Ca²⁺ signaling in astrocytes from Ip3r2⁻/⁻ mice in brain slices and during startle responses in vivo

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Intracellular Ca²⁺ signaling is considered to be important for multiple astrocyte functions in neural circuits. However, mice devoid of inositol triphosphate type 2 receptors (IP3R2) reportedly lack all astrocyte Ca²⁺ signaling, but display no neuronal or neurovascular deficits, implying that astrocyte Ca²⁺ fluctuations are not involved in these functions. An assumption has been that the loss of somatic Ca²⁺ fluctuations also reflects a similar loss in astrocyte processes. We tested this assumption and found diverse types of Ca²⁺ fluctuations in astrocytes, with most occurring in processes rather than in somata. These fluctuations were preserved in Ip3r2⁻/⁻ (also known as Ip2r2⁻/⁻) mice in brain slices and in vivo, occurred in end feet, and were increased by G protein–coupled receptor activation and by startle-induced neuromodulatory responses. Our data reveal previously unknown Ca²⁺ fluctuations in astrocytes and highlight limitations of studies that used Ip3r2⁻/⁻ mice to evaluate astrocyte contributions to neural circuit function and mouse behavior.

Astrocytes are found throughout the brain and possess thousands of processes in well-delineated astrocyte territories¹,² that form the anatomical basis for interactions with neurons, other glia and blood vessels³. A major open question in neuroscience concerns how astrocytes contribute to the functioning of the brain and to neurological and psychiatric disorders⁴. From this perspective, much attention has focused on the existence and physiological function(s) of astrocyte intracellular Ca²⁺ signals⁵, which we refer to as Ca²⁺ fluctuations.

Early studies showed that astrocytes displayed intracellular Ca²⁺ fluctuations that were both spontaneous and triggered by neurotransmitters⁶. Subsequently, organic Ca²⁺ indicator dyes have been used extensively to study astrocyte Ca²⁺ fluctuations in brain slices and in vivo during various types of pharmacological and sensory stimuli (reviewed in refs. 5,7). In these settings, Ca²⁺ fluctuations were used in a correlative manner with simultaneous measurements from neurons or the vasculature. On the basis of such experiments, evidence indicates that astrocyte Ca²⁺ fluctuations occur during neurotransmitter release and affect neuronal⁸ and neurovascular functions⁹. Taken together, these studies suggest that astrocytes contribute to information processing and neurovascular coupling in addition to their trophic and supportive roles.

Several recent studies have questioned the physiological importance of astrocyte Ca²⁺ signaling based in large part on the use of genetically modified mice in which Ca²⁺ fluctuations were reported to be completely absent in all astrocytes⁹–¹ⁱ. In such studies, the genetic deletion of inositol triphosphate type 2 receptor (IP3R2), which is known to be enriched in astrocytes¹², led to the apparent loss of all astrocyte Ca²⁺ fluctuations, but had no effect on behavioral¹³, neuronal¹⁰ or vascular functions¹⁴–¹⁶, leading the authors to conclude that astrocyte Ca²⁺ fluctuations have no role in these functions. However, other studies utilizing similar or complementary approaches have suggested that astrocyte Ca²⁺ fluctuations are involved in blood vessel and neuronal functions¹⁷–²¹. Overall, a confusing picture has emerged on astrocyte intracellular Ca²⁺ fluctuations and their physiological relevance.

We set out to test the assumption that all astrocyte Ca²⁺ fluctuations are abolished in Ip3r2⁻/⁻ mice. We employed fast genetically encoded Ca²⁺ indicators²² that can be selectively expressed in astrocytes and not in other cells²³,²⁴ without causing astrocyte reactivity in the hippocampus²³,²⁴, striatum²⁵ or cortex²⁶. We also used adult mice²⁶ and studied astrocytes in hippocampal slices to be concordant with past work²⁷,²⁸. To avoid the complications caused by anesthesia²⁷,²⁸, we used two-photon microscopy to study astrocytes in awake, non-anesthetized, head-fixed mice that were free to rest or run on a treadmill. Using this procedure, we assessed how astrocytes responded during startle responses mediated by endogenous norepinephrine release²⁹,³⁰. Finally, we employed objective, semi-automated and standardized data analyses using newly developed software. The combination of these approaches revealed that Ca²⁺ fluctuations were not abolished in astrocytes in Ip3r2⁻/⁻ mice as previously thought. Rather, we found a rich variety of previously unknown IP3R2-independent Ca²⁺ fluctuations in astrocyte processes that responded to pharmacological and sensory stimuli in brain slices and in vivo, respectively.

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RESULTS

Astrocyte spontaneous Ca$^{2+}$ fluctuations in hippocampal slices from Ip3r2$^{-/-}$ mice

We used adeno-associated virus (AAVs) of the 2/5 serotype and the minimal astrocyte-specific GfaABC1D promoter to express cytosolic GCaMP6f in astrocytes located in the CA1 region of the adult mouse hippocampus$^{23,24}$. We harvested hippocampal slices 2 weeks after in vivo virus microinjections, identified single GCaMP6f-expressing astrocytes and imaged Ca$^{2+}$ fluctuations with confocal microscopy from wild-type (WT) and Ip3r2$^{-/-}$ mice ($n = 5$ mice of each genotype; Supplementary Videos 1 and 2). GCaMP6f imaging revealed a large number of Ca$^{2+}$ fluctuations in astrocytes, more than previously observed with organic Ca$^{2+}$ indicator dyes$^{9,10,23,31,32}$, illustrating the utility of GCaMP6f$^{22}$. To analyze Ca$^{2+}$ fluctuations identically and objectively for all cells, we developed GECIquant software that permits rapid semi-automated detection of regions of interest (ROIs) containing Ca$^{2+}$ fluctuations (Supplementary Fig. 1). GECIquant performed a series of user-instigated pixel operations for maximum intensity projection, background subtraction, object thresholding, measurement of object areas, object centroids and distance of the object to the somatic centroid (Supplementary Fig. 1a–f and Supplementary Code). GECIquant was rigorously tested for its ability to faithfully identify, measure (Supplementary Fig. 2), trace (Supplementary Fig. 3) and track fluorescence fluctuations (Supplementary Fig. 4) using fluorescent beads, morphologically complex cells and blinking quantum dots (Supplementary Figs. 1–4). After optimization, we used GECIquant to study GCaMP6f-expressing astrocytes (Supplementary Fig. 1g) that were separated from surrounding GCaMP6f-expressing astrocytes, that is, we focused on single cells. This was possible because of the documented sparse nature of GCaMP fluctuations in hippocampal astrocytes from WT and Ip3r2$^{-/-}$ mice. WT and Ip3r2$^{-/-}$ astrocytes were imaged in vivo using confocal microscopy. The data are shown as mean ± s.e.m.

Figure 1 Ca$^{2+}$ fluctuations in hippocampal astrocytes from WT and Ip3r2$^{-/-}$ mice. (a) Schematic illustrating the experimental approach. (b) Representative images and traces for Ca$^{2+}$ fluctuations measured in an astrocyte from a WT mouse. Three predominant types of Ca$^{2+}$ events are demarcated: somatic fluctuations (green), waves (red) and microdomains (yellow). Approximate territory boundaries are outlined in blue, but these were not used for data analyses and are shown only for illustrative purposes. (c) Data are presented as in b, but for two astrocytes from an Ip3r2$^{-/-}$ mouse. Representative movies are shown as Supplementary Videos 1 and 2. (d–f) Average data for Ca$^{2+}$ fluctuation properties in WT and Ip3r2$^{-/-}$ mice ($n = 15$ and 17 astrocytes WT and Ip3r2$^{-/-}$, and 5 mice each for each). For this and all other figures, statistical comparisons were made using unpaired non-parametric Mann-Whitney or unpaired parametric Student’s t tests as deemed appropriate after analyzing the raw data (Online Methods). The $n$ numbers on d–f refer to the numbers of Ca$^{2+}$ fluctuations for the WT and Ip3r2$^{-/-}$ bars, which were averaged for frequency, amplitude and half-width across all cells. The data are shown as mean ± s.e.m.
expression using AAV2/5 and the GαABC1D promoter\textsuperscript{23,24}.

In the case of WT mouse astrocytes expressing GCaMP6f, we were able to readily identify somatic Ca\textsuperscript{2+} fluctuations, which by definition occurred in the anatomically well-defined cell body and covered an area of 80.4 ± 7.8 \(\mu\)m\textsuperscript{2} (n = 109 somatic fluctuations, 15 cells, 5 mice). However, we also found numerous fluctuations in processes. One type of fluctuation in processes appeared as expanding and contracting local waves that spread between adjacent pixels; we refer to these as waves. The waves spread to an area of 14.8 ± 1.4 \(\mu\)m\textsuperscript{2} and displayed average centroid distances of 26.4 ± 0.7 \(\mu\)m from the somatic centroid (n = 837 waves, 15 cells, 5 mice). The second type of Ca\textsuperscript{2+} fluctuations in processes, which we refer to as microdomains, were restricted in area to 0.7 ± 0.01 \(\mu\)m\textsuperscript{2} and displayed average centroid distances of 29.2 ± 0.2 \(\mu\)m from the somatic centroid (n = 3,500 microdomains, 15 cells, 5 mice). Thus, the distinction between somatic fluctuations, waves and microdomains in processes was based on their detection using GECIquant (Online Methods), differences in their properties (Fig. 1), significant differences in the areas covered by the fluctuations (Supplementary Fig. 5), and their locations in astrocyte cell bodies or processes (Supplementary Fig. 6).

Once detected using GECIquant (Fig. 1a–c), we analyzed the numerous Ca\textsuperscript{2+} fluctuations in WT and Ip3r2\textsuperscript{−/−} astrocytes and quantified their basic properties (Fig. 1). We found that, although the frequency (Fig. 1d), amplitude (Fig. 1e) and duration (Fig. 1f) of somatic Ca\textsuperscript{2+} fluctuations were significantly reduced in Ip3r2\textsuperscript{−/−} mice, the fluctuations were clearly not abolished (P < 0.05 using unpaired Student’s t tests; Fig. 1b–f and Supplementary Video 2). Moreover, we found only a subtle decrease in the frequency of waves, no change in the frequency of microdomains in processes (Fig. 1d) and a ∼50–60% decrease in their amplitude that was accompanied by a significant increase in their duration (P < 0.05 using unpaired Student’s t tests; Fig. 1e,f). Ca\textsuperscript{2+} waves and microdomains detected in processes were located at equivalent distances from the somata in WT and Ip3r2\textsuperscript{−/−} mice (Supplementary Fig. 5). In addition, for the data shown in Figure 1d–f, the number of ROIs per cell were 19 ± 2 and 196 ± 18 for waves and microdomains, respectively, for WT mice. The equivalent numbers for ROIs per cell were 19 ± 2 and 72 ± 6 for waves and microdomains, respectively, for Ip3r2\textsuperscript{−/−} mice. However, the data report frequency per ROI, and differences in the numbers of ROIs between genotypes therefore cannot explain the differences (Fig. 1d). Nonetheless, it should be noted that, although fewer microdomain ROIs were detected in Ip3r2\textsuperscript{−/−} in relation to WT mice (72 ± 6 versus 196 ± 18), microdomain ROIs were clearly not abolished. These analyses showed that Ca\textsuperscript{2+} signaling was not completely absent in all hippocampal astrocytes as previously reported\textsuperscript{9–11,33}. Moreover, from a population of 15 cells from 5 WT and 17 cells from 5 Ip3r2\textsuperscript{−/−} mice we found that most Ca\textsuperscript{2+} fluctuations occurred in processes rather than the somata (Fig. 1). Thus, in WT mice, the overall numbers of Ca\textsuperscript{2+} fluctuations were 4.7 ± 1.6 and 37.7 ± 8.5 fluctuations per cell per min for somata and processes, respectively. In the case of Ip3r2\textsuperscript{−/−} mice, the equivalent numbers were 0.5 ± 0.2 and 15.6 ± 3.6 fluctuations per cell per min for somata and processes, respectively. This is a notable discovery given that past evaluations using Ip3r2\textsuperscript{−/−} mice have been based on measurement and quantification of somata alone\textsuperscript{9–11,33}.

To further verify our measurements, we performed one specific set of analyses to examine Ca\textsuperscript{2+} fluctuations in entire astrocytes by measuring the mean fluorescence intensity values for traces lasting 300 s. For this specific data set, ROIs that approximately encompassed entire astrocytes (that is, territories) were drawn (Fig. 2a,b); note that the drawing of territory ROIs was approximate and only used for the data set in Figure 2c. We reasoned that, if Ca\textsuperscript{2+} fluctuations were absent in Ip3r2\textsuperscript{−/−} mice, then this analysis would reveal significantly lower mean fluorescence values over time in comparison to WT mice, where there was an abundance of Ca\textsuperscript{2+} fluctuations (Fig. 1). In contrast, we found no significant differences in mean fluorescence over 300-s-long traces between WT and Ip3r2\textsuperscript{−/−} mice (P = 0.63 with an unpaired Mann-Whitney test; Fig. 2c), suggesting that, when averaged across entire astrocytes, fluctuations in astrocyte Ca\textsuperscript{2+} are largely preserved in Ip3r2\textsuperscript{−/−} mice (Fig. 2b,c). A similar result was found when we pooled wave and microdomain fluctuations from processes (Fig. 2d). We found only subtle changes in frequency and duration of these pooled events (Fig. 2d). We did detect a halving of the amplitude in Ip3r2\textsuperscript{−/−} mice (P < 0.00001 with an unpaired Mann-Whitney test; Fig. 2d). However, this was accompanied by an increase in the average area of pooled waves and microdomains per astrocyte in Ip3r2\textsuperscript{−/−} mice (Fig. 2d). The areas of the individual types of Ca\textsuperscript{2+} fluctuations are shown in Supplementary Figure 5. Overall, the loss of IP3R2s resulted in substantial, but incomplete, loss of somatic Ca\textsuperscript{2+} fluctuations. In contrast, Ca\textsuperscript{2+} fluctuations in processes were still present and, when carefully assessed by several metrics, existed in clearly observable numbers in Ip3r2\textsuperscript{−/−} mice (Figs. 1 and 2).
Figure 3 Effect of nominally Ca\(^{2+}\)-free buffer applications on astrocyte Ca\(^{2+}\) fluctuations in WT mice. (a) Traces and average data for the effect of nominally Ca\(^{2+}\) free buffers on the basal fluorescence intensity of ROIs corresponding approximately to entire astrocytes. (b–d) Data are presented as in a, but for somatic fluctuations (b) as well as for wave (c) and microdomain (d) fluctuations in astrocyte processes. The averages are across all cells for the ROIs indicated in each set of traces. The data are shown as mean ± s.e.m.

Notably, many Ca\(^{2+}\) fluctuations in processes were also seen in WT mice after depletion of intracellular Ca\(^{2+}\) stores with cyclopiazonic acid (CPA, \(n = 10\) cells from 3 mice; Supplementary Tables 1 and 2). We next directly compared Ca\(^{2+}\) fluctuations observed in Ip3r2\(^{-/-}\) mice with fluctuations in WT mice after CPA. Overall, these two data sets were similar, but not identical (Supplementary Tables 1 and 2). This may suggest the existence of subtle differences in the ability of CPA and Ip3r2\(^{-/-}\) to unmask the signals that are not dependent on intracellular Ca\(^{2+}\) stores. However, the main point is that many signals can be observed in astrocyte processes of WT mice after depletion of stores with CPA, which recalls, but is not identical to, the Ip3r2\(^{-/-}\)/data (Supplementary Tables 1 and 2).

We next addressed the issue of whether the Ca\(^{2+}\) fluctuations we observed in single astrocytes (Fig. 1) may reflect some fluctuations emanating from other nearby astrocytes that also expressed GCaMP6f. This issue cannot be addressed by filling an astrocyte with a fluorescent dye via a patch pipette to demarcate its territory, as the dye will spread to ~50–200 neighboring astrocytes in the CA1 region of mice do not possess long processes that extend beyond their individual territories.

Ca\(^{2+}\) fluctuations in astrocyte processes are partly dependent on transmembrane Ca\(^{2+}\) fluxes

Our data (Figs. 1 and 2) show that a component of astrocyte Ca\(^{2+}\) fluctuations is dependent on Ca\(^{2+}\) release from intracellular stores. We next explored how the Ca\(^{2+}\) fluctuations were affected by the application of nominally Ca\(^{2+}\) free buffers (Fig. 3). Application of Ca\(^{2+}\) free buffers reversibly reduced basal Ca\(^{2+}\) levels measured in entire astrocytes (Fig. 3a). However, Ca\(^{2+}\) free buffer applications did not significantly change the frequency of somatic Ca\(^{2+}\) fluctuations (Fig. 3b), which is consistent with the observation that somatic fluctuations were significantly reduced in the Ip3r2\(^{-/-}\)/mice (Fig. 1). In contrast, for 37 and 52% of wave and microdomain ROIs located in processes, Ca\(^{2+}\) free buffers reversibly reduced the Ca\(^{2+}\) fluctuation frequency (Fig. 3c,d). The frequencies of fluctuations in the remaining 63% of wave and 48% of microdomain ROIs were not markedly affected (Supplementary Fig. 10). These data suggest that transmembrane Ca\(^{2+}\) fluxes contribute substantially to basal Ca\(^{2+}\) levels and to a detectable proportion of waves and microdomains in processes (Fig. 4). Thus, mechanistically, both intracellular Ca\(^{2+}\) release (Fig. 1) and transmembrane entry (Fig. 3) underlie diverse Ca\(^{2+}\) fluctuations in astrocytes. It is interesting to
Figure 4 GPCR-mediated Ca²⁺ fluctuations in astrocyte processes are largely intact in hippocampal slices from Ip3r2⁻/⁻ mice. (a–c) Representative traces and average data for endothelin-evoked Ca²⁺ fluctuations in astrocyte somata from WT and Ip3r2⁻/⁻ mice. (d–f) Data are presented as in a–c, but for astrocyte processes. Five WT and five Ip3r2⁻/⁻ mice were analyzed for these experiments, and paired Student’s t tests were used when comparing before and during endothelin application. The data are shown as mean ± s.e.m.

note that the effect of Ca²⁺ free buffer on waves and microdomains was completely reversible, that is, the fluctuations returned to their normal levels almost exactly (Fig. 3c,d). Thus, Ca²⁺ fluctuations in processes are reproducible and stable over time.

GPCR-evoked Ca²⁺ fluctuations in astrocytes in hippocampal slices from Ip3r2⁻/⁻ mice
We next used endothelin (200 nM) to activate Gq protein–coupled endothelin receptors (ETRs) on astrocytes to elicit Ca²⁺ elevations. We chose endothelin because it was used in past studies and because RNA-seq analysis has shown that ETRs are enriched in astrocytes relative to neurons, thereby minimizing the potential complication of indirect actions via neurons for our evaluations. We measured strong endothelin-evoked intracellular Ca²⁺ fluctuations in the somata in 14 of 18 WT astrocytes. We quantified these data by measuring the areas under the traces before and during endothelin applications (Fig. 4a). When averaged across all 18 WT astrocytes, endothelin significantly elevated somatic Ca²⁺ levels ($P = 0.00487$, paired Student’s $t$ test; Fig. 4b). By repeating similar experiments with astrocytes from Ip3r2⁻/⁻ mice, we found that endothelin failed to evoke significant somatic Ca²⁺ fluctuations from a population of 23 astrocytes ($P = 0.26252$, paired Student’s $t$ test; Fig. 4c and Supplementary Videos 3 and 4). However, it should be noted that 8 of 23 astrocyte somata from Ip3r2⁻/⁻ mice responded significantly to endothelin, with the response increasing from 7.1 ± 1.9 to 7.4 ± 2.3 dF/F.s ($P = 0.0162$, paired Student’s $t$ test; Fig. 4c).

Next, we examined endothelin-evoked Ca²⁺ fluctuations in astrocyte processes from WT and Ip3r2⁻/⁻ mice in vivo. (a) Schematic illustrating the experimental approach for in vivo imaging in fully awake mice free to rest or run on a spherical treadmill (with no anesthesia). (b) Representative images and traces for Ca²⁺ fluctuations measured in a cortical astrocyte from a WT mouse. Three predominant types of Ca²⁺ events are shown: somatic, waves and microdomains. Approximate territory boundaries are outlined in blue, but were not used for data analyses and are shown only for illustrative purposes. (c) Data are presented as in b, but for an astrocyte from an Ip3r2⁻/⁻ mouse. Representative movies are Supplementary Videos 5 and 6. (d–f) Average data for astrocyte Ca²⁺ fluctuation properties from WT and Ip3r2⁻/⁻ mice during in vivo imaging ($n = 12$ astrocytes and 4 mice for each). As stated in the figure, the $n$ numbers refer to the numbers of Ca²⁺ fluctuations for each bar, which were averaged for frequency, amplitude and half-width across all cells in d–f. The data are shown as mean ± s.e.m.
and Ip3r2−/− mice (Fig. 4d–f) and measured significant Ca2+ elevations in the same proportion of cells from WT and Ip3r2−/− mice (~50%, P < 0.05, paired Student’s t test; Fig. 4d–f). This shows that Ip3r2−/− mice retain GPCR-mediated Ca2+ signaling in astrocyte processes that is indiscernible from WT mice. Thus, our data do not support past work suggesting that GPCR-mediated Ca2+ signaling is completely abolished in Ip3r2−/− mice.4,10 Furthermore, given that astrocytes interact with neurons via processes, and because endothelin responses were observed in processes and not in the somata of Ip3r2−/− mice, our data support the idea24,38 that astrocyte somata cannot be used as a proxy measure of Ca2+ signaling in processes (Fig. 4).

Spontaneous Ca2+ fluctuations in cortical astrocytes in vivo from WT and Ip3r2−/− mice

We considered it important to assess astrocyte Ca2+ signaling in vivo to be sure that our observations with hippocampal slices were representative of signaling in intact brain. Recent studies have shown that cortical astrocyte somata display ongoing spontaneous Ca2+ fluctuations and respond with increased Ca2+ fluctuations during in vivo startle responses29,30. We used this experimental procedure for our evaluations. We microinjected AAV2/S GfaABC1D-GCaMP6f virus into the visual cortex of adult mice, implanted glass cranial windows and then, after 2–3 weeks of recovery, used two-photon microscopy to assess cortical astrocytes in full wake, non-anesthetized, head-fixed mice that were free to run or rest on a spherical treadmill (Fig. 5a).

We focused first on detailed analyses of somata and processes. We assessed spontaneous Ca2+ fluctuations in astrocytes in the visual cortex of four WT and four Ip3r2−/− mice (during stationary periods when the mice were not in locomotion29). We observed spontaneous somatic fluctuations as well as waves and microdomains in processes (Supplementary Videos 5 and 6), with properties that largely resembled those observed in hippocampal slices (Figs. 1 and 2). Compared with WT mice, Ip3r2−/− mice displayed markedly fewer Ca2+ fluctuations in the somata, and these fluctuations were reduced in frequency and amplitude relative to WT, although their durations were not substantially different (compared with unpaired Mann–Whitney tests; Fig. 5d–f). In contrast with somatic Ca2+ fluctuations, waves and microdomains in processes were not markedly affected. We observed only ~5% and ~40% decreases in the frequency of microdomains and waves, respectively, and no decreases in their amplitude or duration (Fig. 5d–f). In fact, the amplitudes of microdomain Ca2+ fluctuations were significantly increased compared with those of WT mice (Fig. 5e). Hence, overall Ca2+ fluctuations were reduced largely in the somata of cortical astrocytes and were largely spared in astrocyte processes (Fig. 5). This result is apparent from Supplementary Videos 5 and 6, from the representative traces of Ca2+ fluctuations (Fig. 5b,c) and from the average data with statistical comparisons between WT and Ip3r2−/− mice (Fig. 5d–f). Moreover, the type and subcellular pattern of Ca2+ fluctuations in astrocytes was similar between hippocampal astrocytes in vitro and cortical astrocytes in vivo for both WT and Ip3r2−/− mice. This suggests that the Ca2+ fluctuations were not a result of the method employed to study them.

Startle-evoked Ca2+ fluctuations in cortical astrocytes from WT and Ip3r2−/− mice

Startle-evoked Ca2+ fluctuations that cover all astrocytes in an imaging field of view are mediated by endogenous norepinephrine release from noradrenergic fibers emanating from the locus coeruleus and acting on astrocyte α1 adrenoceptors29,30. We refer to these as global Ca2+ fluctuations to discriminate the terminology from that used for the subcellular Ca2+ fluctuations referred to above. We next tested whether cortical astrocytes of non-anesthetized awake behaving mice responded to startle, which was elicited by a gentle puff of air to the face (Supplementary Video 7). WT and Ip3r2−/− mice were exposed to a brief (3 s) air puff to the face and Ca2+ fluctuations were recorded in GCaMP6f-expressing cortical astrocytes using two-photon microscopy (Fig. 7a). We readily observed startle-evoked increases in global Ca2+ fluctuations in astrocytes from WT mice, which were associated with an increase in the locomotion of the mouse on the spherical treadmill (n = 4 mice of each genotype; Fig. 7). Such startle-evoked global Ca2+ fluctuations encompassed essentially all of the astrocytes in the field of view29 (Fig. 7a). For these analyses, we evaluated astrocyte somatic and process compartments separately.
before, during and after startle responses. The somata were easy to identify and process ROIs were chosen to be within ~40 µm of the cell body. However, because all astrocytes in the field of view responded to startle, it was not possible to approximately demarcate whole astrocytes similar to what we did for the hippocampal slice experiments in Figure 2c. Thus, for the in vivo experiments, we were unable to assess Ca2+ fluctuations in whole astrocytes (that is, territories) and restrict our analyses to ROIs in somata and processes.

Startle-evoked global Ca2+ fluctuations in cortical astrocytes are reported in Supplementary Video 8. These data are presented as still frames before (0–75 s), immediately during (225–300 s) and at two subsequent time periods after the startle (300–375 and 375–450 s) in Figure 7a. The exemplar traces in Figure 7b show locomotion activity on the spherical treadmill along with representative traces for astrocyte somatic and process Ca2+ fluctuations. In the case of somata, fast Ca2+ fluctuations were observed ~3 s after startle, which recalls past work29. These fluctuations decreased over time in the representative traces and lasted, on average, ~14 ± 1 s (Fig. 7a–c). In relation to this, the startle-evoked increase in locomotion lasted 20 ± 10 s (n = 4 mice, range of 3–70 s). Startle-evoked somatic Ca2+ fluctuations were completely abolished in Ip3r2−/− mice (Fig. 7c), but the startle-evoked locomotion lasted 13 ± 4 s (n = 4 mice).

In contrast with somatic fluctuations, the Ca2+ fluctuations measured in astrocyte processes in response to startle were multiphasic. Soon after startle, the response comprised a fast component that displayed a similar time course to the somatic response (that is, it peaked in ~3 s and lasted 13 ± 4 s). However, in processes, this fast component was followed by a delayed response (late component) that leveled off at ~50 s after the startle and was maintained for the duration of the recording and returned back to baseline slowly over 5–10 min, that is, with a time course far in excess of the startle-evoked locomotion of 20 ± 10–s duration. The late component of the process responses could be easily seen in the representative traces and in the average data (Fig. 7b–d). The differences in time course of the fast and late components of the process response (Fig. 7d), as well as the lack of the late component in the somata (Fig. 7c), suggests that the two components may be mediated by distinct mechanisms. Consistent with this, the fast component of the process response was abolished in Ip3r2−/− mice (Fig. 7d). In contrast, the late component was completely unaffected in Ip3r2−/− mice (Fig. 7d).

Supplementary Figure 11 summarizes average differences and statistical comparisons (using paired Mann-Whitney tests) between WT and Ip3r2−/− mice at three time periods relative to baseline for startle responses. Overall, the somatic response was almost completely abolished (Supplementary Fig. 11a), whereas the startle-evoked response in processes was not affected at the time points examined (Supplementary Fig. 11b). These data provide compelling evidence that astrocyte physiological Ca2+ fluctuations persist in vivo in Ip3r2−/− mice.

Prazosin did not inhibit startle-evoked astrocyte process Ca2+ fluctuations in WT or Ip3r2−/− mice

We assessed the effect of the α1-adrenoceptor antagonist prazosin on startle-evoked Ca2+ fluctuations in WT and Ip3r2−/− mice (Fig. 8). Cranial window-implanted WT and Ip3r2−/− mice expressing GCaMP6f in astrocytes were subjected to a 3-s air puff to the face, and baseline startle responses were recorded from cortical astrocytes (n = 4 mice of each genotype). These mice were then injected with prazosin (1 mg per kg of body weight, intraperitoneal) and 30 min later, startle responses were recorded in the same field of view of astrocytes to allow direct comparison of control versus prazosin effects in the same cells (Fig. 8a). Prazosin completely inhibited startle-induced Ca2+ fluctuations in astrocyte somata of WT mice (Fig. 8b), but, as expected, did not alter the lack of Ca2+ fluctuations observed from the somata of Ip3r2−/− mice (Fig. 8c). In the processes of WT astrocytes, prazosin selectively inhibited the fast component of the startle-evoked Ca2+ event and had no effect on the late component (Fig. 8d). Similarly in the Ip3r2−/− mice, we measured no significant effect of prazosin on the astrocyte process Ca2+ fluctuations or on the late component following startle (Fig. 8e). Taken together, our in vivo evaluations show
that a previously unknown late component of the Ca\textsuperscript{2+} fluctuations measured in cortical astrocytes after startle persist in \textit{Ip3r2}\textsuperscript{−/−} mice and are not mediated by α\textsubscript{1}-adrenoceptors or by IP3R2 receptors.

**DISCUSSION**

We used state-of-the-art methods to image and analyze astrocyte Ca\textsuperscript{2+} fluctuations with GCaMP6f in astrocytes in brain slices and \textit{in vivo}. There are four main findings from the work. First, in WT mice, the majority of Ca\textsuperscript{2+} fluctuations occurred in astrocyte processes and not in the somata. Second, Ca\textsuperscript{2+} fluctuations were not abolished in any astrocyte compartment and were not markedly altered in astrocyte processes of \textit{Ip3r2}\textsuperscript{−/−} mice. Third, a model GPCR agonist (endothelin) reliably evoked Ca\textsuperscript{2+} fluctuations in the processes of astrocytes from WT and \textit{Ip3r2}\textsuperscript{−/−} mice, although the somatic response was significantly reduced in \textit{Ip3r2}\textsuperscript{−/−} mice. Fourth, startle-evoked Ca\textsuperscript{2+} fluctuations in astrocyte processes \textit{in vivo} consisted of two phases: an early component mediated by α\textsubscript{1}-adrenoceptors\textsuperscript{29,30} and a late component that was independent of α\textsubscript{1}-adrenoceptors and IP3R2-mediated signaling.

As shown in several recent studies\textsuperscript{23,24,29,39}, genetically encoded calcium indicators (GECIs) are excellent tools for studying astrocyte Ca\textsuperscript{2+} fluctuations and have shed new light on areas of the cells such as processes that have previously been difficult to explore. We used GCaMP6f, which is as fast as organic Ca\textsuperscript{2+} indicator dyes\textsuperscript{22}, although this speed was not crucial for measuring astrocyte Ca\textsuperscript{2+} fluctuations, which lasted hundreds of milliseconds to seconds\textsuperscript{24}. Evaluations have shown that bulk loading is not appropriate for studying the vast majority of an astrocyte's area\textsuperscript{32}. We suggest that the previous reliance on bulk loading of organic Ca\textsuperscript{2+} indicator dyes has underestimated the true extent of astrocyte Ca\textsuperscript{2+} signaling and missed the vast majority of fluctuations that occur in processes. This view is supported by recent studies in which main astrocyte processes\textsuperscript{32,38,40,41} and entire astrocytes\textsuperscript{23,24} have been examined. Data gathered with organic Ca\textsuperscript{2+} indicator dyes has led to the erroneous conclusion that all spontaneous and GPCR-mediated Ca\textsuperscript{2+} signaling is abolished in hippocampal astrocytes in \textit{Ip3r2}\textsuperscript{−/−} mice\textsuperscript{9–11}. Because of this finding, subsequent studies have suggested that astrocyte Ca\textsuperscript{2+} fluctuations have no detectable role in neural functions, even though these conclusions are at odds with data using different approaches\textsuperscript{3,7,8}. However, we found that measuring Ca\textsuperscript{2+} fluctuations in physiological astrocyte compartments is necessary to understand how astrocytes contribute to brain function, an aspect not addressed previously. This is analogous to the need to understand signaling in dendritic spines and nerve terminals, which are distal compartmentalized subcellular structures akin to astrocyte processes.

Our evaluations revealed that the overall pattern of Ca\textsuperscript{2+} fluctuations within astrocytes was similar between hippocampal astrocytes in brain slices and cortical astrocytes \textit{in vivo} for WT and IP3R2\textsuperscript{−/−} mice, implying that the measured Ca\textsuperscript{2+} fluctuations were not the consequence of the method employed to study them. Moreover, wave and microdomain Ca\textsuperscript{2+} fluctuations similar to those observed in IP3R2\textsuperscript{−/−} mice could also be seen in WT mice after intracellular store depletion. Additionally, a significant proportion of wave and microdomain Ca\textsuperscript{2+} fluctuations were due to transmembrane Ca\textsuperscript{2+} fluxes. Thus, careful analysis of WT and \textit{Ip3r2}\textsuperscript{−/−} mice revealed Ca\textsuperscript{2+} fluctuations within astrocyte processes that are IP3R2 independent.

What are the relative contributions of the startle response and locomotion for the observed Ca\textsuperscript{2+} fluctuations? Our data show that the fast component of the Ca\textsuperscript{2+} fluctuations in somata and processes lasted \(~14\) s and thus displayed a similar duration to locomotion\textsuperscript{29}, which lasted \(13–20\) s. However, the late component of the Ca\textsuperscript{2+} fluctuations...
lasted far longer (>5 min) than the 13–20 s-long locomotion evoked by startle. Moreover, cortical astrocytes are directly targeted by locus coeruleus projections29,30,42, but the relatively long (~3 s) latency between startle onset and astrocyte Ca2+ fluctuations in processes and somata seems too slow to be causal for the fast tens-of-milliseconds timescale norepinephrine-mediated responses in cortical neurons43. It seems likely that astrocyte Ca2+ fluctuations may drive slow tens-of-seconds timescale changes in K+ concentration44, regulate blood flow3 via Ca2+-dependent phospholipase A2, control neurotransmitter uptake45,46 or respond to the release of neuromodulators8. We hypothesize that the slow elevations in basal Ca2+ in processes may also regulate the tonic release of D-serine47 and hence set a prolonged time window for NMDA receptor–dependent plasticity to occur in microcircuits that received norepinephrine as a volume transmitter.

Consistent with this proposal, resting D-serine levels in cortex are regulated by astrocytes and the availability of D-serine gates synaptic potentiation48. In this scenario, astrocytes would function as intermediary neuromodulators, that is, they would bridge diffuse norepinephrine volume transmission and its effects on synapses. More generally, exploration of the signaling potential and downstream effects of astrocyte Ca2+ fluctuations requires the development of new methods to abolish all of the types of Ca2+ fluctuations that we have described. As recently discussed2, when this new method is available and rigorously validated, it may then be opportune to determine the effects of total loss of astrocyte Ca2+ fluctuations on blood vessels, neurons and mouse behavior. From these perspectives, rigorous biophysical models and further experiments are necessary to explore astrocyte Ca2+ signaling and to identify the sources of the previously unknown Ca2+ fluctuations that we report, which include intracellular release and extracellular entry.

We conclude that the paucity of data on processes that comprise entire astrocytes has contributed to the current controversies in the field on the relevance of Ca2+ signaling. Our findings call for a necessary degree of caution when interpreting past studies that concluded astrocyte Ca2+ fluctuations had no role in neuronal and blood vessel function9,10,14–16, as those studies relied on the assumption that all Ca2+ signaling was abolished in Ip3r2−/− mice9–11. Our data, gathered in brain slices and in vivo, invalidate this view and reveal a previously unknown form of astrocyte signaling that is independent of both IP3R2 and α1-adrenoceptors and is found in astrocyte processes.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.S. carried out the molecular biology, hippocampal stereotaxic injections and most of the slice experiments with help from A.D.J. and H.C. B.S.H. performed all of the cortical virus injections and cranial window implantations for the in vivo experiments. B.S.H. and R.S. did the in vivo imaging together. S.V. wrote the GEClquant software and R.S. tested it. P.G. shared expertise on in vivo calcium imaging. H.Z. made and shared GCaMP6f knock-in mice. R.S. and B.S.K. analyzed data. B.S.K. directed the experiments, assembled the figures and wrote the paper. All of the authors contributed to the final version.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS
All experimental procedures were approved by the University of California Los Angeles Office for Protection of Research Subjects and the Chancellor's Animal Research Committee. All the mice were housed on a 12-h light-dark cycle with no more than five mice per cage. All of the experiments were done between 9 a.m. and 9 p.m.

Mice, molecular biology and adeno-associated virus. 
Ip3r2−/− mice were obtained from J. Chen (University of California at San Diego) and maintained as a heterozygous line. Homozygotes and WT littermates were used for experiments when they reached age postnatal day 46–67 (P46–P67). Briefly, we removed the CMV promoter flanked by BglII and HindIII sites and replaced it with the minimal (∼700 bp) GfaABC1D astrocyte-specific promoter, which was amplified by PCR from Addgene plasmid #19974. We then cloned GCAmp6f into this modified pZac2.1 vector between EcoRI and XbaI sites to generate plasmids we called pZac2.1 GfaABC1D GCaMP6f. The fully sequenced pZac2.1 plasmids were sent to the Penn Vector Core, which used them to generate AAV2/5 for each construct at a concentration of ∼2 × 1013 genome copies per ml (gc ml−1). All our virus constructs have been deposited at Addgene in the Khakh laboratory repository for unrestricted distribution (http://www.addgene.org/Baljit_Khakh/). The plasmid for pZac2.1 GfaABC1D GCaMP6f has an Addgene ID number of 52925. The AAV is also available for purchase from the UPenn Vector Core.

One specific experiment (Supplementary Fig. 8) used GCaMP6fκx mice (JAX #024105) that were crossed with GLAST-Cre/ERT2 mice (JAX #012586), as reported in detail in previous work23. As a heterozygous line. Homozygotes and WT littermates were used for experiments when they reached age postnatal day 46–67 (P46–P67). To generate a heterozygous line. Homozygotes and WT littermates were used for experiments when they reached age postnatal day 46–67 (P46–P67). To generate as a heterozygous line. Homozygotes and WT littermates were used for experiments when they reached age postnatal day 46–67 (P46–P67).

In vivo microinjections for hippocampal astrocyte imaging in situ. Postnatal day 46–67 (P46–P67) male and female Ip3r2−/− mice and WT littermate mice were used. All surgical procedures were conducted under general anesthesia using continuous isoflurane (induction at 5%, maintenance at 1–2.5%, vol/vol). Depth of anesthesia was monitored continuously and adjusted when necessary. After induction of anesthesia, the mice were fitted into a stereotaxic frame, with their heads secured by blunt ear bars and their noses placed into an anesthesia and ventilation system (David Kopf Instruments). Mice were administered 0.05 ml of buprenorphine (0.1 mg ml−1; Buprenex) subcutaneously before surgery. The surgical incision site was then cleaned three times with 10% povidone iodine and 70% ethanol. Skin incisions were made, followed by craniotomies of 2–3 mm in diameter above the left parietal cortex using a small steel burr (Fine Science Tools) powered by a high speed drill (K.1070; Foredom). Saline (0.9%) was applied onto the skull to reduce heating caused by drilling. Unilateral viral injections were performed by using stereotactic apparatus (David Kopf Instruments) to guide the placement of beveled glass pipettes (World Precision Instruments) into the left hippocampus (2 mm posterior to bregma, 1.5 mm lateral to midline, and 1.6 mm from the pial surface). 1.5 μl AAV2/5 GfaABC1D GCaMP6f (at 2.4 × 1013 gc ml−1) was injected using a syringe pump (Pump11 PicoPlus Elite, Harvard Apparatus). Glass pipettes were left in place for at least 10 min. Surgical wounds were closed with single external 5–0 nylon sutures. After surgery, animals were allowed to recover overnight in cages placed partially on a low voltage heating pad. Buprenorphine was administered two times per day for up to 2 days after surgery. In addition, trimethoprim and sulfamethoxazole (40 and 200 mg, respectively, per 500 ml water) were dispensed in the drinking water for 1 week. Mice were killed at 14–21 days after surgery for imaging (typically 14 days). We chose this period because generally it takes 2–3 weeks to achieve GECI expression in cells by AAV infection and because of past experiences23,24,47.

Preparation of brain slices and confocal Ca2+ imaging. Coronal slices of hippocampus (300 μm) were cut in solution comprising (mM): 87 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 25 d-glucose, 7 MgCl2 and 0.5 CaCl2, saturated with 95% O2 and 5% CO2. Slices were incubated at ∼34 °C for 30 min and subsequently stored at 21–23 °C in artificial cerebrospinal fluid (aCSF) comprising (mM): 126 NaCl, 2.5 KCl, 1.3 MgCl2, 10 d-glucose, 2.4 CaCl2, 1.24 NaH2PO4, and 26 NaHCO3, saturated with 95% O2 and 5% CO2. All other slice procedures were exactly as described previously23. All imaging was performed using commercially available off-the-shelf and standard confocal microscopes. In brief, cells were mostly imaged using a confocal microscope (Fluoview 300; Olympus) with a 40× water-immersion objective lens with a numerical aperture of 0.8, and a few cells were imaged with another confocal microscope (Fluoview 1000; Olympus) using the same lens. We used the 488-nm line of an Argon laser, with the intensity adjusted to 0.5–5% of the maximum output, which was 16.9 mW in the case of the Fluoview 300 and 10 mW in the case of the Fluoview 1000. The emitted light pathway consisted of an emission high pass filter (>510 nm) before the photomultiplier tube. These settings were chosen based on the known properties of GCMPs23. Astrocytes were selected from the CA1 stratum radiatum region and were typically ∼40 μm from the slice surface. Endothelin was applied in the bath at ∼3 ml min−1 using a peristaltic pump. In the case of the CPA experiments, baseline movies without CPA were acquired at 1 frame per s for 5 min. CPA was applied at 20 μM for 30 min, after which a second movie of the same cell was acquired in the presence of CPA at 1 frame per s for 5 min. For the Ca2+ free experiments shown in Figure 3, the conditions were exactly as described above except that no Ca2+ was added to the Ca2+ free buffer.

Head-bar installation, virus injection, and cranial window implantation for in vivo imaging. Adult (P46–67) male and female Ip3r2−/− mice and WT littermates were anesthetized with isoflurane (4% for induction, 1–1.5% vol/vol for maintenance) and placed in a stereotaxic frame (Kopf), with body temperature kept at ∼37 °C with a feedback-controlled heating pad (Harvard Apparatus). After removing the scalp and clearing the skull of connective tissues, a custom-made lightweight metal head-bar was fixed onto the skull with cyanoacrylate adhesive (Krazy Glue) and covered with black dental cement (Ortho-Jet). A circular craniotomy (3-mm diameter) was then performed above the primary visual cortex V1 (centered at −2.5 mm lateral from lambda). With the skull opened and the dura intact, the AAV2/5 GfaABC1D GCaMP6f virus was injected at two sites (1.5 μl each) near the center of the craniotomy, at a depth of 150–200 μm. After the injections, a glass cranial window consisting of a 3-mm diameter round #1 coverslip was implanted in the craniotomy, flush with the skull surface, and sealed in place using tissue adhesive (Vetbond). The exposed skull surrounding the cranial window was then completely covered with black dental cement to build a small chamber for imaging with a water-immersion objective. After surgery, animals were returned to their home cages for 2–3 weeks for recovery and viral gene expression before subjecting to imaging experiments. Extreme care was taken to ensure that the dura experienced no damage or major bleeding before and after cranial window implantation. Mice with damaged dura or unclear window were discarded and not used for imaging experiments.

In vivo two-photon imaging and mouse movement tracking. Two-photon laser-scanning microscopy was performed with a moveable objective microscope (Sutter MOM) using a Ti-Sapphire laser (Coherent Ultra II) at 920 nm, through a 40× 0.8 NA water-immersion objective (Olympus). The objective was mounted at a tilt of 30 degrees to the vertical axis in order to image with the light path perpendicular to the cranial window and the cortical surface. Images were acquired using the ScanImage software (Vidrio Technologies)29 and processed with ImageJ (NIH). Fully awake mice, without any anesthesia, were mounted on top of a spherical treadmill by securing its head-bar onto a custom-made head-bar holder under the microscope. The treadmill consisted of an 8-inch diameter Styrofoam ball resting inside another Styrofoam hollow half-sphere (Graham Sweet Studios) into which a constant stream of compressed air was blown to keep the ball afloat, allowing mice to freely run or rest on top. Images were acquired every 750 ms (1.33 Hz). To track the animals' locomotion, the treadmill motion was measured every 25 ms (40 Hz) by a custom-designed optical sensor whose signals were converted into two servo pulse analog signals (front-back and left-right) using an external PIC microcontroller. The locomotion data were acquired simultaneously with the calcium imaging data and synchronized through the scanning mirror signals. These analog signals were digitized with a NIDAQ board...
and the selection was added to the ImageJ ROI manager. Note that the assignment of territory was approximate and was not used for analysis except for the specific data set shown in Figure 2b. All ROIs falling within the range of 30 µm² to infinity inside the polygon selection were detected by GEClquant and added to the ROI manager. An area range of 30 µm² to infinity allowed detection of the astrocyte soma in all cases. The resulting detection was visually checked in every case.

To detect wave and microdomain ROIs, we first demarcated and deleted the soma from original image stacks using the clear selection feature in ImageJ. This was done because the increased basal fluorescence from the astrocyte soma relative to the processes prevented accurate thresholding of images for detection of ROIs in astrocyte processes. The ROI detection module in GEClquant was launched and the microdomain ROI option was selected. Microdomains and expanding wave ROIs were detected in separate analysis sessions. We used an area range of 0.5–4 µm² to detect microdomains and an area range of 5–2,000 µm² for waves. These values were chosen after initial examination of the movie frames and by using several initial ‘best guess’ test values as a guide. Other researchers who use GEClquant will also need to invest time initially to try several best guess values as a way to know what values will work best for the particular cell and fluctuation they are interested in measuring. The values we report here were appropriate for our experiments. For ROI detection, GEClquant generated a temporal maximum intensity projection image from the provided image stack with the deleted cell body. The projection image was manually thresholded by the user and a polygonal selection was manually drawn around the astrocyte of interest. GEClquant automatically detected microdomain and expanding wave ROIs based on the provided area criteria and the ROIs were added to the ImageJ ROI manager. Intensity values for each ROI were extracted in ImageJ and converted to dF/dF values. For each ROI, basal F was determined during 50-s periods with no fluctuations. MiniAnalysis 6.0.07 (Synaptosoft) software was used to detect and measure amplitude, half width and frequency values for the somatic, wave and microdomain transients.

We comment on how we analyzed data for the experiments shown in Figure 2. First, for the analyses shown in Figure 2c, we made approximate ROIs that encompassed whole territories and then plotted the intensity of these regions over 300 s. From these traces, we measured the mean fluorescence intensity values over the 300-s period for each cell, and then averaged these values across all cells to generate the graphs in Figure 2c for WT and Ipsi3zr+/- mice. In the case of the graphs shown in Figure 2d, we pooled the individual microdomain and wave Ca²⁺ fluctuations per cell, obtained the average value per cell of these pooled fluctuations and repeated this procedure for all cells. Then we averaged across all cells to generate the graphs that are shown in Figure 2c for WT and Ipsi3zr+/- mice.

Graphs were made with Origin 8.1 and the figures assembled in CorelDraw 12 (Corel). No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those generally employed in the field. Randomization and blinding was not employed. Statistical comparisons were made using unpaired non parametric Mann-Whitney or unpaired parametric Student’s t tests as deemed appropriate after analyzing the raw data to ascertain whether they were normally-distributed using the Dallal and Wilkinson approach to Lilliefors’ method within Instat. When a statistical test was used, the precise P value and the test employed are reported in the text and/or figures legends. If the P was less than 0.00001, then it is reported as P < 0.00001. Otherwise, precise P values are provided in each case.

A Supplementary Methods Checklist is available.

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