Targeted Path Scanning: An Emerging Method for Recording Fast Changing Network Dynamics across Large Distances

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Abstract

Attention is increasingly focused on the dynamical behavior of large networks of neurons and astrocytes and the changes in these dynamics that occur during the progression of diseases like epilepsy. Recording from large numbers of identified cell types has been traditionally difficult, but the advent of fluorescent indicators capable of detecting changes in the internal calcium levels of cells has led to the ability to visually record the activity of large numbers of cells. However, for most imaging techniques the temporal resolution is sharply limited by the time it takes lasers to traverse the typical raster scan. As network dynamics can evolve quite rapidly, this is a serious limitation. The present paper describes the Targeted Path Scan technique, which dramatically increases the scanning frequency by allowing the user selection of trajectories through cells of interest. TPS is discussed in the context of a study of altered network dynamics in a common rat model of epilepsy.

The present paper describes the Targeted Path Scan technique, which dramatically increases the scanning frequency by allowing the user selection of trajectories through cells of interest. TPS is discussed in the context of a study of altered network dynamics in a common rat model of epilepsy. In this study, traveling waves of calcium transients that were frequently encountered in astrocytes imaged in brain slices obtained from control rats were dramatically reduced in astrocytes imaged in brain slices obtained from rats that had experienced status epilepticus. The speed of these traveling waves would have made them impossible to identify using traditional scanning techniques.

Keywords: Neurons; Astrocytes; Calcium imaging; Hippocampus; Targeted path scan technique

Introduction

The dynamic formation of cell assemblies defined by their mutual patterns of activity is thought to be fundamental for many neural processes [1-3]. Additionally, neurons can interact with glia, particularly astrocytes, potentially forming complex and poorly understood neuron-astrocyte dynamical systems [4-8]. Distinct changes in patterns of network activity have been correlated with a number of diseases including Parkinson’s disease [9-11] schizophrenia [12] autism [13], and epilepsy [14-16]. While much of the work to date has focused on synchronous activity, other dynamic network patterns have received increasing attention such as traveling waves, which are prominent in astrocytic networks [7,17-19]. As the astrocytic traveling waves can be quite fast [7], it is important to have a fast scanning technique to distinguish them from purely synchronous network activity. The determination of such complicated network activity requires experimental techniques that can record activity from a large number of cells over distances spanning the size of networks, and to accomplish this task quickly enough to capture the relevant dynamics. As calcium levels in cells are correlated with activity in both neurons and astrocytes, the imaging of fluorescent calcium indicators has the potential of satisfying the aforementioned requirements. However, to date, imaging the calcium dynamics in numerous cells (10s to 100s) over the relevant distances of at least 100s of micrometer is quite difficult for frame rates above a few Hz.

A technique, Targeted Path Scanning (TPS), for the imaging of calcium changes in a large number of cells over extended distances with high spatiotemporal resolution has been developed by Lillis et al. [20] TPS is an extension of previous studies [21,22] and uses standard two-photon microscopy hardware. TPS improves on the inherently slow nature of raster scanning for generating image sequences by allowing the selection of specific trajectories for the two-photon beam through the cells of interest. The scanning speed is further enhanced by maintaining a user-defined sampling rate within cells of interest while accelerating the beam between cells of interest, thus minimizing dead time. This technique results in scan rates up to 100s of Hz for millimeter-scale distances and maintains the superior signal-to-noise ratio of two-photon microscopy. The technique is discussed in the present paper for the application of determining changes in astrocytic network activity in brain slices prepared from rats that have experienced kainic acid (KA)-induced status epilepticus (SE), a common animal model of epilepsy that results in reactive astrogliosis and changes in astrocyte function. A primary finding in the present set of experiments is that fast traveling waves of calcium activity in astrocytes in the CA1 region of the hippocampus are less prominent in rats that experienced early life SE. Furthermore, the speed of these traveling waves would have made them difficult to distinguish from synchronous activity if traditional imaging techniques had been used. Therefore, TPS will be instrumental in identifying rapid calcium signaling events that are changed as a consequence of neural and glia pathology.

Methods

Slice preparation and labeling with SR101 and Indo-1

All experimental protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Sprague-Dawley rats (P17-P20) were injected I.p with 7.5 mg/kg kainic acid and observed for

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3 hrs following injection. Seizures were classified on the Racine scale (1-5) and animals with significant seizures, class 4-5, were selected for study [23]. Following treatment, rats were returned to their home cages with their mother. Control animals were sham injected with vehicle (0.9% saline). After 3-5 days recovery, rats were deeply anesthetized with isoflurane and brains were rapidly dissected and placed in ice-cold (4°C) oxygenated sucrose Ringer’s solution (95% O2/5% CO2) containing (in mM): 200 sucrose, 26 NaHCO3, 10 glucose, 3 KCl, and 1.4 NaH2PO4. Brains were then trimmed, and glued ventral side down to the mounting disk of a Leica VT1200 (Leica Microsystems, Wetzlar DE). Horizontal brain slices (400μm) containing both the medial entorhinal cortex and hippocampus were cut in oxygenated sucrose Ringer’s solution (4°C). Following sectioning, slices were transferred to a holding chamber that contained 1 μM SR101 (Sigma-Aldrich, St. Louis, MO) [24,25] in normal, oxygenated Ringer’s solution (34°C) containing (in mM): 126 NaCl, 26 NaHCO3, 10 glucose, 3 KCl, 1.4 NaH2PO4, 2 CaCl2, and 2 MgCl2. Following incubation in SR101 (10-12 min), slices were transferred to a separate holding chamber containing oxygenated Ringer’s solution without SR101 (room temperature) for 10 min. The slices were then incubated in 100 μg/ml Indo-1 AM (Invitrogen) in ACSF containing 0.04% Pluronic (Molecular Probes) at 38°C for at least 1 hr. The higher temperature (personal communication with Dr. Doug Coulter) helped with the notoriously difficult labeling of brain slices prepared from young adult rats [26,27]. Following bulk dye labeling the slices were incubated in Ringer’s solution at room temperature for at least 30 min to allow for cleavage of the AM ester group thus trapping the calcium indicator in the cells.

**Calcium imaging**

For imaging, slices were transferred to an immersion style recording chamber (Warner Instruments) and perfused with oxygenated Ringer’s at 32°C. Calcium imaging was performed using a custom-built two-photon microscope [20] built around a mode-locked Ti: Sapphire laser source emitting 140 fs pulses at an 80 MHz repetition rate with a wavelength adjustable from 690-1040 nm (Chameleon Ultra I; Coherent, Santa Clara, CA). The ability to adjust the laser wavelength gives us considerable flexibility in choosing fluorescent dyes. In this setup, laser power is attenuated using an electro-optic modulator (ConOptics, Danbury, CT) and scanning is accomplished using x-y galvonometer-mounted mirrors (GSI Lumonics, Billerica, MA) controlled by custom LabView software [20]. Glial cells are identified as cells containing both Indo-1 (460-580 nm bandpass filter) and sulforhodamine 101 (600-660 nm bandpass filter) in an XY raster scan using a 20X 0.95 NA water-immersion objective (Olympus, Tokyo, Japan). Fluorescently identified glial cells containing both SR101 and Indo-1 in stratum radiatum of the CA1 region of the hippocampus were selected for the TPS trajectories [20]. The CA1 region is thought to be involved in seizure generation in animal models of epilepsy and was the first region we examined using the TPS technique.

**Data analysis**

A fluorescence time series can be extracted from the path scan for all cells by averaging the fluorescence intensity of the pixels belonging to each cell, removing spurious exponential trends introduced by photobleaching, and calculating the change in fluorescence at each time point normalized by resting fluorescence. Additionally, the time series can be filtered to reduce noise using a 4-pole Butterworth filter with a cutoff frequency of 5 Hz. Calcium ‘spikes’ in glial cells are identified as calcium transients having rapid onsets, decay time constants of >2 seconds, and ΔF/F amplitudes greater than 10%. This data analysis is performed with custom-written scripts in Matlab (Math Works, Natick, MA).

**Results**

**Recording from networks of astrocytes**

The dynamics of neural networks result from complex interactions of a variety of types of neurons and glia. TPS is utilized in the present paper to specifically examine the internal calcium dynamics of astrocytes. The astrocytes were identified by double labeling the freshly prepared rat brain slices with SR101, the astrocyte specific fluorescent marker [24,25] and Indo-1 AM, a fluorescent indicator that changes level of fluorescence in response to changes in calcium concentration. An example of SR101 labeling is shown in (Figure 1A) and Indo-1 labeling in (Figure 1B). The fluorescent dyes and indicators were introduced into the tissue by incubating the tissue in solutions containing the fluorophores. This resulted in the labeling of approximately the superficial 50 microns of tissue in the 400 micrometer thick brain slices, a limitation when bulk labeling brain slices prepared from older animals, greater than postnatal day 17 [26]. The co-labeling of glial cells by these two fluorescent dyes is shown in (Figure 1C). The majority of cells are co-labeled indicating that in the conditions of dye labeling in the present work, Indo-1 preferentially,
but not exclusively, labeled astrocytes. The imaged network shown in (Figure 1) spans an area of approximately 600 x 600 μm. This is the size of astrocytic network that has traditionally been imaged with a frame rate on the order of 1 Hz [7,28].

**Selection of the trajectory and recording calcium events**

The selection of the trajectory for the targeted path scan starts with a x-y raster scan of the cells of interest (Figure 2B). Software implemented in Labview [20] then allows for a point-and-click selection of the trajectory with high resolution lines of interest (Figure 2A and Figure 4A) alternating with the accelerated portion of the trajectory between cells. This alternating high and low resolution between the cells of interest and the space between them, respectively, improves the frequency with which each cell is sampled, allows for greater distances to be recorded, and because of the increased time spent in the region of interest improves the signal-to-noise ratio. The path shown in (Figure 2A) is arbitrary and contains a number of less-than-optimal sharp angles. Figure 4A is an example an approximately spiral path that reduces the number of sharp angles and improves the accuracy of the line path at very fast scanning speeds. The frequency with which the two-photon beam completes the selected path depends on the length of the selected path and the number of cells [20].

A time series of changes in calcium levels is calculated by averaging the fluorescence intensity of the pixels belonging to each cell and calculating the change in fluorescence at each time point normalized by resting fluorescence (∆F/F, Figure 2B). The four traces in (Figure 2B) show examples of spontaneous astrocytic calcium transients with their typical slow kinetics on the order of seconds. An example of the raw data collected using the TPS technique is shown in (Figure 2C). Each horizontal line of pixels is an unwrapped and straightened line scan trajectory, in this case the trajectory shown in Figure 2A. Time proceeds downward on the vertical axis with the increment of time defined by the time taken to complete one line scan which is dependent on the details of each targeted path. The location of four astrocytes in the unwrapped line scans are highlighted by arrows at the bottom of (Figure 2C). In this two-photon application, Indo-1 is a calcium indicator that decreases the level of fluorescence in response to an increase in internal calcium, thus the calcium transients can be identified by the darker locations in the data set. While the spontaneous calcium transients were generally quite rare in these recordings, only occurring a few times over the course of minutes, the transients did include 10s of astrocytes per network in networks of CA1 astrocytes in brain slices prepared from young adult rats was the traveling calcium wave. An example of a traveling wave recorded from a sham-injected rat is shown in (Figure 3). The plot of unwrapped trajectories versus time is shown in (Figure 3A) and the red oval outlines the distinctive features of the traveling wave in this form of the data set. The pattern repeats to the left and right of the oval. This is simply due to the spiraling nature of the trajectory, which traverses the same region of the slice multiple times. (Figure 3B) shows a three-dimensional rendering of the trajectory over an image of the slice. The blue line is the trajectory and the colored spheres are the higher resolution regions along the trajectory overlaying astrocytes. The height represents the relative fluorescence. The distinct slope of the relative fluorescence, rising from right to left in (Figure 3B), highlights the traveling wave at a particular time point. The time point for this rendering is marked by the red arrow on the data in (Figure 3A).

The characteristics of the traveling wave can be further examined by plotting the fluorescent time series from astrocytes participating in the wave (Figure 4). As shown in (Figure 4A), 10 astrocytes that participated in the wave were selected for a plot of fluorescent changes over time. These astrocytes are marked with colored circles along the targeted path and overlaid on the x-y image that was used for cell selection. The corresponding fluorescent time series are shown in (Figure 4B). The first peak encountered occurs on the astrocyte marked with the green circle on the right side of (Figure 4A). The corresponding trace in (Figure 4B) is shown at the bottom. From right to left, the peaks in ∆F/F occur at later time points as the astrocytes are positioned more to the left, indicating a traveling wave from right to left in this example.

The incidence of rapid traveling waves was different in brain slices obtained from control rats versus brain slices prepared from rats that...
experienced KA-induced SE. Out of 10 slices prepared from sham injected animals 7 exhibited traveling waves (Figure 5A). However, out of 21 slices prepared from rats that experienced SE, traveling waves could be found in only 2. These ratios were significantly different (P < 0.05; two-proportion test). The speed of the traveling waves in the control animals ranged from 122 to 680 μm/sec and averaged 309 μm/sec (Figure 5B). The speeds of the 2 waves recorded from brain slices prepared from treated animals were 334 and 230 μm/sec, within the range of control speeds. The speed of these traveling is the same order of magnitude, but somewhat faster, than similar traveling waves observed in rat in vivo [7] also using two-photon microscopy with a limited field of view of only 10x instead of 100s of μm. Waves traveling at these speeds would enter and exit fields of view of the 100s of μm squared in approximately a second making sampling rates well above 1 Hz crucial for distinguishing the traveling wave from true synchronous activity. The decrease in structured activity in slices from KA-treated rats may represent a good biomarker for epilepsy-induced changes in network activity. The proper characterization of network activity, traveling wave versus synchronous for example, is important for the formation and testing of hypotheses about pathological changes in network function. Important distinctions could be obscured with slower scanning rates making techniques like TPS an important tool.

Discussion

The present study used targeted path scanning (TPS) to characterize rapid calcium waves across large networks of astrocytes. We found that ultra rapid waves were only detectable as a consequence of the sampling frequency conferred by TPS and that these waves were significantly diminished in incidence in slices obtained from KA-treated rats. It is hypothesized that an important aspect of astrocyte signaling and subsequent modulation of the surrounding neural network is the pattern of calcium activity through many astrocytes in the syncytium. Indeed, recent experiments performed in vivo suggests that Ca2+ responses in astrocytes may be as fast as their neuronal counterparts, and therefore relevant during fast synaptic transmission [29]. Two-photon microscopy is a powerful visualization technique that gives excellent spatial resolution in three dimensions of fluorescent signals. However, scanning an entire two-dimensional image by moving the laser in a classic raster fashion is too slow to capture calcium transients associated with action potentials in neurons or the calcium transients in astrocytes independent of neural activity, extracellular diffusion of ATP and IP3 diffusion through gap junctions, do not support waves this fast. It is not currently clear why astrocytes in slices obtained from KA-induced SE treated rats lacked these fast waves. Indeed, KA-induced SE at the young ages used in this study does not typically result in the extensive cell death that is observed in adult animals. Furthermore, in contrast to SE in adult animals, early life SE does not tend to result in the development of epilepsy, although seizure susceptibility to a second insult is dramatically increased [33]. Nevertheless, astrocytes demonstrate properties of reactive astroglisis, with increases in expression of glial fibrillary acidic protein (GFAP) following early life seizures. Thus the astrocytes in slices from the KA-induced SE treated rats suggests that this treatment is sufficient to induce alterations in network calcium signaling.

The imaging and analysis of network activity is potentially a very powerful experimental tool for studying network pathologies as changes in synchronization of neurons and glial cells have been hypothesized to be involved in a number of disorders including Parkinson’s disease, autism, epilepsy, Alzheimer’s disease, and schizophrenia [13,34]. Because changes in synchronization and other patterns of network behavior are seen in a variety of neurologic disorders in the absence of overt cell death, a technique recording network patterns is potentially more sensitive to changes following damage. Distinct and complex network-level dynamics precede the onset of pathological synchrony observed in patients [16], animal models of epilepsy [35], and in neuronal networks in vitro [36]. As has been shown for astrocytes previously [7] and in the present work, the speed of the astrocyte dynamics can test the limit of traditional imaging techniques making techniques like TPS important for these applications.

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