In vivo profiling of astrocytic calcium excitability and downstream vascular effects: fluorescence intensity and lifetime measurements

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Introduction

Astrocytes express a number of metabotropic receptors for messenger molecules, including glutamate and ATP, and can exhibit elevations of intracellular Ca^{2+} upon their activation. An increase in astrocytic intracellular calcium concentration ($[Ca^{2+}]$), in turn, can lead to release of gliotransmitters implicated in neuroglial and gliovascular interactions (for recent reviews see (*1-8*)). Our goal is to quantitatively assess *in vivo* astrocytic Ca^{2+} dynamics and their downstream vascular effects in response to well-controlled manipulations.

References:
1. C. Agulhon *et al.*, *Neuron* **59**, 932 (Sep 25, 2008). 2. D. Attwell *et al.*, *Nature* **468**, 232 (Nov 11, 2010). 3. R. C. Koehler, R. J. Roman, D. R. Harder, *Trends Neurosci* **32**, 160 (Mar, 2009). 4. C. Iadecola, M. Nedergaard, *Nat Neurosci* **10**, 1369 (Nov, 2007). 5. A. Nimmerjahn, *J Physiol* **587**, 1639 (Apr 15, 2009). 6. G. C. Petzold, V. N. Murthy, *Neuron* **71**, 782 (Sep 8, 2011). 7. O. B. Paulson, S. G. Hasselbalch, E. Rostrup, G. M. Knudsen, D. Pelligrino, *J Cereb Blood Flow Metab* **30**, 2 (Jan, 2010). 8. A. Volterra, J. Meldolesi, *Nat Rev Neurosci* **6**, 626 (Aug, 2005).

Methods

All experiments were done in mice. Anesthesia (α-chloralose) and monitoring performed as described in (*1*).

Dyes: Calcium indicators: OGB BAPTA-1 AM (OGB1) was bolus loaded as described in (*2*); Fluo-4 AM was loaded topically (*3*). An astrocyte marker SR101 was applied to the surface (*4*). Alexa Fluor 350 was added to the pipette solution to visualize "puffs". Fluorescein-conjugated dextran (FITC) in physiological saline was injected intravenously for vascular diameter measurements (*1*).

Imaging: Images were obtained using Ultima-2 2-photon microscopy system from Prairie Technologies. Illumination was provided by Ultra II pulsed laser from Coherent.

Fluorescence Lifetime Imaging Microscopy (FLIM): Fluorescence lifetime data was acquired in time-correlated single photon counting (TCSPC) mode using a FLIM module (Becker & Hickl) integrated into Ultima-2. Calibration was performed using buffer solutions as described previously (*5,6,7*).

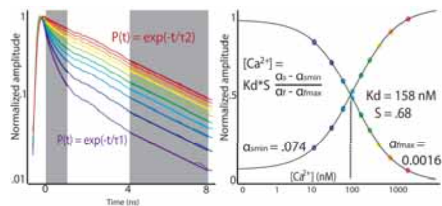

Fig. 1 Calibration of OGB1 in buffers. Left: Fluorescence lifetime curves for buffers with known $[Ca^{2+}]$. The fit is overlaid. Gray rectangles indicate the segments used for the fit. **Right:** Calibration curve.

To fit biological decays, we implemented the maximum likelihood estimator incorporating the model of shot (counting) noise. Spiral scanning trajectories were defined using custom software to optimize temporal resolution.

References:
1. P. Tian *et al.*, *Proc Natl Acad Sci U S A* **107**, 15246 (Aug 24, 2010). 2. C. Stosiek, O. Garaschuk, K. Holthoff, A. Konnerth, *Proc Natl Acad Sci U S A* **100**, 7319 (Jun 10, 2003). 3. X. Wang *et al.*, *Nat Neurosci* **9**, 816 (Jun, 2006). 4. A. Nimmerjahn, F. Kirchhoff, J. N. Kerr, F. Helmchen, *Nat Methods* **1**, 31 (Oct, 2004). 5. K. V. Kuchibhotla, C. R. Lattarulo, B. T. Hyman, B. J. Bacskai, *Science* **323**, 1211 (Feb 27, 2009). 6. C. Lattarulo, D. Thyssen, K. V. Kuchibholta, B. T. Hyman, B. J. Bacskaiq, *Methods Mol Biol* **793**, 377 (2011). 7. C. D. Wilms, H. Schmidt, J. Eilers, *Cell Calcium* **40**, 73 (Jul, 2006).

Simultaneous measurements of astrocytic calcium and arteriolar diameter during local application of transmitters *in vivo*

Objectives:
1) Profile astrocytic Ca^{2+} excitability *in vivo*
2) Compare the *temporal profile* of astrocytic Ca^{2+} and vasodilation

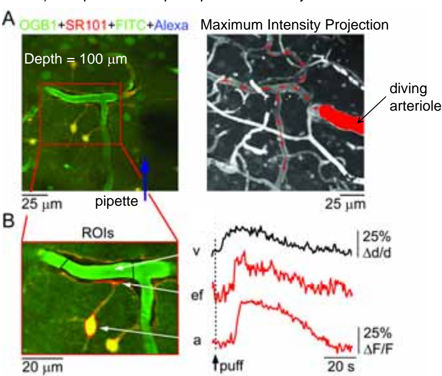

Fig. 2 Vasodilation and astrocytic Ca^{2+} increase in response to 1 mM ATP. A. Left: The imaging plane containing a branching arteriole and a micropipette (blue arrow). Right: MIP from the surface to the imaging plane containing the diving arteriole (red). **B.** Left: a zoomed-in image of regions of interest (ROIs) used to extract time-courses on the right.

See also response to glutamate on poster 590.16

HOW LARGE IS ASTROCYTIC Ca^{2+} INCREASE?

Improvement of FLIM technology

Objectives:
1) Improve temporal resolution of FLIM to allow dynamic quantification of Ca^{2+} *in vivo*
2) Validate the performance of fast FLIM by imaging single neuronal spikes

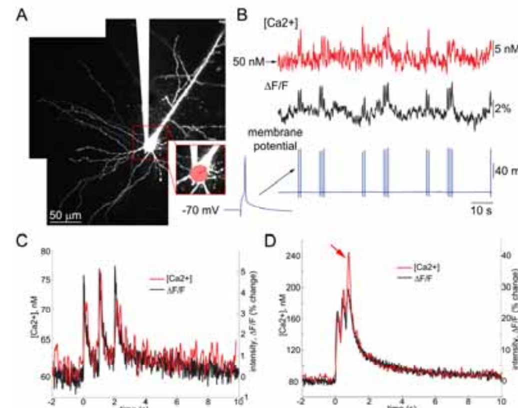

Fig. 3 Quantification of spike-induced $[Ca^{2+}]$ in brain slices. A. Pyramidal cell (with a patch electrode) labeled with OGB1. **B.** Time-course of $[Ca^{2+}]$ (top), intensity (middle), and membrane potential (bottom). **C.** Superimposed $[Ca^{2+}]$ and intensity for 5-ms current injections eliciting 3 spikes. **D.** As in (C) in a different PC for 3 consecutive 100-ms current injections eliciting multiple spikes each.

Application of FLIM for quantification of astrocytic $[Ca^{2+}]$ *in vivo*

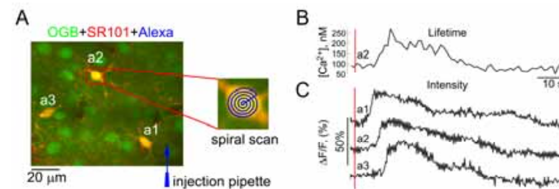

Fig. 4 ***In vivo*** **FLIM $[Ca^{2+}]$ measurements. A.** A "spiral" trajectory calculated within a single astrocytic cell body. **B-C.** FLIM (B) and intensity (C) time-course in response to micropuff of 1mM ATP. Red vertical lines indicate the timing of the puffs.

How to estimate $[Ca^{2+}]$ increase in "astropil"?

Problem: OGB1 labels both astrocytes and neurons. Astrocytic processes are intermingled with neuropil and cannot be resolved.

Solution: Perform $\Delta F/F$ measurement under conditions of preferred astrocytic labeling. Use FLIM measurement from cell bodies to calibrate the $\Delta F/F$ measurement.

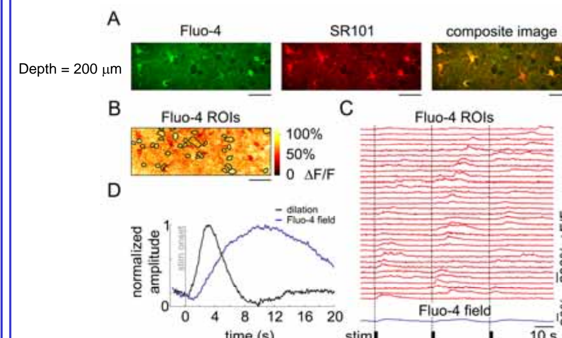

Fig. 5 Fluo-4 imaging of Ca^{2+} "microdomains". A. Reference images. **B.** Subcellular ROIs. **C.** Ca^{2+} time-courses from ROIs in (B). **D.** Trial-averaged response. Scale bars are 50 μm.

IS ASTROCYTIC Ca^{2+} INCREASE NECESSARY FOR DILATION?

Transgenic IP3R2-KO mice as a means to abolish astrocytic Ca^{2+} excitability

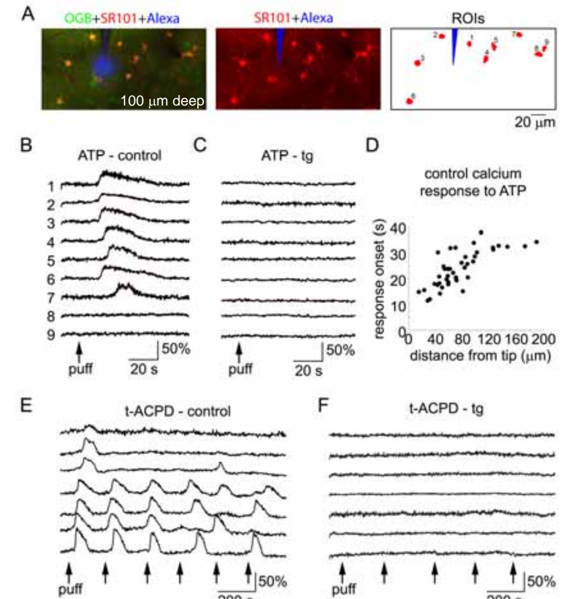

Fig. 6 Astrocytic Ca^{2+} in response to microinjection of ATP (1 mM) or t-ACPD (10-μM). A. Reference images. **B-C.** Ca^{2+} signal time-courses in response to ATP. **D.** Onsets of the response to ATP in control subjects as a function of distance from the injecting pipette. **E-F.** Ca^{2+} signal time-courses in response to t-ACPD.

IP3R2-KO mice exhibit normal stimulus-induced vasodilation

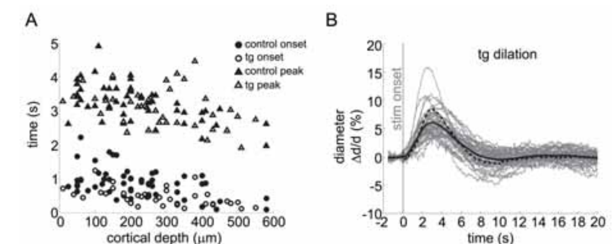

Fig. 7 Transgenic IP3R2-KO mice exhibit normal vasodilation response. A. Onset and time-to-peak for arteriolar diameter changes as a function of the cortical depth. **B.** Time-courses of arteriolar diameter change in IP3R2-KO subjects. The average from control subjects is superimposed in dashed line.

Conclusions and further directions

1. Direct application of transmitters *in vivo* is feasible and useful as a tool for profiling astrocytic Ca^{2+} "responsiveness" including the temporal characteristics of the response.

2. FLIM can be used to perform time-resolved quantitative $[Ca^{2+}]$ imaging *in vivo* with single-cell resolution.

3. IP3R2-KO transgenic mice exhibit an intact stimulus-induced vasodilation suggesting that elevation of astrocytic calcium is not required for neurovascular coupling.

4. The onset of astrocytic Ca^{2+} response is slow in comparison with vasodilation and is unlikely to serve as a triggering mechanism.

5. Future directions: compile a list of biologically-relevant compounds that do (and do not) induce astrocytic Ca^{2+} increase while paying attention to the amplitude and temporal characteristics.

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