Single-Voxel Recording of Voltage Transients in Dendritic Spines

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ABSTRACT We report sensitive recording of membrane potential in single dendritic spines in cortical neurons within a brain slice using two-photon excitation and a new, fluorinated, intracellularly loaded organic dye, di-2-AN(F)EPPTEA. With a two-photon excitation wavelength of 1060 nm, we achieve voltage sensitivity of >16% change in fluorescence per 100 mV. By targeting single spines in single-voxel recordings, we attain excellent single/noise quality, with back-propagating action potentials (bAPs) visible in single sweeps while recording at 10 kHz. This recording rate allows us to reliably assess fast bAP dynamics on single sweeps including bAP rise times of 0.5 ms. The amplitude and propagation delays of the bAPs are similar among different spines located within the same dendritic region, and this is true despite large differences in spine size. The interregion differences in bAP waveforms in spines vary in relation to their distance from the soma and the caliber of their parent dendrites.

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Voltage-sensitive dye (VSD) imaging of electrical activity permits the high spatial resolution recording of voltage changes when classical electrodes or patch pipettes are too bulky (1). Perhaps the ultimate application of optical voltage recording, requiring excellent spatial and temporal resolution, is to probe voltage changes at individual dendritic spines, which are the fundamental neuronal units for the initial processing of synaptic inputs. This has been recently achieved using second harmonic generation (2), confocal linescans (3), and a fast charge-coupled device (CCD) camera (4) to image spines near the surface of a brain slice. In each case, the VSD was applied intracellularly and allowed to diffuse into the dendritic arbor. In the latter study, a dramatic increase in sensitivity and signal/noise permitted visualization of spine voltage changes in single trials. Applying this approach to two-photon imaging of VSDs (5) could improve the measurements still further by permitting deeper penetration of the brain while preserving the sensitivity of fluorescence-based detection.

Here we introduce a new, VSD optimized for two-photon imaging, combining it with a single voxel recording method targeted to individual spines. This combination allowed us to obtain recordings with sufficient temporal resolution to record fast voltage transients in single spines with single-sweep sensitivity. We use this approach to examine how back-propagating action potentials (bAPs) recorded in spines vary at different locations along the dendritic tree of a pyramidal neuron in a mouse cortical brain slice.

Our lab is actively developing new voltage-sensitive dyes to improve the attainable signal/noise quality of fluorescence recordings by improving the voltage sensitivity and photostability of the dyes. The dye we introduce here is di-2-AN(F)EPPTEA (synthetic procedure is provided in the Supporting Material), which has a structure shown in Fig. 1 *A*. It can be compared to the popular di-2-ANEPEQ from our lab (known also as JPW1114) with the following differences: there is a fluorine substitution on the naphthalene group; and a propyltriethylammonium rather than an ethyltrimethylammonium group at the polar head. Fluorination is known to improve the photostability of organic dyes (6). Substituting fluorine on the π-electron donor side of the chromophore shifts the absorption and emission spectra to the blue compared to the unsubstituted chromophore. The one-photon absorption spectrum of the fluorinated di-2-AN(F)EPPTEA shows a peak at 452 nm (Fig. 1 *B*), compared with 480 nm for nonfluorinated di-2-ANEPEQ.

We measured the two-photon excitation spectrum by tuning the Ti-Sapphire laser in 20-nm increments and found a peak excitation wavelength of 920 nm (Fig. 1 *B*). To maximize sensitivity in VSD imaging of this class of hemicyanine dyes, it is necessary to select excitation wavelengths significantly longer than the peak (the so-called "red-edge excitation") (7,8). For di-2-AN(F)EPPTEA, we observed that voltage-sensitivity rises nearly linearly with increasing two-photon excitation wavelength (Fig. 1 *D*). This was established by repeating recordings of bAPs in single spines and varying excitation wavelength from 940 to 1060 nm. Laser power was adjusted (increased with increasing wavelength) to match baseline signal levels for a fair comparison of signal/noise, which also increased with wavelength (Fig. 1 *E*). At 1060 nm, the voltage-sensitivity in the spine was determined to be 16.1% per 100 mV change at the soma. These recordings were performed at the most proximal spines on basal dendrites (<30 μm). Because there may be a small decrement in the bAP amplitude at the

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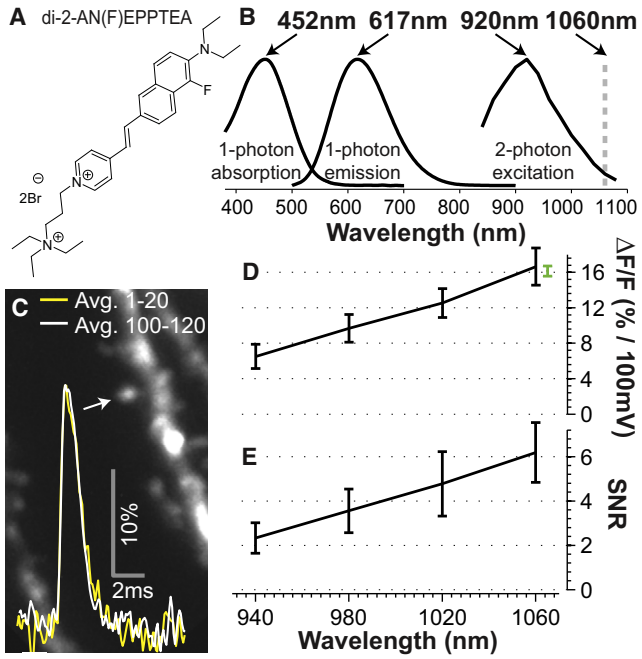

FIGURE 1 Stability, voltage-sensitivity of di-2-AN(F)EPPTEA. (*A*) Chemical structure of the dye. (*B*) One-photon absorption and emission spectra in multilamellar lipid vesicles with peak wavelengths shown. Two-photon excitation spectrum measured in a brain slice—excitation power (after objective) was 2.5 mW for all wavelengths. (*C*) Overlapped averages of first and last 20 (of 120) recordings of back-propagating action potentials (bAPs) in a single spine, with no change in signal. (*D*) Two-photon voltage sensitivity as a function of excitation wavelength, measured using bAP amplitudes at proximal spines, normalized by somatic AP amplitude (100 mV somatic). For each of $N = 9$ spines (five cells), all four wavelengths were tested (error bars are SD). Separate green point: all proximal spine measurements at 1060 nm (16.1%, $N = 15$ spines, seven cells, error bar is mean ± SE). (*E*) Single-sweep signal/noise ratios (SNRs). SNR values were normalized by $\sqrt{N}$ (number of sweeps averaged). Typical laser powers: 1 mW at 940 nm, 4 mW at 1060 nm. Bleaching is negligible in all instances.

proximal dendrites (9,10), this may be slight underestimate of the actual voltage sensitivity. We saw no signs of photodamage or toxicity, even when recording over 100 sweeps from individual spines at 1060 nm (Fig. 1 *C*, more on phototoxicity in Supporting Material).

Single-voxel recordings at 10 kHz (see Supporting Material for details) triggered by electrophysiology were performed to track bAPs in individual spines at different regions along the dendritic tree. Dye was loaded internally via the somatic whole cell patch electrode. The dye-filled pipette was removed and the cell was repatched with a dye-free pipette after a period of 35 min (9), a procedure that provided adequate staining of the dendritic arbor (Fig. 2 *A*). Action potentials (APs) were elicited by somatic current injection via the patch electrode. At the most proximal recording location (an apical oblique dendrite, ≈65-μm path distance from the center of the cell body, ignoring small scale curvature and changes in depth), the physiology of two different spines (interspine distance ≈8 μm) was explored using single-voxel two-photon VSD imaging (Fig. 2 *B*). Despite significantly different baseline intensities (bottom targeted spine, 35% as bright as top spine, presumably due to different spine sizes or membrane areas) the measured $\Delta F/F$ amplitudes were similar, 17.4 and 19.7%. Recordings from the larger spine produced good quality waveforms in single sweeps (signal/noise ratio, $SNR = 8.3$ for single sweep; see Fig. 2 *B*). The average of eight sweeps, temporally aligned to the peak of the first AP in the electrical recording, produced a SNR of 27.7.

Two spines at a distal region along the apical trunk yielded $\Delta F/F$ amplitudes of 16.7 and 14.6% (Fig. 2 *C*). Again, the amplitudes seen at two separate spines in the same region were similar; however, they were significantly smaller than the amplitudes observed at the apical oblique recording site. Finally, at a distal recording location in the apical tuft, signals were clearly diminished (Fig. 2 *D*); although the distance to this tuft region is not much further from the soma than in Fig. 2 *C*, the caliber of the tuft dendrite is smaller than the trunk. Signal amplitudes from all spines recorded are given in Fig. 2 *E*. The simplest explanation for the observed consistency between fluorescence changes in pairs of spines within local dendritic regions is that:

1. The attenuation of the bAP with distance is gradual (11) such that the parent dendritic segments for the different spines within each separate region see approximately the same bAP amplitude.
2. bAPs invade different spines with little or no attenuation ((2–4), and see also Fig. S1 in the Supporting Material).
3. The sensitivity of the VSD recordings from spines is not significantly corrupted by differences in spine size, or differences in internally bound dye (contributing to background fluorescence).

As with the bAP amplitude, propagation delays were also consistent between spines in the same region with more proximal regions showing 0.3-ms delays, whereas the most distal spines showed 1.1 and 1.5 ms delays (Fig. 2 *F*). These delays are readily resolved because of our high time resolution and are consistent with measurements of bAP delays in the dendrite, measured either electrically (12) or optically (13). Our recordings from spines also indicate that back-propagation is decremental in this example—a conclusion that is consistent with the observed changes in waveform shape across dendritic regions. In particular, half-widths increase from an average of 1.0 to 1.7 ms from the more proximal apical oblique region to the more distal apical tuft region (Fig. 2 *G*).

To summarize, this work presents a new VSD and brain slice optical recording method, which are both optimized for two-photon measurements of electrical activity in single spines with high spatial and temporal resolution. We demonstrate decremental propagation of the back-propagating

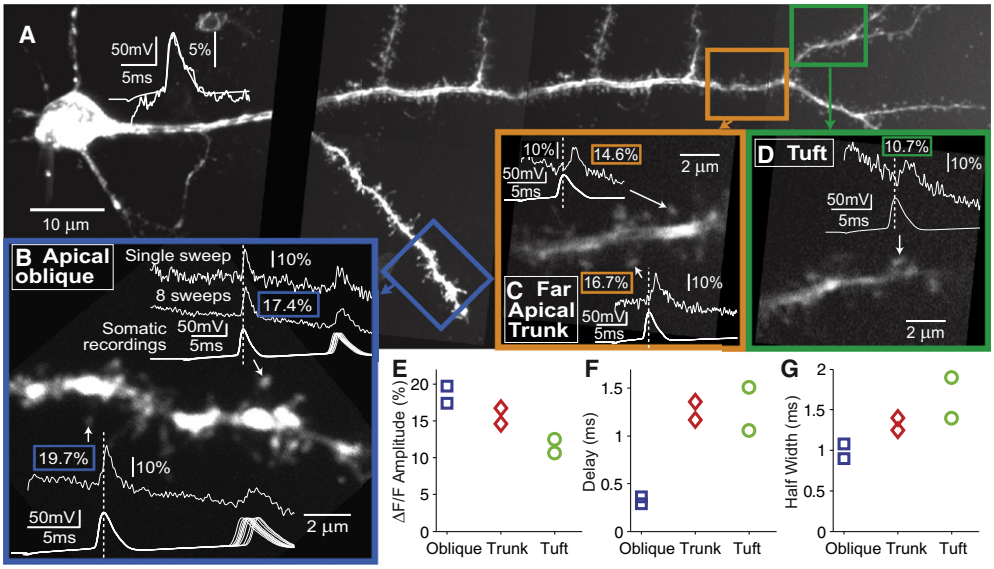

FIGURE 2 Imaging of action potential backpropagation and invasion of dendritic spines using voltage-sensitive dye (VSD) di-2-AN(F)EPPTEA. (*A*) An image montage of a cortical pyramidal neuron loaded via somatic patch pipette with the VSD. The traces at the upper left show superimposed somatic electrical and perisomatic VSD records, both at 10 kHz, demonstrating precise temporal synchronization. (*B*) Recordings of back-propagating action potentials (bAPs, two spikes, analysis applies to first spike only), elicited using somatic current injection, at two different spines in the same region on an apical oblique dendrite. Somatic electrical waveforms with multiple recordings aligned at the first spike are shown below single-voxel imaging data from spines. (*Inset boxes*) Amplitudes of the optically recorded bAP waveforms. For the top spine, a single optical sweep is shown along with the average of eight temporally aligned sweeps. Single-sweep measurements: 18.0% amplitude, 0.29 ms delay, 0.32 ms rise time, 0.83 ms half-width, SNR = 8.3). (*C*) Same as panel *B*, except two spines are targeted on a distal apical trunk region. (*D*) VSD recording from a spine on the apical tuft. (*E*–*G*) Summary of amplitudes, propagation delay times (optical relative to electrical peak time), and half-widths for bAPs recorded in spines in all three regions. In all cases, spines (indicated by *arrows*) were placed in focus and targeted at their centers. (*Vertical dashed lines*) Peak times of somatic action potentials. Laser power (measured after objective, above slice): 4–5.5 mW (proximal to distal locations).

action potential (bAP) into spines at remote regions of the dendritic arbor. These results are also consistent with the conclusion of others (2–4) that bAPs invade spines from the adjacent dendrite.

SUPPORTING MATERIAL

Additional information with a synthesis and one figure and MATLAB code for voxel recordings is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00719-3.

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