Pulsed infrared radiation excites cultured neonatal spiral and vestibular ganglion neurons by modulating mitochondrial calcium cycling

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Lumbreras V, Bas E, Gupta C, Rajguru SM. Pulsed infrared radiation excites cultured neonatal spiral and vestibular ganglion neurons by modulating mitochondrial calcium cycling. J Neurophysiol 112: 1246–1255, 2014. First published June 11, 2014; doi:10.1152/jn.00253.2014.—Cochlear implants are currently the most effective solution for profound sensorineural hearing loss, and vestibular prostheses are under development to treat bilateral vestibulopathies. Electrical current spread in these neuroprostheses limits channel independence and, in some cases, may impair their performance. In comparison, optical stimuli that are spatially confined may result in a significant functional improvement. Pulsed infrared radiation (IR) has previously been shown to elicit responses in neurons. This study analyzes the response of neonatal rat spiral and vestibular ganglion neurons in vitro to IR (wavelength = 1,863 nm) using Ca2+ imaging. Both types of neurons responded consistently with robust intracellular Ca2+ ([Ca2+]i) transients that matched the low-frequency IR pulses applied (4 ms, 0.25–1 ppm). Radiant exposures of ~637 mJ/cm2 resulted in continual neuronal activation. Temperature or [Ca2+]i variations in the media did not alter the IR-evoked transients, ruling out extracellular Ca2+ involvement or primary mediation by thermal effects on the plasma membrane. While blockage of Na+, K+, and Ca2+ plasma membrane channels did not alter the IR-evoked response, blocking of mitochondrial Ca2+ cycling with CGP-37157 or ruthenium red reversibly inhibited the IR-evoked [Ca2+]i transients. Additionally, the magnitude of the IR-evoked transients was dependent on ryanodine and cyclopiazonic acid-dependent Ca2+ release. These results suggest that IR modulation of intracellular calcium cycling contributes to stimulation of spiral and vestibular ganglion neurons. As a whole, the results suggest selective excitation of neurons in the IR beam path and the potential of IR stimulation in future auditory and vestibular prostheses.

infrared radiation; optical stimulation; mitochondria; calcium photostimulation; cochlear implants; vestibular; neuroprostheses


PREVIOUS WORK SUGGESTS THAT OPTICAL THERMAL EFFECTS OVERTIDE EFFECTS OF PRESSURE, ELECTRIC FIELDS OR PHOTOCHEMISTRY IN PULSED IR STIMULATION (WELLS et al. 2007). IT HAS BEEN SHOWN THAT IR RESULTS IN A CAPACITIVE PHOTOTHERMAL MEMBRANE CURRENT (LIU et al. 2014; OKUNADE AND SANTOS-SACCHI 2013; SHAPIRO et al. 2012), WHICH IS LIKELY A UNIVERSAL PHENOMENON. IN THE
relatively small amplitude of depolarization reported in these studies would not be sufficient to trigger action potentials in many neurons. The cellular mechanism(s) underlying the IR stimulation of neurons and synaptic vesicular release remains unclear. Recent experiments have shown that applied IR pulses modulate intracellular calcium ([Ca\(^{2+}\)]\(_{\text{im}}\)) fluxes and that Ca\(^{2+}\) signaling is primarily responsible for somatic IR excitability (Iwanaga et al. 2006; Smith et al. 2001, 2006). Pharmacological and Ca\(^{2+}\) imaging evidence on cardiomyocytes indicates that IR modulated Ca\(^{2+}\) cycling in mitochondria by activating both mitochondrial calcium uniporter (mCU) and Na\(^{+}\)/Ca\(^{2+}\) exchanger (Dittami et al. 2011). For advancing the clinical utility of IR stimulation of neurons, it is important to characterize the cellular events induced by pulsed IR.

The present study examines whether cultured neonatal spiral and vestibular ganglion neurons respond to IR (wavelength \(\lambda = 1,863\) nm) in vitro. Precise characterization of the IR-evoked response in these neurons is fundamental to achieve therapeutic use of IR in auditory and vestibular neuroprostheses. The IR-evoked [Ca\(^{2+}\)] transient elicited in these neurons were recorded and analyzed for that purpose. To determine the Ca\(^{2+}\) source of the observed transients, the neurons were treated with an ample pharmacological array. Ascertaining whether intracellular or extracellular Ca\(^{2+}\) contribute to the IR response would clarify how IR excites neurons.

**MATERIALS AND METHODS**

**Spiral and Vestibular Ganglion Neurons Isolation and Culture**

All animal procedures were approved by the University of Miami Institutional Animal Care and Use Committee. The spiral and vestibular ganglia were isolated from 3- to 4-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) (Fig. 1A). Tissue was dissociated by incubation in 50 g/ml trypsin (Life Technologies, Carlsbad, CA) at 37°C for 10 min and by being passed through an 18G needle (BD, Franklin Lakes, NJ), followed by centrifugation at 4°C. The neurons were cultured in glass-bottom dishes coated with poly-D-lysine (MatTek, Ashland, MA) with complete Dulbecco’s modified Eagles’s medium [supplemented with 1% N1 (Sigma-Aldrich, St. Louis, MO), 500 U/ml penicillin and total 6 g/l glucose] and incubated at 37°C, 5% CO\(_2\) for 4 days.

**Immunocytochemistry**

The neurons were fixed with 100% methanol chilled at \(-20^\circ\)C (VWR, Radnor, PA) for 6 min, rinsed three times with 0.1 M phosphate-buffered saline (PBS, pH = 7.4) buffer, permeabilized with 1% Triton X-100 (Shelton Scientific, Shelton, CT), and blocked with 5% normal goat serum (Vector Labs, Burlingame, CA) in PBS for 1 h at room temperature. The slides were incubated with anti-β-tubulin (TUJ1; Covance, Princeton, NJ), in 5% normal goat serum and 0.25% Triton X-100 overnight at 4°C. After three rinses with PBS, the neurons were incubated with the secondary antibody, goat anti-mouse IgG Alexa-Fluor 594 (Life Technologies, Carlsbad, CA), in 5% normal goat serum, and 0.25% Triton X-100 for 1 h at 25°C. Staining control slides were incubated only with the secondary antibody. Finally, after three rinses with PBS, the neurons were incubated with 600 nM 4’,6-diamino-2-phenylindole (Life Technologies, Carlsbad, CA) solution, rinsed once more, coverslipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63× oil immersion objective.
resonant scanner and a 20× water immersion objective. Different 512 × 512 pixels/frame sequences of the neurons exposed to pulsed IR stimuli were recorded for 1 min (14–28 frames/s, sufficient to sample without aliasing the IR-evoked [Ca^{2+}]_i transients).

Pulsed Infrared Stimulation

IR stimulation was delivered with a multimodal 400 µm diameter optical fiber (Ocean Optics, Dunedin, FL) connected to a Capella laser (Lockheed Martin Acelight, Bothell, WA). The fiber was held and controlled with a micromanipulator, allowing IR to be focused 300 µm away from the target cells (Fig. 1C). A pilot light was used as a guide to position and focus the laser beam. The laser source was configured to emit 4-ms pulses, λ = 1,863 nm, with frequencies ranging from 0.25 to 5 Hz. IR-evoked responses are wavelength dependent, and the selection of 1,863 nm was done following previous experiments in cardiomyocytes (Dittami et al. 2011) and vestibular afferents (Rajguru et al. 2011). For the results presented here, the radiant exposure was configured to emit 4-ms pulses, λ = 1,863 nm, with frequencies ranging from 0.25 to 5 Hz. IR-evoked responses are wavelength dependent, and the selection of 1,863 nm was done following previous experiments in cardiomyocytes (Dittami et al. 2011) and vestibular afferents (Rajguru et al. 2011). For the results presented here, the radiant exposure varied from 398 to 809 mJ/cm^2, depending on the frequency and radiant energy of the laser pulses applied, and has been reported in the results where appropriate. The energy output by the fiber was measured in air using a digital optical power/energy meter (FieldMaxIt, Coherent, Santa Clara, CA). In these conditions, thermal but no stress confinement is expected. These stimulation parameters have been proved sufficient and safe for stimulation in previous work (Goyal et al. 2012; Rajguru et al. 2010).

Pharmacological Array

Pharmacological studies were conducted on seven different groups of cultures. The neurons were incubated for 30 min at room temperature in the presence of either ammoniated ruthenium oxychloride (ruthenium red), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157), 2-aminoethoxydiphenylborane (2-APB), tetrodotoxin, ryanodine, 4-aminopyridine, bepridil hydrochloride, and cyclopiazonic acid (all 50 µM, and from Tocris Bioscience, Ellisville, MO) in artificial perilymph (with the exception of ammoniated ruthenium oxychloride, which was diluted in Ca^{2+}-free DPBS). IR stimulation was delivered to the neurons every 10 min during incubation, and fluorescence changes were recorded. Then the cells were washed, and fresh artificial perilymph was added to remove the pharmacological compound. IR stimulation was repeated ~10 min following washout.

Image Processing

Image sequences were processed using ImageJ (National Institutes of Health, Bethesda, MD) to adjust brightness and contrast. Average fluorescence values of the neurons in each frame were computed using region of interest analysis. For comparison, several regions of interest selecting the background fluorescence were added. These intensity values were imported in Matlab (MathWorks, Natick, MA) to plot the normalized fluorescence variations of each neuron in every sequence. Normalization was done with respect to the average fluorescence value of the first frame, which represented fluorescence intensity of the cell at rest (ΔF/ΔF_0, where ΔF = F – F_0).

Statistical Models

Binary mixed logit model analysis with random effects was performed to test whether laser parameters, medium properties and specific drugs play a role in the number and magnitude of the IR-evoked transients. Selected fixed effects were the frequency of the laser pulses, the radiant exposure, the media temperature, the type of media, and the pharmacological array tested. Because of the consideration of random effects, conditional dependencies among data points from the same plate and stimulation trial can be accounted for.

Outcomes. In the first set of models, the outcome variable (the number of evoked transients) was dichotomized as high or low activation, depending on whether 95% or more of the possible transients were triggered. In the second set of models, the outcome variable (the transient magnitude) was dichotomized as large or small, depending on whether the change in fluorescence was less than 0.1 ΔF/ΔF_0. These thresholds were chosen given the distributions of the number and magnitude of the transients observed.

Predictors. The frequency (0.25, 0.5, 1 Hz) was treated as a continuous predictor. The radiant exposure (398, 477, 557, 637, 796 mJ/cm^2) was coded as a categorical predictor with three levels: low, medium, and high. Low settings were treated as the baseline against which treatment of medium and high settings were compared. The type of extracellular medium (Artificial Perilymph, Ca^{2+}-free DPBS) and its temperature (8, 18, 25, 37°C) were modeled as categorical predictors. 25°C, the room temperature, was treated as the baseline against which 8°C, 18°C, and 37°C were compared. For the pharmacological analysis, each drug was treated as a categorical predictor, and the control group without drug is the baseline for comparison at different times (before loading the drug, 10 and 30 min of drug incubation and 10 min after washout of the drug).

Results of each model are reported as the regression coefficient β, SE (β), and the P value. Among the models tried, those with highest Bayesian information criterion were selected to ensure robust fitting.

A custom-written R script (The R Project for Statistical Computing, Vienna, Austria, www.R-project.org) was coded to perform all calculations and this proposed statistical modeling.

RESULTS

Immunocytochemical Analysis

As an average, 99% of the cells stained positive for the neuronal marker TUJ1 (Fig. 2). Control cultures (not shown) for both types of neurons confirmed that the labeling observed was due only to specific binding of the secondary to the primary antibody.

Ca 520 AM and Culture Controls

Ionomycin was used as a positive control of Ca 520 AM. This drug depletes [Ca^{2+}]_i pools increasing cytosolic Ca^{2+} and...
results in a significant fluorescence change in the target cell. As expected, the responses observed in the neurons consisted of transients of large magnitude lasting for a few minutes (Fig. 3A). Since low K\(^+/\) extracellular concentration induces apoptosis in neurons manifested in a large response lasting for hours, a K\(^+/\) solution was used as a second control to test the neuronal nature of the cells cultured at the moment of imaging. The responses observed after adding the solution were of a large magnitude and persistent until the end of recordings (Fig. 3B).

**Response of Neurons to IR Stimulation**

IR stimulation delivered to the quiescent neurons evoked controllable, pulse-by-pulse \([\text{Ca}^{2+}]_{i}\) responses. Both auditory and vestibular neurons responded with relatively large shifts in \([\text{Ca}^{2+}]_{i}\) fluorescence matched to the low-frequency IR pulses applied (0.25–1 pps). \([\text{Ca}^{2+}]_{i}\) signal dropped in the neurons with each pulse and then returned back to baseline levels. Varying the frequency of the laser pulses applied changed the temporal occurrence of the transients (Fig. 4). The limitation in temporal resolution of the confocal microscope prevented resolving of the time course of the fast transients and sampling of responses without aliasing when more than 2 pps were applied.

**Statistical Analysis**

Fluorescence variations of 405 neurons in 69 stimulation trials from 11 culture plates were analyzed to account for the effects of the radiant exposure and the frequency of the laser pulses applied. Frequency does not predict the number of evoked transients (\(\beta = 0.83, \text{SE} = 0.96, P = 0.39\)) or their magnitude (\(\beta = -0.06, \text{SE} = 1.56, P = 0.97\)). Frequency variations only affect the temporal occurrence of the IR-evoked transients. The radiant exposure does predict outcome. For the results presented here, radiant exposure was coded as low (398 mJ/cm\(^2\)), medium (477 mJ/cm\(^2\)), and high (557, 637, 716, 796 mJ/cm\(^2\)) in the statistical model. When comparing low to medium settings, there were no significant differences in the number of evoked transients (\(\beta = 0.97, \text{SE} = 0.74, P = 0.19\)) or their magnitude (\(\beta = 1.18, \text{SE} = 1.01, P = 0.24\)). On the other hand, when comparing low to high settings, there was a very significant change in the number of evoked transients (\(\beta = 3.79, \text{SE} = 0.62, P < 0.001\)) and their magnitude (\(\beta = 4.01, \text{SE} = 0.83, P < 0.001\)). Regression coefficients become large with positive sign, meaning that the higher the radiant exposure, the more likely it is to evoke more and larger transients. The number of IR-evoked transients increased with radiant exposure in a sigmoidal fashion (Fig. 5A), and their magnitude linearly (Fig. 5B).

Fluorescence variations of 208 neurons in 33 stimulation trials from 5 culture plates were analyzed to account for the

![Fig. 3. Ca 520 AM and culture controls. The data are expressed as the 95% confidence interval of the mean (N = 10 neurons). A: 1 \(\mu\)M ionomycin was used as a positive control of Ca 520 AM. The neurons responded with large transients lasting up to a few minutes. B: a 30 mM K\(^+\) solution was used to test the neuronal nature of the cells at the moment of imaging. The responses obtained were persistent until the end of recordings. \(\Delta F/F_0\), normalized fluorescence with respect to the fluorescence intensity of the cell at rest.](image-url)

![Fig. 4. IR stimulation of the spiral and vestibular ganglion neurons. IR stimulation (\(\lambda = 1,863\) nm) delivered for 1 min to the neurons evoked controllable, pulse-by-pulse intracellular \([\text{Ca}^{2+}]_{i}\) responses. A–C: depiction of how varying the frequency of the IR pulses applied (1, 0.5 and 0.25 pps, respectively) changes the temporal occurrence of the IR-evoked \([\text{Ca}^{2+}]_{i}\) transients.](image-url)
irradiation (s) showed no significant differences in the number of evoked transients (β = 0.1, SE = 0.50, P = 0.84) and their magnitude (β = -0.15, SE = 0.57, P = 0.79).

Pharmacological Tests

Regarding the pharmacological tests, both ryanodine (n = 120 neurons analyzed from k = 4 culture plates) and cyclopiazonic acid (n = 208, k = 4) reduced the magnitude of the IR-evoked transients compared with the control group (n = 62, k = 3). Ryanodine is a blocker of ryanodine receptors (RyRs) inhibiting calcium-induced calcium release from the sarcoplasmic/endoplasmic reticulum (Sutko et al. 1997). The effect of this drug on the magnitude could be observed after 30 min of incubation (β = -2.58, SE = 1.20, P = 0.03) (Fig. 6A). Cyclopiazonic acid is a reversible inhibitor of sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (Plenege-Tellechea et al. 1997). This drug was diluted in Ca2+-free DPBS to further confirm involvement of Ca2+ extruded by the endoplasmic reticulum in the IR-evoked response. The effect of this drug on the magnitude could be observed after 30 min of incubation (β = -2.27, SE = 0.91, P = 0.01) (Fig. 6B). Both CGP-37157 (n = 116, k = 5) and ruthenium red (n = 111, k = 4) reversibly reduced the number and magnitude of the IR-evoked transients compared with the control group. CGP-37157 is a selective antagonist of mitochondrial Na+/Ca2+ exchanger blocking mitochondrial Ca2+ extrusion (Baron and Thayer 1997). Ruthenium red inhibits the activity of the mCU (Moore 1971), the rapid mode (RaM) of mitochondrial Ca2+ uptake (Gunter et al. 2000; Sparagna et al. 1995), and Letm1 Ca2+/H+ antiporter (Jiang et al. 2009) blocking mitochondrial Ca2+ uptake. The effect of both these two drugs could be observed after 10 and 30 min of incubation (Fig. 6, C and D). However, at 30 min, the effects of ruthenium red on the number of evoked transients (β = -2.45, SE = 0.62, P < 0.001) and their magnitude (β = -2.84, SE = 1.34, P = 0.03) were the most significant of all drugs tested. CGP-37157 effects were not as potent (β = -1.60, SE = 0.66, P = 0.02; β = -2.74, SE = 1.23, P = 0.03). 2-APB (n = 99, k = 4), an inositol 1,4,5-trisphosphate (IP3) antagonist, tetrodotoxin (n = 94, k = 3), a blocker of voltage-gated Na+ channels, 4-aminopyridine (n = 52, k = 2), a blocker of voltage-gated K+ channels, and bepridil hydrochloride (n = 54, k = 3), a blocker of membrane Ca2+ channels, all failed to inhibit the IR response (P > 0.05 after 30 min; Fig. 7).

DISCUSSION

Pulsed IR Evoked [Ca2+]i Transients

This study shows that low-frequency IR pulses (0.25–1 pps; λ = 1,863 nm) entrain controllable, pulse-by-pulse [Ca2+]i transients in cultured neonatal spiral and vestibular ganglion neurons. The results confirm that IR evoked Ca2+ signaling is responsible for somatic IR excitability in these neurons. IR-evoked [Ca2+]i events have been described before in HeLa cells (Iwanaga et al. 2006; Smith et al. 2001) and cardiomyocytes (Dittami et al. 2011; Smith et al. 2008). Statistical analysis of the Ca2+ fluorescence recordings indicated that the number and magnitude of the IR-evoked Ca2+ events were not

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dependent upon the presence of extracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_o$), as in HeLa cells and wild type oocytes (Shapiro et al. 2012; Smith et al. 2001). Pharmacological results further suggest that the IR-evoked transients are caused by flow of intracellular Ca$^{2+}$ rather than carrier-mediated transport across the plasma membrane (Dittami et al. 2011).

Changes in the temperature of the extracellular medium did not have a significant effect on the IR-evoked transients. It is known that temperature has an effect on Ca$^{2+}$-sensitive fluorescent probes binding and fluorescence (Oliver et al. 2000), but the fact that the observed responses were similar at both low and high temperatures suggests that they are primarily IR driven and not just a temperature-related effect on the probe. Pulsed IR induces a transient increase in temperature up to $\Delta T \approx 22.2^\circ C$ for a 10-ms pulse (7.3 mJ, 5.8 J/cm) (Liljemalm et al. 2013; Shapiro et al. 2012). In the present study, the maximum radiant energy was 1.017 mJ (809 mJ/cm$^2$) for a 4-ms pulse. A significant change in temperature could result in the Ca$^{2+}$ response observed in the neurons by activating transient receptor potential vanilloid (TRPV) channels (Albert et al. 2012) or changing the capacitance of the plasma membrane (Liu et al. 2014; Okunade and Santos-Sacchi 2013; Shapiro et al. 2012).

Nevertheless, cooling down the extracellular medium to $8^\circ C$ did not have a significant effect on the IR-evoked transients. Similar results have been described in vestibular afferents in vivo (Rajguru et al. 2011), where the IR-evoked response was persistent and qualitatively similar at low temperatures (6–7°C), and whole organ temperature increases did not evoke robust responses on their own. TRPV4 channel has been previously proposed as a primary mediator in the IR response (Albert et al. 2012). However, TRPV4 would be expected to remain under its heat activation threshold ($T < 27^\circ C$) (Guler et al. 2002) in the low-temperature conditions of these experiments. Additionally, the persistence of the IR response even in the absence of $[\text{Ca}^{2+}]_o$ further discourages a key role of temperature-gated ion channels. Pulsed IR thermal effect has also been reported to induce changes in the electrical capacitance of the plasma membrane. Voltage changes induced by this capacitive effect were up to 9 mV in artificial bilayers. Given this small change, it was hypothesized that only cells close to threshold would fire an action potential (Liu et al. 2014; Shapiro et al. 2012). Computational analysis of this capacitive change concluded that this effect on its own is unlikely to be primarily responsible for the IR-evoked response (Peterson and Tyler 2012).

Fig. 6. IR modulates mitochondrial Ca$^{2+}$ cycling. Treatment was with 50 μM ryanodine (RYN; A), cyclopiazonic acid (CPA; B), CGP-37157 (CGP; C), and ruthenium red (RR; D). Each graph is a composite that depicts the response of a representative neuron before loading the drug, after 30 min of drug incubation, and 10 min after washing the drug. Both blockers of Ca$^{2+}$ cycling of the endoplasmic reticulum, RYN and cyclopiazonic acid reduced the magnitude of the IR-evoked transients. Both blockers of mitochondrial Ca$^{2+}$ cycling, CGP and RR reversibly inhibited the IR-evoked response.
95% of the possible [Ca\(^{2+}\)]\(_{\text{neurons}}\) exists, which we considered to happen when at least 10% of Spikes

The number and magnitude of the IR-evoked transients triggered were only dependent on the radiant exposure (398–796 mJ/cm\(^2\)). The number of transients increased with radiant exposure in a sigmoidal fashion, and their magnitude linearly. Previous in vivo recordings also showed radiant exposure dependence of the IR-evoked response in the auditory nerve (Izzo et al. 2006) and vestibular afferents (Rajguru et al. 2011). Our in vitro recordings indicate that, with radiant exposure of ~637 mJ/cm\(^2\), a high probability of maximal activation of the neurons exists, which we considered to happen when at least 95% of the possible [Ca\(^{2+}\)]\(_{\text{neurons}}\) transients are evoked with amplitudes larger than 0.1 \(\Delta F/F_0\). Lowering the radiant exposure to ~398 mJ/cm\(^2\) reduced the number of neurons stimulated, but the IR response persisted in neurons near the center of the beam. This could be explained by the fact that the optical beam of a multimode fiber tends toward a Gaussian-shaped envelope

(Norton et al. 2013), and the radiant energy is highest at the center of the beam. Based on these results, it is unlikely that the [Ca\(^{2+}\)] transients were a result of activation of mechanosensitive Ca\(^{2+}\) influx led by an optoacoustic phenomenon (Teudt et al. 2011).

Role of Mitochondrial Ca\(^{2+}\) Cycling

The results suggest an endogenous sensitivity of these neurons to IR radiation, with [Ca\(^{2+}\)] signaling likely modulated by IR interaction with the mitochondrion. The onset of an intracellular mechanism after IR exposure is supported by previous research of long latencies between laser pulses applied and consequent evoked responses: ~7.6 ms in vestibular afferents (Rajguru et al. 2011) and ~2.5 ms in the cochlea (Richter et al. 2013).

To confirm the possibility of an intracellular origin, we treated the neurons with pharmacological agents. Both blockers of mitochondrial Ca\(^{2+}\) cycling ruthenium red and CGP-37157 reversibly inhibited the IR response, and ryanodine reduced its magnitude. The results strongly suggest that mitochondria play a key role in the infrared-evoked intracellular Ca\(^{2+}\) response of the neurons. The role of Ca\(^{2+}\) signaling in controlling biological processes such as muscle contraction and neurotransmission is well established (Berridge et al. 2000, 2003). Intracellular Ca\(^{2+}\) is controlled by endoplasmic reticulum, the primary stores in cells, and mitochondria, which shape and decode cellular Ca\(^{2+}\) signals by uptake and extrusion of Ca\(^{2+}\). The Ca\(^{2+}\) transients observed here may be interpreted as the rapid uptake of cytosolic Ca\(^{2+}\) with each pulse of IR, followed by extrusion back into the cytoplasm by the Na\(^+\)/Ca\(^{2+}\) exchanger restoring the electrochemical equilibrium.

The inhibition of pulsed IR evoked Ca\(^{2+}\) signaling by ruthenium red suggest the involvement of the mitochondrial uniporter (mCU) in uptake of Ca\(^{2+}\) (Moore 1971; Pitter et al. 2002). Unfortunately, given that ruthenium red is known to inhibit multiple uptake pathways, it is difficult to ascertain precisely if IR activated more than one mitochondrial Ca\(^{2+}\) transport pathway. Ruthenium red has been reported to interfere with the binding of Ca\(^{2+}\) to calmodulin and inhibit several types of ion channels: RyRs, TRPs, as well as the Letm1 (Chen and MacLennan 1994; Jiang et al. 2009; Ma 1993; Sasaki et al. 1992; Vriens et al. 2009). The Letm1 Ca\(^{2+}\)/H\(^+\) antiporter drives the slow entry of calcium into mitochondria in exchange for protons. The major component of mitochondrial electrochemical potential gradient of protons is the mitochondrial membrane potential (\(\Delta \Psi_m\)). Recent findings have expanded this list of Ca\(^{2+}\) transport mechanisms to include the RaM of Ca\(^{2+}\) uptake that is kinetically distinct and is faster than the Ca\(^{2+}\) uptake by the mCU (Gunter et al. 2000; Sparagna et al. 1995). The RaM is activated only transiently at the beginning of the Ca\(^{2+}\) pulses and is rapidly recovered between pulses, enabling mitochondria to respond to repetitive Ca\(^{2+}\) transients. Each of these mitochondrial Ca\(^{2+}\) uptake mechanisms can be driven by changes in \(\Delta \Psi_m\) and have been reported to occur in energized mitochondria (Santo-Domingo and Demaurex 2010). Recent results from our laboratory suggest that IR changes \(\Delta \Psi_m\) in these neurons, leading to mitochondrial Ca\(^{2+}\) uptake and extrusion (Lumbreras and Rajguru 2014).

The observed effect of ryanodine and cyclopiazonic acid on the magnitude of the IR response may be explained by Ca\(^{2+}\)...
microdomain formation. Mitochondria close to endoplasmic or sarcoplasmic RyRs are exposed to higher \([\text{Ca}^{2+}]\), making them more sensitive to higher rates of \([\text{Ca}^{2+}]\) uptake (David et al. 1998), so blocking \([\text{Ca}^{2+}]\) extrusion from intracellular stores could reduce \([\text{Ca}^{2+}]\) buffering in mitochondria. Since delivery of cyclopiazonic was done in \([\text{Ca}^{2+}]\)-free DPBS, it is likely that \([\text{Ca}^{2+}]\) sequestered by mitochondria during IR stimulation may come primarily from the endoplasmic reticulum in these neurons. In cardiomyocytes, \(\text{IP}_1\)-channel antagonist 2-APB also inhibited the IR response (Dittami et al. 2011). However, intramitochondrial \(\text{Ca}^{2+}\) signaling following \(\text{IP}_1\)-induced \([\text{Ca}^{2+}]\) release seems unlikely in these neurons, as 2-APB did not have the same effect on the IR response.

The present study concludes that IR modulates mitochondrial \(\text{Ca}^{2+}\) cycling, but how IR energizes mitochondria remains uncertain. One possibility could be that cytochrome-c oxidase absorbs IR accelerating respiratory metabolism (Karú 1999). IR \((\lambda = 700–2,000 \text{ nm})\) has been shown to induce cytochrome c release in isolated liver mitochondria (Frank et al. 2004). A second possibility could be that IR also induces a capacitive effect on mitochondrial membrane, leading to changes in \(\Delta \Psi_m\) (Shapiro et al. 2012). The experiments presented here have been carried out in neonatal neurons grown in culture, and this in vitro model has its experimental limitations. However, the consistency of our results suggests that the model is reliable to project into adult and in vivo models.

**Potential Applications and Future Directions**

One of the primary challenges in the design of neuroprosthetic devices includes improving spatial selectivity (Grill et al. 2009). For example, CIls have up to 22 electrodes, but clinical and psychophysical studies show that users do not receive functional benefit on all channels (Busby and Clark 1997; Chatterjee and Shannon 1998; Collins et al. 1997; Shannon et al. 2004). Additionally, tissue-electrode interactions cause fibrosis with deleterious effects (Bas et al. 2012). The loss of perceptual channels in CIls results in a poor performance for music perception and word recognition in noisy environments (Drennan and Rubinstein 2008; McDermott 2004; Spahr et al. 2008). Successful design and implantation of vestibular prosthetics encoding transduction of head rotation by semicircular canals have been achieved (Merfeld and Lewis 2012; Mitchell et al. 2013; Nìe et al. 2013), although they may also be limited by electrical current spread due to the close proximity of the three ampullary and two macular branches of the vestibular nerve (Della Santina et al. 2007; Fridman et al. 2010). This electrical current spread causes misalignment between the axis of the cupula and head rotation. Encoding of otolith endorgan transduction using electrical stimulation has proven even more difficult (Goto et al. 2003, 2004), probably because axons representing different directions are very close together within each macule and macular nerve. Postsynaptic vestibular afferent responses to IR have been shown to be excitatory, inhibitory or mixed (Rajguru et al. 2011), so feasible and advantageous use of IR in vestibular prostheses requires further research into what determines one type of response or the others. The goal of this research is to develop optical neuroprostheses, providing better frequency resolution and dynamic range than conventional stimuli. Optogenetics and thermogenetics are other possible optical techniques to address these problems; however, IR stimulation may be advantageous since it does not require genetic or pharmacological pretreatment.

Pressure wave generation and propagation have been reported to occur in thermal confinement (Teudt et al. 2011) and have been proposed as a primary mediator of the IR response, relying on functional hair cells in the cochlea (Schultz et al. 2012). Although the results presented here do not demonstrate a mechanical effect of IR on the neurons, future studies should confirm whether these IR-evoked \([\text{Ca}^{2+}]\) responses are also elicited in pressure confinement with a nanosecond laser.

IR stimulation may also be utilized as a tool to study the role of mitochondria in \([\text{Ca}^{2+}]\) dynamics, respiratory metabolism, and events controlling synaptic transmission in the inner ear. Changes in the elements of mitochondrial retrograde signaling \((\text{Ca}^{2+}, \Delta \Psi_m, \text{reactive oxygen species, nitric oxide, and fission-fusion of mitochondria})\) activate or suppress signal molecules in the cytoplasm and subsequent changes of downstream cascades (Gao and Xing 2009). Future studies should analyze whether IR induces changes in other elements of mitochondrial retrograde signaling apart from \([\text{Ca}^{2+}]\). Future studies should also research into long-term effects of IR on mitochondria-mediated apoptosis and major regulators of the apoptotic process (e.g., BCL-2 family members, heat shock proteins) in these neurons.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


The versatility and universality of 1254 PULSED IR MODULATES [Ca2+] in NEURONS


