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## Dendritic Enlightenment: Using Patterned Two-Photon Uncaging to Reveal the Secrets of the Brain's Smallest Dendrites

It has been a longstanding challenge for experimentalists to manipulate precisely the spatial and temporal patterns of synaptic input to the dendritic tree in order to mimic activity occurring in the intact brain and determine their importance for synaptic integration. In this issue of *Neuron*, Losonczy and Magee have used rapid multisite two-photon uncaging of glutamate to define patterns of synaptic input on a submillisecond and micron scale to investigate the rules for summation of synaptic inputs in the fine oblique dendrites of pyramidal neurons.

Most synapses are made onto the dendrites of neurons. This is essential for the wiring up of the brain (Chklovskii, 2004) but also has direct consequences for the way individual synaptic inputs are integrated by the postsynaptic neuron to generate its action potential (AP) output (Häusser and Mel, 2003). The integrative properties of dendrites are governed by their passive cable properties, but dendrites are also known to contain various types of voltage- and calcium-dependent conductances (Johnston et al., 1996). Together they can shape the rules for synaptic integration such that synaptic inputs summate sublinearly or approximately linearly at the soma (Cash and Yuste, 1999; Urban and Barrionuevo, 1998). However, under some conditions, particularly when

many synaptic contacts are concurrently active in a small region of the dendritic tree, regenerative activation of dendritic  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and NMDA receptor channels may occur, resulting in a supralinear response: a dendritic spike. The exact conditions for the generation of these local spikes, in particular in the thin basal and oblique branches of pyramidal neurons are unclear. How many synaptic inputs need to arrive on a small dendritic branch, and in which time interval, for the branch to exit the approximately linear operating regime and generate a local dendritic spike? Does the threshold for evoking such a spike, or its spatial extent and peak amplitude, depend strongly on the exact spatial pattern of inputs onto a single branch? And once a spike is evoked in a particular dendrite, how does its effect spread to the soma, and how does this affect the AP output of the neuron?

The ability of the thin dendritic branches of hippocampal and neocortical pyramidal neurons to support initiation of local dendritic spikes has been known for some time. Schiller et al. (2000) demonstrated that local dendritic spikes can be evoked in the basal branches of layer 5 pyramidal neurons by focal extracellular stimulation of nearby axons, and by one-photon uncaging of glutamate. Using a combination of pharmacology and modelling they showed that these spikes are carried by  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and predominantly by NMDA receptor conductances. Applying the same techniques to the basal dendrites of hippocampal CA1 pyramidal neurons, Ariav et al. (2003) showed that these, too, support local dendritic spikes, which in this case are dominated by a fast,  $\text{Na}^+$ -based initial component followed by a slow, NMDA receptor-dependent component. Again in layer 5 pyramidal neurons, Polsky et al. (2004) demonstrated using extracellular stimulation at two locations that nearby inputs on the same branch summated supralinearly as they cooperated in the initiation of a local dendritic spike in that branch, while spatially separated inputs to different branches summated linearly. These experiments provide support for a two-layer “neural network” model of synaptic integration in the dendritic tree of a pyramidal neuron, in which the individual thin dendritic branches correspond to the first layer of thresholding units whose output is then relayed to the second-layer thresholding unit corresponding to the AP initiation site near the soma of the neuron (Häusser and Mel, 2003; Mel, 1993; Poirazi et al., 2003).

In order to understand the “arithmetic” of dendrites in realistic detail, experiments are required which provide more quantitative control over the spatiotemporal organization of synaptic input patterns delivered to individual dendritic trees. Existing methods either do not permit fine control of the spatial pattern of synaptic input, or do not provide a physiological time course or AMPA/NMDA ratio for individual synaptic conductance inputs. For example, experiments using focal extracellular stimulation of nearby axons cannot accurately control which and how many synapses are activated on the postsynaptic neuron in question. Furthermore, dendritic spikes are typically elicited only after two successive extracellular stimulation events (Schiller et al., 2000), as this helps to increase recruitment of NMDA receptor-mediated conductances. Similarly, one-photon uncaging tends to activate glutamate-gated conductances

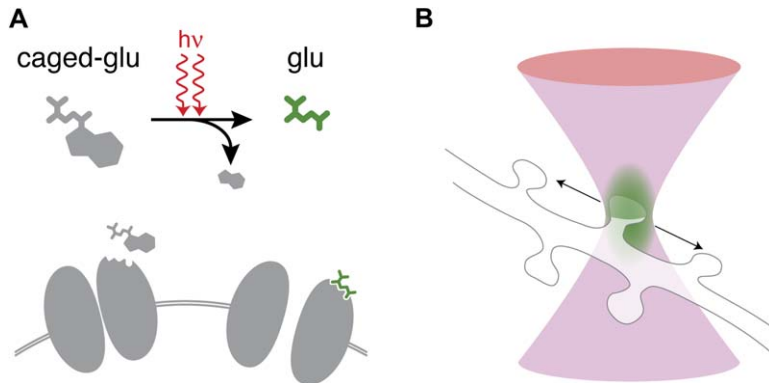


Figure 1. Targeted Activation of Synapses Using Two-Photon Uncaging of Glutamate  
(A) Caged glutamate is photoconverted into its bioactive form by the near coincident absorption of two IR photons. (B) Because the photon density required for two-photon uncaging is only reached in a small focal volume, glutamate can be released with single-spine resolution.

with a time course much slower than that of unitary synaptic inputs, and with a reduced AMPA/NMDA ratio (see below).

The dream experiment for elucidating the arithmetic of dendrites would therefore provide realistic spatiotemporal patterns of synaptic inputs (whose amplitude, time course and composition is close to physiological) in a controlled way, at single-spine spatial and high temporal resolution, while monitoring the neuron both electrophysiologically and optically. This was previously possible only using computer models (e.g., Poirazi et al., 2003). Now, Losonczy and Magee (2006), along with a recent publication by the same lab (Gasparini and Magee, 2006), have made a major step toward this goal. The technology that enabled them to accomplish this feat is rapid, multisite two-photon uncaging of glutamate.

Two-photon uncaging combines the advantages of two powerful and established approaches: photolysis of caged compounds and multiphoton excitation. Caged compounds are derived from bioactive molecules and are usually synthesized by the addition of chemical groups, which mask the biological function of the molecule, e.g., by sterically preventing the interaction of a molecule with its receptor (Figure 1A). When absorbed photons deliver sufficient energy to cleave the inactivating chemical group, the bioactive form of the molecule is restored and metaphorically released from its cage. This is commonly achieved by flashes of focused UV laser light. Because caged molecules can be preloaded to achieve uniform concentrations within deep tissue and because they can be released within a few  $\mu$ s after a light pulse, they have been widely used in biology since the first application of caged ATP (Kaplan et al., 1978). Caged neurotransmitters have proved particularly interesting to neuroscientists since they offer a unique opportunity to artificially simulate synaptic communication between neurons (e.g., Schiller et al., 2000).

However, when UV light is used for uncaging, the hourglass shape of a focused laser beam precludes localized release in volumes as small as dendritic spines, because too much glutamate is uncaged along the laser path above and below the focus. This is likely to activate extrasynaptic receptors, influence the ratio of evoked AMPA/NMDA currents, and even activate glutamate receptors on other neurons – and is therefore likely to represent an unphysiological stimulus, especially deep within a slice or in vivo. This problem can be overcome

by taking advantage of two-photon excitation, which is applied in two-photon microscopy (Denk and Svoboda, 1997). The near coincident absorption of two infrared (IR) photons raises the energy state of a caged compound by an amount that is equivalent to their added energies or the energy of one UV photon. Because the photon density required for two-photon excitation is only reached in a very small and defined focal volume, two-photon uncaging offers a powerful method to deliver neurotransmitters near single synaptic spines with high spatial resolution (Figure 1B). Caged MNI-glutamate is so far the only caged neurotransmitter with a sufficient two-photon cross-section and has been successfully used to map the locations of glutamate receptors on hippocampal CA1 pyramidal neurons with single-spine resolution (Matsuzaki et al., 2001). By using fast beam-deflecting mirrors, focal two-photon uncaging can be achieved at multiple locations within a few milliseconds. Although this is not a new idea, it has been used for the first time by the Magee lab (Gasparini and Magee, 2006; Losonczy and Magee, 2006) to explore how different spatio-temporal patterns of synaptic input are integrated in the dendritic tree.

Using multisite two-photon uncaging of MNI-glutamate, Losonczy and Magee artificially simulated patterns of excitatory synaptic input on radial oblique dendrites of CA1 pyramidal neurons in hippocampal slices while measuring uncaging-evoked changes in membrane potential (gluEPSPs) with an intracellular electrode at the soma. Radial oblique dendrites are of particular interest because they receive most of the synaptic input to CA1 pyramidal neurons while their small diameter has so far precluded direct dendritic patch-clamp recordings. To examine temporal integration of synaptic inputs, the authors evoked gluEPSPs on multiple spines along an oblique branch and found that synaptic inputs summate linearly or supralinearly depending on the input pattern. Inputs summed supralinearly, i.e., they evoked dendritic spikes, when the depolarization by gluEPSPs was sufficiently rapid and large, with a threshold corresponding to about 20–25 average-sized synaptic inputs arriving within a time window of less than 6 ms (Figure 2). Because each oblique branch is contacted by about 300–400 synapses (Megias et al., 2001) Losonczy and Magee estimated that at least 5% of all synapses on a branch must be activated within a time period of 6 ms or less to evoke a dendritic spike. Less synchronous patterns summated linearly.

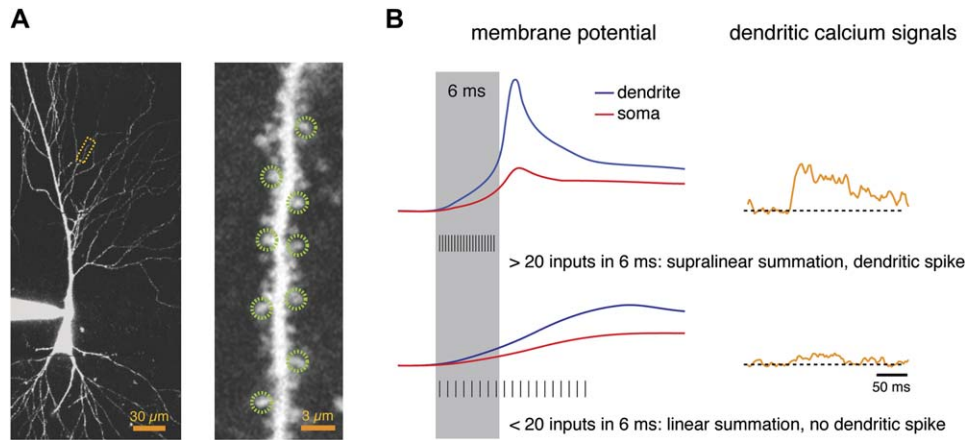


Figure 2. Defining Rules for Summation of Synaptic Inputs Using Patterned Uncaging

(A) left panel, two-photon image stack of a CA1 pyramidal neuron filled with OGB-1. Right panel, close-up single image showing the radial oblique branch highlighted by the dotted orange box in the left panel showing sites for two-photon glutamate uncaging (green) (Losonczy and Magee, unpublished data). (B) schematic representation of somatic (red) and dendritic (blue) membrane potentials and dendritic calcium signals (orange) for input patterns suprathreshold (top) and subthreshold (bottom) for initiation of a dendritic spike.

Using glutamate uncaging it is possible to use pharmacological tools to dissect the different postsynaptic mechanisms contributing to dendritic spike generation. The authors show that the rapid initial phase of the dendritic spike is mostly carried by dendritic  $\text{Na}^+$  channels, while the slow component primarily responsible for the supralinear input-output relation at the soma is predominantly generated by NMDA receptor-mediated conductances, in agreement with the findings of Ariav et al. (2003) in basal dendrites. Voltage-gated  $\text{Ca}^{2+}$  channels provide most of the dendritic  $\text{Ca}^{2+}$  influx but contribute little to the voltage transient associated with the dendritic spike. Interestingly, transient outward currents such as A-type  $\text{K}^+$  currents are involved in setting the duration of the time window during which inputs must arrive in order to evoke a dendritic spike.

Losonczy and Magee have thus delineated the ionic mechanisms and temporal constraints for triggering dendritic spikes with synaptic input to oblique dendrites. Their approach in principle also allows them to examine the spatial constraints for dendritic spike initiation. Along a small stretch of an oblique dendrite, changing the spatial distribution of inputs did not influence the probability to evoke a dendritic spike, suggesting that oblique branches can act as single integrative compartments. However, the current method has some limitations for spatial sampling of input, dictated both by technology and biology. First, no more than 3-4 locations can be visited per millisecond using their multisite uncaging approach. The limiting factors are the speed at which the uncaging laser beam can be deflected by the scan mirrors ( $\sim 100 \mu\text{s}$ ) and the exposure time necessary to uncage sufficient amounts of MNI-glutamate ( $\sim 200 \mu\text{s}$ ). While the travelling time between uncaging locations can be reduced to a minimum (a few  $\mu\text{s}$ ) by replacing conventional scan mirrors with acoustooptical deflectors (Shoham et al., 2005), reducing the necessary exposure time at tolerable light intensities would require new caged compounds with higher two photon cross-sections. Second, the current method only allows uncaging within a given XY plane, whereas dendrites are

rarely confined to a single plane. Activation of multiple spines across large sections of the dendritic tree therefore also requires a fast method for scanning in the Z axis, e.g., using piezo displacement or another acoustooptical deflector (Reddy and Saggau, 2005). These technical improvements are currently on the horizon.

Despite these limitations, Losonczy and Magee demonstrate that multisite two-photon uncaging is a highly promising tool for exploring conditions for nonlinear summation in dendrites by mimicking spatial and temporal patterns of synaptic input. Future studies using this approach, particularly in combination with the expected technical refinements, will be able to address a range of key open questions in dendritic physiology. While the spatial patterning along a single oblique branch did not seem to have a strong influence on the probability of dendritic spike generation, it will be interesting to explore how different spatially patterned inputs summate if they arrive on different branches, or in the presence of ongoing background synaptic input in the entire dendritic tree, as expected to occur in vivo. This will allow us to test whether dendritic branches act as independent integration units (Mel, 1993) or whether spikes on different branches can influence each other. If the spatial patterning of inputs indeed proves to be important when input is delivered across different branches, this multidimensional input space must be explored in order to understand the interaction between spatial and temporal dendritic integration. This should also include an assessment of the contribution of synaptic inhibition to limiting the threshold, spread and interactions of dendritic spikes in different branches, since feedforward inhibition is a critical element of synaptic excitation (Pouille and Scanziani, 2001). This will require further development of caged inhibitory neurotransmitters suitable for two-photon uncaging. The context-dependence of synaptic integration can also be assessed by using uncaging of neurotransmitters in vivo, which will allow us to study the state-dependent dendritic integration of neurons embedded in functioning networks. Finally, the local dendritic spikes triggered

by synchronous synaptic input lead to large dendritic calcium signals, which are potent stimuli for triggering synaptic plasticity (Golding et al., 2002). The readout of synaptic integration by the dendritic calcium signal can therefore provide a link to long-term storage of activity patterns in dendrites. Thus, the approach pioneered by Losonczy and Magee now allows us to probe one of the dark corners of the brain, the fine oblique dendrites, and promises to provide a more enlightened view of dendritic function.

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## Inducible and Cell-Type Restricted Manipulation in the Entorhinal Cortex

The entorhinal cortex functions as the gateway to the hippocampal formation. However, its role in formation and consolidation of hippocampus-dependent memory remains relatively unexplored. In this issue of *Neuron*, Yasuda and Mayford report an elegant cell-type

restricted inducible transgenic mouse overexpressing a mutant form of CaM kinase II selectively in superficial layers of medial entorhinal cortex and its upstream regions. These animals display a selective spatial memory deficit during the immediate posttraining period as well as during acquisition in the Morris water maze. Similar to the hippocampus, this time-limited involvement of entorhinal cortex in spatial memory processing suggests a crucial role for hippocampal-entorhinal circuitry in spatial memory formation.

Studies of stroke and other brain-damaged patients have shown a localization of many brain functions, including specific forms of learning and memory. The most compelling evidence that memory formation and recall of daily life depends on the medial temporal lobe came from neuropsychological studies of the amnesic patient H.M. (Scoville and Milner, 1957), who received bilateral temporal lobectomy after medically intractable epilepsy. Although the severity of H.M.'s seizures was reduced by the surgery, H.M. instead suffered from characteristic memory impairments. Although his ability to learn basic motor skills and short-term memory was preserved, he was unable to form new declarative memories that can readily be brought to conscious recollection. Moreover, he could not recall events that transpired within about 11 years preceding his surgery (Sagar et al., 1985). Later, amnesia patients suffering from ischemic injury limited only to the hippocampus were also found to be impaired in the acquisition of new memories but not to the severe degree experienced by H.M. (Squire and Zola-Morgan, 1991). Because his bilateral medial temporal lobe resection included the hippocampal formation and adjacent structures, including most of the amygdala and entorhinal cortex, differential and substantial roles of the parahippocampal regions in some memory processes have long been suggested.

For some years now it has been known that the majority of the cortical input to the hippocampus is funneled through the association cortices that surround the hippocampus. In particular, the entorhinal cortex receives inputs from various cortical areas, including the perirhinal, parahippocampal, pre- and parasubiculum, piriform, orbitofrontal, and retrosplenial cortices (Witter et al., 1989). Therefore, one would expect that selective lesions of the entorhinal cortex could severely impair hippocampus-dependent memory. However, the majority of recent studies have suggested that selective hippocampal lesions result in more profound acquisition deficits in spatial navigation tasks, such as the Morris water maze, than do selective entorhinal cortex lesions (Aggleton et al., 2000; Jarrard et al., 2004). This line of evidence has also been replicated by selective electrolytic lesions of temporoammonic (TA) pathway from entorhinal layer III cells to hippocampal CA1 in rats (Remondes and Schuman, 2004). Importantly, the TA lesion 24 hr after, but not 3 weeks after, the training of hidden platform tasks impaired memory recall later, suggesting that the TA-conveyed cortical activity is required for memory consolidation that occurs within 3 weeks after the training. But why are conventional selective fiber-sparing lesions of entorhinal cortex so controversial in mimicking the acquisition deficit in Morris water maze tasks