Neuroscience

A spatial threshold for astrocyte calcium surge

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Abstract

Astrocytes are active cells involved in brain function through the bidirectional communication with neurons, in which the astrocyte calcium signal plays a crucial role. Synaptically-evoked calcium increases can be localized to independent subcellular domains or expand to the entire cell, i.e., calcium surge. In turn, astrocytes may regulate individual synapses by calcium-dependent release of gliotransmitters. Because a single astrocyte may contact ~100,000 synapses, the control of the intracellular calcium signal propagation may have relevant consequences on brain function by regulating the spatial range of astrocyte neuromodulation of synapses. Yet, the properties governing the spatial dynamics of the astrocyte calcium signal remains poorly defined. Imaging subcellular responses of cortical astrocytes to sensory stimulation in mice, we show that sensory-evoked astrocyte calcium responses originated and remained localized in domains of the astrocytic arborization, but eventually propagated to the entire cell if a spatial threshold of >23% of the arborization being activated was surpassed. Using transgenic $IP_3R2^{-/-}$ mice, we found that type-2 IP_3 receptors were necessary for the generation of the astrocyte calcium surge. We finally show using in situ electrophysiological recordings that the spatial threshold of the astrocyte calcium signal consequently determined the gliotransmitter release. Present results reveal a fundamental property of astrocyte calcium physiology, i.e., a spatial threshold for the astrocyte intracellular calcium signal propagation, which depends on astrocyte intrinsic properties and governs the astrocyte integration of local synaptic activity and the subsequent neuromodulation.

One-Sentence Summary

There is a spatial threshold for the astrocyte intracellular calcium signal propagation that is determined by astrocyte intrinsic properties and controls gliotransmission.

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Building on their own prior work, the authors present **valuable** findings that add to our understanding of cortical astrocytes, which respond to synaptic activity with calcium release in subcellular domains that can proceed to larger calcium waves. The proposed concept of a spatial "threshold" is based on **solid** evidence from in vivo and ex vivo imaging data and the use of mutant mice. However, details of the specific threshold should be taken with caution and appear **incomplete** unless supported by additional experiments with higher resolution in space and time.

Introduction

Accumulating evidence indicates that astrocytes play active roles in synaptic function and neural information processing by exchanging signals with neurons. They respond to synaptic activity with intracellular calcium elevations, which stimulate the release of gliotransmitters that regulate neuronal and synaptic function (Araque et al., 2014; Perea et al., 2009). Hence, the astrocyte calcium signal is a crucial signaling event in the bidirectional communication between neurons and astrocytes. Moreover, astrocyte calcium manipulations have been shown to regulate neuronal network function (Lee et al., 2014; Lines et al., 2020; Mederos et al., 2021; Perea et al., 2016; Poskanzer and Yuste, 2016) and animal behavior (Adamsky et al., 2018; Corkrum et al., 2020; Kofuji and Araque, 2021; Martin-Fernandez et al., 2017; Monai et al., 2016; Nagai et al., 2021; Oliveira et al., 2015), and disruption of astrocyte calcium has been proposed to contribute to brain diseases (Delekate et al., 2014; Jiang et al., 2016; Kuchibhotla et al., 2009; Lines et al., 2021; Tian et al., 2005; Yu et al., 2018).

Astrocyte calcium variations represent a complex signal that exists over a wide range of spatial and temporal scales (Bazargani and Attwell, 2016; Rusakov, 2015; Semyanov et al., 2020; Shigetomi et al., 2016; Volterra et al., 2014). Spatially, the astrocyte calcium signal may occur at discrete subcellular regions, termed domains, in the astrocyte arborization, or may encompass large portions of the cell or even the entire astrocyte (Bazargani and Attwell, 2016; Rusakov, 2015; Semyanov et al., 2020; Shigetomi et al., 2016; Volterra et al., 2014). Subcellular astrocyte calcium events have been recently proposed to be involved in the representation of spatiotemporal maps (Curreli et al., 2022; Doron et al., 2022; Serra et al., 2022) and to contribute to long-term information storage (Curreli et al., 2022; Doron et al., 2022; Georgiou et al., 2022; Vignoli et al., 2021). Calcium activity in astrocytes is believed to originate in domains within astrocytic processes that contact and bi-directly communicate with nearby synapses termed microdomains (Arizono et al., 2020; Bindocci et al., 2017; Di Castro et al., 2011; Grosche et al., 1999; Khakh and Sofroniew, 2015; Panatier et al., 2011). While calcium transients in discrete domains have been found to be independent events within astrocyte arborizations (Chen et al., 2020; Stobart et al., 2018; Ung et al., 2021), they can also occur in concert (Agarwal et al., 2017; Georgiou et al., 2022; Otsu et al., 2015; Shigetomi et al., 2013) and eventually expand to a larger cellular region, including the astrocytic soma, a phenomenon termed astrocyte calcium surge (Hirase et al., 2004).

Moreover, the regulation of the amplitude and spatial extension of the astrocyte calcium signal by the coincident activity of different synaptic inputs has been proposed to endow astrocytes with integrative properties for synaptic information processing (Durkee and Araque, 2019; Perea and Araque, 2005) Because a single astrocyte may contact ~100,000 synapses (Bushong et al., 2002), the integrative properties of a single astrocyte and the control of the intracellular calcium signal propagation may have relevant consequences by regulating the spatial range of astrocyte

influence on synaptic terminals (Fellin et al., 2004; Gordleeva et al., 2019). While there have been recent works describing molecular underpinnings of microdomain calcium transients (Diaz et al., 2019; Ma and Freeman, 2020; Montagna et al., 2019), the underlying processes governing the connection between the two subcellular activity states —independent or concerted events— and the spatial extension of the intracellular calcium signal remain unknown.

To address these issues, we have monitored sensory-evoked astrocyte calcium activity in the mouse primary somatosensory cortex in vivo, combining astrocyte structural imaging data and subcellular imaging analysis. Here, we use an unbiased and semi-automatic algorithm to perform high-throughput analysis across a large number of astrocytes (~1000) to discover a subcellular property. We have found that astrocyte calcium responses originate in the surrounding arborizations and propagate to the soma if over 23% of the surrounding arborization is activated. If the astrocyte calcium spatial threshold is overcome, this spurs a surge of calcium into the surrounding arborization. Using transgenic *IP$_3$R2$^{-/-}$* mice, we found that the activation of type-2 IP$_3$ receptors is necessary for the generation of astrocyte calcium surge. Patch-clamp recordings of neurons near activated astrocytes showed an increase in slow-inward currents (SICs), detailing an output of astrocyte calcium surge. Using a combination of structural and functional two-photon imaging of astrocyte activity, we define a fundamental property of astrocyte calcium physiology, i.e., a spatial threshold for astrocyte calcium propagation.

Results

Imaging astrocyte structure and function simultaneously in vivo

We simultaneously monitored calcium activity in identified SR101-labelled astrocytes in the primary somatosensory cortex using two-photon microscopy in vivo. We used transgenic mice expressing GCaMP6f under the *GFAP* promoter (Bindocci et al., 2017; Lines et al., 2020) to monitor sensory-evoked intracellular astrocyte calcium dynamics in combination with sulforhodamine 101 (SR101)-labeling to monitor astrocyte morphology (Nimmerjahn et al., 2004) (**Figure 1A,B**). Regions of interest (ROIs) were computationally determined from SR101-positive structural imaging (Bindocci et al., 2017) by outlining individual astrocytes and performing semi-automatic segmentation into somas and arborizations (**Figure 1C**; see **Figure S1** for an in depth description of segmentation). Next, subcellular quantification of soma and arborization calcium signals from individual astrocytes was evaluated in response to peripheral electrical stimulation of the hindpaw (2 mA at 2 Hz for 20 s; **Figure 1D**). Following the segmentation of an individual astrocyte into soma and arborization, the astrocyte arborization was further discretized into a grid of maximally-sized 4.3 μm x 4.3 μm square regions of interest, which we define as astrocyte domains (**Figure 1E,F**) (Agarwal et al., 2017; Di Castro et al., 2011; Grosche et al., 1999; Shigetomi et al., 2013). Thus, we were able to quantify the sensory-evoked calcium responses in individual domains, as well as in the arborization and soma (**Figure 1G**).

The analysis of the sensory-evoked calcium activity from astrocyte arborization and soma uncovered that 1) the majority of responses occurred in both the soma and arborization (57.7 ± 4.5%; n = 30 populations, 3 animals); 2) some responses occurred only in the arborization (15.1 ± 1.6%; n = 30 populations, 3 animals; **Figure 2A-D**); 3) a small minority of responses included activity in the soma but not the arborization (3 ± 0.5%; n = 30 populations, 3 animals; **Figure 2D**); and 4) some astrocytes did not respond (24.1 ± 3.6%; n = 30 populations, 3 animals). Since the majority of cells showed responses in both the soma and arborization, we hypothesized that the proportion of domain activity within the arborization and the somatic calcium activity were correlated across a population of astrocytes. To test this hypothesis, we first examined the average percentage of responding arborizations versus the percentage of soma activation within a population and found a significant linear correlation between these subcellular measures of

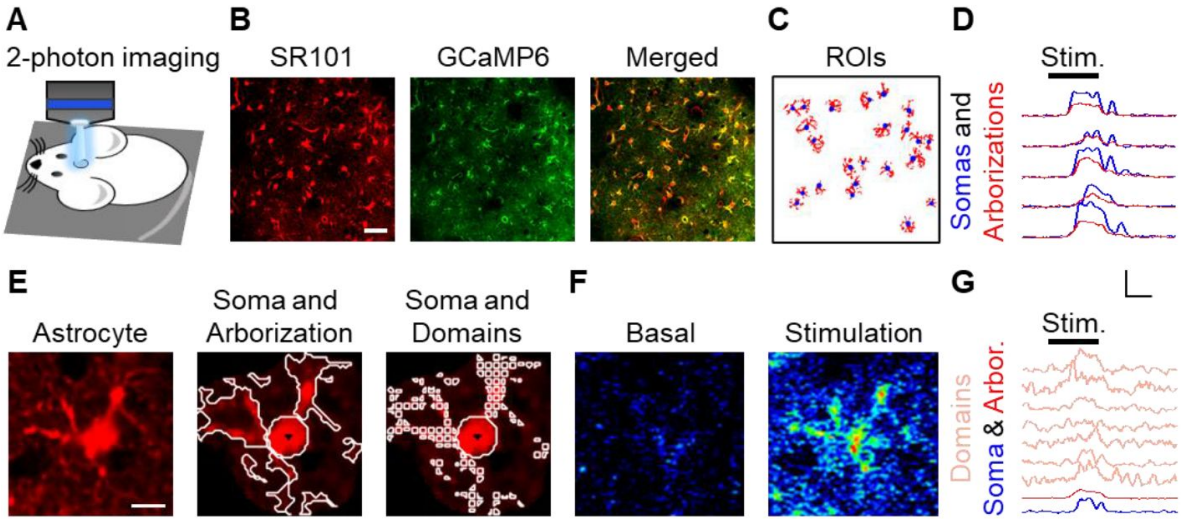

Figure 1.

Imaging astrocyte structure and function simultaneously in vivo.

(A), Scheme of in vivo preparation to image astrocyte Ca^{2+} and structure. (B), SR101-stained astrocyte structure, GCaMP6 to monitor astrocyte Ca^{2+} signal, and merge. Scale bar = 50 μm. (C), Regions of interest (ROIs) from SR101-stained structure of somas (blue) and arborizations (red). (D), Ca^{2+} traces from B from somas (blue) and arborizations (red). Scale = F/F_o, 10 s. (E), SR101-stained astrocyte (left), ROIs outlining soma and arborization (center) and ROIs defining the soma and domains (right). Scale bar = 10 μm. (F), Pseudocolor Ca^{2+} image during basal (left) and hindpaw electrical stimulation (right). (G), Ca^{2+} traces from f from domains (salmon), arborization (red) and soma (blue). Scale = F/F_o, 10 s.

activity (linear correlation: $p < 0.001$, $R^2 = 0.90$; n = 30 populations, 3 animals; **Figure 2E**). Additionally, averaging the percentage of active domains per cell over a population versus the percentage of somas active showed a significant linear correlation (linear correlation: $p < 0.001$, $R^2 = 0.87$; n = 30 populations, 3 animals; **Figure 2F**). These results indicate that, on average, subcellular calcium events located in astrocyte arborizations are related to soma activation.

Subcellular astrocyte calcium originates in the arborization

We then analyzed the spatial and temporal properties of the intracellular calcium dynamics in astrocytic somas and arborizations (**Figure 3A-D**). First, we determined the kinetics of the sensory-evoked astrocyte calcium signal. Sensory-evoked calcium rises in arborizations occurred with a delay of 11.1 ± 0.3 s from the onset of the peripheral stimulation and significantly preceded those occurring in the soma with a 13.2 ± 0.2 s delay from stimulus onset ($p < 0.001$; n = 30 populations, 3 animals; **Figure 3D-F**). Moreover, rise time to peak and decay time back to baseline of the calcium traces were faster in somas than arborizations (10-90% rise time: 5.7 ± 0.2 s in arborizations vs. 3.5 ± 0.2 s in somas; $p < 0.001$; 90-10% decay time: 4.8 ± 0.2 s in arborizations vs. 4.3 ± 0.2 s in somas; $p < 0.01$; n = 30 populations, 3 animals; **Figure 3E,F**). These results indicate that astrocyte responses occurred initially in the arborizations, which is consistent with the idea that synapses are likely to be accessed at the astrocyte arborization (Arizono et al., 2020; Papouin et al., 2017).

A spatial threshold to activate the soma and calcium surge

Next, we determined the relative spatial relationship of calcium activity of domains within the arborizations and somas of individual astrocytes (**Figure 4A**). We quantified the proportion of subcellular domains in individual astrocytic arborizations that responded to electrical stimuli with varied parameters and assessed whether the corresponding soma responded (**Figure 4A-C**). When changing the stimulus parameters (duration, frequency, and intensity), the number of responding domains increased as the stimulus duration, frequency, and intensity increased (ANOVA: duration: $p < 0.001$, frequency: $p < 0.001$, intensity: $p < 0.001$; n = 11 populations, 4 animals; **Figure 4D**). As described above, the probability of soma activation vs. the percentage of active domains could be accurately fit to a linear regression (see **Figure 2F**) indicating a correlation between these variables. To further characterize this relationship, we plotted paired values of on/off active soma (i.e., activated or not) vs. the proportion of active domains from individual astrocytes. We found that the activation of a relatively low proportion of domains occurred without activation of the soma (**Figure 4E**). Conversely, large proportions of activated domains were accompanied by a calcium elevation in the soma (**Figure 4E**). Fitting these values to the Heaviside step function (Davies, 2002) indicated that somas were active when at least 22.6% of their respective domains were active ($R^2 = 0.42$; n = 995 astrocytes from 30 populations and 3 animals; **Figure 4E**). This spatial threshold value was independent of the sensory input because similar values were found across various stimulus parameters (1-way ANOVA: duration: $p = 0.50$, frequency: $p = 0.29$, intensity: $p = 0.38$; n = 11 populations, 4 animals; **Figure 4F**), suggesting that it is determined by intrinsic astrocyte properties. Consolidating spatial threshold measurements from various stimulation parameters we quantified the spatial threshold to be within 95% confidence intervals of [21.2%, 24.0%]. Moreover, plotting the percent of active domains for an individual astrocyte versus the amplitude of the somatic calcium response was fit to a sigmoid curve ($R^2 = 0.58$; n = 995 astrocytes from 30 populations and 3 animals; **Figure 4G**). These fits to cellular data as well as the large cluster of unchanged somatic amplitude with subthreshold domain activity further confirms that nonresponsive somas were not just below event detection, but indeed the soma does not become active. Taken together, these results indicate the existence of a spatial threshold for soma activation determined by astrocyte intrinsic properties and the proportion of active domains.

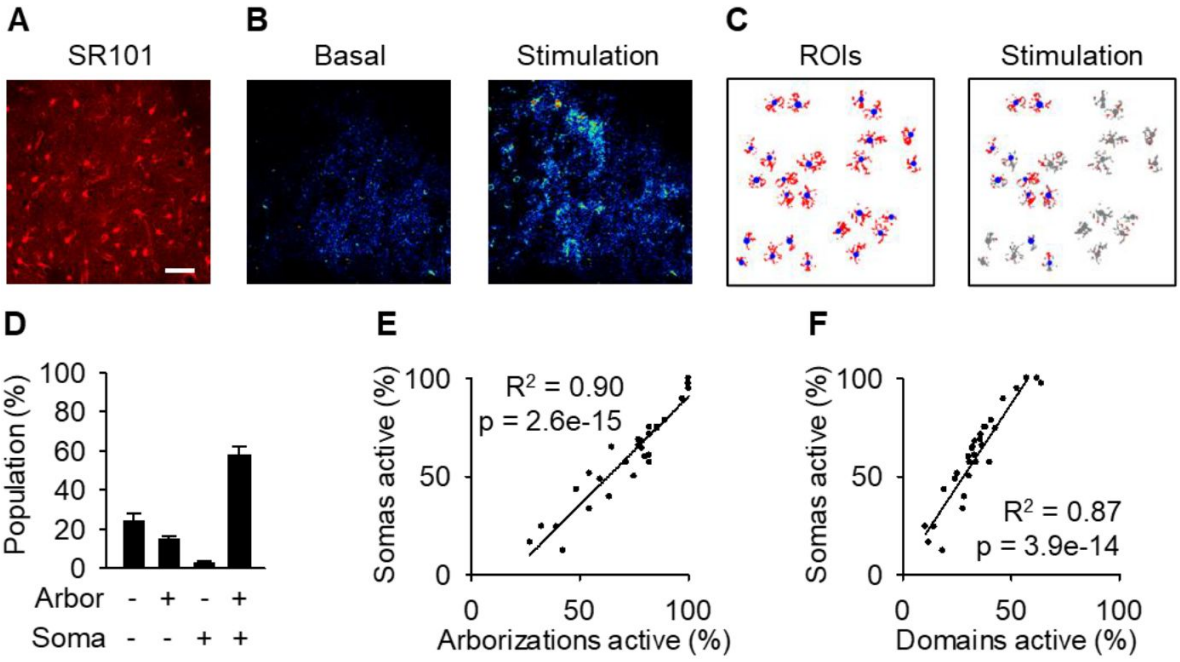

Figure 2.

Population arborization calcium is correlated to population soma activity.

(A), SR101 staining. Scale bar = 50 μm. (B), pseudocolor Ca^{2}+ images at basal and stimulation. (C), ROIs of soma and arborizations/domains along with activity during stimulation. (D), Proportion of subcellular responses to stimulation. (E), Percentage of active arborizations vs. percent of somas active. (F), Percentage of domains active vs. percent of somas active. Mean ± SEM. Pearson correlation.

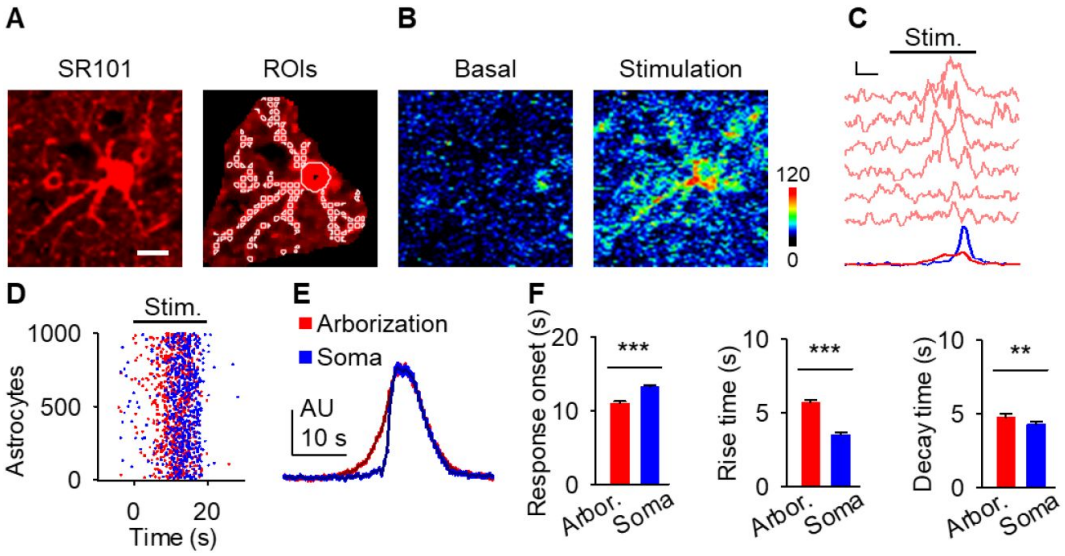

Figure 3.

Astrocyte calcium responses originate in the arborization before the soma.

(A), Astrocyte with regions of interest (ROIs). Scale bar = 10 μm. (B), Pseudocolor Ca^{2+} image. (C), Ca^{2+} traces in B from domains (pink), arborization (red), and the soma (blue). Scale = F/F_o, 5 s. (D), Raster plot of astrocyte somas (blue) and arbors (red) in response to stimulation (gray). (E), Average calcium traces from somas (blue) and arborizations (red) aligned to their respective soma onset. (F), Soma and arbor latency to response (left), event rise time (center) and event decay time (right). Mean ± SEM. '**' ≡ $p < 0.01$ and '***' ≡ $p < 0.001$ using paired student t-test.

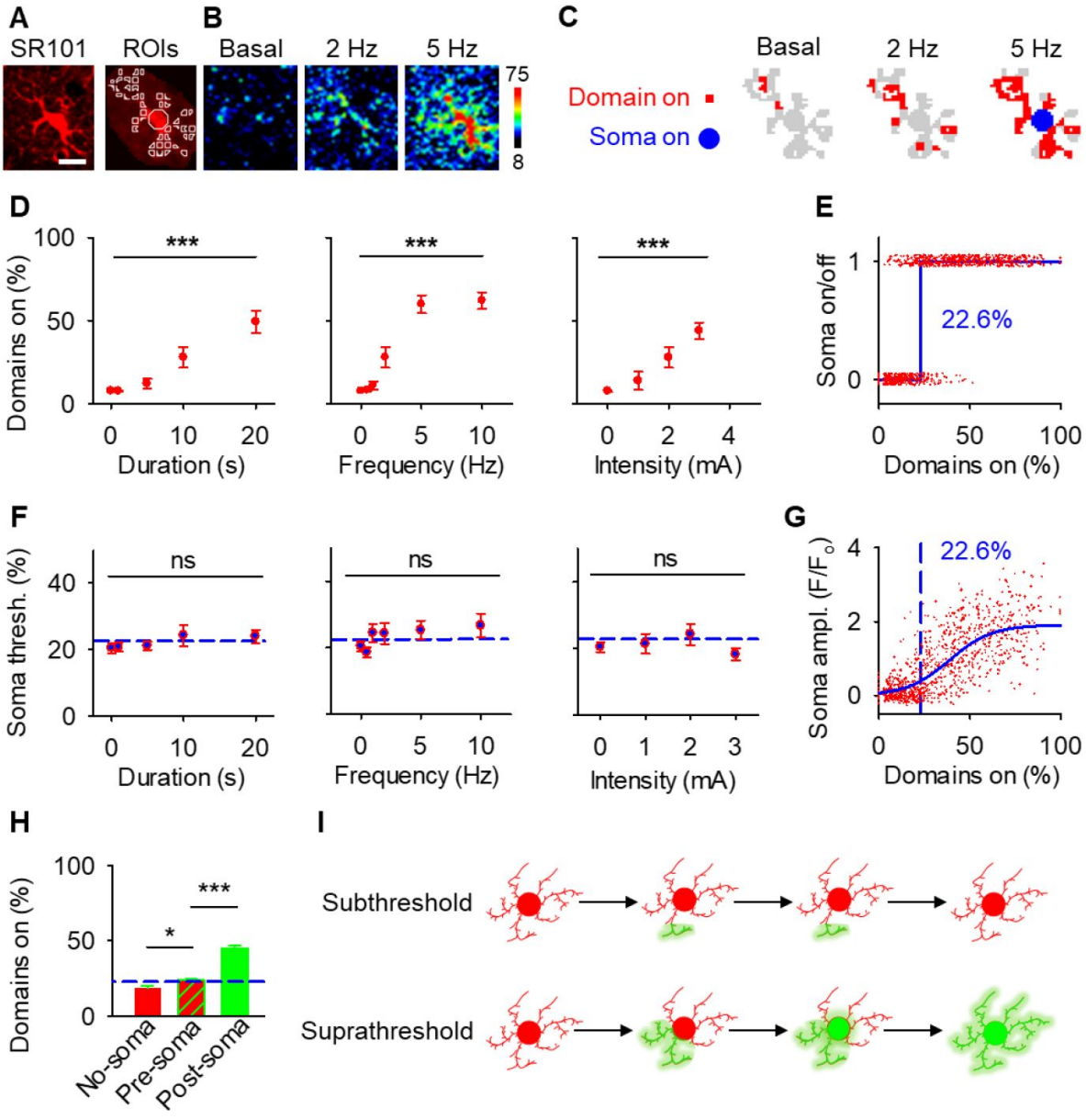

Figure 4.

A spatial threshold for astrocyte calcium in the soma to reach astrocyte calcium surge.

(A), Astrocyte and ROIs. Scale bar = 10 μm. (B), Pseudocolor Ca^{2+} images during basal and different frequency of stimulations. (C), Scheme of domains (red) and soma (blue) Ca^{2+} activity from B. (D), Percentage of active domains vs. stimulus duration, intensity and frequency. (E), Active state of soma for individual astrocytes vs. percentage of active domains (red). Data were fit to a Heaviside step function (blue dotted line). (F), Percentage of active domains necessary to elicit soma activation vs. stimulus duration, intensity and frequency. Blue dotted lines denote 22.6% spatial threshold. (G), Soma fluorescence versus percentage of active domains (red). Data were fit to a sigmoidal function (blue) and a blue dotted line denotes 22.6% spatial threshold. (H), Percentage of active domains in the absence of soma activation versus active domains before and after soma activation. Blue line denotes 22.6% spatial threshold. (I), Schematic showing subthreshold and suprathreshold astrocyte calcium activity. Mean ± SEM. '***' ≡ $p < 0.001$ and 'ns' ≡ $p > 0.05$ using 1-way ANOVA or student t-test.

The presence of a spatial threshold for somatic responses suggests that cells that respond with soma activity would have a higher response of domains prior to the soma response (Pre-Soma) when compared to astrocytes without a soma response (No-Soma). Indeed, when comparing these populations we found that astrocytes with responding somas had a significantly larger proportion of surrounding domains active prior to soma activation (Pre-Soma) when compared to cells without a somatic response (No-Soma), confirming our hypothesis of a spatial cellular threshold (18.5 ± 1.7% of active domains in No-Soma versus 23.9 ± 0.8% of domains in Pre-soma cells, $p < 0.05$; n = 607 active vs n = 388 not active, 30 populations and 3 animals; **Figure 4H**). Further, we found that astrocytes with an excess of 22.6% of domain activity, that induced a somatic response, led to increased domain activation throughout the remaining arborization (Post-Soma), i.e. domain activity before soma activation (Pre-Soma) versus domain activity after soma activation (Post-Soma), (23.9 ± 0.8% in Pre-Soma firing compared to 45.0 ± 1.8% in Post-Soma activation, $p < 0.001$; n = 607 astrocytes, 30 populations in 3 animals; **Figure. 4H**, **Figure S2**). Together, these results confirm that astrocytes that respond with domain activity in excess of the spatial threshold precipitates somatic activity and a calcium surge of expanded responses throughout the astrocytic arborization (**Figure 4I**).

The type-2 IP_3 receptor is necessary for astrocyte calcium surge

Previous reports have demonstrated in transgenic mice with type-2 IP_3 receptors knocked out ($IP_3R2^{-/-}$ mice) have ablated somatic calcium activity, but preserved certain domain calcium events (Agarwal et al., 2017; Lines et al., 2020; Schmidt and Oheim, 2020; Srinivasan et al., 2015). Since IP_3R2-mediated calcium mobilization is an important signaling pathway in astrocyte calcium dynamics (Guerra-Gomes et al., 2017; Lim et al., 2021), we hypothesized that IP_3R2 activity was necessary for astrocyte calcium surge. To test this, we injected an adeno-associated virus to express GCaMP6f within astrocytes under the astroglial *GfaABD1d* promoter (AAV-*GfaABC1d*-GCaMP6f) into the primary somatosensory cortex of $IP_3R2^{-/-}$ mice. We then quantified the calcium activity within GCaMP6f-expressing SR101-labeled cortical astrocytes before and after sensory stimulation (2 mA, 2 Hz for 20 sec; **Figure 5A-C**). In agreement with previous results (Agarwal et al., 2017; Lines et al., 2020; Srinivasan et al., 2015; Stobart et al., 2018), astrocytes in $IP_3R2^{-/-}$ mice responded to stimulation within the domains, but not the arborizations (i.e., average signal over the entire astrocyte arborization) or the somas (in domains: 6.0 ± 0.5% in basal vs 9.0 ± 0.6% in stimulation, $p < 0.001$; n = 2450 domains; in arborizations: 1.8 ± 1.3% in basal vs 5.4 ± 2.1% in stimulation, $p = 0.15$; n = 112 arborizations; in somas: 3.6 ± 1.8% in basal vs 4.5 ± 2.0% in stimulation, $p = 0.74$; n = 112 somas, 5 populations in 2 animals; **Figure 5D**). Moreover, within individual astrocytes, the percentage of activated domains in $IP_3R2^{-/-}$ mice in response to stimulation was reduced compared to wildtype mice (34.5 ± 0.8% in wildtype mice vs 14.5 ± 1.0% in $IP_3R2^{-/-}$ mice, $p < 0.001$; n = 995 astrocytes in 30 populations in 3 wildtype mice vs n = 112 astrocytes in 5 populations in 2 $IP_3R2^{-/-}$ mice; **Figure 5E**). Notably, while domain activity in $IP_3R2^{-/-}$ mice increased upon stimulation, the level of activation remained below the defined spatial threshold of 22.6% (**Figure 5E**; dashed blue line), which astrocytes in $IP_3R2^{-/-}$ mice were unable to overcome. Further confirming this, the probability of astrocyte somatic responses to stimulation was dramatically reduced in $IP_3R2^{-/-}$ mice compared to wildtype mice (61.0 ± 1.6% in wildtype mice vs 4.5 ± 2.2% in $IP_3R2^{-/-}$ mice, $p < 0.001$; n = 995 somas in 30 populations in 3 wildtype mice vs n = 112 somas in 5 populations in 2 $IP_3R2^{-/-}$ mice; **Figure 5E**). Taken together, these results indicate that IP_3R2-mediated calcium internal release is necessary for astrocyte calcium surge and further support the idea of the spatial threshold for astrocyte calcium spread.

Astrocyte calcium surge is associated with gliotransmission

We finally investigated whether the spatial threshold for astrocyte calcium impacted gliotransmission. We performed patch-clamp recordings of layer 2/3 cortical neurons in cortical brain slices to monitor the NMDAR-mediated slow inward currents (SICs), a biological assay of glutamate gliotransmission (Araque et al., 2000; Gomez-Gonzalo et al., 2018) and applied

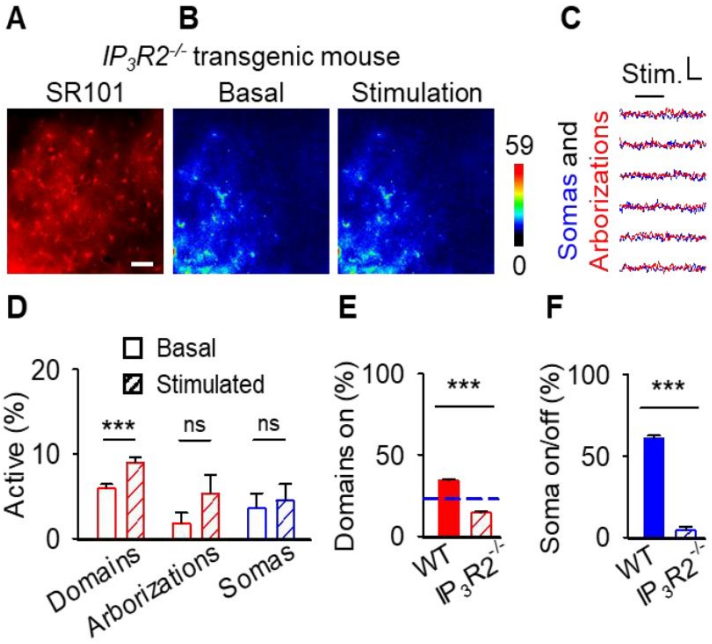

Figure 5.

The spatial activation of domain Ca^{2+} remains below the spatial threshold in mice lacking the IP_3 receptor Type-2.

(A), SR101 staining. Scale bar = 50 µm. (B), Pseudocolor Ca^{2+} images at basal and stimulation. (C), Traces from astrocytes in B. Scale = F/F_o, 10 s. (D), Percentage of domains (left) arborizations (center) and somas (left) active at basal (open) and stimulation (hashed) in $IP_3R2^{-/-}$ mice. (E), Percentage of domains active in wildtype (filled) and $IP_3R2^{-/-}$ mice (hashed). Blue line denotes 22.6% spatial threshold. (F), Probability of soma activation in wildtype (filled) and $IP_3R2^{-/-}$ mice (hashed). Mean ± SEM. '***' ≡ $p < 0.001$ using paired and unpaired student t-test.

different amounts of Adenosine triphosphate (ATP) from a local micropipette with pressure pulses of different durations to gradually activate astrocytes (**Figure 6A-C**). Fluorescence imaging of astrocyte calcium in brain slices confirmed the existence of an astrocyte spatial threshold for calcium surge (22.9%; **Figure 6C,D**), that is within 95% confidence of our in vivo quantification [21.2%, 24.0%]. Beyond the threshold, increasing the duration of ATP puffs increased the proportion of activated astrocytic domains (1-way ANOVA: $p < 0.001$, n = 11 populations, 7 animals; **Figure 6E**; blue line indicates the threshold value obtained in **Figure 6D**). Likewise, similar to the domain activation, the SIC frequency increased as the duration of ATP puffs increased (1-way ANOVA: $p < 0.001$, n = 9 neurons, 9 animals; **Figure 6F**). Moreover, SIC frequency correlated with astrocyte domain activity (Pearson correlation: $p < 0.001$, $R^2 = 0.95$; **Figure 6G**) but SIC frequency increased only beyond the spatial threshold of the astrocyte calcium signal (blue line in **Figure 6G**), indicating that the spatial threshold of the astrocyte calcium is correspondingly manifested in gliotransmitter release. These results indicate that spatial threshold of the astrocyte calcium surge has a functional impact on gliotransmission, which have important consequences on the spatial extension of the astrocyte-neuron communication and synaptic regulation.

Discussion

In the present study, we imaged calcium activity in identified SR101-labelled astrocytes of the primary somatosensory cortex in vivo, and developed and used an unbiased computational algorithm to integrate these data at different cellular levels, i.e., domains, arborizations and somas. Here, we show that sensory-evoked astrocyte calcium responses originated in the arborization and were followed by delayed soma activation. A detailed examination of the domains within arborizations uncovered a correlation between domain activity and soma responses, and we were able to quantify a spatial threshold of activated domains necessary to produce soma activation (~23%). Domain activation was found to be stimulus dependent, however the spatial threshold for somatic response remained unchanged to various stimulus parameters, indicating that spatial threshold was determined by astrocytic intrinsic properties rather than synaptic inputs. We also found that soma responses preceded an increase in the spread of intracellular calcium activation across the arborization (i.e. calcium surge). In $IP_3R2^{-/-}$ mice, we found sensory-evoked calcium responses in astrocyte domains, albeit significantly reduced compared to wildtype mice and never reaching the defined spatial threshold to spur somatic activation. Finally, in cortical brain slices, we found that astrocyte calcium surge is related to nearby neuronal modulation as seen in the presence of slow inward currents. Anesthesia has been shown to reduce astrocyte activity (Thrane et al., 2012), however we suppose that subcellular machinery is intact, and this is further supported in our slice experiments void of anesthesia. These results demonstrate that astrocytic responses to synaptic inputs were initiated in arborizations, extend intracellularly after reaching a spatial threshold of concomitant domain activation reliant on IP_3R2, and impact astrocyte to neuronal signaling.

Our results support the idea that neurotransmitters released at tripartite synapses act on microdomains at astrocytic arborizations (Di Castro et al., 2011; Lia et al., 2021; Otsu et al., 2015; Panatier et al., 2011) because they responded to sensory stimulation prior to astrocyte somas. Reports have found astrocyte domains can become active independently without recruiting neighboring arborizations or the soma, and domains also can become active en masse (Agarwal et al., 2017; Shigetomi et al., 2013). Our findings add to this, defining a spatial threshold of domains that needs to be reached in order to lead to soma activation and a calcium surge that propagates to the rest of the astrocyte arborization.

Several lines of evidence indicate that the spatial threshold does not result from increased stimulus parameters. First, the stimulus-dependence of domain activity shows continuous activation with no threshold. Second, the spatial threshold identified using the Heaviside step function depends on domain activation and not stimulus parameters. Third, the spatial threshold

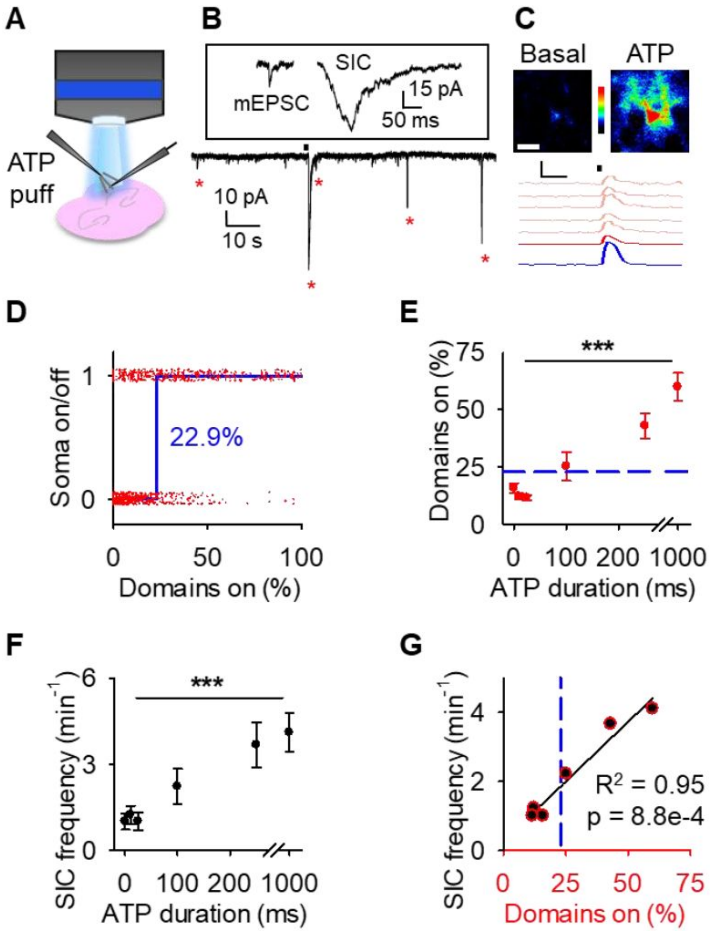

Figure 6.

Increases in slow-inward currents occurs with astrocyte calcium surge.

(A), Scheme of cortical brain slice experiments to image astrocyte Ca^{2+} and record slow-inward currents (SICs) with ATP application. (B), Example traces of a miniature excitatory post synaptic current (mEPCS) and a slow inward current (SIC) (upper) and SICs following ATP puff (black bar) (lower). (C), Pseudocolor Ca^{2+} images at basal and ATP with traces of responses to puff (black bar) in the soma (blue), arbor (red), and domains (salmon). Scale bar = 10 μm. Scale = F/F_o, 10 s. (D), Active state of soma for individual astrocytes vs. percentage of active domains (red), with fit to a Heaviside step function (blue line). (E), Percentage of domains active in response to ATP puff. Blue dotted line denotes spatial threshold from D. (F), SIC frequency in response to ATP puff. (G), Pearson correlation between percent active domains vs. SIC frequency. Blue dotted line denotes spatial threshold from D. Mean ± SEM. '***' ≡ $p < 0.001$ using 1-way ANOVA and t-test of Pearson correlation.

is independent of the stimulus parameter, including no stimulation. Finally, using a different set of experiments in slice recreated the spatial threshold. Overall, this evidence indicates the existence of a spatial threshold that is determined by intrinsic properties of the astrocyte.

Present results indicate that if the activation of a spatially localized astrocyte arborization by a localized set of synapses reaches the spatial threshold, the calcium signal is then globally expanded to modulate different synapses and neurons. The present demonstration of a spatial threshold to astrocyte calcium surge suggests that astrocytes spatially integrate information from multiple synaptic inputs. While previous reports have shown information integration of different neurotransmitters and synaptic inputs by astrocytes in situ (Durkee et al., 2019; Perea and Araque, 2005), which may coordinate networks of neurons in silico (Gordleeva et al., 2019), our findings reveal novel integrative properties of spatial information by astrocytes in vivo.

Detailed examinations of the subcellular roots of population activity is important in understanding network activity (Buzsaki et al., 2012; Yuste, 2015). Examining single-unit neuronal activity from a specific brain region and comparing to low frequency recordings of the local field potential creates a reductionist description of network-mediated brain function (Buzsaki and Draguhn, 2004). The discovery and detailing of the action potential was landmark to understanding neuronal information processing capabilities as integrators at the single cell (Yuste and Tank, 1996). The determination of an astrocyte subcellular spatial threshold that underlies a calcium surge is an analogue to the action potential threshold found in neurons. Much like in neurons, the astrocyte spatial threshold is shown as a transformation of subcellular activity that underlies integrative properties.

IP_3R2-dependent calcium release has been shown to critically contribute to G protein-coupled receptor-induced astrocyte calcium activity in the age range of mice imaged here (Agulhon et al., 2008; Kofuji and Araque, 2020). Using $IP_3R2^{-/-}$ mice, we found that IP_3R2 are required for somatic calcium responses and that they are necessary for the sensory-evoked responses to surpass the spatial threshold. Indeed, close examinations of astrocytic arborizations in $IP_3R2^{-/-}$ mice showed domains still responded with calcium events, albeit at a reduced percentage compared to wildtype mice. Moreover, this reduction of domain activity in $IP_3R2^{-/-}$ mice was steadily below the spatial threshold for calcium surge, suggesting a role for IP_3 in this physiological property. The subcellular calcium dynamics of astrocytes in $IP_3R2^{-/-}$ mice have been shown previously (Agarwal et al., 2017; Lines et al., 2020; Schmidt and Oheim, 2020; Srinivasan et al., 2015; Stobart et al., 2018), yet present data further demonstrate that IP_3R2 are necessary for the propagation of astrocyte calcium surge. Outside of IP_3R2 mediated intracellular Ca^{2+} increases, extracellular Ca^{2+} entry into the cell has been shown (Rungta et al., 2016), and our study does not rule out this possibility.

Astrocyte calcium activity induces multiple downstream signaling cascades, such as the release of gliotransmitters (Araque et al., 2014). Using patch-clamp recordings of nearby neurons we showed that astrocyte calcium surge is also related to the increase in slow inward currents, previously demonstrated to be dependent on astrocytic vesicular release of glutamate (Araque et al., 2000; Durkee et al., 2019; Fellin et al., 2004). The output of astrocyte calcium surge is equally important to network communication as the labeling of astrocyte calcium surge, as it identifies a biologically relevant effect onto nearby neurons. Many downstream signaling mechanisms may be activated following astrocyte calcium surge, and the effect of locally concentrated domain activity vs astrocyte calcium surge should be studied further on different astrocyte outputs.

In addition to normal brain function, many neurological disorders have been shown to have a cause at the cellular level that translate up to aberrant network brain function. Examples include: closer inspections of Alzheimer's disease have uncovered aberrant synaptic activity early on in the disease that may underly network dysfunction and cognitive processes (Selkoe, 2002), increased

cellular excitability contributes to epileptic seizure activity (Cohen et al., 2002), and NMDA dysfunction in schizophrenia impairs long-range neuronal synchronization contributing to altered cognitive states (Olney et al., 1999). Closer examinations into altered subcellular astrocyte activity may also uncover contributions to neurological disorders. By understanding the root of the cause, novel translational diagnostics and therapeutics for brain disorders may be found.

Considering that a single astrocyte can contact ~100,000 synapses (Bushong et al., 2002) that can independently trigger the astrocyte calcium signal (Covelo and Araque, 2018; Panatier et al., 2011) and that can be independently regulated by gliotransmitters released through calcium dependent mechanisms (Araque et al., 2014; Savtchouk and Volterra, 2018), the processes governing the intracellular expansion of the calcium signal may have relevant consequences on brain function by determining the spatial extension of astrocytic neuromodulation of synapses. In conclusion, by showing novel integrative properties of spatial information by astrocytes and the existence of a spatial threshold for the spread of the calcium signal and the subsequent gliotransmission, which is determined by astrocyte intrinsic properties, present findings identify novel physiological properties of astrocyte function that may add computational capabilities to brain information processing.

Methods

Proper animal use and care

All the procedures for handling and sacrificing animals were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals. We used both female and male transgenic animals (GFAP-GCaMP6f) that were 2-4 months of age, kept on a continuous 12h light/dark cycle and freely available to food and water. Transgenic mice were created from crossing GFAP-Cre (*https://www.jax.org/strain/024098*) mice with floxed GCaMP6f mice (*https://www.jax.org/strain/028865*).

Stereotaxic surgery for in vivo recordings

Mice were anesthetized with 1.8 mg/kg urethane administered intraperitoneally (IP). Anesthetized mice were placed in a stereotaxic atop a heating pad controlled with an anal probe feedback to maintain body temperature, and faux tears were applied to prevent corneal dehydration. An incision was made down the midline of the scalp and the skin was parted to expose the skull. Screws were placed over the right frontal plate and interparietal plate. A craniotomy was made no more than 2 mm in diameter centered over the primary somatosensory cortex (S1; in mm from bregma: $-1_{a\text{-}p}$, $1.5_{m\text{-}l}$) (Franklin, 2019). After the dura was removed, sulforhodamine 101 (SR101) was topically applied to the exposed cortex to label astrocytes (50 µM for 20 minutes) (Rasmussen et al., 2016). Agarose (1%) was made from artificial cerebrospinal fluid (containing in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, EDTA 1, HEPES-K 8.6, Glucose 10) and placed on the exposed cortex before fixing a glass coverslip over the craniotomy using dental cement. Finally, a frame was mounted onto the exposed skull using dental cement. In experiments testing IP_3R2 in calcium surge, two weeks before imaging mice were injected with adenovirus encoding GCaMP6f under the GfaABC1d (AAV5-GfaABC1d-GCaMPf) into S1.

In vivo two-photon calcium fluorescence imaging

In vivo imaging was performed in layers 2/3 (100 – 300 µm below the cortical surface) of the exposed mouse cortex with a Leica SP5 multiphoton upright microscope. Videos were obtained for 60 s over an area of 366 × 366 µm at either 256 × 256 or 512 × 512 sized images with a sampling interval of 0.2 – 0.5 s. Red and green fluorescence was obtained in parallel to image calcium activity in identified SR101-labelled astrocytes.

Peripheral stimulation

A bipolar electrode needle was placed in the hindpaw contralateral to the recorded cortical hemisphere. Square electrical pulses with 0.5 ms width and increasing intensities (1, 2, 3 mA at 2 Hz for 10 s) and variable frequencies (0.5, 1, 2, 5, 10 Hz at 2 mA for 10 s) were applied in sustained durations (1, 5, 10, 20 s at 2 mA and 2 Hz). Stimulus parameters were pseudorandomly ordered to differentially activate and characterize different levels of activation of astrocytes.

Calcium image processing and analysis

All image processing and analysis was performed in the novel graphical user interface (GUI) Calsee (**Figure S1**; *https://www.araquelab.com/code*). Within Calsee, functional and structural video files can be loaded simultaneously. Regions of interest can be defined based on structural or functional imaging. In this study, structural images of SR101-stained astrocytes were used to outline individual astrocyte territories (Bindocci et al., 2017). These regions of interest were refined using Calsee to limit regions of interest to SR101-positive pixels. Next, astrocyte territories were further segmented into soma and arborization regions of interest. Astrocyte process arborization was then discretized into a grid of maximally-sized 4.3 µm x 4.3 µm square regions of interest (ROIs). At fine distal processes, ROIs were automatically reduced in size to only include SR101-positive pixels. These regions of interest based on SR101 labeling were then used to quantify calcium activity from the simultaneously recorded green channel. Event detection of calcium fluorescence was determined when the amplitude of the response was 3 times the standard deviation away from the average baseline amplitude.

Every event happening in the domains of an astrocyte before its soma becomes active is referred as pre-soma events. Events happening in the domains after soma activation are referred as post-soma events. Accordingly, a cell whose soma becomes active at a given moment can be subdivided into pre-soma cell (all the activity of the cell prior to soma activation) and post-soma cell (all the activity of the cell following soma activation).

Slice experiments

Following rapid decapitation, brains were extracted and placed in a vibratome to create 350 µm thick brain slices that included the primary somatosensory cortex. Brain slices were left to incubate in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 2.69, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 26, $CaCl_2$ 2, ascorbic acid 0.4, and glucose 10, and continuously bubbled with carbogen (95% O_2 and 5% CO_2) (pH 7.3). After incubation, brain slices were placed in a chamber with a perfusion system to image astrocytes as well as record neuronal membrane potential via patch clamp. To stimulate astrocytes locally, a pipette tip was lowered above the slice and used to apply a puff of 0.5 mM ATP.

Statistical testing

Astrocyte calcium quantifications were averaged over all astrocytes of a single video and these values were used in statistical testing. Paired and unpaired two-tailed student t-tests were performed with $\alpha = 0.05$ against the null hypothesis that no difference exists between the two groups. Correlations were confirmed using a student's t-test against the null hypothesis that no correlation exist. To test the stimulus dependence of a stimulus-response curve, 1-way ANOVAs were performed with $\alpha = 0.05$ against the null hypothesis that no dependence exists. In the comparisons of two groups' response curves a 2-way ANOVA was performed with $\alpha = 0.05$ against the additional null hypotheses that the two groups are the same and no interaction exists. In some examinations following a significant ANOVA, multiple comparison testing was performed using Tukey's range test using $\alpha = 0.05$ against the null hypothesis that no samples are different from each other.

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Author Contributions

M.N., A.A., P.K., E.D.M, J.A and J.L. contributed to project conception, project design, and manuscript writing. J.L. performed the experiments and analyzed the results. J.L. and A.B. contributed to the creation and development of Calsee.

Competing Interests statement

The authors declare no competing interests.

Figure Legends

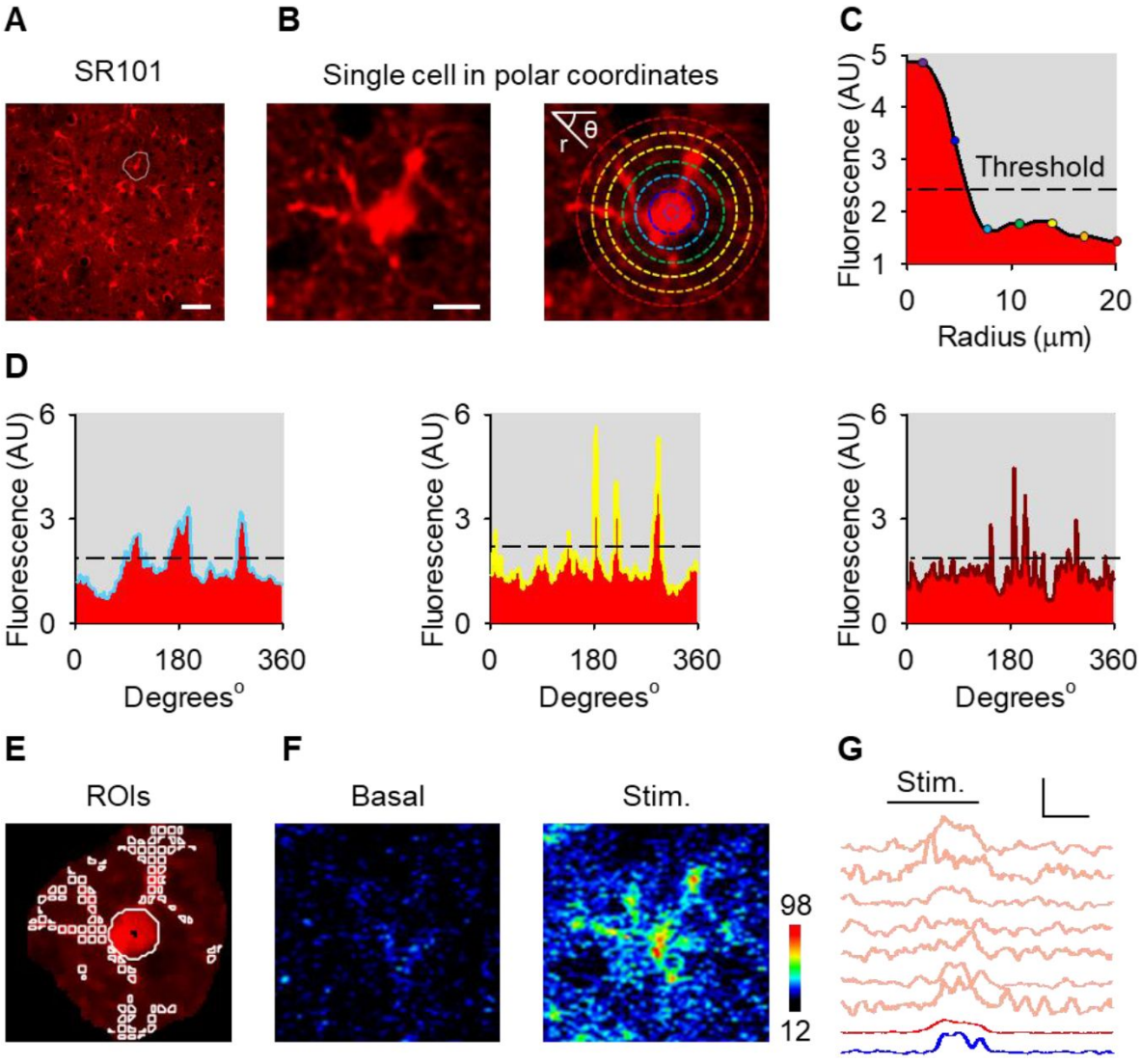

Figure S1.

Semi-automatic method for the segmentation of astrocyte morphology.

(A), SR101 stained astrocyte population with an outlined cell. Scale bar = 50 μm. (B), Selected astrocyte placed in polar coordinates with rings overlaid to assess structural fluorescence. Scale bar = 10 μm. (C), Average fluorescence of rings centered on astrocyte soma as radius is extended outward. Note, overlay of values from rings in B. (D), Fluorescence of rings in panel B as a function of angle. (E), Regions of Interest (ROIs) from algorithm. (F), Calcium pseudocolor image during basal and stimulation. (G), Calcium traces from f during stimulation. Scale = F/F_o, 10 s.

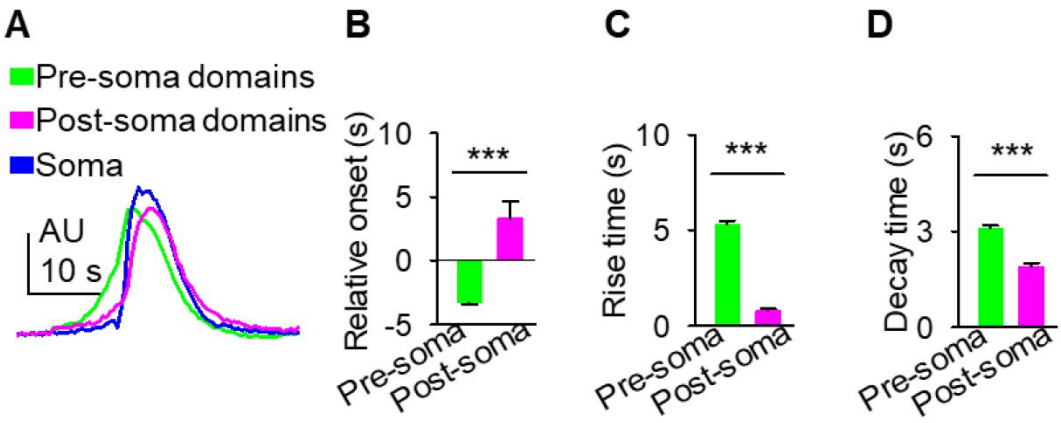

Figure S2.

Distinct dynamics of domains before and after soma onset.

(A), Average calcium traces from somas (blue) and domains activating before the soma (pre-soma; green) and after the soma (post-soma; pink) aligned to their respective soma onset. (B), Pre-soma and post-soma latency to response relative to their respective soma onset. (C), Event rise time. (D), Event decay time. Mean ± SEM. '***' ≡ $p < 0.001$ using paired student t-test.

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Reviewer #1 (Public Review):

Lines et al., provide evidence for a sequence of events in vivo in adult anesthetized mice that begin with a foot-shock driving activation of neural projections into layer 2/3 somatosensory

cortex, which in turn triggers a rise in calcium in astrocytes within "domains" of their "arbor". The authors segment the astrocyte morphology based on SR101 signal and show that the timing of "arbor" Ca2+ activation precedes somatic activation and that somatic activation only occurs if at least {greater than or equal to}22.6% of the total segmented astrocyte "arbor" area is active. Thus, the authors frame this {greater than or equal to}22.6% activation as a spatial property (spatial threshold) with certain temporal characteristics - i.e., must occur before soma and global activation. The authors then elaborate on this spatial threshold by providing evidence for its intrinsic nature - is not set by the level of neuronal stimulus and is dependent on whether IP3R2, which drives Ca2+ release from the endoplasmic reticulum (ER) in astrocytes, is expressed. Lastly, the authors suggest a potential physiologic role for this spatial threshold by showing ex vivo how exogenous activation of layer 2/3 astrocytes by ATP application can gate glutamate gliotransmission to layer 2/3 cortical neurons - with a strong correlation between the number of active astrocyte Ca2+ domains and the slow inward current (SIC) frequency recorded from nearby neurons as a readout of glutamatergic gliotransmission. This is interesting and would potentially be of great interest to readers within and outside the glia research community, especially in how the authors have tried to systematically deconstruct some of the steps underlying signal integration and propagation in astrocytes. Many of the conclusions posited by the authors are potentially important but we think their approach needs experimental/analytical refinement and elaboration.

The primary issue for us, and which we would encourage the authors to address, relates to the low spatial-temporal resolution of their approach. This issue does not necessarily compromise the concept of a spatial threshold, but more refined observations and analyses are likely to provide more reliable quantitative parameters and a more comprehensive view of the mode of Ca2+ signal integration in astrocytes. For this reason, and because their observations might be perceived as both a conceptual and numerical standard in the field, we believe that the authors should proceed with both experimental and analytical refinement. Notably, we have difficulty with the reported mean delays of astrocyte Ca2+ elevations upon sensory stimulation. The 11s delay for response onset in "arbor" and 13s in the soma are extremely long, and we do not think they represent a true physiologic latency for astrocyte responses to the sensory activity. Indeed, such delays appear to be slower even than those reported in the initial studies of sensory stimulation in anesthetized mice with limited spatial-temporal resolution (Wang et al. Nat Neurosci., 2006) - not to say of more recent and refined ones in awake mice (Stobart et al. Neuron, 2018) that identified even sub-second astrocyte Ca2+ responses, largely preserved in IP3R2KO mice. Thus, we are inclined to believe that the slowness of responses reported here is an indicator of experimental/analytical issues. There can be several explanations of such slowness that the authors may want to consider for improving their approach: (a) The authors apparently use low zoom imaging for acquiring signals from several astrocytes present in the FOV: do all of these astrocytes respond homogeneously in terms of delay from sensory stimulus? Perhaps some are faster responders than others and only this population is directly activated by the stimulus. Others could be slower in activation because they respond secondarily to stimuli. In this case, the authors could focus their analysis specifically on the "fast-responding population". (b) By focusing on individual astrocytes and using higher zoom, the authors could unmask more subtle Ca2+ elevations that precede those reported in the current manuscript. These signals have been reported to occur mainly in regions of the astrocyte that are GCaMP6-positive but SR101-negative and constitute a large percentage of its volume (Bindocci et al., 2017). By restricting analysis to the SR101-positive part of the astrocyte, the authors might miss the fastest components of the astrocyte Ca2+ response likely representing the primary signals triggered by synaptic activity. It would be important if they could identify such signals in their records, and establish if none/few/many of them propagate to the SR-101-positive part of the astrocyte. In other words, if there is only a single spatial threshold, the one the authors reported, or two or more of them along the path of signal propagation towards the cell soma that leads eventually to the transformation of the signal into a global

astrocyte Ca2+ surge. In this context, there is another concept that we encourage the authors to better clarify: whether the spatial threshold that they describe is constituted by the enlargement of a continuous wavefront of Ca2+ elevation, e.g. in a single process, that eventually reaches 22.6% of the segmented astrocyte, or can it also be constituted by several distinct Ca2+ elevations occurring in separate domains of the arbor, but overall totaling 22.6% of the segmented surface? Mechanistically, the latter would suggest the presence of a general excitability threshold of the astrocyte, whereas the former would identify a driving force threshold for the centripetal wavefront. In light of the above points, we think the authors should use caution in presenting and interpreting the experiments in which they use SIC as a readout. Their results might lead some readers to bluntly interpret the 22.6% spatial threshold as the threshold required for the astrocyte to evoke gliotransmitter release. Indeed, SIC are robust signals recorded somatically from a single neuron and likely integrate activation of many synapses all belonging to that neuron. On the other hand, an astrocyte impinges in a myriad of synapses belonging to several distinct neurons. In our opinion, it is quite possible that more local gliotransmission occurs at lower Ca2+ signal thresholds (see above) that may not be efficiently detected by using SIC as a readout; a more sensitive approach, such as the use of a gliotransmitter sensor expressed all along the astrocyte plasma-membrane could be tested to this aim.

Additional considerations are that the authors propose an event sequence as follows: stimulus - synaptic drive to L2/3 - arbor activation - spatial threshold - soma activation - post soma activation - gliotransmission. This seems reminiscent of the sequence underlying neuronal spike propagation - from dendrite to soma to axon, and the resulting vesicular release. However, there is no consensus within the glial field about an analogous framework for astrocytes. Thus, "arbor activation", "soma activation", and "post soma activation" are not established ẗerms-of-art.ˊ Similarly, the way the authors use the term "domain" contrasts with how others have (Agarwal et al., 2017; Shigetomi et al., 2013; Di Castro et al., 2011; Grosche et al., 1999) and may produce some confusion. The authors could adopt a more flexible nomenclature or clarify that their terms do not have a defined structural-functional basis, being just constructs that they justifiably adapted to deal with the spatial complexity of astrocytes in line with their past studies (Lines et al., 2020; Lines et al., 2021).

Our previous points suggest that the paper would be significantly strengthened by new experimental observations focusing on single astrocytes and using acquisitions at higher spatial and temporal resolution. If the authors will not pursue this option, we encourage them to at least improve their analysis, and at the same time recognize in the text some limitations of their experimental approach as discussed above. We indicate here several levels of possible analytical refinement.

The first relates to the selection of astrocytes being analyzed, and the need to focus on a much narrower subpopulation than (for example) 987 astrocytes used for the core data. This selection would take into greater consideration the aspects of structure and latency. With the structural and latency-based criteria for selection, the number of astrocytes to analyze might be reduced by 10-fold or more, making our second analytical recommendation much more feasible.

For structure-based selection - Genetically-encoded Ca2+ indicators such as GCaMP6 are in principle expressed throughout an astrocyte, even in regions that are not labelled by SR101. Moreover, astrocytes form independent 3D territories, so one can safely assume that the GCaMP6 signal within an astrocyte volume belongs to that specific astrocyte (this is particularly evident if the neighboring astrocytes are GCaMP6-negative). Therefore, authors could extend their analysis of Ca2+ signals in individual astrocytes to the regions that are SR101-negative and try to better integrate fast signals in their spatial threshold concept. Even if they decided to be conservative on their methods, and stick to the astrocyte segmentation based on the SR-101 signal, they should acknowledge that SR101 dye staining quality can vary

considerably between individual astrocytes within a FOV - some astrocytes will have much greater structural visibility in the distal processes than others. This means that some astrocytes may have segmented domains extending more distally than others and we think that authors should privilege such astrocytes for analysis. However, cases like the representative astrocytes shown in Figure 4A or Figure S1B, have segmented domains localized only to proximal processes near the soma. Accordingly, given the reported timing differences between "arbor" and "soma" activation, one might expect there to be comparable timing differences between domains that are distal vs proximal to the soma as well. Fast signals in peripheral regions of astrocytes in contact with synapses are largely IP3R2-independent (Stobart et al., 2018). However, the quality of SR101 staining has implications for interpreting the IP3R2 KO data. There is evidence IP3R2 KO may preferentially impact activity near the soma (Srinivasan et al., 2015). Thus, astrocytes with insufficient staining - visible only in the soma and proximal domains - might show a biased effect for IP3R2 KO. While not necessarily disrupting the core conclusions made by the authors based on their analysis of SR101-segmented astrocytes, we think results would be strengthened if astrocytes with sufficient SR101 staining - i.e. more consistent with previous reports of L2/3 astrocyte area (Lanjakornsiripan et al., 2018) - were only included. This could be achieved by using max or cumulative projections of individual astrocytes in combination with SR101 staining to construct more holistic structural maps (Bindocci et al., 2017).

For latency-based selection - The authors record calcium activity within a FOV containing at least 20+ astrocytes over a period of 60s, during which a 2Hz hindpaw stimulation at 2mA is applied for 20s. As discussed above, presumably some astrocytes in a FOV are the first to respond to the stimulus series, while others likely respond with longer latency to the stimulus. For the shorter-latency responders <3s, it is easier to attribute their calcium increases as "following the sensory information" projecting to L2/3. In other cases, when "arbor" responses occur at 10s or later, only after 20 stimulus events (at 2Hz), it is likely they are being activated by a more complex and recurrent circuit containing several rounds of neuron-glia crosstalk etc., which would be mechanistically distinct from astrocytes responding earlier. We suggest that authors focus more on the shorter latency response astrocytes, as they are more likely to have activity corresponding to the stimulus itself.

The second level of analysis refinement we suggest relates specifically to the issue of propagation and timing for the activity within "arbor", "soma" and "post-soma". Currently, the authors use an ROI-based approach that segments the "arbor" into domains. We suggest that this approach could be supplemented by a more robust temporal analysis. This could for example involve starting with temporal maps that take pixels above a certain amplitude and plot their timing relative to the stimulus-onset, or (better) the first active pixel of the astrocyte. This type of approach has become increasingly used (Bindocci et al., 2017; Wang et al., 2019; Ruprecht et al., 2022) and we think its use can greatly help clarify both the proposed sequence and better characterize the spatial threshold. We think this analysis should specifically address several important points:

1. Where/when does the astrocyte activation begin? Understanding the beginning is very important, particularly because another potential spatial threshold - preceding the one the authors describe in the paper - could gate the initial activation of more distal processes, as discussed above. This sequentially earlier spatial threshold could (for example) rely on microdomain interaction with synaptic elements and (in contrast) be IP3R2 independent (Srinivasan et al., 2015, Stobart et al., 2018). We would be interested to know whether, in a subset of astrocytes that meet the structure and latency criteria proposed above and can produce global activation, there is an initial local GCaMP6f response of a minimal size that must occur before propagation towards the soma begins. The data associated with varying stimulus parameters could potentially be useful here and reveal stimulus intensity/duration-dependent differences.

2. Whether the propagation in the authors' experimental model is centripetal? This is implied throughout the manuscript but never shown. We think establishing whether (or not) the calcium dynamics are centripetal is important because it would clarify whether spatially adjacent domains within the "arbor" need to be sequentially active before reaching the threshold and then reaching the soma. More broadly, visualizing propagation will help to better visualize summation, which is presumably how the threshold is first reached (and overcome). The alternative hypothesis of a general excitability threshold, as discussed above, would be challenged here and possibly rejected, thereby clarifying the nature of the Ca2+ process that needs to reach a threshold for further expansion to the soma and other parts of the astrocyte.

3. In complement to the previous point: we understand that the spatial threshold does not per se have a location, but is there some spatial logic underlying the organization of active domains before the soma response occurs? One can easily imagine multiple scenarios of sparse heterogeneous GCaMP6f signal distributions that correspond to {greater than or equal to}22.6% of the arborization, but that would not be expected to trigger soma activation. For example, the diagram in Figure 4C showing the astrocyte response to 2Hz stim (which lacks a soma response) underscores this point. It looks like it has {greater than or equal to}22.6% activation that is sparsely localized throughout the arborization. If an alternative spatial distribution for this activity occurred, such that it localized primarily to a specific process within the arbor, would it be more likely to trigger a soma response?

4. Does "pre-soma" activation predict the location and onset time of "post-soma" activation? For example, are arbor domains that were part of the "pre-soma" response the first to exhibit GCaMP6f signal in the "post-soma" response?

Reviewer #2 (Public Review):

Lines et al investigated the integration of calcium signals in astrocytes of the primary somatosensory cortex. Their goal was to better characterize the mechanisms that govern the spatial characteristics of calcium signals in astrocytes. In line with previous reports in the field, they found that most events originated and stayed localized within microdomains in distal astrocyte processes, occasionally coinciding with larger events in the soma, referred to as calcium surges. As a single astrocyte communicates with hundreds of thousands of synapses simultaneously, understanding the spatial integration of calcium signals in astrocytes and the mechanisms governing the latter is of tremendous importance to deepen our understanding of signal processing in the central nervous system. The authors thus aimed to unveil the properties governing the emergence of calcium surges. The main claim of this manuscript is that there would be a spatial threshold of ~23% of microdomain activation above which a calcium surge, i.e. a calcium signal that spreads to the soma, is observed. Although the study provides data that is highly valuable for the community, the conclusions of the current version of the manuscript seem a little too assertive and general compared with what can be deduced from the data and methods used.

The major strength of this study is the experimental approach that allowed the authors to obtain numerous and informative calcium recordings in vivo in the somatosensory cortex in mice in response to sensory stimuli as well as in situ. Notably, they developed an interesting approach to modulating the number of active domains in peripheral astrocyte processes by varying the intensity of peripheral stimulation (its amplitude, frequency, or duration).

The major weakness of the manuscript is the method used to analyze and quantify calcium activity, which mostly relies on the analysis of averaged data and overlooks the variability of the signals measured. As a result, the main claims from the manuscript seem to be incompletely supported by the data. The choice of the use of a custom-made semi-automatic ROI-based calcium event detection algorithm rather than established state-of-the-art

software, such as the event-based calcium event detection software AQuA (DOI: 10.1038/s41593-019-0492-2), is insufficiently discussed and may bias the analysis. Some references on this matter include: Semyanov et al, Nature Rev Neuro, 2020 (DOI: 10.1038/s41583-020-0361-8); Covelo et al 2022, J Mol Neurosci (DOI: 10.1007/s12031-022-02006-w) & Wang et al, 2019, Nat Neuroscience (DOI: 10.1038/s41593-019-0492-2). Moreover, the ROIs used to quantify calcium activity are based on structural imaging of astrocytes, which may not be functionally relevant.

For the reasons listed above, the manuscript would probably benefit from some rephrasing of the conclusions and a discussion highlighting the advantages and limitations of the methodological approach. The question investigated by this study is of great importance in the field of neuroscience as the mechanisms dictating the spatio-temporal properties of calcium signals in astrocytes are poorly characterized, yet are essential to understand their involvement in the modulation of signal integration within neural circuits.

Reviewer #3 (Public Review):

Summary:
The study aims to elucidate the spatial dynamics of subcellular astrocytic calcium signaling. Specifically, they elucidate how subdomain activity above a certain spatial threshold (~23% of domains being active) heralds a calcium surge that also affects the astrocytic soma. Moreover, they demonstrate that processes on average are included earlier than the soma and that IP3R2 is necessary for calcium surges to occur. Finally, they associate calcium surges with slow inward currents.

Strengths:
The study addresses an interesting topic that is only partially understood. The study uses multiple methods including in vivo two-photon microscopy, acute brain slices, electrophysiology, pharmacology, and knockout models. The conclusions are strengthened by the same findings in both in vivo anesthetized mice and in brain slices.

Weaknesses:
The method that has been used to quantify astrocytic calcium signals only analyzes what seems to be a small proportion of the total astrocytic domain on the example micrographs, where a structure is visible in the SR101 channel (see for instance Reeves et al. J. Neurosci. 2011, demonstrating to what extent SR101 outlines an astrocyte). This would potentially heavily bias the results: from the example illustrations presented it is clear that the calcium increases in what is putatively the same astrocyte goes well beyond what is outlined with automatically placed small ROIs. The smallest astrocytic processes are an order of magnitude smaller than the resolution of optical imaging and would not be outlined by either SR101 or with the segmentation method judged by the ROIs presented in the figures. Completely ignoring these very large parts of the spatial domain of an astrocyte, in particular when making claims about a spatial threshold, seems inappropriate. Several recent methods published use pixel-by-pixel event-based approaches to define calcium signals. The data should have been analyzed using such a method within a complete astrocyte spatial domain in addition to the analyses presented. Also, the authors do not discuss how two-dimensional sampling of calcium signals from an astrocyte that has processes in three dimensions (see Bindocci et al, Science 2017) may affect the results: if subdomain activation is not homogeneously distributed in the three-dimensional space within the astrocyte territory, the assumptions and findings between a correlation between subdomain activation and somatic activation may be affected.

The experiments are performed either in anesthetized mice, or in slices. The study would have come across as much more solid and interesting if at least a small set of experiments were performed also in awake mice (for instance during spontaneous behavior), given the profound effect of anesthesia on astrocytic calcium signaling and the highly invasive nature

of preparing acute brain slices. The authors mention the caveat of studying anesthetized mice but claim that the intracellular machinery should remain the same. This explanation appears a bit dismissive as the response of an astrocyte not only depends on the internal machinery of the astrocyte, but also on how the astrocyte is stimulated: for instance synaptic stimulation or sensory input likely would be dependent on brain state and concurrent neuromodulatory signaling which is absent in both experimental paradigms. The discussion would have been more balanced if these aspects were dealt with more thoroughly.

The study uses a heaviside step function to define a spatial 'threshold' for somata either being included or not in a calcium signal. However, Fig 4E and 5D showing how the method separates the signal provide little understanding for the reader. The most informative figure that could support the main finding of the study, namely a ~23% spatial threshold for astrocyte calcium surges reaching the soma, is Fig. 4G, showing the relationship between the percentage of arborizations active and the soma calcium signal. A similar plot should have been presented in Fig 5 as well. Looking at this distribution, though, it is not clear why ~23% would be a clear threshold to separate soma involvement, one can only speculate how the threshold for a soma event would influence this number. Even if the analyses in Fig. 4H and the fact that the same threshold appears in two experimental paradigms strengthen the case, the results would have been more convincing if several types of statistical modeling describing the continuous distribution of values presented in Fig. 4E (in addition to the heaviside step function) were presented.

The description of methods should have been considerably more thorough throughout. For instance which temperature the acute slice experiments were performed at, and whether slices were prepared in ice-cold solution, are crucial to know as these parameters heavily influence both astrocyte morphology and signaling. Moreover, no monitoring of physiological parameters (oxygen level, CO2, arterial blood gas analyses, temperature etc) of the in vivo anesthetized mice is mentioned. These aspects are critical to control for when working with acute in vivo two-photon microscopy of mice; the physiological parameters rapidly decay within a few hours with anesthesia and following surgery.

Author Response

eLife assessment

Building on their own prior work, the authors present valuable findings that add to our understanding of cortical astrocytes, which respond to synaptic activity with calcium release in subcellular domains that can proceed to larger calcium waves. The proposed concept of a spatial "threshold" is based on solid evidence from in vivo and ex vivo imaging data and the use of mutant mice. However, details of the specific threshold should be taken with caution and appear incomplete unless supported by additional experiments with higher resolution in space and time.

We thank the reviewers and editors for the positive assessment of our work as containing valuable findings that add to our understanding of cortical astrocytes. We also appreciate their positive appraisal of the proposed concept of a spatial threshold supported by solid evidence.

Regarding their specific comments, we truly appreciate them because they have helped to clarify issues and to improve the study. Provisional point-by-point responses to these comments are provided below. Regarding the general comment on the spatial and temporal resolution of our study, we would like to clarify that the spatial and temporal resolution used in the current study (i.e., 2 - 5 Hz framerate using a 25x objective with 1.7x digital zoom with

pixels on the order of 1 μm2) is within the norm in the field, does not compromise the results, nor diminish the main conceptual advancement of the study, namely the existence of a spatial threshold for astrocyte calcium surge.

We respect the thoughtfulness of the reviewers and editors and look forward to improving the paper to fully answer both public and private comments with a revised manuscript.

> ### *Reviewer #1 (Public Review):*
>
> *Lines et al., provide evidence for a sequence of events in vivo in adult anesthetized mice that begin with a footshock driving activation of neural projections into layer 2/3 somatosensory cortex, which in turn triggers a rise in calcium in astrocytes within "domains" of their "arbor". The authors segment the astrocyte morphology based on SR101 signal and show that the timing of "arbor" Ca2+ activation precedes somatic activation and that somatic activation only occurs if at least {greater than or equal to}22.6% of the total segmented astrocyte "arbor" area is active. Thus, the authors frame this {greater than or equal to}22.6% activation as a spatial property (spatial threshold) with certain temporal characteristics - i.e., must occur before soma and global activation. The authors then elaborate on this spatial threshold by providing evidence for its intrinsic nature - is not set by the level of neuronal stimulus and is dependent on whether IP3R2, which drives Ca2+ release from the endoplasmic reticulum (ER) in astrocytes, is expressed. Lastly, the authors suggest a potential physiologic role for this spatial threshold by showing ex vivo how exogenous activation of layer 2/3 astrocytes by ATP application can gate glutamate gliotransmission to layer 2/3 cortical neurons - with a strong correlation between the number of active astrocyte Ca2+ domains and the slow inward current (SIC) frequency recorded from nearby neurons as a readout of glutamatergic gliotransmission. This is interesting and would potentially be of great interest to readers within and outside the glia research community, especially in how the authors have tried to systematically deconstruct some of the steps underlying signal integration and propagation in astrocytes. Many of the conclusions posited by the authors are potentially important but we think their approach needs experimental/analytical refinement and elaboration.*

We thank the reviewer for her/his positive appraisal and comments that has helped us to improve the study. In response to their insights, we aim to address the key points raised below:

1. Sequence of Events: We acknowledge the reviewer's interest in our findings regarding the sequence of events. We will provide a more detailed description of the methods and results to clarify the temporal relationships between neural activation, astrocyte calcium dynamics, and astrocyte morphology segmentation.

2. Spatial Threshold: The reviewer accurately identifies our characterization of a spatial threshold (≥22.6% activation) with temporal characteristics as a crucial aspect of our study. We will expand upon this concept by offering a clearer illustration of how this threshold relates to somatic and global activation.

3. Intrinsic Nature of Spatial Threshold: The reviewer's insightful observation regarding the inherent quality of the spatial threshold, regardless of its dependence on neuronal stimuli is noteworthy. We will provide additional details to substantiate this claim, shedding more light on the fundamental nature of this phenomenon.

4. Physiological Implications: The reviewer rightly highlights the potential physiological significance of our findings, particularly in relation to gliotransmission in cortical neurons. We will enhance our discussion by elaborating on the implications of these observations.

> *The primary issue for us, and which we would encourage the authors to address, relates to the low spatialtemporal resolution of their approach. This issue does not necessarily compromise the concept of a spatial threshold, but more refined observations and analyses are likely to provide more reliable quantitative parameters and a more comprehensive view of the mode of Ca2+ signal integration in astrocytes.*

We agree with the reviewer that our spatial-temporal resolution (2 – 5 Hz framerate using a 25x objective and 1.7x digital zoom with pixels on the order of 1 μm) does not compromise the proposed concept of the existence of a spatial threshold for the intracellular calcium expansion.

> *For this reason, and because their observations might be perceived as both a conceptual and numerical standard in the field, we believe that the authors should proceed with both experimental and analytical refinement. Notably, we have difficulty with the reported mean delays of astrocyte Ca2+ elevations upon sensory stimulation. The 11s delay for response onset in "arbor" and 13s in the soma are extremely long, and we do not think they represent a true physiologic latency for astrocyte responses to the sensory activity. Indeed, such delays appear to be slower even than those reported in the initial studies of sensory stimulation in anesthetized mice with limited spatial-temporal resolution (Wang et al. Nat Neurosci., 2006) - not to say of more recent and refined ones in awake mice (Stobart et al. Neuron, 2018) that identified even sub-second astrocyte Ca2+ responses, largely preserved in IP3R2KO mice. Thus, we are inclined to believe that the slowness of responses reported here is an indicator of experimental/analytical issues. There can be several explanations of such slowness that the authors may want to consider for improving their approach: (a) The authors apparently use low zoom imaging for acquiring signals from several astrocytes present in the FOV: do all of these astrocytes respond homogeneously in terms of delay from sensory stimulus? Perhaps some are faster responders than others and only this population is directly activated by the stimulus. Others could be slower in activation because they respond secondarily to stimuli. In this case, the authors could focus their analysis specifically on the "fast-responding population". (b) By focusing on individual astrocytes and using higher zoom, the authors could unmask more subtle Ca2+ elevations that precede those reported in the current manuscript. These signals have been reported to occur mainly in regions of the astrocyte that are GCaMP6-positive but SR101-negative and constitute a large percentage of its volume (Bindocci et al., 2017). By restricting analysis to the SR101-positive part of the astrocyte, the authors might miss the fastest components of the astrocyte Ca2+ response likely representing the primary signals triggered by synaptic activity. It would be important if they could identify such signals in their records, and establish if none/few/many of them propagate to the SR-101-positive part of the astrocyte. In other words, if there is only a single spatial threshold, the one the authors reported, or two or more of them along the path of signal propagation towards the cell soma that leads eventually to the transformation of the signal into a global astrocyte Ca2+ surge.*

We thank the reviewer for these excellent and important comments. The qualm with the mean delays of astrocyte activation is indeed a result of averaging together astrocyte responses to a 20 second stimulus. Indeed, astrocyte responses are heterogeneous and many astrocytes respond much quicker, as can be seen in example traces in Figs. 1D, 1G, and 3C.

Indeed, with any biological system variability exists, however here we take the averaged responses in order to identify a general property of astrocyte calcium dynamics: the existence of the concept of a spatial threshold for astrocyte calcium surge.

Further, we used a lower stimulus frequency (2Hz) than Stobart et al. (90 Hz) to assess subthreshold activities. We found that stronger stimuli decreased response delays and will include this result in the revised manuscript. Interestingly, from Fig 4F, higher stimulus did not significantly alter the spatial threshold. In the revised version of the manuscript, we will provide a more detailed analysis and the consequent discussion of this analysis.

> *In this context, there is another concept that we encourage the authors to better clarify: whether the spatial threshold that they describe is constituted by the enlargement of a continuous wavefront of Ca2+ elevation, e.g. in a single process, that eventually reaches 22.6% of the segmented astrocyte, or can it also be constituted by several distinct Ca2+ elevations occurring in separate domains of the arbor, but overall totaling 22.6% of the segmented surface? Mechanistically, the latter would suggest the presence of a general excitability threshold of the astrocyte, whereas the former would identify a driving force threshold for the centripetal wavefront. In light of the above points, we think the authors should use caution in presenting and interpreting the experiments in which they use SIC as a readout. Their results might lead some readers to bluntly interpret the 22.6% spatial threshold as the threshold required for the astrocyte to evoke gliotransmitter release. Indeed, SIC are robust signals recorded somatically from a single neuron and likely integrate activation of many synapses all belonging to that neuron. On the other hand, an astrocyte impinges in a myriad of synapses belonging to several distinct neurons. In our opinion, it is quite possible that more local gliotransmission occurs at lower Ca2+ signal thresholds (see above) that may not be efficiently detected by using SIC as a readout; a more sensitive approach, such as the use of a gliotransmitter sensor expressed all along the astrocyte plasma-membrane could be tested to this aim.*

The reviewer raised an excellent point. Whether the spatial threshold of 22.6% occur in the segmented astrocyte or may be reached occurring in separate domains of the arbor, is an important question and we aim to address this by novel analysis that will be provided in the revised version of the manuscript.

Regarding comments on SIC, we fully agree with the reviewer. In the revised version of the manuscript, we will include text in the discussion to ensure the correct interpretation of the results, i.e., the observed 22.6% spatial threshold for the SIC does not necessarily indicates an intrinsic property of gliotransmitter release; rather, since SICs have been shown to be calcium-dependent, it is not surprising that their presence, monitored at the whole-cell soma, matches the threshold for the intracellular calcium extension.

> *Additional considerations are that the authors propose an event sequence as follows: stimulus - synaptic drive to L2/3 - arbor activation - spatial threshold - soma activation - post soma activation - gliotransmission. This seems reminiscent of the sequence underlying neuronal spike propagation - from dendrite to soma to axon, and the resulting vesicular release. However, there is no consensus within the glial field about an analogous framework for astrocytes. Thus, "arbor activation", "soma activation", and "post soma activation" are not established ṫerms-of-art.´Similarly, the way the authors use the term "domain" contrasts with how others have (Agarwal et al., 2017; Shigetomi et al., 2013; Di Castro et al., 2011; Grosche et al., 1999) and may produce some confusion. The authors could adopt a more flexible nomenclature or clarify that their terms do not have a defined structural-functional basis, being just constructs that they justifiably adapted to deal with the spatial complexity of astrocytes in line with their past studies (Lines et al., 2020; Lines et al., 2021).*

We agree there is no consensus within the glial field about this event sequence. One major difference between this sequence of events and neuronal spike propagation is directionality from dendrite to soma to axon. It is unknown whether directionality of the calcium signal exists in astrocytes. The term “microdomain” is used in the references above to define distal subcellular domains in contact with synapses, and in order to dissociate from this term we adopt the nomenclature “domain” to define all subcellular domains in the astrocyte arborization. These items will be discussed and clarified in the revised version of the manuscript.

> *Our previous points suggest that the paper would be significantly strengthened by new experimental observations focusing on single astrocytes and using acquisitions at higher spatial and temporal resolution. If the authors will not pursue this option, we encourage them to at least improve their analysis, and at the same time recognize in the text some limitations of their experimental approach as discussed above. We indicate here several levels of possible analytical refinement.*

We believe our spatial (25x objective and 1.7x digital zoom with pixels on the order of 1μm) and temporal (2 – 5 Hz framerate) resolution is within the range used in the glial field. In any case the existence of a spatial threshold for astrocyte calcium surge is not compromised with the use of this imaging resolution.

> *The first relates to the selection of astrocytes being analyzed, and the need to focus on a much narrower subpopulation than (for example) 987 astrocytes used for the core data. This selection would take into greater consideration the aspects of structure and latency. With the structural and latency-based criteria for selection, the number of astrocytes to analyze might be reduced by 10-fold or more, making our second analytical recommendation much more feasible.*

We agree that individual differences exist, however, establishing a general concept requires the sampling of many astrocytes. Nevertheless, we aim to further address this issue in the revised version of the manuscript by analyzing the calcium dynamics in individual domains.

> *For structure-based selection - Genetically-encoded Ca2+ indicators such as GCaMP6 are in principle expressed throughout an astrocyte, even in regions that are not labelled by SR101. Moreover, astrocytes form independent 3D territories, so one can safely assume that the GCaMP6 signal within an astrocyte volume belongs to that specific astrocyte (this is particularly evident if the neighboring astrocytes are GCaMP6negative). Therefore, authors could extend their analysis of Ca2+ signals in individual astrocytes to the regions that are SR101-negative and try to better integrate fast signals in their spatial threshold concept. Even if they decided to be conservative on their methods, and stick to the astrocyte segmentation based on the SR-101 signal, they should acknowledge that SR101 dye staining quality can vary considerably between individual astrocytes within a FOV - some astrocytes will have much greater structural visibility in the distal processes than others. This means that some astrocytes may have segmented domains extending more distally than others and we think that authors should privilege such astrocytes for analysis. However, cases like the representative astrocytes shown in Figure 4A or Figure S1B, have segmented domains localized only to proximal processes near the soma. Accordingly, given the reported timing differences between "arbor" and "soma" activation, one might expect there to be comparable timing differences between domains that are distal vs proximal to the soma as well. Fast signals in peripheral regions of astrocytes in contact with synapses are largely IP3R2-independent (Stobart et al., 2018). However, the quality of SR101 staining has implications for interpreting the IP3R2 KO data. There is evidence IP3R2 KO may preferentially impact activity near the soma*

> *(Srinivasan et al., 2015). Thus, astrocytes with insufficient staining - visible only in the soma and proximal domains - might show a biased effect for IP3R2 KO. While not necessarily disrupting the core conclusions made by the authors based on their analysis of SR101-segmented astrocytes, we think results would be strengthened if astrocytes with sufficient SR101 staining - i.e. more consistent with previous reports of L2/3 astrocyte area (Lanjakornsiripan et al., 2018) - were only included. This could be achieved by using max or cumulative projections of individual astrocytes in combination with SR101 staining to construct more holistic structural maps (Bindocci et al., 2017).*

We agree with the ideas concerning SR101, and indeed there could be variability in the origins of the astrocyte calcium signal. Astrocyte territory boundaries can be difficult to discern when both astrocytes express GCaMP6. Here we take a conservative approach to constrain ROIs to SR101-positive astrocyte territory outlines without invading neighboring cells in order to reduce error in the estimate of a spatial threshold. The effect of IP3R2 KO preferentially impacting activity near the soma is interesting, and in line with our conclusions. We agree that the findings from SR101-negative pixels would not necessarily disrupt the core conclusions of the study, and the additional analysis suggested would further strengthen results.

> *For latency-based selection - The authors record calcium activity within a FOV containing at least 20+ astrocytes over a period of 60s, during which a 2Hz hindpaw stimulation at 2mA is applied for 20s. As discussed above, presumably some astrocytes in a FOV are the first to respond to the stimulus series, while others likely respond with longer latency to the stimulus. For the shorter-latency responders <3s, it is easier to attribute their calcium increases as "following the sensory information" projecting to L2/3. In other cases, when "arbor" responses occur at 10s or later, only after 20 stimulus events (at 2Hz), it is likely they are being activated by a more complex and recurrent circuit containing several rounds of neuron-glia crosstalk etc., which would be mechanistically distinct from astrocytes responding earlier. We suggest that authors focus more on the shorter latency response astrocytes, as they are more likely to have activity corresponding to the stimulus itself.*

We agree that different times of astrocyte calcium increases may be due to different mechanisms outside of the astrocyte. We believe the spatial threshold will be intrinsic to these external variables; yet we believe that longer latency responses are physiological and may carry important information to determining the astrocyte calcium responses.

> *The second level of analysis refinement we suggest relates specifically to the issue of propagation and timing for the activity within "arbor", "soma" and "post-soma". Currently, the authors use an ROI-based approach that segments the "arbor" into domains. We suggest that this approach could be supplemented by a more robust temporal analysis. This could for example involve starting with temporal maps that take pixels above a certain amplitude and plot their timing relative to the stimulus-onset, or (better) the first active pixel of the astrocyte. This type of approach has become increasingly used (Bindocci et al., 2017; Wang et al., 2019; Ruprecht et al., 2022) and we think its use can greatly help clarify both the proposed sequence and better characterize the spatial threshold. We think this analysis should specifically address several important points:*

We agree that the creation of temporal maps from our own data will be interesting. We will provide the results of the suggested analysis in the revised version of the manuscript.

1. *Where/when does the astrocyte activation begin? Understanding the beginning is very important, particularly because another potential spatial threshold - preceding the one the authors describe in the paper - could gate the initial activation of more distal processes, as discussed above. This sequentially earlier spatial threshold could (for example) rely on microdomain interaction with synaptic elements and (in contrast) be IP3R2 independent (Srinivasan et al., 2015, Stobart et al., 2018). We would be interested to know whether, in a subset of astrocytes that meet the structure and latency criteria proposed above and can produce global activation, there is an initial local GCaMP6f response of a minimal size that must occur before propagation towards the soma begins. The data associated with varying stimulus parameters could potentially be useful here and reveal stimulus intensity/duration-dependent differences.*

This is a very important point. It is difficult to pinpoint the beginning of the signal, which is why we rely on the average of responses.

1. *Whether the propagation in the authors' experimental model is centripetal? This is implied throughout the manuscript but never shown. We think establishing whether (or not) the calcium dynamics are centripetal is important because it would clarify whether spatially adjacent domains within the "arbor" need to be sequentially active before reaching the threshold and then reaching the soma. More broadly, visualizing propagation will help to better visualize summation, which is presumably how the threshold is first reached (and overcome). The alternative hypothesis of a general excitability threshold, as discussed above, would be challenged here and possibly rejected, thereby clarifying the nature of the Ca2+ process that needs to reach a threshold for further expansion to the soma and other parts of the astrocyte.*

We agree that our view is centripetal. Indeed, we have found arborization activity precedes soma activity. However, whether this is intrinsic or due to the fact that synapses are more likely to occur in the periphery requires further studies.

1. *In complement to the previous point: we understand that the spatial threshold does not per se have a location, but is there some spatial logic underlying the organization of active domains before the soma response occurs? One can easily imagine multiple scenarios of sparse heterogeneous GCaMP6f signal distributions that correspond to {greater than or equal to}22.6% of the arborization, but that would not be expected to trigger soma activation. For example, the diagram in Figure 4C showing the astrocyte response to 2Hz stim (which lacks a soma response) underscores this point. It looks like it has {greater than or equal to}22.6% activation that is sparsely localized throughout the arborization. If an alternative spatial distribution for this activity occurred, such that it localized primarily to a specific process within the arbor, would it be more likely to trigger a soma response?*

This is an interesting point and an analysis of spatial clustering on pre-soma domain activation may be useful to answer it.

1. *Does "pre-soma" activation predict the location and onset time of "post-soma" activation? For example, are arbor domains that were part of the "pre-soma" response the first to exhibit GCaMP6f signal in the "post-soma" response?*

This is another interesting analysis that can be done with a spatial clustering analysis.

Reviewer #2 (Public Review):

Lines et al investigated the integration of calcium signals in astrocytes of the primary somatosensory cortex. Their goal was to better characterize the mechanisms that govern the spatial characteristics of calcium signals in astrocytes. In line with previous reports in the field, they found that most events originated and stayed localized within microdomains in distal astrocyte processes, occasionally coinciding with larger events in the soma, referred to as calcium surges. As a single astrocyte communicates with hundreds of thousands of synapses simultaneously, understanding the spatial integration of calcium signals in astrocytes and the mechanisms governing the latter is of tremendous importance to deepen our understanding of signal processing in the central nervous system. The authors thus aimed to unveil the properties governing the emergence of calcium surges. The main claim of this manuscript is that there would be a spatial threshold of ~23% of microdomain activation above which a calcium surge, i.e. a calcium signal that spreads to the soma, is observed. Although the study provides data that is highly valuable for the community, the conclusions of the current version of the manuscript seem a little too assertive and general compared with what can be deduced from the data and methods used.

The major strength of this study is the experimental approach that allowed the authors to obtain numerous and informative calcium recordings in vivo in the somatosensory cortex in mice in response to sensory stimuli as well as in situ. Notably, they developed an interesting approach to modulating the number of active domains in peripheral astrocyte processes by varying the intensity of peripheral stimulation (its amplitude, frequency, or duration).

We thank the reviewer for their kind and thoughtful review of our study.

The major weakness of the manuscript is the method used to analyze and quantify calcium activity, which mostly relies on the analysis of averaged data and overlooks the variability of the signals measured. As a result, the main claims from the manuscript seem to be incompletely supported by the data. The choice of the use of a custom-made semi-automatic ROI-based calcium event detection algorithm rather than established state-of-the-art software, such as the event-based calcium event detection software AQuA (DOI: 10.1038/s41593-019-0492-2), is insufficiently discussed and may bias the analysis. Some references on this matter include: Semyanov et al, Nature Rev Neuro, 2020 (DOI: 10.1038/s41583-020-0361-8); Covelo et al 2022, J Mol Neurosci (DOI: 10.1007/s12031-022-02006-w) & Wang et al, 2019, Nat Neuroscience (DOI: 10.1038/s41593-019-0492-2). Moreover, the ROIs used to quantify calcium activity are based on structural imaging of astrocytes, which may not be functionally relevant.

Unfortunately, there is no general consensus for calcium analysis in the astrocyte or neuronal field, and many groups use custom made software made in lab or custom software such as GECIquant or AQuA. While AQuA is an event-based calcium event detection software, it may be that not including inactive domains that are SR101 positive could underestimate the spatial threshold for calcium surge. Our data is not based on the functional events but is based on calcium with structural constraints within a single astrocyte. This is crucial to properly determine the ratio of active vs inactive pixels within a single astrocyte.

For the reasons listed above, the manuscript would probably benefit from some rephrasing of the conclusions and a discussion highlighting the advantages and limitations of the methodological approach. The question investigated by this study is of great importance in the field of neuroscience as the mechanisms dictating the spatio-

temporal properties of calcium signals in astrocytes are poorly characterized, yet are essential to understand their involvement in the modulation of signal integration within neural circuits.

We thank the reviewer for their suggestions to benefit the conclusions and discussion.

Reviewer #3 (Public Review):

Summary:

The study aims to elucidate the spatial dynamics of subcellular astrocytic calcium signaling. Specifically, they elucidate how subdomain activity above a certain spatial threshold (~23% of domains being active) heralds a calcium surge that also affects the astrocytic soma. Moreover, they demonstrate that processes on average are included earlier than the soma and that IP3R2 is necessary for calcium surges to occur. Finally, they associate calcium surges with slow inward currents.

Strengths:

The study addresses an interesting topic that is only partially understood. The study uses multiple methods including in vivo two-photon microscopy, acute brain slices, electrophysiology, pharmacology, and knockout models. The conclusions are strengthened by the same findings in both in vivo anesthetized mice and in brain slices.

We thank the reviewer for the positive assessment of the study and his/her comments.

Weaknesses:

The method that has been used to quantify astrocytic calcium signals only analyzes what seems to be a small proportion of the total astrocytic domain on the example micrographs, where a structure is visible in the SR101 channel (see for instance Reeves et al. J. Neurosci. 2011, demonstrating to what extent SR101 outlines an astrocyte). This would potentially heavily bias the results: from the example illustrations presented it is clear that the calcium increases in what is putatively the same astrocyte goes well beyond what is outlined with automatically placed small ROIs. The smallest astrocytic processes are an order of magnitude smaller than the resolution of optical imaging and would not be outlined by either SR101 or with the segmentation method judged by the ROIs presented in the figures. Completely ignoring these very large parts of the spatial domain of an astrocyte, in particular when making claims about a spatial threshold, seems inappropriate. Several recent methods published use pixel-by-pixel event-based approaches to define calcium signals. The data should have been analyzed using such a method within a complete astrocyte spatial domain in addition to the analyses presented. Also, the authors do not discuss how two-dimensional sampling of calcium signals from an astrocyte that has processes in three dimensions (see Bindocci et al, Science 2017) may affect the results: if subdomain activation is not homogeneously distributed in the three-dimensional space within the astrocyte territory, the assumptions and findings between a correlation between subdomain activation and somatic activation may be affected.

In order to reduce noise from individual pixels, we chose to segment astrocyte arborizations into domains of several pixels. As pointed out previously, including pixels outside of the SR101-positive territory runs the risk of including a pixel that may be from a neighboring cell, and we chose to avoid this source of error. We agree that the results have limitations from being acquired in 2D instead of 3D, but it is likely to assume the 3D astrocyte is homogeneously distributed and that the 2D plane is representative of the whole astrocyte.

Indeed, no dimensional effects were reported in Bindocci et al, Science 2017. We plan to include a paragraph in the discussion to address this limitation in our study.

> *The experiments are performed either in anesthetized mice, or in slices. The study would have come across as much more solid and interesting if at least a small set of experiments were performed also in awake mice (for instance during spontaneous behavior), given the profound effect of anesthesia on astrocytic calcium signaling and the highly invasive nature of preparing acute brain slices. The authors mention the caveat of studying anesthetized mice but claim that the intracellular machinery should remain the same. This explanation appears a bit dismissive as the response of an astrocyte not only depends on the internal machinery of the astrocyte, but also on how the astrocyte is stimulated: for instance synaptic stimulation or sensory input likely would be dependent on brain state and concurrent neuromodulatory signaling which is absent in both experimental paradigms. The discussion would have been more balanced if these aspects were dealt with more thoroughly.*

Yes, we agree that this is a limitation, and we will acknowledge this is in the discussion.

> *The study uses a heaviside step function to define a spatial 'threshold' for somata either being included or not in a calcium signal. However, Fig 4E and 5D showing how the method separates the signal provide little understanding for the reader. The most informative figure that could support the main finding of the study, namely a ~23% spatial threshold for astrocyte calcium surges reaching the soma, is Fig. 4G, showing the relationship between the percentage of arborizations active and the soma calcium signal. A similar plot should have been presented in Fig 5 as well. Looking at this distribution, though, it is not clear why ~23% would be a clear threshold to separate soma involvement, one can only speculate how the threshold for a soma event would influence this number. Even if the analyses in Fig. 4H and the fact that the same threshold appears in two experimental paradigms strengthen the case, the results would have been more convincing if several types of statistical modeling describing the continuous distribution of values presented in Fig. 4E (in addition to the heaviside step function) were presented.*

We agree with the reviewer that we should add to the paper a discussion for our justification on the use of the Heaviside step function, and plan to include this. We chose the Heaviside step function to represent the on/off situation that we observed in the data. We agree with the reviewer that Fig. 4G is informative and demonstrates that under 23% most of the soma fluorescence values are clustered at baseline. We agree that a similar graph should be included in Fig. 5 as well. We agree that a different statistical model describing the data would be more convincing and also confirmed the spatial threshold with the use of a confidence interval in the text.

> *The description of methods should have been considerably more thorough throughout. For instance which temperature the acute slice experiments were performed at, and whether slices were prepared in ice-cold solution, are crucial to know as these parameters heavily influence both astrocyte morphology and signaling. Moreover, no monitoring of physiological parameters (oxygen level, CO2, arterial blood gas analyses, temperature etc) of the in vivo anesthetized mice is mentioned. These aspects are critical to control for when working with acute in vivo two-photon microscopy of mice; the physiological parameters rapidly decay within a few hours with anesthesia and following surgery.*

We will increase the thoroughness of our methods section. Especially including that body temperature and respiration were indeed monitored throughout anesthesia.