MNI-glutamate, 10 mM). Scale bar: 2 µm. Adapted with permission from[46]. Right: super-resolution image obtained using stimulated emission depletion (STED) showing uneven distribution of P2Y1 receptors along a multi-branched astrocytic process in the adult mouse hippocampus. The maximum z-projection image shows the 3D contour of the process (grayscale) according to glutamine synthase labeling and P2Y1 receptor labeling (red) within the process. Scale bar: 2 µm. On the side: 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. Scale bar: 2 µm. Ca^{2+} signal bar: 20% $\Delta G/R$. Adapted with permission from[39].

Figure 3. Different astrocytic Ca^{2+} signaling pathways may contribute to different forms of hippocampal LTP. The figure presents a schematic summary of the current hypothesis concerning the astrocytic involvement which integrates the results in the mouse hippocampus obtained in Refs.28, 48, 50, 51 (see text and Box1 for details). Astrocytes would mediate cholinergic LTP (cLTP) at CA3-CA1 synapses (neuron 1) as follows: acetylcholine (Ach) released from afferent fibers coming from the alveus activates astrocytic muscarinic ACh receptors (mAChR) coupled to IP_3R2 receptors and Ca^{2+} release from the ER stores. This Ca^{2+} elevation leads to release of glutamate from the astrocyte that activates pre-synaptic mGluRs, potentiating synaptic release (see text). In contrast, astrocytes would contribute to classical NMDAR-dependent LTP (nLTP) at the same CA3-CA1 synapses (neuron 2) via a different

mechanism. Their activation during LTP induction would cause influx of external Ca^{2+}, possibly through plasma membrane TRPA channels. This Ca^{2+} elevation (apparently not dependent on IP_3R2) results in D-serine release from the astrocyte that acts as co-agonist to potentiate activation of post-synaptic NMDAR.

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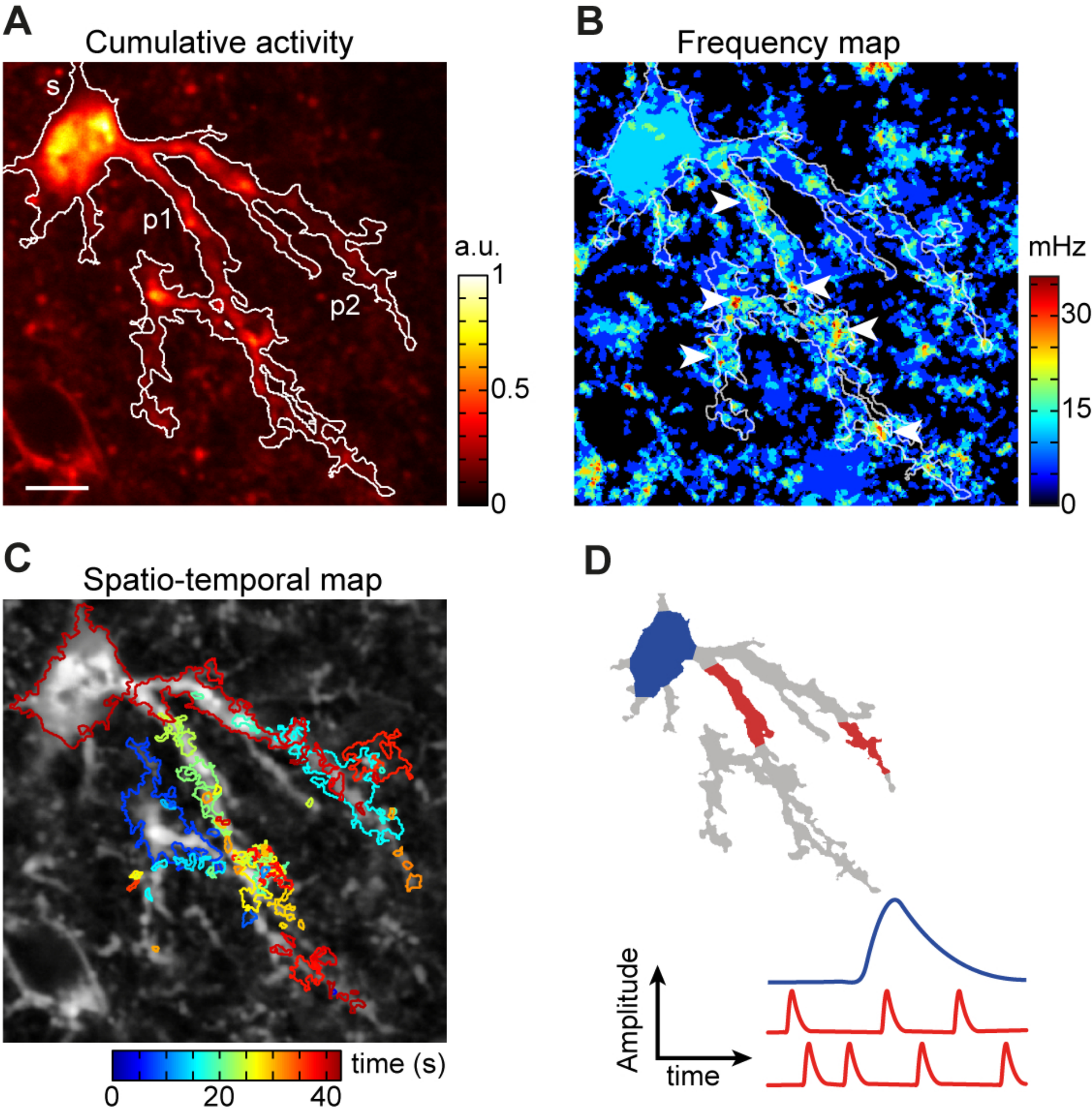
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Cumulative activity
s
p1
p2
a.u.
1
0.5
0
B
Frequency map
mHz
30
15
0
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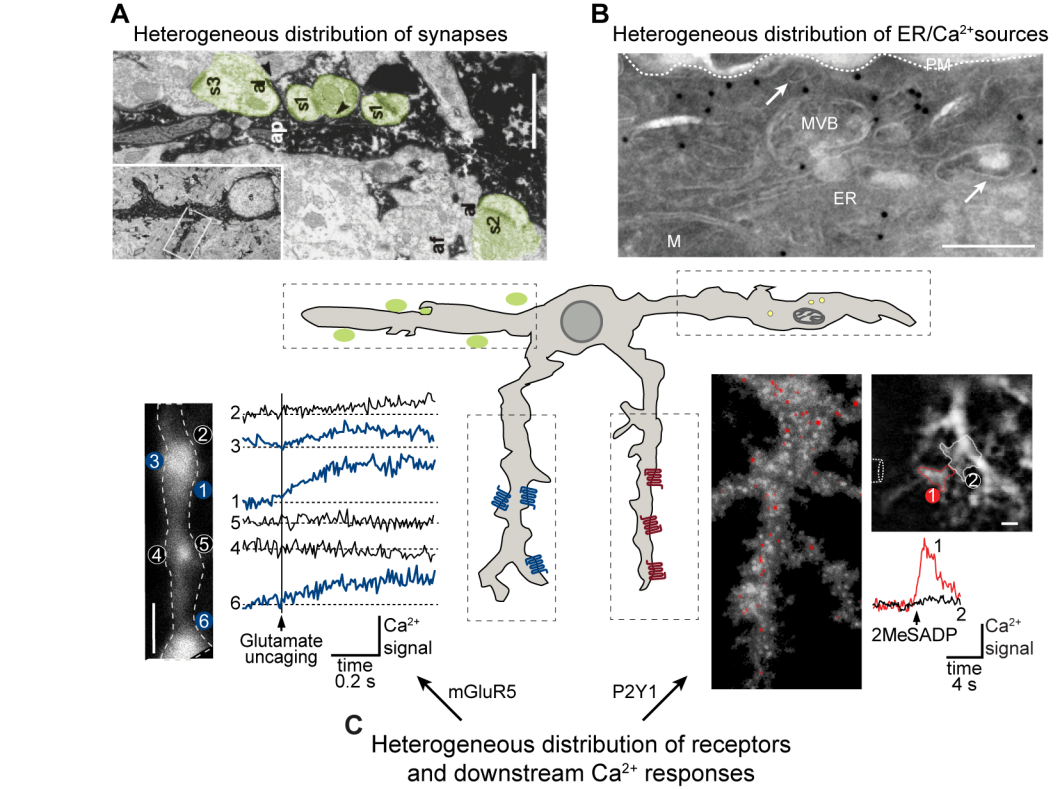
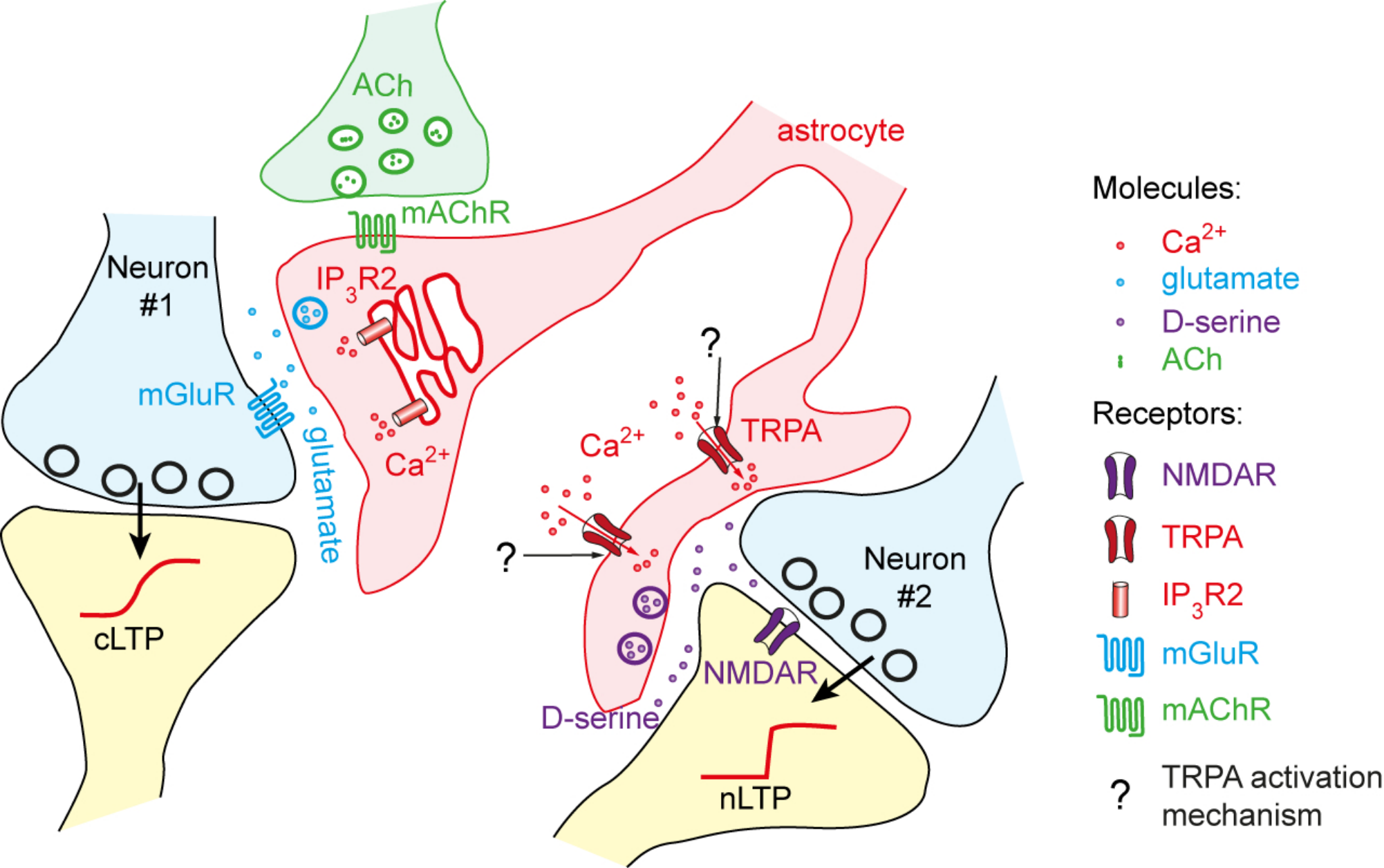
time (s)
0
20
40
D
Amplitude
time


A
Heterogeneous distribution of synapses
s3
ad
s1
ap
s1
ad
s2
ad
B
Heterogeneous distribution of ER/Ca2+sources
PM
MVB
ER
M
2
3
1
5
4
6
Glutamate uncaging
Ca2+ signal
time 0.2 s
mGluR5
P2Y1
1
2
2MeSADP
Ca2+ signal
time 4 s
C
Heterogeneous distribution of receptors and downstream Ca2+ responses


ACh
astrocyte
mAChR
Neuron
#1
IP3R2
mGluR
glutamate
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cLTP
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TRPA
Ca2+
?
Neuron
#2
NMDAR
D-serine
nLTP
Molecules:
Ca2+
glutamate
D-serine
ACh
Receptors:
NMDAR
TRPA
IP3R2
mGluR
mAChR
?
TRPA activation
mechanism

BOX 1 - Role of astrocytic Ca^{2+} signalling in hippocampal LTP: different approaches give different outcomes

	A (Ref.50)	B (Ref.48)	C (Ref. 28)	D (Ref.51)
Key experimental observations:				
Astrocytic Ca^{2+} involved in LTP?	✓ Yes	× No	✓ Yes	✓ Yes
Identified astrocytic Ca^{2+} source:	Not defined	—	External, via TRPA1	ER, via IP_3R_2 (mAChR)
Downstream gliotransmitter:	D-serine	—	D-serine	Glutamate
Experimental settings:				
Age and species	Adult 4-8 week old rats	Young P16-17 mice	Adult 8-18 week old mice	Young P13-18 mice, P12-17 rats
Method of LTP induction:	SC tetanic stimulation, 100 Hz, 1 s x 3	SC tetanic stimulation, 100 Hz, 1 s x 2	SC tetanic stimulation, 100 Hz, 1 s x 2	Alveus theta-burst stimulation (ACh fibers)
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:				
Tested approaches:				
Astrocytic Ca^{2+} chelation (all sources):	LTP disrupted	*not tested*	*not tested*	LTP disrupted
IP_3R_2 KO mice:	*not tested*	LTP not affected	LTP not affected	LTP disrupted
TRPA1 KO mice:	*not tested*	*not tested*	LTP disrupted	*not tested*
Effect of artificially induced Ca^{2+} elevation in the astrocytes:				
Tested approach:				
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

Abbreviations: TRPA1 = Transient receptor potential cation channel, member A1; IP_3R_2 = IP_3 receptor type 2; mAChR = muscarinic acetylcholine receptor; SC = CA3 Schaffer collaterals; GPCR = G protein-coupled receptor; UV uncaging = UV light Ca^{2+} uncaging.

All four LTP studies were performed in the CA3-CA1 hippocampal circuit, but reached different conclusions regarding involvement of astrocytic Ca^{2+} signaling. This could be explained by several important experimental differences among studies including:

1. Use of preparations of different age (adult for **A**,**C;** young for **B, D**): coupling between neuronal activity and astrocytic Ca^{2+} signaling is likely different in the mature and immature circuitry (see text).
2. Use of different LTP induction protocols (**A,B,C** vs. **D**), resulting in the recruitment of different forms of LTP which differently rely on astrocytic mechanisms: high-frequency stimulation of SC (**A,B,C**) induces NMDAR-dependent post-synaptic LTP possibly requiring release of astrocyte D-serine as co-agonist (nLTP); theta-burst stimulation of alveus cholinergic afferents (**D**) induces cholinergic LTP (cLTP), possibly presynaptic [51], requiring astrocytic mACh activation and glutamate release (see text).
3. Use of different strategies for blocking astrocyte Ca^{2+} signaling (**A** vs. **B** vs. **C**): astrocytic Ca^{2+} chelation (**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+} (**B, C**).
4. Use of different protocols to test the effect of artificial astrocytic $[Ca^{2+}]_i$ elevation on LTP (**B** vs. **D**): in **B**, astrocytic $[Ca^{2+}]_i$ was elevated via transgenic GPCR stimulation prior to neuronal stimulation and remained steadily elevated during neuronal stimulation: this protocol did not enhance LTP. However, native astrocytic Ca^{2+} signals produced during LTP induction were not blocked in parallel, with a possible occlusive effect. Moreover, the evoked $[Ca^{2+}]_i$ elevations had much longer duration (~5 min) than the native signals (~30 s, see text). In **D**, astrocytic $[Ca^{2+}]_i$ was elevated via Ca^{2+} uncaging coincidentally with the start of sub-threshold neuronal stimulation and after blocking native GPCR-dependent Ca^{2+} signaling: this protocol induced LTP. The uncaging method produced pulsatile, not steady, $[Ca^{2+}]_i$ elevations in astrocytes[47].

BOX2 - 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 suitable for studies in dendritic spines[113] are not easily adaptable to 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 (e.g., where GPCRs are located) are not yet clear (see **Fig 2**). Nevertheless, by adopting different strategies, two studies[46,55] succeeded in recording fast Ca^{2+} dynamics in astrocytic processes. Box **Fig2-A** 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 3D map of the astrocyte morphology (grey). Ca^{2+} dynamics were recorded in a process lying favorably in the focal volume (dark red, magnified below) with the two-photon "line-scan" approach, which samples activity at ultra-fast speed along a single line rather than in a volume. The scan line (white arrow, bottom) was positioned along the process length, allowing imaging of all the Ca^{2+} activity (black traces) evoked by the surrounding synaptic activity. The 3D morphological map was then used to distinguish truly local Ca signals, confined to process regions fully located in the imaged volume (dark red), from those occurring in branching regions (green). This approach led to identify two types of local Ca^{2+} signals, dubbed "focal" and "expanded" (**Fig 2-B**). "Focal" events (a1) are spatially-restricted (~4 µm), small and fast events, whereas "expanded" ones (a2) are spatially-enlarged (~13 µm), bigger and longer-lasting events. "Focal" and "expanded" events are pharmacologically distinguishable: only "expanded" ones are sensitive to tetrodotoxin (TTX, blocker of neuronal firing that abolishes evoked synaptic release). Adapted with permission from[55]. **Fig2-C** depicts the alternative approach used in Ref [46]. Here researchers patch-clamped a neuron (azure, electrode e1) and neighbouring astrocyte (dark red, electrode e2) while using a third electrode (e3) to stimulate nearby axons. They minimally stimulated axons and in parallel scanned a small region of the proximal astrocytic process, looking for temporally-correlated Ca^{2+} events (lower image). They often observed (**Fig2-D)** a single-synapse excitatory post-synaptic 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 **Fig2-B** and, likewise, is TTX-sensitive. Adapted with permission from[46]. The dual indicator approach[113] and selection of the appropriate dyes[46,55] contributed to the success of both studies. Whole-cell patch-clamp dye infusion in an astrocyte, allowed resolution of even very tiny Ca^{2+} events as far as 35 µm from the soma during several min periods. In parallel, it allowed constant control of the cell's membrane electrical properties and loading of pharmacological agents. A disadvantage of this method is, however, the possible dialysis of intracellular components. The advent of dye loading via cell electroporation[114,115], potentially less intrusive, or use of GECI[65] (see text), provide plausible alternatives.