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Calcium imaging of infrared-stimulated activity in rodent brain

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Summary

Infrared neural stimulation (INS) is a promising neurostimulation technique that can activate neural tissue with high spatial precision and without the need for exogenous agents. However, little is understood about how infrared light interacts with neural tissue on a cellular level, particularly within the living brain. In this study, we use calcium sensitive dye imaging on macroscopic and microscopic scales to explore the spatiotemporal effects of INS on cortical calcium dynamics. The INS-evoked calcium signal that was observed exhibited a fast and slow component suggesting activation of multiple cellular mechanisms. The slow component of the evoked signal exhibited wave-like properties suggesting network activation, and was verified to originate from astrocytes through pharmacology and 2-photon imaging. We also provide evidence that the fast calcium signal may have been evoked through modulation of glutamate transients. This study demonstrates that pulsed infrared light can induce intracellular calcium modulations in both astrocytes and neurons, providing new insights into the mechanisms of action of INS in the brain.

Keywords

infrared neural stimulation; calcium signaling; somatosensory cortex; optical imaging; calcium dye imaging; neurons; astrocytes

1. Introduction

Infrared neural stimulation (INS) has been demonstrated to be capable of activating peripheral nerves [1–4], auditory ganglion cells in the cochlear [5–7], the cristas ampullaris in the vestibular system [8], and can be used as a method for cardiac pacing [9]. The high

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spatial selectivity of INS suggests that this technique has the potential to improve upon current clinical care in a range of settings. However, one area that has been underexplored is the potential application of INS for stimulation of the central nervous system, for example for deep brain stimulation (DBS) in Parkinson's disease, or for mapping eloquent cortex during brain tumor resection. An all-optical neurostimulation method that does not require exogenous agents could have numerous advantages in clinical settings.

Our group first demonstrated INS in the central nervous system by evoking compound action potentials in thalamocortical brain slices *in vitro* [10]. In a separate study, we discovered that pulsed infrared light increases GABA release in cultured neurons, suggesting that INS can modulate inhibitory synaptic activity [11]. The first *in vivo* application of INS in somatosensory cortex employed optical intrinsic signal imaging (OISI) to detect a decrease in the optical reflectance of 632 nm light in response to INS that appears similar to cortical reflectance changes evoked by peripheral tactile stimulation. However, single unit recordings during cortical INS revealed inhibition of spontaneous firing rates of cortical neurons, contradicting the excitatory neural responses evoked by somatosensory stimulation typically observed in OISI [12]. This result prompts the question of what effects INS has at the cellular level in the living brain, a question that must be answered if the utility of INS for clinical use in the brain is to be determined.

INS is known to evoke action potentials through a thermal gradient that causes depolarization [13]. Shapiro and colleagues provided evidence that INS depolarizes lipid membrane bilayers via a thermally mediated change in membrane capacitance that requires no specific ion channels. This theory suggests that any cell membrane can be depolarized by pulsed infrared light, which is consistent with responses seen in oocytes and mammalian HEK cells [14]. It is reasonable to expect therefore, in addition to neurons, that other cell types abundant in the brain, such as astrocytes could influence the cortical response to INS in ways not seen in the peripheral nervous system.

In contrast to our prior OISI measurements, aimed at capturing the secondary hemodynamic effects of INS, the current study utilizes multiscale calcium sensitive dye imaging as a direct measure of cellular responses. Calcium sensitive dyes exhibit an increase in fluorescence in the presence of increased calcium, so when loaded into a cell, the change in fluorescence can be used as a direct measure of intracellular calcium responses. Calcium sensitive dyes can be imaged in exposed brain on an ensemble level using wide-field optical imaging methods similar to OISI, or on a cellular level using *in vivo* two-photon microscopy [15–18]. Here, both imaging methods are used in addition to pharmacology to identify the predominant cell types responding to INS in the living brain.

In our studies, wide-field calcium responses to INS, applied to the exposed somatosensory cortex, were found to exhibit a fast and slow component. Wave-like properties were also observed, suggesting possible network activation. Cellular imaging and pharmacology confirmed that the predominant cellular mechanisms responsible for INS-evoked calcium signals are primarily astrocytic; however, small underlying neuronal influences are present. The relationship between INS parameters and evoked calcium signals were characterized to

obtain a complete picture of the observed response. This study demonstrates that INS invokes physiological responses in both astrocytes and neurons.

2. Methods

2.1 Surgical procedures

All procedures were performed in accordance with protocols approved by the Vanderbilt University or Columbia University (two-photon experiments only) IACUC. Briefly, male Sprague-Dawley rats ($n = 32$; 300 – 500 g) were anesthetized with a 50% urethane (Sigma-Aldrich, St. Louis MO) solution (I.P. 1.4 g/kg). Toe-pinch was used to ensure that the animal was in an adequate state of anesthesia. The animal was placed in a stereotactic frame and a craniotomy and durotomy were conducted to expose somatosensory cortex (+2 to –5 mm anterior/posterior, and 7 mm lateral to bregma) [19, 20]. Mannitol (1.0 ml, 20% concentration) was given I.P. to prevent potential brain edema. Oregon Green 488 BAPTA-1 AM calcium dye (O-6807, Life Technologies, Grand Island NY) was mixed with 8 μ l pluronic acid in DMSO and 32 μ l of Ringer's solution. This dye was loaded into a pulled glass capillary pipette and a microinjector (Picospritzer, Parker, Cleveland OH) was used to inject the dye into the cortex throughout the craniotomy (5 – 10 locations, 20 – 40 psi, 200 – 1000 ms) at depths up to 1000 μ m. A 400 μ m diameter optical fiber (Ocean Optics, St. Petersburg FL) to deliver INS light was placed directly onto the cortex, and warm ($\sim 37^\circ\text{C}$) 3% agar (Sigma-Aldrich) in saline and a glass coverslip were used to stabilize the cortex and create an imaging window (Fig. 1a). Imaging began 30 – 60 minutes after dye loading to ensure proper labeling of cells.

2.2 Optical imaging methods

A multispectral imaging system based on the design of Bouchard et al. and Sun et al. was used to perform calcium sensitive dye imaging [15, 21]. Briefly, a high-power light emitting diode (LED) centered at 488 nm with a 460 ± 30 nm bandpass filter (Thorlabs, MCLED, Newark NJ, Semrock, FF01-460/60-20, Rochester NY) was focused onto the cortex with a bi-convex lens (Thorlabs, BK7, focal length = 10.0 mm). Images of the cortex were acquired using a 480 $\times$ 640 pixel Pike fire-wire camera (AVT, Newburyport MA) using a Zoom 7000 macro lens (Navitar, Rochester NY). A 500 nm longpass filter (BLP01-488R-25, Semrock) was placed between the lens and camera to reject the excitation light. The camera and LED were controlled by the SPLASH software package [21].

For each experiment, one imaging run was composed of multiple (5 – 10) imaging trials, where a stimulus was presented once in each trial. Each imaging trial consisted of 30 seconds of imaging at 30 fps, with 6–10 seconds of baseline acquired before stimulation and images were binned 2 $\times$ 2. Image acquisition was synchronized with the start of INS (or electrical) stimulation, and LED illumination was driven from the camera's 'expose' output signal to minimize photobleaching and exposure of the camera during read-out.

2.3 Laser stimulation parameters

Infrared neural stimulation was performed using a $1.875 \mu\text{m} \pm 0.02 \mu\text{m}$ diode laser (Capella neural stimulator, Lockheed Martin Aculight, Bothel WA). This laser was chosen because

previous studies indicated that the optical penetration depth (300 – 600 μm) was optimal for stimulation of neural tissue [2, 10]. Light was delivered to cortex via a 400 μm diameter low OH fiber optic (Ocean Optics, St. Petersburg FL) with a numerical aperture (NA) of 0.22. The fiber was positioned onto somatosensory cortex with a micromanipulator (Stoelting, Wood Dale IL). Average power was measured using a PM3 detector head attached to a Power Max 500 laser power meter (Coherent, Santa Clara CA) from the tip of the fiber after the completion of each experiment. Radiant exposure was calculated by assuming the spot size was the diameter of the fiber optic (400 μm). Radiant exposure varied for each experiment dependent on individual experimental parameters but ranged between 0.1 – 0.88 J/cm^2 . Stimulation with infrared light was primarily applied at 200 Hz for a stimulation duration of 500 ms with a pulse width of 250 μs . Any variations in laser parameters are indicated in the descriptions of results and figure legends.

2.4 Electrical stimulation parameters

Electrical stimulation of cortex was performed with 3 M Ω tungsten microelectrodes (FHC, Bowdoin ME). The tip of the electrode was inserted into somatosensory cortex (d = 100 μm) using a micromanipulator (Stoelting, Wood Dale IL). Electrical pulses were generated using an IsoSTIM A320 (World Precision Instruments, Sarasota FL) isolator. Electrical stimulation parameters were set to match INS parameters for repetition rate (200 Hz), pulse width (250 μs), and pulse train length (500 ms). Current amplitude was constant at 0.3 mA.

2.5 Data Analysis

Image processing was performed using Matlab[™] (Mathworks, Natick MA). The frames encompassing the first second before stimulation in each trial were averaged together to establish a baseline image before stimulation was applied. The baseline image was used to visualize differences in images acquired during and after stimulation through difference maps and time-course analysis. Activation maps were calculated by dividing images at particular times post-stimulation by images from 1 second before stimulation began (and converting to % change). The time course of the signal was analyzed using blockview visualization (Fig. 1d) and average pixel value in a region of interest (ROI) versus time. Blockview analysis was performed by binning images for each half second. These images were averaged together and an activation map was calculated for each bin to show the spatiotemporal components of the INS evoked calcium signal. Regions of interest were selected automatically by first identifying the pixel with the greatest percent change in the calculated activation map and centering a 3 $\times$ 3 pixel ROI around the identified pixel. The pixels in the ROI were averaged together for each timepoint and plotted against time. To analyze the spatial and temporal components of the evoked calcium signal, a ROI was translated in the anterior/posterior and medial/lateral directions. To compare results across all animals, distance points were binned together to obtain seven averaged distance bins from the center of activation, and the corresponding time to peak values, in each bin, were averaged together. Time to peak signal was plotted as a function of distance for temporal analysis, and peak signal was plotted as a function of distance for spatial analysis.

2.6 Pharmacological studies

Fluoroacetate and CNQX were obtained from Sigma (St. Louis MO). Fluoroacetate was with a concentration of 1 mM and CNQX was prepared with a concentration of 50 μ M in PBS. In these experiments, a water tight imaging chamber was constructed using a small plastic ring (diameter: 10 mm, height: 5 mm) and 4% agar solution. Baseline imaging was acquired with ACSF in the chamber using the following laser parameters: RR= 200 Hz, PW = 250 μ s, STL = 500 ms, and RE= 0.51–0.65 J/cm². The ACSF was replaced by FAC or CNQX after acquisition of baseline imaging and incubated for 30–60 minutes. Peak signal evoked by infrared stimulation after application of pharmacological agents was compared to the peak signal acquired during baseline imaging to quantify effects of CNQX or FAC. Data from each animal was normalized to compare data across animals. A paired t-test was used to establish statistical significance between experimental conditions.

2.7 Two-photon imaging

All two-photon imaging experiments (n=3) were performed using a custom two-photon microscope [22]. The exposed somatosensory cortex was labeled with Oregon Green 488 BAPTA-1 AM calcium sensitive dye using methods previously discussed. Sulforhodamine 101 (0.04 mM in ACSF, Invitrogen, Grand Island NY) was dropped onto the surface of the exposed cortex to selectively label astrocytes. After 30 minutes, the cortex was rinsed with ACSF and a cranial window was built using agar and a glass coverslip as described above. The animal was carefully positioned under the microscope's upright objective lens, and the INS delivery fiber optic was positioned to allow stimulation of cortex adjacent to the imaging field of view. Imaging was performed using 800–850 nm light generated with a Ti:Sapphire laser (Mai Tai HP Deep-See, Spectra Physics, Mountain View CA). Emitted fluorescent light was collected in three emission channels (350 to 505, 505 to 560, and 560 to 650 nm). A 20x objective lens (XLUMlanFL 20x 0.95W; Olympus, Tokyo Japan) was used to image cortex at a depth between 100 – 300 μ m. Advancement of the microscope objective to image at depths deeper than 300 μ m caused the objective to come in contact with the fiber optic deforming the fiber and moving the fiber tip out of the area of interest that was imaged. Therefore, depths greater than 300 μ m could not be imaged due to the position of the INS fiber optic and working distance constraints imposed by the microscope objective. Dynamic images of a selected 2D plane were acquired at 7.5 fps for 30 seconds during synchronized delivery of INS. Control measurements were performed where images were acquired with no INS. Image analysis was performed using MatlabTM. Regions of interest were selected over astrocytes and the surrounding neuropil to permit extraction of the time-course of intracellular calcium in response to INS.

3. Results

3.1 Infrared neural stimulation activates complex calcium waves *in vivo*

Our initial experiments aimed to demonstrate that pulsed infrared light evokes intracellular calcium changes in rat somatosensory cortex *in vivo*. Infrared neural stimulation was applied to OBG labeled rodent somatosensory cortex through a stimulating optical fiber placed on the cortical surface, and high-speed fluorescence images were acquired at 30 frames per second during INS. As shown in Fig. 1B and supplementary movie 1, INS evoked a focal

calcium response in rodent somatosensory cortex. For all experiments, the peak percent change in fluorescence signal ranged from 0.2–1.7% and was dependent on the laser parameters used during the individual experiment. Calcium signals evoked by INS had an average duration, measured from stimulation onset to the time point where fluorescence signal returns to 5% of the maximum signal, of 7.54 ± 1.39 seconds following stimulus onset and an averaged peak signal at 2.40 ± 1.01 seconds for INS at 200 Hz for 500 ms duration ($n=12$) (Fig. 1C&D). It should be noted that the brief decrease in fluorescence observed at the onset of laser stimulation was determined to be thermal artifact caused by temperature dependent quenching of fluorescence, and was confirmed in calcium rich agar based phantoms and post-mortem animals [23, 24]. Detailed characterization of the spatial extent of the signal and a parametric investigation of the effects of INS laser parameters on response amplitudes are provided in the supplemental information.

A two component calcium signal was observed in 75% (19/25) of the animals that can be classified into two distinct temporal components (Fig. 1B and C) suggesting activation of multiple cellular pathways. The first, fast temporal component is characterized by a rapid rise in fluorescence signal that peaks 716 ± 33 ms after the start of laser stimulation. This fast component of the INS evoked calcium signal is similar to calcium responses associated with neuronal activity [17, 25, 26]. The second, slow temporal component of the INS evoked intracellular calcium signal is generally greater in magnitude than the fast component and corresponds to the overall peak signal (at 2.40 ± 1.01 s) and total duration (7.54 ± 1.39 s) of the calcium signal. Additionally, the slow component was present in all animals ($n=25$). Slow calcium responses have been reported to occur in astrocytes and other glial cells [17, 18, 27, 28]. Several studies have also demonstrated that intracellular calcium signals generated by astrocytes, interneurons, and dendrites can contain both fast and slow temporal components similar to those evoked by INS [17, 29, 30]. The presence of the fast and slow components and the overall long duration of the calcium response in relation to the short duration of INS (500 ms) was a surprising result, forcing the consideration of other involved cellular mechanisms in addition to neuronal firing.

3.2 Comparison of INS calcium response to direct electrical stimulation

Direct electrical stimulation of the cortex is expected to primarily excite neuronal firing. We therefore sought to compare the intracellular calcium response to INS with the response to electrical cortical stimulation in the same animal (with matched parameters as described in Methods). Electrical stimulation (0.3 mA, 200 Hz for 500 ms) was found to generate a more spatially distributed calcium response than INS (radiant exposure: 0.68 J/cm^2 , 200 Hz for 500 ms), $2 \text{ mm} \times 3 \text{ mm}$ versus $0.5 \times 1 \text{ mm}$ respectively (Fig. 2A and B). Calcium responses to electrical stimulation peaked, on average, at 168 ± 118 ms and returned to 0% at 2.15 ± 0.26 seconds after start of stimulation ($n=4$), whereas the fast component of the INS-evoked calcium signal peaked later at 716 ± 33 ms and had a secondary slow component peak at 2.4 ± 1.01 secs. The total duration of the INS-evoked signal was five seconds longer than the electrically evoked calcium signal. The peak amplitude of the change in fluorescence for electrical stimulation was generally twice that of INS, as shown in Fig. 2C. The difference between peak times and durations of the INS and the electrically evoked signal indicate that different primary cellular mechanisms are responsible for generating calcium signals. The

lack of a slow component in the electrically evoked signal provides evidence that two separate cellular mechanisms are involved in generation of INS evoked signals.

3.3 Calcium signals evoked by INS propagate across cortex

Temporal analysis of INS-evoked calcium signals identified propagation of the calcium signal radiating out from the center of activation in cortex (n=8 out of 12). Fig. 3B–C displays the response time-course for different locations medial and anterior to the stimulation point. Fig. 3D shows the time taken for these calcium signals to reach their maximum amplitude and has a linear relationship. The averaged time to peak data from the eight animals approximates the propagation velocity of the response to be $313 \pm 130 \mu\text{m/s}$ across the surface of cortex (Fig 3E), which is slower than calcium waves associated neuronal signaling (10–20 mm/s) [31] but faster than velocities associated with astrocyte signaling (10 – 60 $\mu\text{m/s}$) [18]. The observed velocity of the INS evoked calcium wave, and the differences in velocity associated with neurons and astrocytes motivates further investigation into the cellular processes activated by the infrared light.

3.4 Cellular contributions to INS evoked calcium signals

To further investigate the primary cellular components activated by INS, we conducted pharmacological studies using fluoroacetate (FAC), an astrocyte poison, and CNQX, a glutamate antagonist. The goal of these experiments was to isolate the roles of astrocytes and neurons in generating INS evoked calcium signals. For these experiments (n=8), a watertight imaging chamber was used to allow application of pharmacological agents to the surface of cortex. Control measurements of the calcium sensitive dye fluorescence response to INS with artificial cerebral spinal solution in the chamber were acquired first, to establish baseline signal (Fig. 4A&E). A solution containing either CNQX (50 μM in PBS) or FAC (1 mM in PBS) was then introduced into the imaging chamber and allowed to incubate for 30 – 60 minutes.

Glutamate antagonist CNQX reduced the amplitude of the fast component (identified by peak signal between 0–1 seconds following start of stimulation) by $41.09 \pm 7.65\%$ (n=3 rats, $p=0.033$, single sample t-test), but only reduced the slow component signal (averaged signal between 1 – 5 seconds post stimulation) by $10.0 \pm 8.5\%$, a decrease that was not statistically significant (n=3 rats, $p=0.35$, single sample t-test) (Fig. 4A, B, D, H, I). This finding suggests that the fast component of the INS evoked calcium signal is related to excitatory glutamatergic neuronal activity while the slow component has minimal contributions from glutamatergic activity. Application of FAC reduced the fast component by $36.67 \pm 6.6\%$ (n=4 rats, $p=0.01$, single sample t-test) and reduced the slow component by $57.2 \pm 6.9\%$ (n=4 rats, $p=0.004$, single sample t-test); however, the significant reduction in the fast component by FAC was hypothesized to be an overall reduction of the slow component that is superimposed with the fast component. This hypothesis is supported by the observation that FAC significantly reduced slow component when compared to the non-significant reduction caused by CNQX alone ($p=0.021$, paired t-test). These findings are consistent with astrocytes being a major cellular contributor to the slow INS-evoked calcium signal (Fig. 4E–I). Combined effects of CNQX and FAC (studied by adding FAC to the cortical bath after CNQX imaging) decreased the fast component by $71.9 \pm 9.37\%$ (n=4 rats, $p=0.005$,

single sample t-test) and the slow component by $80.0 \pm 8.2\%$ ($n=4$ rats, $p=0.002$, single sample t-test) (Fig. 4C, D, H, I) leaving only a small decrease in fluorescence caused by thermal effects as described previously. A paired t-test was performed between each pharmacological condition to determine statistically significant differences. For the fast component of the calcium signal, the decrease in observed fluorescence for combined CNQX+FAC was found to be significantly different when compared to FAC alone ($p=0.003$) but not significant for CNQX alone ($p=0.15$). The combined effect of CNQX +FAC significantly decreased the magnitude of the slow component when compared to CNQX alone ($p=0.013$), but only near significant differences were observed between FAC alone and CNQX+FAC ($p=0.093$). This further decrease in fluorescence signal observed under CNQX+FAC conditions when compared to the effects of FAC and CNQX alone for both the slow and fast components suggests that a complementary mechanism, involving astrocytes and neurons, is responsible for producing the calcium response to INS.

To directly examine the cellular manifestations of the INS-evoked intracellular calcium response, in-vivo two-photon microscopy was used ($n=3$). In addition to the OGB calcium indicator, the cortex was labeled with astrocyte indicator Sulforhodamine 101 (0.04 mM in ACSF) [25, 32]. After confirming wide-field calcium responses to INS, 2-photon imaging was performed using a custom-built in-vivo 2-photon microscope capable of resolving astrocytes, neurons and the microvasculature (Fig. 5A, C) [22]. Fig. 5A, shows a region of the somatosensory cortex 100 μm below the surface, with regions of interest selected to correspond to astrocytes. Fig. 5B shows the time-courses of OGB fluorescence recorded from each of these astrocyte-specific ROIs during INS. The sequential increase in intracellular calcium seen in each cell body, in order of their distance from the site of INS excitation is consistent with the propagation of a calcium wave within the astrocyte network (Fig. 5B). The velocity of this observed wave was determined to be $23.3 \pm 4.4 \mu\text{m/s}$ that is within the reported velocity range (10 – 60 $\mu\text{m/s}$) for astrocyte calcium waves observed with 2-photon imaging [18]. When regions of interest are selected to correspond to the surrounding neuropil, for the same data set (Fig. 5C), a more uniform response to INS is observed, consisting of a small, fast increase in OGB fluorescence (Fig. 5D). The neuropil response does not exhibit the wave propagation-like properties as seen in individual astrocytes. The INS evoked propagating calcium wave seen in the astrocyte network confirms sensitivity of astrocytes to pulsed infrared light; however, the local response seen in the neuropil indicates that neurons are activated by INS as the neuropil response is a confluence of signals generated by apical dendrites, interneurons, and astrocyte processes.

4. Discussion

The goal of this study was to evaluate and identify the cellular mechanisms activated by INS applied directly to exposed cortex, through the investigation of intracellular calcium dynamics *in vivo*. INS was demonstrated to evoke spatially localized calcium signals that propagate across the cortex. In 75% of the experiments, INS evoked calcium signals contained a fast component and a slow component suggesting multiple cellular types are involved in generating INS calcium responses. A side-by-side comparison of INS to electrical stimulation revealed that INS evoked calcium signals were different, spatially and temporally, from calcium responses evoked by electrical stimulation, indicating that

different cellular mechanisms are activated by each stimulation modality. Pharmacological studies demonstrated that CNQX significantly decreased the magnitude of the fast component of the calcium signal while FAC significantly reduced the overall signal; however, the effect of FAC was determined to be primarily associated with the slow component. Combined effects of CNQX and FAC further reduced the averaged calcium response demonstrating that INS induces responses in both astrocytes and neurons. Two-photon microscopy experiments revealed cellular-level responses in individual astrocytes, with sequenced calcium increases consistent with propagation of a calcium wave within astrocyte networks. The faster, and more instantaneous component was localized to the neuropil, likely corresponding to calcium activation in dendrites, axons, and possibly glial processes in response to INS, consistent with the effects of CNQX on the fast response as described above. The results of this study validate the ability of pulsed infrared light to evoke intracellular calcium transients in both astrocytes and neurons *in vivo*.

The biophysical mechanisms associated with INS helps to explain how INS activates both astrocytes and neurons and the location of these activated cells. Infrared neural stimulation is a thermally driven process that relies on the absorption of infrared light by water to induce a thermal gradient in neural tissue [13]. Shapiro et al. recently identified that the thermal gradient evoked by INS depolarizes lipid membrane bi-layers through a thermally mediated change in capacitance and demonstrated this concept by using pulsed infrared light to depolarize non-excitable cells [14]. In this study, the effective penetration depth of INS is important in determining the cellular sources of the INS evoked calcium response. The wavelength of light (1.875 μm) used in this study penetrates approximately 300 – 600 μm into tissue, where intensity decays exponentially following Beer's law indicating that light is mainly absorbed in layers I and II of cortex with few photons reaching layer III [10, 33]. The predominant cell types/processes located in layers I and II of cortex are astrocytes and apical dendrites [27, 30]. The distribution of infrared light in cortex combined with the capacitance mechanism of INS validates our pharmacological and two-photon imaging results where astrocytic and neuronal calcium responses were observed in response to infrared light stimulation.

Astrocytes were determined to be the primary cellular component behind the slow component of the INS evoked calcium signal, which have been well characterized for generating spontaneous and evoked intracellular calcium signals [34]. The slow component time-course of the INS-evoked calcium response observed with both wide-field and two-photon imaging closely resembled the reported propagating calcium waves observed in astrocytes using *in vivo* two-photon microscopy [18, 27]. Additionally, recent reports of astrocyte photosensitivity to near infrared light (800 nm) further support our findings that astrocytes are sensitive to INS [18, 35]. These similarities between astrocyte calcium signals observed previously and the slow component of the INS evoked calcium signal confirm that astrocytes play a significant role in generating calcium signals in response to INS. Calcium transients in apical dendrites located in the superficial layers of cortex represent the most likely source for neuronal contribution to the observed INS-evoked calcium signal. Researchers using imaging methods sensitive to dendritic calcium activity have demonstrated dendritic calcium signals evoked by hindpaw stimulation exhibit fast and slow

components that are similar to the fast and slow components observed in INS-evoked signals [29, 30]. While the total duration of the calcium signal evoked by hindpaw stimulation (1 – 2 secs) is shorter than INS-evoked calcium signals (7.54 ± 1.39 secs), the fast component evoked by the two stimulation methodologies is similar in duration (73 – 120 ms for hindpaw stimulation and 132 ± 68 ms for INS) [29, 30]. These similarities between the fast temporal components of calcium signals evoked by hindpaw and INS provide support for apical dendritic glutamate activity as the neural source of INS-evoked calcium signals in this study.

With these results, we demonstrate that INS directly evokes calcium transients in astrocytes and in apical dendrites of neurons. We propose the hypothesis that these two methods of calcium signal generation interact giving rise to the confluence of calcium signals documented here. Further detailed cellular physiology studies are needed to fully understand the mechanisms behind INS evoked calcium transients and how neuronal and astrocytic calcium signals interact. While this study did not investigate subsurface stimulation with INS, we hypothesize that calcium signals evoked by subsurface INS in deeper layers of cortex will be dominated by neuronal contributions as neuron somas will be directly stimulated. Future studies that employ two-photon imaging in brain slice and *in vivo* preparations will be used to address this hypothesis and identify new applications for INS in the brain. This study highlights the utility of INS as a minimally invasive tool that can be used to further our understanding of interactions between neurons and astrocytes in disease processes associated with epilepsy [36], Alzheimer's disease [37], Parkinson's disease [38], and other movement disorders [39], and the potential of INS to effectively treat these neurological diseases, such as INS based deep brain stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Pulsed infrared light evokes calcium activity in cortex. (A) Reference image displaying fiber position and location of calcium dye in cortex. (B) Activation map of INS evoked calcium signal and region of interest locations for timecourse analysis (C) Timecourse of INS evoked signal (D) Spatiotemporal components of INS evoked signal.

Figure 2.

Direct comparison of electrical and INS-evoked calcium signals. (A) Top panel: Blood vessel map displaying placement of electrode in cortex. Bottom panel: Activation map of calcium signal evoked by direct electrical stimulation of cortex. Image corresponds to 5 – 6 seconds on timecourse for electrical stimulation. Electrical stimulation parameters: Current Amplitude: 0.3 mA, repetition rate: 200 Hz, pulse width: 250 μ s, pulse train length: 500 ms. (B) Top panel: Blood vessel map displaying location of fiber optic in cortex. Bottom Panel: Activation map of calcium signal evoked by infrared stimulation in same animal. Image corresponds to 6 – 11 seconds on timecourse for INS. Laser parameters: λ : 1.875 μ m, repetition rate: 200 Hz, pulse width: 250 μ s, pulse train length: 500 ms, radiant exposure: 0.68 J/cm². (C) Timecourses of electrical and INS-evoked calcium signals.

Figure 3.

Pulsed infrared light evokes propagating calcium wave in cortex. (A) Activation map of INS evoked calcium signal. Arrows (black) indicate direction of ROI translation using 3X3 pixel box spaced 2 pixels apart in medial and anterior directions. (B&C.) Smoothed timecourse signals for each positional ROI in the medial and anterior directions signifying a shift in time to peak of the calcium signal as distance increases from the stimulation site. (D) Time to peak signal as a function of distance for data displayed in b&c. Laser parameters: λ : 1.875 μm , repetition rate: 200 Hz, pulse width: 250 μs , pulse train length: 500 ms, radiant exposure: 0.86 J/cm². (E) Averaged time to peak signal as a function of distance for all animals (n = 8). Wave propagates linearly from center of activation with an approximate velocity of 313 ± 130 $\mu\text{m/s}$. Error bars are $\pm$ s.e.m.

Figure 4.

Pharmacological analysis demonstrates INS-evoked calcium signal is generated by combination of astrocytes and neurons. (A – C) Activation maps evoked by INS for (A.) control, (B.) CNQX, and (C.) combined FAC and CNQX. (D.) Timecourse of signal after application of pharmacological agents (60 minutes). (E–F.) Activation maps evoked by INS in separate experiment for (E.) control and (F.) FAC alone. (G.) Timecourse of signals comparing control signal to signal after FAC application (60 minutes). (H) Difference between peak signal corresponding to the fast component (0 – 1.0 seconds on timecourse in D&G) for control signal and signal during pharmacological conditions. The combination of FAC and CNQX decreases the fast component calcium signal more than FAC alone ($p=0.003$) (I) Difference between averaged signal (2 – 7 secs on timecourse in D&G) corresponding to the slow component for control and pharmacological conditions. The combination of FAC+CNQX ($p=0.013$) and FAC alone ($p=0.021$) decreases calcium signal more than CNQX alone (all error bars=s.e.m.)

Figure 5.

Two-photon imaging of INS-evoked calcium signals. (A&C) Two photon image in cortex corresponding to a depth of 100 μm with ROI over responsive (A) astrocytes and (C) neuropil. White arrow indicates location of stimulation (fiber off screen). (B) Timecourse of calcium signal in astrocytes highlighted by colored ROI demonstrating propagating calcium wave in the astrocytic network evoked by infrared stimulation. The wave propagation velocity is $23.3 \pm 4.4 \mu\text{m/s}$. (D) Calcium activity in neuropil surrounding activated astrocytes. Red ROI indicates localized neuropil response. Laser Parameters: λ : 1.875 μm , repetition rate: 200 Hz, pulse width: 250 μs , pulse train length: 500 ms, radiant exposure: 0.8 J/cm²