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In Vivo Imaging of Ca²⁺ Signaling in Astrocytes Using Two-Photon Laser Scanning Fluorescent Microscopy

Shinghua Ding

Abstract

Astrocytes are the predominant nonneuronal cell type in the central nervous system. Although they are electrically nonexcitable, they have been found to play an active role in modulation of neuronal function and plasticity through Ca²⁺ excitability. Thus, Ca²⁺ signaling in astrocytes serves as a mediator of bidirectional interactions between neurons and astrocytes. Although astrocytic Ca²⁺ signaling has been extensively studied in cultured cells, the recent development of two-photon laser scanning fluorescent microscopy and astrocyte-specific dye labeling make it possible to study astrocytic Ca²⁺ signaling in live animals. Here we describe a detailed protocol for in vivo Ca²⁺ imaging of astrocytes in mice.

Keywords

Two-photon microscopy; Ca²⁺ imaging; GPCR; Craniotomy; Fluo-4 AM

1. Introduction

Astrocytes are the predominant glial cell type in the central nervous system (CNS) (1, 2). They provide nutritional and structural support for neurons, and act as a K⁺ sink to maintain extracellular K⁺ homeostasis (3). In addition to these passive roles, it is also established that they can remove glutamate from the synaptic cleft through glutamate transporters, thus avoiding glutamate toxicity (4, 5). The discovery that astrocytes can mediate Ca²⁺ signaling suggests that they could play even more active roles in the CNS (6). Astrocytic Ca²⁺ signaling can be stimulated in a variety of ways including neuronal input, direct G-protein-coupled receptor (GPCR) activation, mechanical stimulations, photolysis of caged glutamate, and inositol 1,4,5-triphosphate (IP₃) (7). Spontaneous excitation of astrocytes has been observed in vivo using 2-photon microscopy (8, 9). Our own and other results show that under normal conditions, cortical astrocytes in adult mice exhibit low frequency of Ca²⁺ oscillations which are confined to the microdomains in the cellular processes (10, 11). Pharmacological or sensory stimulations can induce intercellular Ca²⁺ waves in astrocytes within a brain slice and in vivo (6, 10–18). Although few studies have been done on the properties of Ca²⁺ signaling in vivo under pathological situations, our studies and others demonstrate cell-wide Ca²⁺ signals and intercellular waves in astrocytes following status epilepticus, ischemia and in a mouse model of Alzheimer's disease (10, 19, 20). Ca²⁺ signaling in astrocytes is now considered to be a primary form of cellular excitability that can be determined by fluorescent imaging using a Ca²⁺ indicator.

Astrocytes generally employ metabotropic receptors that are coupled with G_{q/11} to activate phospholipase C (PLC) to liberate IP₃ that activates IP₃ receptor (IP₃R) to release Ca²⁺ from the internal store (1, 21, 22). Among the three types of IP₃R (IP₃R1-3), IP₃R2 is

primarily expressed and is the predominant IP₃R in astrocytes in the rodent brain (23–25). IP₃R2 knock-out (IP₃R2 KO) mice do not exhibit GPCR agonist-evoked increase in astrocytic Ca²⁺, thus providing compelling evidence that IP₃R2 is a key mediator of astrocytic intracellular Ca²⁺ release (21). Astrocytes express a variety of GPCRs, e.g., glutamate, gamma-aminobutyric acid (GABA), adenosine-5'-triphosphate (ATP), serotonin, norepinephrine, and dopamine, which can all mediate astrocytic Ca²⁺ signaling through the PLC/IP₃ pathway by activation of their respective receptors, including P2Y receptors, metabotropic glutamate receptors (mGluRs), GABA_B receptors and dopamine receptors (26, 27). A number of studies in cultured astrocytes (28) as well as in acute brain slice preparations (12, 29, 30) have linked the increase in astrocytic Ca²⁺ to the release of chemical transmitters. Thus, Ca²⁺ signaling in astrocytes serves as a mediator of bidirectional interactions between neurons and astrocytes.

Although astrocytic Ca²⁺ signaling has been extensively studied in cultured cells, relatively few studies have been done in vivo. The recent development of two-photon laser scanning fluorescent microscopy and astrocyte-specific dye labeling make it possible to study the Ca²⁺ signaling and structure of astrocytes in live animals (8, 10, 11, 19, 31, 32). This chapter describes a detailed protocol for in vivo imaging of Ca²⁺ signaling in astrocytes.

2. Materials

2.1. Animals

Adult FVB/NJ or C57/6BJ mice (5–7 weeks of age) were purchased from The Jackson Laboratory. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the University Of Missouri Office Of Animal Care Quality Assurance.

2.2. Chemicals and Reagents

1. Urethane, at a working concentration of 200 mg/mL in artificial cerebral spinal fluid (ACSF). Dose: 1.75–2 mg/g body weight.
2. Artificial cerebral spinal fluid (ACSF): 120 mM NaCl, 3.1 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, and 10 mM Hepes (pH 7.4).
3. Fluorescent dyes: Sulforhodamine 101 (SR101), Calcium indicator fluo-4 AM, and Dextran-Rhodamine (all from Invitrogen).
4. Low melting point agarose.
5. Pluronic F-127 (Sigma).
6. Dexamethasone sodium phosphate (APP Pharmaceuticals LLC). Working solution 4 mg/mL. Dose: 20 µL per mouse.
7. Buprenorphine (Henryschein), working solution: 2 mg/mL in saline. Dose: 0.5 mg/kg body weight.
8. Vaseline or eye ointment.

2.3. Surgical Tools

1. High-speed micro drill (Fine Science Tools).
2. Trephine with side opening for clearing of material. 2.3 mm shaft diameter, 44 mm overall length (Fine Science Tools).
3. Self-regulating heating pad.

4. Dumont forceps with fine tip.
5. Straight stainless steel 12 cm surgical scissors.
6. Cyanoacrylate glue (3M Vetbond Adhesive, World Precision Instruments).
7. Glass coverslips, 0.13–0.17 mm thick.
8. Custom-made metal frame (see Fig. 1) to which brain is attached (with glue) and fixed on microscope stage.
9. Dissecting stereomicroscope.

2.4. Two-Photon Microscope

Previously, two-photon microscopes usually were custom made in the laboratory. They are now commercially available from several companies. Usually, the upright fluorescence microscope is used for in vivo imaging. Two photomultiplier tubes (PMT) are generally installed for detecting green and red fluorescence, achieved by using a 575LP nm dichroic mirror. 40×/60× long working distance water-immersion objectives are suitable for in vivo imaging. We use a 60×, NA0.9 Olympus water-immersion objective for astrocytic Ca^{2+} imaging.

3. Methods

3.1. Cranial-Window Surgery

1. To prevent or reduce brain edema, dexamethasone is usually injected 30 min prior to surgery.
2. Anesthetize mouse with an intraperitoneal (IP) injection of urethane (1.5–2.0 mg/g body weight) dissolved in ACSF.
3. After mouse reaches a surgical level of anesthesia, shave the fur over the scalp.
4. Using scissors, make an incision in the midline of the scalp and remove a flap of skin (see Note 1).
5. Using a high-speed drill over the somatosensory cortex at coordinates -0.8 mm from bregma and 2.0 mm lateral to the midline, perform a circular craniotomy 2.0 mm in diameter (see Fig. 2b) (see Note 2).
6. Attach the custom-made metal frame (see Fig. 1) to the skull with cyanoacrylate glue, then carefully remove the dura with fine forceps under a dissecting stereomicroscope (see Note 3).
7. Add a drop of ACSF solution over the cranial window (see Note 4).

3.2. Fluorescent Dye Labeling of Astrocytes In Vivo

3.2.1. Fluo-4 Labeling of Astrocytes In Vivo—To load the Ca^{2+} indicator fluo-4 into astrocytes, 50 μg acetoxymethyl (AM) ester of fluo-4 (i.e., fluo-4 AM) is dissolved in 5 μL

¹ Ensure that the mouse has reached the surgical level of anesthesia by pinching the tail.

² Bone debris on the trephine drill bit should be removed regularly, and the bit should be replaced when it is performing poorly. The drill must be adequately charged for high-speed operation. To avoid heat generated during drilling, ACSF must be intermittently added in the drilling region.

³ Extreme care must be taken at all times not to damage the cortical tissue, especially during the removal of the dura. Good quality of the cranial window is necessary to insure that dyes can be loaded into astrocytes in the following steps. Fine-tipped forceps are required for removing the dura. Be sure the tip does not touch hard surfaces. If the tip is bent or blunt, it can be sharpened using a sharpening stone.

⁴ To avoid drying of the brain tissue after removal of the dura, add a drop of ACSF.

20% DMSO solution of Pluronic F-1287 (e.g., 0.2 mg of Pluronic F-127 in 1 mL DMSO) to obtain a 10 $\mu\text{g}/\mu\text{L}$ stock solution. This stock solution (2.5 μL) is mixed with 40 μL ACSF and following the craniotomy, is applied to the dura-free cortical surface for 1 h (see Note 5). The residue dye is then washed away with ACSF. This procedure leads to the selective labeling of astrocytes with fluo-4 (see Fig. 3) (8–10, 19). After dye labeling, a glass coverslip is glued over the cranial window on the metal frame, and the gap between glass and cranial window is filled with 2% agarose premelted in ACSF solution. This greatly reduces movement artifacts resulting from respiration and heartbeat. The mouse is then transferred to the stage of a two-photon microscope for in vivo imaging.

3.2.2. SR101 Labeling of Astrocytes In Vivo—Selectivity of labeling of astrocytes can be confirmed using sulforhodamine101 (SR101). In this procedure, 100 μL of 100 μM SR101 dissolved in ACSF is applied on the cortical surface in craniotomy for 1–5 min and then is washed away with ACSF. After 40–60 min, astrocytes are selectively labeled with SR101. Using two wide-field detectors, co-labeling of astrocytes with fluo-4 and SR101 can be confirmed (see Fig. 3) (see Note 6).

3.3. In Vivo Ca^{2+} Imaging in Astrocytes

After dye labeling, the mouse is transferred to the stage of microscope for imaging. The cranial window is covered by ACSF for objective immersion. Time-lapse imaging is performed to monitor Ca^{2+} oscillation in astrocytes. Astrocytes usually exhibit low frequency Ca^{2+} oscillations in anesthetized mice under normal conditions (10, 19, 33) (see Note 7). ATP can trigger robust Ca^{2+} oscillation and waves. Iontophoretic application of ATP induces a synchronous Ca^{2+} elevation in a number of astrocytes (34), while in the continuous presence of ATP, astrocytes exhibit synchronous and regenerative Ca^{2+} oscillations as waves (10, 19). For ATP stimulation, we apply 0.5 mM ATP in ACSF to the cortex. The cranial window is then filled with 2% agarose containing 0.5 mM ATP. ACSF containing the same concentration is applied on the surface of the solidified agarose before imaging. Imaging is usually performed on astrocytes 80–100 μm below the cortical surface within 15–60 min after ATP administration. Multiple time-lapse imaging is performed to monitor Ca^{2+} signals for a period of 7.5 min, with acquisition rates of one image in every 2 s. For each mouse, 15–25 astrocytes in 4–5 fields are imaged and all astrocytes imaged are used for analysis. Throughout the experiment (about 3–4 h from the beginning of surgery to the end of imaging), the mouse is maintained at 37°C using a heating pad and at a surgical level of anesthesia. If the preparation is good, one should see bright astrocytes labeled with fluo-4 and repetitive and synchronous Ca^{2+} oscillations in astrocytes can be observed (see Fig. 4).

Some experiments require the relocation of individual astrocytes to determine whether the addition of agonists and antagonists affects astrocytic Ca^{2+} oscillations. Because of the three-dimensional nature of the cortex, it is difficult to identify and relocate the same astrocytes previously imaged in the other fields or at different depths. However, this relocation can be achieved using vasculature as landmarks. To label vasculature, we inject Dextran-Rhodamine (200 μL of 20 mg/mL solution), which highlights blood plasma, into the tail vein (see Fig. 5a, b). This approach can relocate the same astrocytes so that we are

⁵For fluo-4 loading in astrocytes, dura removal is required as fluo-4 AM cannot penetrate the dura. During labeling, cover the cranial window with parafilm to prevent evaporation of dye solution and examine frequently to determine whether the dye is dried. Pressure injection of dye can be used for labeling fluo-4 both in astrocytes and neurons. Details can be found in refs. (10, 35). Usually SR101 will be co-labeled to distinguish astrocytes from neurons although astrocytes and neurons are morphologically different.

⁶For SR101 loading in astrocytes, it is not necessary to remove dura since SR101 can readily penetrate the dura.

⁷Minimal laser power should be used to avoid photobleaching and phototoxicity during time-lapse imaging.

able to image individual astrocytes in different brain regions before and after pharmacological agents are administered.

3.4. Analysis of Astrocytic Ca^{2+} Signal

The fluorescent signals can be quantified by measuring the mean pixel intensities of the cell body of each astrocyte using MetaMorph software (Universal Imaging, CA). Ca^{2+} changes are expressed as $\Delta F/\text{Fo}$ values vs. time, where Fo is the background subtracted baseline fluorescence. To calculate the magnitude of Ca^{2+} signals without subjective selection of threshold values, we integrate the $\Delta F/\text{Fo}$ signal over the imaging period using Origin software (OriginLab Corporation, MA). The resulting value is expressed as $\Delta F/\text{Fo}$ s. Data collected from multiple cells from each individual mouse are averaged and the averaged value of these cells is used as a single value for that mouse. The summary data should be the average value from four to five mice.

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References

1. Agulhon C, Petravic J, McMullen AB, Sweiger EJ, Minton SK, Taves SR, Casper KB, Fiacco TA, McCarthy KD. What is the role of astrocyte calcium in neurophysiology? *Neuron*. 2008; 59:932–946. [PubMed: 18817732]
2. Barres BA. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron*. 2008; 60:430–440. [PubMed: 18995817]
3. Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD. Conditional Knock-Out of Kir4.1 Leads to Glial Membrane Depolarization, Inhibition of Potassium and Glutamate Uptake, and Enhanced Short-Term Synaptic Potentiation. *J. Neurosci*. 2007; 27:11354–11365. [PubMed: 17942730]
4. Huang YH, Bergles DE. Glutamate transporters bring competition to the synapse. *Current Opinion in Neurobiology*. 2004; 14:346–352. [PubMed: 15194115]
5. Bergles DE, Diamond JS, Jahr CE. Clearance of glutamate inside the synapse and beyond. *Current Opinion in Neurobiology*. 1999; 9:293–298. [PubMed: 10395570]
6. Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*. 1990; 247:470–473. [PubMed: 1967852]
7. Fiacco TA, Agulhon C, McCarthy KD, Fiacco TA, Agulhon C, McCarthy KD. Sorting out astrocytes physiology from pharmacology. *Annu Rev. Pharmacol. Toxicol*. 2009; 49:151–174. [PubMed: 18834310]
8. Hirase H, Qian L, Bartho P, Buzsaki G. Calcium dynamics of cortical astrocytic networks in vivo. *Plos Biology*. 2004; 2:E96. [PubMed: 15094801]
9. Nimmerjahn A, Kirchhoff F, Kerr JN, Helmchen F. Sulforhodamine 101 as a specific marker of astroglia in the neocortex in vivo. *Nature Methods*. 2004; 1:31–37. [PubMed: 15782150]
10. Ding S, Fellin T, Zhu Y, Lee SY, Auberson YP, Meaney DF, Coulter DA, Carmignoto G, Haydon PG. Enhanced Astrocytic Ca^{2+} Signals Contribute to Neuronal Excitotoxicity after Status Epilepticus. *J. Neurosci*. 2007; 27:10674–10684. [PubMed: 17913901]
11. Wang X, Lou N, Xu Q, Tian GF, Peng WG, Han X, Kang J, Takano T, Nedergaard M. Astrocytic Ca^{2+} signaling evoked by sensory stimulation in vivo. *Nature Neuroscience*. 2006; 9:816–823.
12. Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron*. 2004; 43:729–743. [PubMed: 15339653]
13. Charles AC, Merrill JE, Dirksen ER, Sanderson MJ. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron*. 1991; 6:983–992. [PubMed: 1675864]

14. Dani JW, Chernjavsky A, Smith SJ. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron*. 1992; 8:429–440. [PubMed: 1347996]
15. Chuquet J, Hollender L, Nimchinsky EA. High-Resolution In Vivo Imaging of the Neurovascular Unit during Spreading Depression. *J. Neurosci*. 2007; 27:4036–4044. [PubMed: 17428981]
16. Bowser DN, Khakh BS. Vesicular ATP Is the Predominant Cause of Intercellular Calcium Waves in Astrocytes. *J. Gen. Physiol*. 2007; 129:485–491. [PubMed: 17504911]
17. Schummers J, Yu H, Sur M. Tuned Responses of Astrocytes and Their Influence on Hemodynamic Signals in the Visual Cortex. *Science*. 2008; 320:1638–1643. [PubMed: 18566287]
18. Petzold GC, Albeanu DF, Sato TF, Murthy VN. Coupling of neural activity to blood flow in olfactory glomeruli is mediated by astrocytic pathways. *Neuron*. 2008; 58:897–910. [PubMed: 18579080]
19. Ding S, Wang T, Cui W, Haydon PG. Photothrombosis ischemia stimulates a sustained astrocytic Ca^{2+} signaling in vivo. *GLIA*. 2009; 57:767–776. [PubMed: 18985731]
20. Kuchibhotla KV, Goldman ST, Lattarulo CR, Wu HY, Hyman BT, Bacskaï BJ. Abeta plaques lead to aberrant regulation of calcium homeostasis in vivo resulting in structural and functional disruption of neuronal networks. *Neuron*. 2008; 59:214–225. [PubMed: 18667150]
21. Petravic J, Fiacco TA, McCarthy KD. Loss of IP₃ Receptor-Dependent Ca^{2+} Increases in Hippocampal Astrocytes Does Not Affect Baseline CA1 Pyramidal Neuron Synaptic Activity. *J. Neurosci*. 2008; 28:4967–4973. [PubMed: 18463250]
22. Haydon PG. GLIA: listening and talking to the synapse. *Nature Reviews Neuroscience*. 2001; 2:185–193.
23. Hertle DN, Yeckel MF. Distribution of inositol-1,4,5-trisphosphate receptor isotypes and ryanodine receptor isotypes during maturation of the rat hippocampus. *Neuroscience*. 2007; 150:625–638. [PubMed: 17981403]
24. Holtzclaw LA, Pandhit S, Bare DJ, Mignery GA, Russell JT. Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. *GLIA*. 2002; 39:69–84. [PubMed: 12112377]
25. Sharp AH, Nucifora FCJ, Blondel O, Sheppard CA, Zhang C, Snyder SH, Russell JT, Ryugo DK, Ross CA. Differential cellular expression of isoforms of inositol 1,4,5-trisphosphate receptors in neurons and glia in brain. *Journal of Comparative Neurology*. 1999; 406:207–220. [PubMed: 10096607]
26. Haydon PG, Carmignoto G. Astrocyte Control of Synaptic Transmission and Neurovascular Coupling. *Physiol. Rev*. 2006; 86:1009–1031. [PubMed: 16816144]
27. Ni Y, Malarkey EB, Parpura V. Vesicular release of glutamate mediates bidirectional signaling between astrocytes and neurons. *Journal of Neurochemistry*. 2007; 103:1273–1284. [PubMed: 17727631]
28. Parpura V, Basarsky TA, Liu F, Jęftinija K, Jęftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. *Nature*. 1994; 369:744–747. [PubMed: 7911978]
29. D'Ascenzo M, Fellin T, Terunuma M, Revilla-Sanchez R, Meaney DF, Auberson YP, Moss SJ, Haydon PG. mGluR5 stimulates gliotransmission in the nucleus accumbens. *PNAS*. 2007; 104:1995–2000. [PubMed: 17259307]
30. Angulo MC, Kozlov AS, Chrapak S, Audinat E. Glutamate released from glial cells synchronizes neuronal activity in the hippocampus. *J. Neurosci*. 2004; 24:6920–6927. [PubMed: 15295027]
31. Anderson CM, Nedergaard M. Astrocyte-mediated control of cerebral microcirculation. *Trends in Neurosciences*. 2003; 26:340–344. [PubMed: 12850427]
32. Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. Astrocyte-mediated control of cerebral blood flow. *Nat Neurosci*. 2006; 9:260–267. [PubMed: 16388306]
33. Tian GF, Takano T, Lin JHC, Wang X, Bekar L, Nedergaard M. Imaging of cortical astrocytes using 2-photon laser scanning microscopy in the intact mouse brain. *Advanced Drug Delivery Reviews*. 2006; 58:773–787. [PubMed: 17045697]
34. Tian GF, Azmi H, Takano T, Xu Q, Peng W, Lin J, Oberheim N, Lou N, Wang X, Zielke HR, Kang J, Nedergaard M. An astrocytic basis of epilepsy. *Nature Medicine*. 2005; 11:973–981.
35. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. In vivo two-photon calcium imaging of neuronal networks. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:7319–7324. [PubMed: 12777621]

Fig. 1. The custom-made metal frame. The mouse brain is attached to the frame with cyanoacrylate glue. The depth of chamber for retaining ACSF solution is 1 mm. The thickness of the plate is 1.5mm.

Fig. 2.

Schematic diagram of in vivo 2-P imaging. **(a)** Diagram of the optical pathway of the Ultima dual scanning 2-P microscope. Two Ti:Sapphire laser sources: one can be tuned to 720 nm for photolysis and the other to 820 nm to excite Fluo-4 for calcium imaging. A scan control system allows performing 2-P imaging and photolysis simultaneously. **(b)** In vivo 2-P fluorescent imaging. *Left*: The head of mouse was attached to a metal plate beneath objective for 2-P imaging. *Right*: A craniotomy was performed on the mouse cortex, where fluorescent dyes can be loaded and imaging can be performed.

Fig. 3. Selective labeling of astrocytes with calcium indicator fluo-4 using surface incubation method. Astrocytes were labeled with the astrocyte-specific dye SR101 (*left*) and fluo-4 (*middle*). *Right panel* shows the merged image. The image is modified from Ding et al. (19), and is used with permission of *Glia*.

Fig. 4.

In vivo imaging of Ca^{2+} signaling in astrocytes induced by ATP stimulation. **(a)** Representative images showing the fluo-4 fluorescence changes in response to 0.5 mM ATP. **(b)** Time courses of fluo-4 fluorescence ($\Delta F/F_o$) *in vivo* from somata of individual astrocytes indicated by the numbers in **(a)** where $t = 20$ s, in the presence of 0.5 mM ATP. The *boxed region* is corresponding to the images in **(a)**. Notice that Ca^{2+} signals are highly synchronized among the astrocytes.

Fig. 5.

Vasculature as landmarks to relocate the same astrocytes. (a) 3D construction with maximum projection of vasculature loaded with Dextran-Rhodamine. (b) A color-combined image from single frame images of vasculature (*red*) and astrocytes (*green*) labeled with fluo-4 AM showing the relative positions of vasculature and astrocytes. (c, d) shows the fluo-4 fluorescent images of the same astrocytes before (c) and after (d) they were labeled with SR101. With vasculature as landmarks, it is easy to identify the same astrocytes even after the animal is reattached to the imaging platform after drug administration.