In vivo calcium imaging of cerebellar astrocytes with synthetic and genetic indicators

Kuhn[1], Bernd

Hoogland[1], Tycho M.

Wang[1,2], Samuel S.-H.

[1] Department of Molecular Biology and Princeton Neuroscience Institute, Princeton University, Princeton, NJ 08544, USA.

[2] Corresponding author (sswang@princeton.edu)

ABSTRACT

The two astrocyte types of the cerebellar cortex are Bergmann glia of the molecular layer and velate protoplasmic astrocytes of the granule cell layer. In vivo, these cell types generate both subcellular calcium transients and transglial calcium waves. In the molecular layer, transglial waves have unusual radial symmetry, encompassing many astrocyte processes in a near-ellipsoidal volume without filling any one cell in full. Here we describe two protocols to study glial signals using synthetic and genetically encodable calcium indicators. Multicell bolus loading (MCBL) in the molecular layer of the synthetic calcium indicators fluo-5F/AM and fluo-4/AM preferentially labels Bergmann glia. Because bolus loading tends to additionally label neurons, we have also used the fluorescent calcium indicator protein G-CaMP2. The use of a replication-incompetent recombinant adenovirus containing a cytomegalovirus (CMV) immediate-early (IE) promoter confines expression of G-CaMP2 to astrocytes. Expression is sufficiently high to allow calcium signals to be recorded in Bergmann glial processes as well as the processes and somata of velate protoplasmic astrocytes. To obtain structural information, G-CaMP2 fused with the brighter chromophore DsRed allows three-dimensional reconstruction of cells. G-CaMP2 expression lasts for at least three weeks, enabling long-term functional imaging in both anesthetized and awake, behaving animals.

INTRODUCTION

Recent work adds to a growing body of evidence that astrocytes are active players in brain function (Allen and Barres 2009). Astrocytes regulate blood flow (Iadecola and Nedergaard 2007), reflect neuronal activity after whisker stimulation in barrel cortex (Wang et al. 2006), show orientation selectivity in visual cortex (Schummers et al. 2008), regulate sleep homeostasis (Halassa et al. 2009), participate in circadian rhythmicity in Drosophila (Suh and Jackson 2007) and perhaps mammals (Prolo et al. 2005), and are necessary for sensory organ function in C. elegans (Bacaj et al. 2008).

In the cerebellar cortex, Bergmann glia (Figure 1A-E) of the molecular layer and velate protoplasmic astrocytes (Figure 1G) of the granule cell layer may play active signaling roles. Using in vivo calcium imaging with two-photon microscopy we have found (Hoogland et al. 2009) that both types of astrocytes show spontaneous compartmental calcium signals that fill small parts of individual cells, as they do in brain slices (Grosche et al. 1999), as well as transcellular calcium waves (Figure 1F, H). Waves are particularly novel because they span processes of many astrocyte processes in approximately ellipsoidal domains without fully encompassing any one cell. These waves spread at rates consistent with diffusion and depend on the P2 purine receptor, whose native agonists include ATP, ADP, and adenosine. The role of these waves in cerebellar function is a subject of active inquiry and may include regional regulation of excitability or blood flow.

In this chapter we describe protocols for in vivo calcium imaging in cerebellar astrocytes. First, we describe the injection of a replication-incompetent recombinant adenovirus for gene transfer of the fluorescent calcium indicator protein (FCIP) G-CaMP2. Second, we describe a

cerebellar craniotomy procedure which is used days after virus injection to image astrocyte activity. The same procedure is used to prepare an animal for preferential multi-cell bolus loading (Stosiek et al. 2003) of Bergmann glia with fluo-5F or fluo-4 (Figure 1E), for which a protocol is given in the third part.

PROTOCOLS

All procedures must be approved by the local Institutional Animal Care and Use Committee.

Protocol 1 - Injection of recombinant adenovirus for gene transfer of FCIP G-CaMP2 to astrocytes of the cerebellar cortex

MATERIALS

Reagents

Buprenorphine (e.g. Buprenex 0.3 mg per ml, for mice dilute 20-fold with saline to a final concentration of 15 µg/ml)

Cotton-tipped applicators

Cyanoacrylate ("super") glue

Hair removal cream (e.g. Nair (TM)), or alternatively a hair trimmer for small animals

Ketamine (80 mg/ml) / xylazine (12 mg/ml) hydrochloride solution (K113, Sigma); for mice dilute 10-fold with saline to a final concentration of 8 mg/ml ketamine and 1.2 mg/ml xylazine

Mannitol (optional; 25% in water, heat up to dissolve)

Parafilm (TM)

Pressurized air

Recombinant adenovirus containing the FCIP sequence, here G-CaMP2 fused to DsRed <R>

Saline (0.9% NaCl in water)

Equipment

Absorption triangles (Sugi Saugtupfer, Kettenbach Medical)

Box for quartz pipette storage

Dissection microscope (e.g. Leica MZ16)

Electrode beveller (Sutter instruments or W.P.I.)

Electrode puller (Sutter Instruments or W.P.I.) suitable for preparing sharp pipettes, optimally from quartz tubing (Sutter Instruments)

Heating blanket, rectal temperature probe, temperature controller

Low vibration dental drill (High speed micro drill, F.S.T.; or EXL-M40, Osada Dental Drill) with fine (0.5 mm) carbide drill burs

Micromanipulator with electrode holder, tubing, syringe for pressure control, and pressure gauge mounted in surgery area

Needle, 30 gauge

Quartz tubing, outer diameter 1.0 mm, inner diameter 0.3 mm, no capillary. Alternatively thick-walled borosilicate glass without capillary. If volumes above 200 nl are injected, a wider inner diameter should be used. The smaller surface-to-volume ratio reduces surface absorption of virus.

Stereotaxic apparatus

Surgery area, optimally on a vibration isolated table, biosafety level 2 (BSL-2) approved

Surgical tools, including scissors, micro curette, scalpel, and forceps #5 and #55 (F.S.T.)

Permanent marker, fine tip

METHOD

1 Pull sharp (greater than 10 MΩ) electrodes from quartz or glass tubing and bevel at an angle of about 20 degrees to an inner tip diameter of 5-10 µm. Calibrate and control the diameter of the tip during beveling by applying pressure in the range of 5-10 psi (0.35-0.7 bar) to the pipette.

Quartz tubing is preferred over glass tubing because the resulting electrode shanks are stiffer.

2 Using a permanent marker draw tick marks at 1 mm intervals on the beveled pipettes. These marks allow control over the injected volume. For electrodes with inner diameter of 0.3 mm the marks correspond to ~70 nl increments.

3 Keep pipettes in a clean and closed box to prevent contamination with dust.

4 Anesthetize animal with ketamine/xylazine (stock concentration 80 mg/12 mg per ml). Inject 1 ml per 1 kg body weight intraperitoneally. For mice use ketamine/xylazine 10-fold diluted in saline to achieve a more precise dosage, and inject 16 ml per 1 kg body weight.

5 Optionally, to infect a wider area of cells, inject mannitol intraperitoneally, 30 ml of 25% mannitol per 1 kg body weight, and wait 15 minutes before starting surgery.

This increases the extracellular space and promotes better virus diffusion (Burger et al. 2005).

6 Remove hair over the site where the craniotomy will be made with a hair removal cream or hair trimmer.

7 Fix the head of the animal in a stereotaxic apparatus so that the intended injection site is accessible. Insert rectal temperature probe and turn on heating blanket to keep animal temperature at 37 °C. Continue on a vibration insolated surgery table with dissection microscope.

8 Using scissors open the skin over the brain area of interest to a length of about 1 cm and pull the skin to the side with #5 forceps.

9 If muscles cover the skull over the brain area of interest, carefully loosen tendons from the underlying bone with a micro curette or scalpel.

Avoid damage to muscles as much as possible.

10 Dry skull with cotton-tipped applicators, or absorption triangles and gentle application of pressurized air.

11 Drill a circular region in the skull 1.5 times the size of the drill (i.e. 0.75 mm), leaving a thin bone layer intact.

Remove the last layer of bone by cutting a slit with the sharp edge of a 30 gauge needle tip and then remove the disk of thinned bone with #55 forceps. Keep the dura dry.

Do not push the bone into the underlying brain tissue. Make sure that the dura mater stays intact and no cerebrospinal fluid leaks out.

12 Mount a beveled pipette on a pipette holder. Attach tubing with pressure meter and syringe to the back of the pipette.

13 Under visual control through the dissection microscope, tip-load the beveled pipette with virus stock by putting a 2 to 5 µl droplet of virus stock on Parafilm (TM), immersing

the tip of the pipette, and applying suction until the pipette has taken up the virus stock. Follow progress by looking at the tick marks.

Make sure that the electrode does not draw in any air.

14 Under visual control through the dissection microscope, approach the craniotomy with the pipette tip, applying 0.3 psi (0.02 bar) of positive pressure before touching. When the pipette tip reaches the dura, zero the micromanipulator position indicator.

A dry dura will minimize the amount of virus spilled.

15 Enter the brain at an angle of about 45 degrees to the skull surface. Slowly (10 µm/s) penetrate in the direction of the pipette by 240 µm, which corresponds to a depth of about 170 µm below the dura mater.

16 Increase the pressure to about 0.5 psi (0.035 bar). Using the tick marks drawn on the pipette for guidance, inject 50-400 nl of virus stock over a period of ~15 minutes. Check the progress frequently and adjust pressure in a range of 0.1 to 1 psi (0.007 – 0.07 bar).

Low pressure and low volume injection will give a more local infection; conversely, high pressure and large volume injection will give more widespread infection.

See Troubleshooting.

17 Reduce the pressure to 0.1 psi (0.007 bar) and retract the pipette slowly. Keep pipette mounted as it can be used for further injections.

We usually inject 6 to 8 animals in a session.

18 Remove the mouse from the stereotaxic apparatus, wet the skull with saline, and close the skin by sealing it with superglue.

19 For post-operative analgesia give buprenorphine at 20-50 µg per kg body weight. Keep the mouse on a heating blanket at 37˚C until it recovers. Check it regularly afterwards and administer more buprenorphine if necessary.

20 Keep the mouse for 1 to 25 days after virus injection to allow protein expression.

21 To prepare the animal for in vivo imaging, use the protocol described in the next section, “Cerebellar craniotomy for in vivo imaging”

22 For two–photon calcium imaging of G-CaMP2, use an excitation wavelength of 920 nm with an emission filter of 500 to 550 nm. For cell reconstruction with DsRed, use an excitation wavelength between 990 and 1050 nm and an emission filter of 550 to 650 nm. Objectives of high numerical aperture should be used.

See Troubleshooting.

Troubleshooting

Problem: Pipette with virus solution is clogged.

Step 16

Solution: This is the most critical problem as the virus stock is in many cases a precious substance and hard to recover from a pipette. While the tip is exposed to air apply high pressure of up to 20 psi (1.4 bar) until a droplet comes out of the tip. If this fails, tissue or blood cells may have clogged the tip. In this case immerse the tip in saline for a few seconds at low pressure, retract the tip, and again increase pressure until a droplet is expelled. Remove the droplet with absorbent triangles to prevent spillage of virus on the dura.

Problem: No infected cells are found during in vivo imaging.

Step 22

Solution: Make sure that the craniotomy for imaging was done at the right coordinates. Especially when the virus was injected in young adult mice (16 to 25 days) the skull will recover almost completely after 10 days. Take good notes of the injection site and draw dura blood vessels after wetting the skull with saline. Remember that the angle of injection was 45 degrees causing the center of infection to be away from the center of the original drilling. If all injected animals fail to show infected cells, check the potency of the adenoviral stock by in vitro infection of HEK293 cells and titering the virus by agar plug assay (Luo et al. 2007).

Protocol 2 - Cerebellar craniotomy for in vivo imaging

This protocol is used to prepare a virus-injected animal (Protocol 1) for in vivo imaging. It is also used to prepare an animal for multi-cell bolus loading (Protocol 3).

MATERIALS

Reagents

Cyanoacrylate ("super") glue

Dental acrylic (e.g. MetaBond, Parker Inc.)

Hair removal cream (e.g. Nair(TM)), or alternatively a hair trimmer for small animals

HEPES-buffered artificial cerebrospinal fluid <R>

Ketamine (80 mg/ml) / xylazine (12 mg/ml) hydrochloride solution (K113, Sigma); for mice dilute 10-fold with saline to a final concentration of 8 mg/ml ketamine and 1.2 mg/ml xylazine, or isoflurane

Lidocaine hydrochloride topical solution (4%)

Pressurized air

Equipment

Absorption triangles (Sugi Saugtupfer, Kettenbach Medical)

Cotton-tipped applicators

Dissection microscope (e.g. Leica MZ16)

Heating blanket, rectal temperature probe, temperature controller

Isoflurane vaporizer (if isoflurane is used) with O_2 tank, plastic box for initial anesthesia, animal mask, and filter

Low vibration dental drill (High speed micro drill, F.S.T., or EXL-M40, Osada Dental Drill) with fine (0.5 mm) carbide drill burs

Mounting frame with custom headplate

Needle, 30 gauge

Stereotaxic apparatus

Surgery area, optimally on a vibration-isolated table

Surgical tools, including scissors, micro curette, scalpel, and forceps #5 and #55 (F.S.T.)

METHOD

1 Anesthetize rats with ketamine/xylazine injection of 1 ml per 1 kg body weight intraperitoneally, for mice use 16 ml per 1 kg body weight of the diluted ketamine/xylazine. If isoflurane is used, put animal in sealed plastic box and connect to 2% isoflurane in O_2.

2 Remove hair over the site where the craniotomy will be made with a hair removal cream or hair trimmer.

3 Mount animal on stereotaxic apparatus, and use rectal probe and heating blanket to maintain an internal body temperature of 37°C. Work on a vibration-isolated surgery table with dissection microscope. If isoflurane is used, mount animal mask and reduce isoflurane to 1.0-1.5% in O_2.

4 Make an incision with scissors over the brain region of interest. Pull skin to the side with #5 forceps and apply lidocaine solution with cotton-tipped applicators topically on incision. If necessary scrape tendons of muscles and connective tissue from the skull with a micro curette or scalpel. Clean the skull with saline and cotton-tipped applicators. Gently dry skull with pressurized air or absorbent triangles.

5 Mount a metal headplate to the skull with a thin, dried layer of superglue followed by dental acrylic.

6 Transfer animal from the stereotaxic apparatus to a frame that holds the headplate in a stable position.

7 Drill a 1-2 mm wide circular region defining the outline of the craniotomy. When the thinned bone is soft and transparent, stop drilling. Use pressurized air to blow away bone dust.

Take care not to put excessive pressure on the skull. Constantly move the drill to avoid drilling-induced local overheating of the brain.

See Troubleshooting.

8 Puncture the thinned bone along the edge of the circle with the tip of a 30 gauge needle or #55 forceps.

9 Put a drop of saline on the area of the drilled region and lift the bone patch with forceps.

Care must be taken to avoid putting pressure on the underlying brain area. If slight dura bleeding occurs, rinse immediately with saline until bleeding stops. If the saline is cloudy,

remove it with absorption triangles and replace with fresh saline. The dura mater should still be intact with no brain swelling. Keep saline on the exposed craniotomy.

10 For experiments in which drugs are topically applied to the brain surface, the dura must be removed. Make a small slit in the dura leaving the arachnoid mater and pia mater intact. Lift the dura with one pair of forceps on each side of the slit simultaneously and gently tear apart toward the edge of the craniotomy, taking care to exert force on dura against dura, not the brain.

This step requires practice. If the brain swells after dura removal, not only the dura but also parts of the arachnoid or pia mater were probably removed. In this case the experiment should be terminated.

11 Move animal from the surgery area to the two-photon microscope setup. If anesthesia has become light, inject additional ketamine/xylazine (50% of initial dose). In case of isoflurane anesthesia a quick transition from the surgery area to the imaging setup is necessary.

Troubleshooting

Problem: Breaking through the bone or bleeding while drilling the craniotomy

Step 5

Solution: The skull over the cerebellum varies in thickness and thicker parts have blood-filled sinuses. For this reason it is necessary to become familiar with the bone thickness over the brain area of interest in a test surgery. Be careful and slow when drilling in thin skull areas. If you drill in a thick part of the skull, sinus bleeding can be stopped by slow

drilling of the bone, which causes the resulting bone-blood mixture to coagulate quickly and stop the bleeding. Be careful with the use of cauterizers, since these can easily cause damage to underlying brain structures, especially in mice.

Protocol 3 - Preferential loading of Bergmann glia with synthetic AM calcium dyes

Multicell bolus loading has been described in previous chapters. In the cerebellum, a degree of cell type-specificity can be achieved by varying the depth of injection. Here we give a short description of our particular loading procedure after a craniotomy has been done as described in Protocol 2.

MATERIALS

Reagents

Agarose (Type III-A, Sigma) 1.5% in saline, heat up until completely dissolved and keep in waterbath at 45˚C

DMSO, desiccated by molecular sieves (3Å pore size)

Filter, pore size 0.22 μm in spinnable centrifuge tube (Ultrafree-MC, Millipore)

Fluo-5F/AM (50 μg, Invitrogen) or Fluo-4/AM (50 μg, Invitrogen)

Pluronic F-127 (Invitrogen or BASF)

Saline (0.9% NaCl in water) or HEPES-buffered artificial cerebrospinal fluid <R> but without $CaCl_2$

Equipment

Borosillicate glass tubing for pipette

Electrode beveller (Sutter instruments or W.P.I.)

Electrode puller suitable for preparing patch pipettes (Sutter instruments or W.P.I.)

Glass cover slip #1 (about 5 mm x 5 mm)

Isoflurane vaporizers with O_2 tanks, if isoflurane is used

Microloader (Eppendorf)

Micromanipulator with a position counter, an electrode holder with tubing, syringe, and pressure gauge on the two-photon microscope system

METHOD

1 For the preparation of the dye stock solution, add 5 µl of 20% Pluronic F-127 freshly dissolved in water-free DMSO to 50 µg fluo-5F/AM or Fluo-4/AM. Vortex, then add 80 µl saline. Vortex and filter dye stock solution through a low-volume 0.22 µm filter.

We find that fluo-5F/AM as well as fluo-4/AM generally allow for measurements with better signal-to-noise in astrocytes than OGB-1/AM. Fluo-5F (K_D ~1 µM) (Sarkisov and Wang 2008) is especially suited to measuring calcium signals of the magnitude produced in transglial waves.

2 Pull patch pipette and back-fill it with the prepared solution with microloader. Bevel at an angle of 20 degrees to a resistance of 3 to 4 MΩ.

3 Mount the beveled pipette in the pipette holder on the micromanipulator and apply 0.5 psi (0.035 bar) positive pressure to the pipette before entering the saline over the craniotomy.

4 Approach the dura under visual control by entering the saline over the craniotomy and zero the manipulator when reaching the brain surface. Check under fluorescence viewing that dye is coming out of the pipette.

5 Advance the pipette into the brain surface. After passing the dura reduce the pressure to 0.3 psi (0.02 bar) and advance to a depth of about 50 µm below the dura. Verify that dye gets ejected from the pipette by visualization of fluorescence. To label Bergmann glia, eject the dye solution at about 2 psi (0.14 bar). A similar injection into the granule cell layer (in mice more than 140 µm below pia mater) will result in relatively increased labeling of Purkinje cells. Apply pressure for 5 min.

6 Reduce pressure to 0.1 psi (0.007 bar) and slowly retract electrode.

7 Add 1.5% agarose at 45°C over the craniotomy and seal with a cover glass while the agarose is still liquid.

8 Wait for about 30 min for dye uptake.

9 Image using an excitation wavelength of 840 or 920 nm and a 500-550 nm bandpass filter for detection.

RECIPES

Recombinant adenovirus containing the FCIP sequence

The recombinant replication-incompetent adenovirus AdEasy is commercially available and supplied as viral backbone and shuttle vector for gene insertion (JHU-23, ATCC). Recombinant viruses can be produced with basic knowledge of molecular cloning by following the protocol (Luo et al. 2007). For virus amplification, a biosafety level 2 (BSL-2) cell culture hood and incubator are required. The viral vectors can be harvested from HEK293 cells after lysis by freeze/thawing and centrifugation. Lysate can be used directly from the supernatant after 0.5 µm filtration or further purification and concentration using a $CsCl_2$ gradient or a commercially available kit (Vivapure AdenoPACK, Satorius Stedim Biotech S.A.). The titer of infectious particles should be in the range of 10^8-10^9 per ml. FCIP constructs are typically generously supplied by their developers, including the labs of R.Y. Tsien, J. Nakai, O. Griesbeck, and others. The construct we used, a fusion protein of G-CaMP2 and DsRed, was designed and made by J. Nakai (Saitama Univ., Japan).

Artificial cerebrospinal fluid

135 mM NaCl, 5.4 mM KCl, 5 mM NaHEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$. Adjust to pH 7.3 with HCl.

DISCUSSION

We have described two protocols to monitor astrocytic calcium signals in the intact cerebellum. Multi-cell bolus loading is reliable and quick and gives preferential labeling of Bergmann glia if the dye is injected into the superficial molecular layer (Fig.1E). If injected into the granule cell layer we have been able to simultaneously observe activity in Bergmann glia, interneurons, and Purkinje cells (Sullivan et al. 2005). The disadvantage of bolus loading is low contrast, so that fine processes in the neuropil cannot be resolved, 3D reconstructions of Bergmann glia are blurry, and signals from small structures cannot be assigned to particular cell types.

To overcome the problem of nonspecific labeling, we used attenuated, nonreplicating adenovirus to express the fluorescent calcium indicator protein (FCIP) G-CaMP2 in Bergmann glia and velate protoplasmic astrocytes. Compared with synthetic dyes such as Oregon Green BAPTA-1, the current generation of FCIPs has slower on and off calcium binding rates and smaller relative fluorescence changes (Hendel et al. 2008). However, neither constraint appears to be a limiting factor in the study of astrocytic calcium signals, which last for seconds and reach micromolar levels. Fluorescence changes in FCIPs are highly nonlinear with respect to calcium concentration, with Hill coefficients greater than 1 (Hendel et al. 2008) so that measurements are, to a degree, a thresholded function of the actual change in free calcium. Signals can be recorded in vivo from even the finest structures such as Bergmann glia microdomains (Grosche et al. 1999) or velate processes of protoplasmic astrocytes. FCIPs are also well suited for long-term imaging (days or longer) while dye-loaded cells lose their labeling after a few hours. Additionally, functionally imaged cells can be reconstructed in three dimensions in vivo with the help of two-photon microscopy (Figure 1A).

The virus-based method presented in this chapter is one of several currently available methods for expressing FCIPs in neurons and astrocytes in situ. Other methods include transgenic animal generation (Hasan et al. 2004; Heim et al. 2007), in utero electroporation (Mank et al. 2008; Mao et al. 2008), single-cell electroporation (Judkewitz et al. 2009), and other recombinant viruses (Mank et al. 2008; Wallace et al. 2008). As adenoviral vectors have long been known to be useful for gene transfer to astrocytes (Le Gal La Salle et al. 1993), in our protocol we used the replication-incompetent recombinant adenovirus AdEasy-1 which has low cytotoxic side effects (He et al. 1998). However, it should be mentioned that other viruses or virus serotypes also infect glia with high efficiency. Examples are baculovirus (Wang and Wang 2006), single and double stranded AAVs of specific serotypes (Shevtsova et al. 2005; Lowery et al. 2009), lentivirus (Croci et al. 2006; Colin et al. 2009), and Semliki Forest virus (Mank et al. 2008).

In vitro, adenoviruses have a much higher probability of infecting glia than neurons (Sato et al. 2004). In addition, the CMV IE promoter results in significantly higher expression levels in astrocytes than in neurons (Boulos et al. 2006). Consistent with these two observations, the approach outlined here leads to expression of G-CaMP2 in Bergmann glia and velate protoplasmic astrocytes. Adenoviruses do not integrate into the genome, and calcium imaging is limited to about 1 to 25 days after virus injection. Sparse expression allows single cells and their processes to be studied (Figure 1A, B), whereas high-density labeling across regions of several hundred micrometers can label all astrocytes (Figure 1C), comparable to a transgenic approach (1E).

In vivo, G-CaMP2 has low resting fluorescence comparable to wild-type GFP (Tallini et al. 2006; Mao et al. 2008). To improve detection of expression, we used a fusion protein of G-CaMP2 with DsRed, facilitating detection and three-dimensional reconstruction using an excitation wavelength optimal for DsRed (990 nm). Two-channel detection also allows for ratiometric measurements and thus compensation of motion artifacts, an important consideration when recording from awake, behaving animals.

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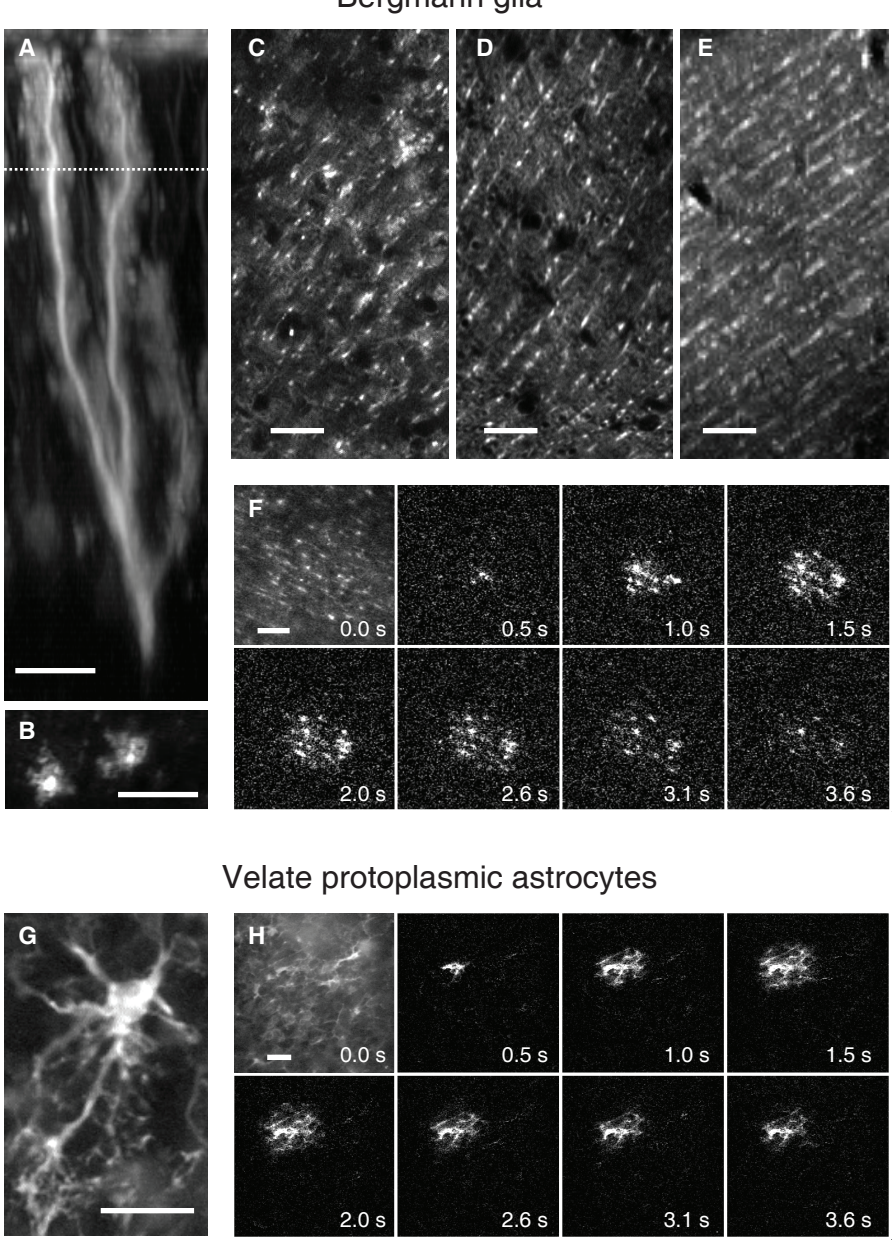
FIGURE CAPTION

Figure 1 Astrocytes of the cerebellar cortex generate transglial calcium waves in vivo. (A) Bergmann glia cell expressing a fusion protein of the FCIP G-CaMP2 and DsRed under the CMV IE promoter after adenoviral gene transfer. (B) The use of FCIP also allows imaging of glial microdomains when cells are sparsely infected or (C) cell populations at higher infection rates which are almost comparable to (D) transgenic mice expressing GFP under the Bergmann glia-specific GFAP promoter. (E) Multicell bolus loading (MCBL) with the synthetic calcium dye fluo-5F/AM shows preferential Bergmann glia labeling if injected superficially into the molecular layer of the cerebellar cortex. (F) In vivo Bergmann glia show radially expanding transglial calcium waves measured with G-CaMP2. The first image shows resting fluorescence; following images show fluorescence changes relative to the first image. (G) Velate protoplasmic astrocyte of the granule cell layer expressing G-CaMP2 after infection by recombinant adenovirus. (H) Velate astrocytes also generate transglial calcium waves in vivo. The first image shows resting fluorescence; following images fluorescence changes relative to the first image. Scale bar in all panels, 20 μm.


Bergmann glia
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Velate protoplasmic astrocytes
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