

Shining light on spiny matters

Karel Svoboda

Two-photon microscopy might be the silver bullet to image calcium in individual dendritic spines, and can provide new information on calcium dynamics during synaptic activity.

Ramon y Cajal was the first to note that most dendrites in the central nervous system are conspicuously studded with thousands of dendritic spines. These are tiny, bulbous protuberances (volumes, $0.01 - 1 \mu\text{m}^3$), which are connected to their parent dendrites via thin necks (Fig. 1). The majority of excitatory synapses terminate on spines. Speculation regarding spine function, mainly based on ultra-structural data from serial electron microscopy, has centered on the electrical and diffusional resistance of the narrow neck connecting the spine head to the dendrite¹. According to these models, spine necks serve to isolate spines chemically or electrically from other spines and from the rest of the cell. Chemical isolation could be important for synapse specificity in long-term plasticity, for example by restricting diffusion of second messengers or activated enzymes to the vicinity of a particular synapse. Electrical isolation could lead to activation of voltage-dependent conductances limited to the spine head and be crucial for the induction of some forms of synaptic plasticity. Spines would therefore function as the smallest units of dendritic integration. However, despite some courageous attempts (for example ref. 2), until recently, most of these models had to remain conjectural for lack of data; this was mainly because their small size ($< 1 \mu\text{m}$) made direct experiments on spines in living tissue extremely difficult. As so often in neuroscience, a well posed problem had to await the emergence of a

new technique for resolution.

Several recent reports³⁻⁵, including an elegant paper on page 114 of the present issue of *Nature Neuroscience*⁶, indicate that two-photon excitation laser-scanning microscopy (TPLSM)⁷ might be the silver bullet for the spine riddle. TPLSM

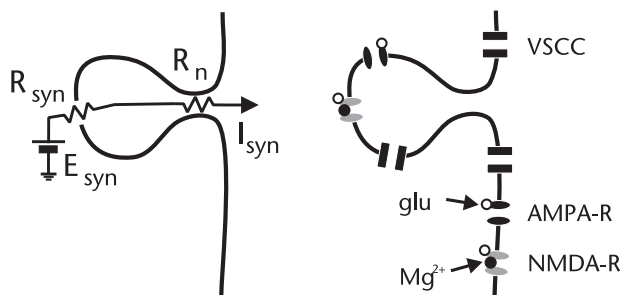


Fig. 1. Spine cartoons. Left, electrical model. The synaptic current (I_{syn}) is driven by the electrochemical driving force, E_{syn} , producing a voltage drop ($I_{syn}R_n$) along the spine neck. This voltage drop could help to open voltage sensitive conductances in the spine head as opposed to the dendrite. Right, receptors and channels that are involved in shaping EPSCs and associated $[\text{Ca}^{2+}]$ transients.

allows high-resolution fluorescence imaging deep in living tissue with minimal photodamage (reviewed in ref. 8). What was the problem with traditional microscopies to begin with? In traditional microscopy, light scatters strongly in brain tissue, degrading resolution and contrast of wide-field microscopy rapidly with distance from the surface of the sample. In confocal microscopy, resolution and contrast are recovered by placing an aperture in front of the detector to reject off-focus and scattered light, but at a terrible price: in addition to rejecting background, the aperture will also reject signal that was scattered on its way out of the tissue. When imaging deep in the brain slice, where the healthy neurons are, most of the signal will thus be rejected; increasing the excitation intensity in compensation leads to photobleaching and

phototoxicity (any imager's nightmare). Spines are separated from dendrites by barely more than the resolving power of the microscope, and in a typical experiment they might contain only a thousand dye molecules. On these size scales, neither spatial resolution nor efficient signal collection can be sacrificed, and TPLSM offers a way to avoid either compromise.

How does TPLSM work? In two-photon excitation, two infrared photons are absorbed nearly simultaneously to produce a visible fluorescence photon. The crucial property of two-photon excitation, from which all of the advantages of TPLSM spring, is the quadratic dependence of the absorption rate on the instantaneous laser intensity. In the case of an infrared laser beam focused by an objective onto a fluorescent specimen, as

in a TPLSM microscope, fluorescence excitation is localized to the tiny focal volume ($\sim 0.5 \times 0.5 \times 1.5 \mu\text{m}^3$), without appreciable background from off-focus locations. This holds even in living tissue, because randomly scattered excitation photons bounce around harmlessly, too dilute to excite. Hence fluorescence can be excited in an individual spine without background from the much larger parent dendrite (Fig. 2). What about efficient signal detection? Because the properties of two-photon excitation ensure that fluorescence originates only in the focal volume, all of it constitutes useful signal. Scattered and ballistic fluorescence photons

can be indiscriminately focused onto photodetectors, resulting in extremely efficient signal detection, without degrading resolution and contrast.

Returning to the problem of synaptic function, TPLSM allows the imaging of calcium concentration ($[\text{Ca}^{2+}]$) dynamics in individual spines. This is of profound interest because synaptic activation produces postsynaptic Ca^{2+} influx, and the resulting Ca^{2+} accumulation drives the biochemical cascades underlying the induction of long-term potentiation (LTP)⁹. In the first application of TPLSM to spine physiology, Denk and collaborators showed that sub-threshold synaptic activity can produce $[\text{Ca}^{2+}]$ transients localized to individual spines^{3,4}, suggesting that spine necks impede diffusion. Chemical compartmentalization was shown directly by

Karel Svoboda is at the Center for Learning and Memory at the Cold Spring Harbor Laboratories, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA
email: svoboda@cshl.org

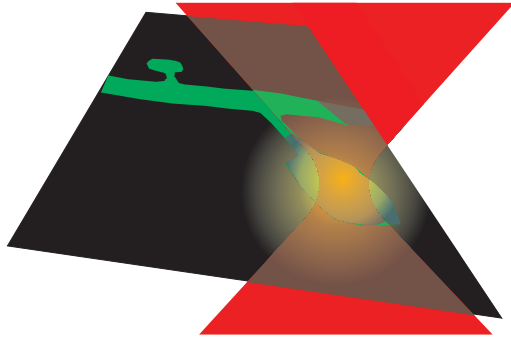


Fig. 2. Localization of excitation by two-photon absorption. An IR laser beam is focused on a dendritic spine, producing fluorescence in the spine, leaving the nearby dendrite unperturbed.

using two-photon excitation photorelease and photobleaching of fluorescence to measure diffusional transport between spine head and dendrite⁵. Action potentials back-propagating into dendrites also produce rapid $[Ca^{2+}]$ transients in spines, demonstrating that action potentials invade spines and that voltage-sensitive Ca^{2+} channels (VSCCs) exist in spines. Pairing of synaptic activity and action potential generates supralinear (more than a linear sum of the individual components) $[Ca^{2+}]$ transients in activated spines, suggesting that spine calcium can encode coincident pre- and postsynaptic activity³.

The prime suspect for generating supralinear Ca^{2+} influx is the NMDA receptor, famous precisely because of its potential for coincidence detection and its necessity for LTP induction¹⁰. This receptor is highly Ca^{2+} permeable and is blocked in a voltage-dependent manner by extracellular magnesium. Synaptic glutamate release and postsynaptic depolarization have to occur simultaneously to generate a large Ca^{2+} influx through this receptor (Fig. 1). However, VSCCs could also act as coincidence detectors of the potentials generated by synaptic currents and action potentials. Distinguishing between these mechanisms by pharmacological techniques alone poses dangerous pitfalls. For example, blockers of VSCCs in many cases conflict with synaptic transmission; they could also spuriously reduce NMDA-receptor-mediated Ca^{2+} influx by blocking currents that normally help to relieve the NMDA receptor voltage blockade. It is for these reasons that the mechanisms shaping spine $[Ca^{2+}]$ dynamics during synaptic activity have so far eluded

experimental attack.

Here is where Schiller and colleagues got clever. They raised the technological ante by combining TPLSM $[Ca^{2+}]$ imaging with focal uncaging of glutamate using an ultraviolet laser (see for example ref. 11). Like many successful experiments, theirs seems deceptively simple in hindsight. Layer V pyramidal cells in neocortical brain slices were filled with $[Ca^{2+}]$ indicators and bathed in caged glutamate. While rapidly measuring fluorescence in the spine and its parent dendrite, a

brief pulse of glutamate was released along a piece ($\sim 10 \mu\text{m}$) of the imaged dendrite, generating an excitatory postsynaptic-like current (EPSCs). Uncaging bypasses the presynaptic machinery, activating postsynaptic receptors directly and thus allowing pharmacological manipulation of postsynaptic VSCCs without the complication of presynaptic effects. They found that blockade of VSCCs dramatically reduced EPSC-induced $[Ca^{2+}]$ transients, suggesting that most of the Ca^{2+} influx is via VSCCs and that NMDA receptors account for only a small proportion (18%) of the total. Pairing EPSCs with action potentials produced supralinear spine $[Ca^{2+}]$ transients that were 60% larger than the computed sum of transients due to EPSCs and action potentials alone. Interestingly, during pairing, 59% of Ca^{2+} influx was mediated by NMDA receptors; this potentiation of the NMDA receptor component can fully account for the observed $[Ca^{2+}]$ supralinearity. It therefore seems that indeed, under at least some conditions, the NMDA receptor mediates coincidence detection in spines by supralinear $[Ca^{2+}]$ accumulation.

Stimulating studies often pose as many questions as they answer. For example, could there be differences between EPSCs and true excitatory postsynaptic currents (EPSCs) with respect to the origin of spine $[Ca^{2+}]$? For EPSCs, relatively large currents are generated by receptors in a few spines. When rushing through the resistive spine neck, these currents can produce considerable voltage drops, elevating the spine-head potential relative to the dendrite (Fig. 1)⁵ and relieving the voltage block of NMDA

receptors. In contrast, for EPSCs, glutamate-evoked currents are generated by distributed activation of synaptic and extrasynaptic glutamate receptors (Fig. 1) involving about 20 synapses¹². Individual spines generate relatively small currents, producing small voltage differentials between spine heads and dendrites, helping little to open NMDA receptors. For EPSCs, the NMDA receptor could therefore carry a larger fraction of the Ca^{2+} current than for EPSCs. This difference might explain the larger supralinearity observed by Schiller and colleagues compared to earlier work³. Finally, a recent study showed that synaptic potentials can actually amplify backpropagating action potentials¹³, producing cooperative dendritic Ca^{2+} influx mediated by an interplay of A-type potassium, sodium and calcium channels¹⁴. Could a part of the supralinearity observed by Schiller and colleagues be ascribed to such a mechanism?

Regardless, there is no doubt that TPLSM will play an important role in settling these and future issues in spine physiology. Beyond spines, TPLSM has already been used to pursue a wide-range of neurobiological problems that benefit from sub-cellular imaging⁸. A particularly exciting future application is the measurement of synaptic function in the intact brain. It could be that soon we will mean 'individual synapses' when we speak of 'single units'.

- Harris, K.M. & Kater, S.B. *Ann. Rev. Neurosci.* **17**, 341–371 (1994).
- Muller, W. & Connor, J.A. *Nature* **354**, 73–76 (1991).
- Yuste, R. & Denk, W. *Nature* **375**, 682–684 (1995).
- Denk, W., Sugimori, M. & Llinas, R. *Proc. Natl. Acad. Sci. USA* **92**, 8279–8282 (1995).
- Svoboda, K., Tank, D.W. & Denk, W. *Science* **272**, 716–719 (1996).
- Schiller, J., Schiller, Y. & Clapham, D.E. *Nature Neurosci.* **1**, 114–118 (1998).
- Denk, W., Strickler, J.H. & Webb, W.W. *Science* **248**, 73–76 (1990).
- Denk, W. & Svoboda, K. *Neuron* **18**, 351–357 (1997).
- Bliss, T.V.P. & Collinridge, G.L. *Nature* **361**, 31–39 (1993).
- Bourne, H.R. & Nicoll, R.A. *Cell* **72**, 65–75 (1993).
- Wieboldt, R., et al. *Proc. Natl. Acad. Sci. USA* **91**, 8752–8756 (1994).
- Braitenberg, V. & Schutz, A. *Anatomy of the Cortex* 1–249 (Springer Verlag, Berlin, 1991).
- Magee, J.C. & Johnston, D. *Science* **275**, 209–213 (1997).
- Hoffman, D.A., Magee, J.C., Colbert, C.M. & Johnston, D. *Nature* **287**, 869–875 (1997).