UNIVERSITY OF MIAMI

MECHANISMS OF INFRARED NEURAL STIMULATION

By

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A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2014
MECHANISMS OF INFRARED NEURAL STIMULATION

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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 device performance. In comparison to conventional electrical stimulation, optical stimuli that are spatially confined may result in a significant functional improvement. Pulsed infrared radiation (IR) allows direct stimulation of tissue without genetic or pharmacological manipulation, and has been shown to excite neurons. This study analyzes if pulsed IR ($\lambda = 1863$ nm) elicits intracellular Ca\textsuperscript{2+} ($[\text{Ca}^{2+}]_i$) transients in cultured neonatal rat spiral and vestibular ganglion neurons. The neurons responded consistently with $[\text{Ca}^{2+}]_i$ transients that matched the low frequency IR pulses applied (4 ms, 0.25-1 pps, 398-796 mJ cm\textsuperscript{-2}). While blockage of Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} plasma membrane channels did not alter the IR-evoked $[\text{Ca}^{2+}]_i$ response, blocking of mitochondrial Ca\textsuperscript{2+} cycling with CGP-37157 or Ruthenium Red reversibly inhibited the IR-evoked response. Additionally, the magnitude of the IR-evoked $[\text{Ca}^{2+}]_i$ transients was dependent on Ca\textsuperscript{2+} extrusion from the endoplasmic reticulum (ER). To clarify the role of mitochondria in these intracellular responses, we
characterized the IR modulation of mitochondrial transmembrane potential, $\Delta \Psi_m$. Two $\Delta \Psi_m$ sensors, TMRE and Rhodamine 123, showed transient mitochondrial hyperpolarization in response to pulsed IR stimuli (100 $\mu$s, 100 pps, 178-374 mJ cm$^{-2}$; 4 ms, 0.25-1 pps, 398-796 mJ cm$^{-2}$) in the neurons. Drugs targeting either mitochondrial Ca$^{2+}$ cycling, intracellular Ca$^{2+}$ or the mitochondrial electron transport chain (ETC) inhibited the IR-evoked increases in $\Delta \Psi_m$. Intracellular pH ($\text{pH}_i$) was acidified in the neurons during stimulation. Regarding long-term effects of IR (1 ms, 30 Hz, 661 mJ cm$^{-2}$, 10 minutes) on the neurons, reactive oxygen species (ROS) levels measured at 6 hours were higher than in non-radiated neurons. However, colocalization of mitochondria and cytochrome c suggested no permanent mitochondrial permeability transition pore (mPTP) opening. Accordingly, JC-1 fluorescence indicated that $\Delta \Psi_m$ was also observed at normal resting level 24 hours after radiation. Minimal active caspase-3 was detected further supporting that IR modulation of $\Delta \Psi_m$ was transient and did not result in irreversible damage. Finally, the present study also examined whether IR-evoked [Ca$^{2+}$]$_i$ events contribute to plasma membrane depolarization. Results indicate that pulsed IR stimuli (100 ms, 100 pps, 178-374 mJ cm$^{-2}$) delivered to the neurons resulted in an increase in fluorescence of plasma membrane potential ($\Delta \Psi_p$) sensor FluoVolt™. These increases in fluorescence suggest $\Delta \Psi_p$ depolarization. Drugs targeting either mitochondrial Ca$^{2+}$ cycling, intracellular Ca$^{2+}$ or Ca$^{2+}$ extrusion from the ER decreased the IR-evoked $\Delta \Psi_p$ depolarization. This pharmacological analysis suggests correlation between the depolarization observed and IR induced intracellular Ca$^{2+}$ release. Overall, the results suggest that pulsed IR stimulation provides a novel optical tool to study the role of
intracellular organelles in manipulating Ca$^{2+}$ cycling and related events. The selective excitation of neurons in the IR beam path suggests the feasibility of infrared neural stimulation (INS) in cochlear and vestibular implants.
ACKNOWLEDGEMENTS

I feel extremely blessed thinking about all the people that have helped me to achieve this important milestone. I want to highlight that my accomplishments are due to the constant support and encouragement of many people over the years.

I would like to thank Dr. Suhrud M. Rajguru, my doctoral adviser, for giving me the chance of joining his research laboratory as his first graduate student. Thank you very much for your thoughtful insights, support, help, teaching, and academic mentoring. Thank you also for your time and dedication to review my manuscripts, and supporting all my academic applications with letters of recommendation. I really appreciate the encouragement to present this work in several conferences and the opportunity of attending the “Biology of the Inner Ear” course at the Marine Biological Laboratory. I am going to miss our discussions over colada and the fun times in and out of the lab. I hope many other graduate students can benefit by working with such a great mentor.

I would like to thank Dr. Esperanza Bas for her invaluable support and friendship. My academic background is on electrical engineering, and when I started working at the University of Miami I had no clue of how to do anything in a basic science laboratory. Thank you very much for taking your time to teach me how to do cell cultures, confocal microscopy, immunocytochemical analysis, and RT-PCR. Dr. Bas has transmitted me her passion for science, and has helped me to become an independent researcher. I am going to miss you a lot in Chicago Espe. Hopefully, one day I can become a great scientist and person as you are.
I would also like to thank the members of my dissertation committee for their academic service: Dr. Claus-Peter Richter, Dr. Fabrice Manns, Dr. Fred Telischi, and Dr. Ozcan Ozdamar. Thank you very much for taking your time to read and comment on my dissertation.

A very special thanks to my University of Miami Ear Institute family. Dr. Thomas Van De Water, thanks for always creating such a pleasant working environment. I am going to miss our fun chats over breakfast and lunch. Thank you very much for all your mentoring tips too, I really appreciate them. Dr. Chhavi Gupta, the lab has not been the same after you left. Thank you very much for being such a loving person. Dr. Jorge Bohorquez, I am going to miss your treats including almojabanas, pan de bonos, and Colombian empanadas. I know you bring them because I love them. Thank you very much for your kindness. Stefania Goncalves, I wish you the best of luck with your future residency. Efrem Robertson, I miss you in the lab. If you ever decide to come back to graduate school for a Ph.D., I am sure that you will do well. Weitao Jiang, Ilmar Tamames, Ravin Sajnani, Michael Finale, Ramanamurthy Mylavarpalu, Sonny Huynh, and Jeenu Mittal, it has been a pleasure working with all of you.

A very special thanks to my Miami family. Sandra Lee, I do not know what I am going to do without your spinning and kickboxing classes. I have had such a great time riding with you and Esperanza Bas on the bikes in the spinning class at the UM Wellness Center. Thank you very much. Every time I have felt overwhelmed, you and your classes were there to cheer me up and put me back on track. Thanks for the good energy coming from your passion as a fitness instructor. I wish one day I can instruct spinning the way you do. Micah Gill, thank you very much for your kind friendship and the fun times in
Miami. Thanks for making me discover the beauty of Miami while running (even at 4:30 am in the morning). Fr. Jose David Padilla, you are another beloved friend I am going to miss in Chicago. Thanks a lot for all the fun times in Miami, and all the invaluable support in the hardest moments. I cannot close this paragraph without thanking my adopted Cuban grandparents, Jose and Leticia. They have taken care of me like a grandson all this time here in Miami.

A very special thanks to all my friends back in Madrid or Chicago. We have had great times together, and I cannot wait to see you all very soon: Virginia Lopez, David Haro, David Malo, Carlos Gonzalez, Alessia Calderalo, Irene del Aguila, Carlota Alonso, Pablo Bartolome, Jesus Talavera, Carmen Bravo, Aitor Aller, Jacobo Blasco, and Cecilia Garcia. Also, a very special thanks to all my extended family, which I will not list due to its big size, but that does not mean they are not equally important.

Lastly and most importantly, I would like to express my gratitude to my parents, Maria and Fernando. This dissertation is the result of their constant love over the past twenty-six years, and is especially dedicated to them. Many of the good things about me definitely come from them. I would also like to express my gratitude to my partner Carlos for loving me the way I am and making me happy every day we pass together. This dissertation is also dedicated to him. I would also like to express my gratitude to my long time best friend Hector Magro (BFF, as they text it now). He is like a brother to me, and his family is my family. This dissertation is also dedicated to him. Finally, this dissertation is also dedicated to my brother Fernando. I am very thankful to him, love him, and I am going to miss having him close when I leave Miami. My favorite person in
this world is my two-year-old nephew Santi. It has been a blessing being able to do my Ph.D. so close to my brother, his lovely wife Melisa, and Santi.

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTORY REMARKS

Cochlear implantation has proven successful in restoring some hearing in severe-to-profound deaf patients. By direct and discrete stimulation of spiral ganglion neurons, cochlear implants (CIs) mimic sound transduction in the auditory pathway providing some patients with better sense of sound (Zeng, Rebscher et al. 2008). Similarly, development of vestibular prostheses encoding transduction of at least three orthogonal components of head rotation by semicircular canals could serve as an effective long-term solution for patients diagnosed with bilateral vestibulopathies (Merfeld and Lewis 2012, Mitchell, Dai et al. 2013, Nie, Ling et al. 2013). However, challenges remain in the design of these and other neuroprosthetic devices, given certain drawbacks of conventional electrical stimulation (Grill, Norman et al. 2009).

The aforementioned drawbacks include tissue-electrode electrochemical interactions that induce fibrosis with deleterious effects (O'Leary, Fayad et al. 1991, Gstoettner, Plenk et al. 1997, Eshraghi, Hoosien et al. 2010, Bas, Gupta et al. 2012), and electrical current spread that causes interference among spectral channels (Busby and Clark 1997, Collins, Zwolan et al. 1997, Chatterjee and Shannon 1998). A normal hearing subject can achieve simple sentence recognition with only three or four channels of spectral information when there is no background noise (Fetterman and Domico 2002). When speech materials become more difficult, thirty or more channels are needed for moderate recognition performance (Shannon, Fu et al. 2004). Clinically available CIs have up to
twenty-two electrodes, but only four to seven operate independently (Dowell, Seligman et al. 1987, Fishman, Shannon et al. 1997) due to channel interference (Shannon 1983, Shannon 1990, Busby, Whitford et al. 1994, Busby and Clark 1997, Collins, Zwolan et al. 1997, Chatterjee and Shannon 1998, Shannon, Fu et al. 2004). In CI patients, this loss of perceptual channels results in a poor performance for music perception and word recognition in noisy environments (McDermott 2004, Drennan and Rubinstein 2008, Spahr, Litvak et al. 2008). Regarding vestibular prostheses, electrical current spread causes misalignment between the axis of the eye and head rotation as the three ampullary and two macular branches of the vestibular nerve are all close together (Della Santina, Migliaccio et al. 2007, Fridman, Davidovics et al. 2010). Encoding of otolith endorgan transduction using electrical stimulation has proven even more difficult (Goto, Meng et al. 2003, Goto, Meng et al. 2004), probably because axons representing different directions are very close together within each macule and macular nerve.

Several strategies have been explored in order to increase selectivity of electrical stimulation, and hence improve spectral channel independence. Multipolar electrode configurations could be used to improve selectivity (Kral, Hartmann et al. 1998, Bierer and Middlebrooks 2002, Snyder, Bierer et al. 2004, Mens and Berenstein 2005) or introduce virtual channels (Koch, Downing et al. 2007, Berenstein, Mens et al. 2008, Choi and Hsu 2009, Landsberger and Srinivasan 2009). However, reported threshold responses using these configurations require more stimulation power (Kral, Hartmann et al. 1998, Bierer and Middlebrooks 2002, Snyder, Bierer et al. 2004). Cuff electrodes have also been used in several studies for stimulating motor neurons and the optical nerve
although nerve damage can result from the cuff (Larsen, Thomsen et al. 1998, Grill and Mortimer 2000). Penetrating electrodes have also proven successful in increasing spatial resolution in stimulation. Thresholds for neural excitation are as much as 50-fold lower and interference between electrodes stimulated simultaneously is markedly reduced (Rousche and Normann 1998, Rousche and Normann 1999, Middlebrooks and Snyder 2007). Nevertheless, insertion of these electrodes may lead to inflammation, edema, and consequently, impaired neural function (Bowman and Erickson 1985, Lefurge, Goodall et al. 1991).

Recent research has focused on using photonics to manipulate and stimulate cells for therapeutic applications. Confined optical stimuli may provide higher spatial selectivity of stimulation compared to standard electrical methods (Grill, Norman et al. 2009). This could result in a significant functional improvement in neuroprostheses like vestibular and cochlear implants. Neural stimulation with light is not a novel concept (Arvanitaki and Chalazonites 1961), but has gained momentum over the last decade shaping the field of neuroscience. Optical control of neural activity with cell-specific depolarization or silencing (Boyden, Zhang et al. 2005, Rajguru, Matic et al. 2010, Fenno, Yizhar et al. 2011, Bernstein, Garrity et al. 2012, Rein and Deussing 2012, Deisseroth and Schnitzer 2013, Richter and Tan 2014) shows promise both as a research tool and for development of optical neuroprosthetics. Optogenetics and thermogenetics (Bernstein, Garrity et al. 2012, Hernandez, Gehrt et al. 2014) rely on expressing light- or temperature-sensitive channels in the target cell to control its excitation or inhibition. In contrast, infrared radiation (IR, typically 1400-1600 nm and 1840-2100 nm) or near infrared radiation
(NIR, typically 790-850 nm) allows selective stimulation of cells without genetic or pharmacological modification (Wells, Kao et al. 2005). In Table 1 in page 5 an overview of the history of optical stimulation of neurons (not including optogenetics) is given (Richter and Tan 2014).

IR and NIR have been previously shown to consistently elicit in vivo and in vitro cell activation. More precisely, research studies suggest that transient responses could be elicited in peripheral and cranial nerves (Izzo, Richter et al. 2006, Teudt, Nevel et al. 2007, Fried, Lagoda et al. 2008), vestibular hair cells (Rajguru, Richter et al. 2011), cultured and stem cell derived neurons (Katz, Ilev et al. 2010, Bec, Albert et al. 2012, Bas, Van De Water et al. 2014), astrocytes (Zhao, Zhang et al. 2009), and cardiomyocytes (Smith, Kumamoto et al. 2008, Dittami, Rajguru et al. 2011). Therapeutic applications of IR and NIR under research include wound healing (Toyokawa, Matsui et al. 2003, Eells, Wong-Riley et al. 2004), surgical use (Kaufmann, Hartmann et al. 1994), dermal protection from ultraviolet light (UV) cytotoxicity (Menezes, Coulomb et al. 1998, Frank, Oliver et al. 2004), treatment of diabetic peripheral neuropathy (Arnall, Nelson et al. 2006), optical pacing of the heart (Jenkins, Duke et al. 2010, Jenkins, Wang et al. 2013), and neuroprostheses (Rajguru, Matic et al. 2010, Matic, Robinson et al. 2013). Previous results suggest that infrared neural stimulation (INS) of the cochlea is spatially selective. Tuning curves obtained via masking experiments (Matic, Walsh et al. 2011) and inferior colliculus measurements in vivo (Richter, Rajguru et al. 2011) showed that the width of the tuning curves obtained with IR stimulation was similar to those of acoustic stimuli. Therefore, the possibility
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Table 1. Overview of the history of optical stimulation of neurons (not including optogenetics) (Richter and Tan 2014).
using INS in neuroprostheses has its appeal, and further research is needed to ascertain whether this could improve the contemporary technology. Previous research suggests that optical thermal effects override effects of pressure, electric fields or photochemistry in pulsed IR stimulation (Wells, Kao et al. 2007). The heating of tissue caused by water absorbing most of the incident energy limits the rate and maximum radiant exposure of stimulation (Wells, Kao et al. 2007, Shapiro, Homma et al. 2012). Still, the cellular mechanism(s) underlying the IR stimulation of neurons and transmitter release remain unclear. Research studies have proposed different mediators of the IR-evoked responses: temperature-sensitive TRPV channels (Albert, Bec et al. 2012), plasma membrane capacitive currents (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014), intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) (Dittami, Rajguru et al. 2011), and optoacoustic byproducts of stimulation (Teudt, Maier et al. 2011, Schultz, Baumhoff et al. 2012). Pulsed IR induces a transient increase in temperature up to \(\sim 22.2^\circ\text{C}\) for a 10 ms pulse (7.3 mJ, 5.8 J cm\(^{-2}\)) (Shapiro, Homma et al. 2012, Liljemalm, Nyberg et al. 2013). Such rise in temperature could result in the activation of TRPV channels (Albert, Bec et al. 2012). IR also results in a capacitive photothermal membrane current (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014), which is likely a universal phenomenon. The relatively small amplitude of depolarization reported (\(\sim 9\text{ mV}\)) due to this capacitive effect would not be sufficient to trigger action potentials persistently in neurons (Peterson and Tyler 2012, Shapiro, Homma et al. 2012). This effect on its own cannot explain the phase locking observed in vestibular afferents under IR stimuli (Figure 1) (Rajguru, Matic et al. 2010). Other research experiments have shown that Ca\(^{2+}\) signaling may be primarily responsible for induced IR excitability.
Pharmacological and Ca\textsubscript{2+} imaging evidence on cardiomyocytes indicated that IR modulates [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 2) with high dependence on mitochondrial Ca\textsuperscript{2+} cycling (Figure 3) (Dittami, Rajguru et al. 2011). Pressure wave generation and propagation has been reported to occur in thermal confinement (Teudt, Maier et al. 2011), and has been proposed as a primary mediator of IR-evoked responses in the cochlea with dependence on functional hair cells (Schultz, Baumhoff et al. 2012). With the objective of developing optical neuroprostheses, the main focus of the present study is to gain insight into the cellular mechanism(s) of INS. The results previously obtained in cardiomyocytes (Dittami, Rajguru et al. 2011) fundament the initial hypothesis of this study that pulsed IR elicits [Ca\textsuperscript{2+}]\textsubscript{i} transients in neurons.

The present study gives evidence that IR (λ = 1863 nm) elicits [Ca\textsuperscript{2+}]\textsubscript{i} transients in cultured neonatal spiral and vestibular ganglion neurons by modulating ER-mitochondria Ca\textsuperscript{2+} transfer. Results suggest that mitochondrial transmembrane potential (ΔΨ\textsubscript{m}) hyperpolarizes under pulsed IR stimuli via acceleration of the mitochondrial electron transport chain (ETC). This increase in [Ca\textsuperscript{2+}]\textsubscript{i} appears to be upstream of an acidic shift in cytoplasmic pH, and contributes to plasma membrane depolarization. Long-term radiation did not cause irreversible neuronal damage in our experimental settings.
Figure 1. “Afferents phase-locked to IR pulse trains ($n = 9$). A, an example of phase locking of afferent discharge at various IR pulse train frequencies (10–33 pps). This particular afferent was nearly silent at rest. B–D, a second example showing phase locking at $\sim 78$ spikes s$^{-1}$ in response to 200 µs-long, 610 µJ pulse$^{-1}$, IR pulses at 78 pps (D) (resting discharge rate: $\sim 72$ spikes s$^{-1}$, cv = 0.24). E, stimulus triggered histogram showing $\sim 8.8$ ms average latency of afferent discharge relative to the IR pulse” (Rajguru, Richter et al. 2011).
Figure 2. “Activation thresholds for IR-evoked Ca\(^{2+}\) release in cardiomyocytes. A, fluorescence intensity trace for a single neonatal cardiomyocyte exposed to varying radiant energy (RE) levels of IR. Release exhibited an all-or-nothing activation dependence with no consistent amplitude variability with RE. B, probability of \([Ca^{2+}]_i\) event (\(P_{\text{event}}\)) for each IR pulse (number of cells per point in parentheses) as a function of pulse RE. Data indicated that the activation threshold for IR-evoked release occurred in a sigmoidal fashion with RE with a half-maximal probability of activation occurring at 6.3 J cm\(^{-2}\) and maximal probability at REs >8.3 J cm\(^{-2}\). Error bars indicate ± SEM” (Dittami, Rajguru et al. 2011).
Figure 3. “Pharmacological Analysis. Representative data for application of ryanodine (100 µM, n = 6 cells), a RyR channel antagonist (blocker of Ca\textsuperscript{2+} extrusion from the sarcoplasmic reticulum) (A), CGP-37157 (20 µM, n = 12), a known inhibitor of mitochondrial Ca\textsuperscript{2+} extrusion (B), Ruthenium Red (40 µM, n = 13) an inhibitor of mitochondrial Ca\textsuperscript{2+} uptake (C), and 2-APB (10 µM, n = 6), an IP\textsubscript{3} channel antagonist (blocker of Ca\textsuperscript{2+} extrusion from the sarcoplasmic reticulum) (D). Each graph is a
composite of the pre-drug cell [Ca$^{2+}$], fluorescence response (‘control’), the response following 10–15 min exposure to the drug (‘drug’), and the response after 4X washout of the drug. (‘washout’) as labeled in A” (Dittami, Rajguru et al. 2011).

### 1.2 CONTENTS OF THIS DISSERTATION

The present study investigates the cellular mechanism(s) underlying infrared neural stimulation (INS).

The primary hypothesis is that infrared radiation (IR) modulates intracellular Ca$^{2+}$ in neurons based on previous pharmacological and Ca$^{2+}$ imaging evidence on cardiomyocytes. In Chapter 2 (Lumbreras, Bas et al. 2014), this hypothesis is examined to find out if cultured neonatal spiral and vestibular ganglion neurons respond to IR ($\lambda = 1863$ nm) in vitro. The IR-evoked intracellular Ca$^{2+}$ transients 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. The main finding was that compounds targeting Ca$^{2+}$ transport of the endoplasmic reticulum and the mitochondria diminished the IR-evoked transients.

These results led to a second hypothesis that IR modulates mitochondrial transmembrane potential ($\Delta\Psi_m$), as mitochondrial Ca$^{2+}$ transport can in turn affect $\Delta\Psi_m$. In Chapter 3 (in review by Journal of Physiology), this hypothesis is examined to find out if pulsed IR modulates $\Delta\Psi_m$. Evidence is presented that the inner mitochondrial membrane hyperpolarizes under pulsed IR stimuli via acceleration of the electron transport chain (ETC). Mitochondrial ETC activation is known to result in energy state
changes in neurons, causing mitochondrial hyperpolarization linked to an increased proton gradient across the inner mitochondrial membrane. Hence, intracellular pH (pH$_i$) changes were monitored during IR stimulation. Long-term effects were also analyzed so as to determine whether IR induces apoptosis and/or mitochondrial permeability transition pore (mPTP) opening.

The third hypothesis of this study is that IR increases plasma membrane potential ($\Delta \Psi_p$). This hypothesis is based on research experiments showing phase locking of neurons under IR stimuli (Rajguru, Richter et al. 2011). In Chapter 4 (in preparation), this hypothesis is examined to find out if pulsed IR depolarizes $\Delta \Psi_p$. Evidence is presented that $\Delta \Psi_p$ depolarizes under pulsed IR stimuli, and that the IR induced intracellular Ca$^{2+}$ release contributes to this depolarization.

Chapter 5 summarizes the main findings of this study, and states the conclusions drawn from the results. IR may provide a tool to stimulate inner ear neurons, and research into the role of intracellular Ca$^{2+}$ in neural excitability, mitochondrial processes, and synaptic transmission.
CHAPTER 2: PULSED IR MODULATES MITOCHONDRIAL Ca^{2+} CYCLING IN CULTURED NEURONS

2.1 INTRODUCTORY REMARKS

Cochlear implants (CIs) have proven successful in restoring some hearing in severe-to-profound deaf patients. By direct and discrete stimulation of spiral ganglion neurons, CIs mimic sound transduction in the auditory pathway providing patients with better sound sensing (Zeng, Rebscher et al. 2008). Similarly, development of vestibular prostheses encoding transduction of at least three orthogonal components of head rotation by semicircular canals could serve as an effective long-term solution for patients diagnosed with bilateral vestibulopathies (Merfeld and Lewis 2012, Mitchell, Dai et al. 2013, Nie, Ling et al. 2013). However, challenges remain in the design of these and other neuroprosthetic devices, given the drawbacks of conventional electrical stimulation (Grill, Norman et al. 2009). The aforementioned drawbacks include tissue-electrode electrochemical interactions that induce fibrosis with deleterious effects (O'Leary, Fayad et al. 1991, Gstoettner, Plenk et al. 1997, Eshraghi, Hoosien et al. 2010, Bas, Gupta et al. 2012), and electrical current spread that causes interference among channels (Eddington 1980, Townshend, Cotter et al. 1987, Mens, Oostendorp et al. 1994, Busby and Clark 1997, Collins, Zwolan et al. 1997, Chatterjee and Shannon 1998). In CI patients, the loss of perceptual channels results in a poor performance for music perception and word recognition in noisy environments (McDermott 2004, Drennan and Rubinstein 2008, Spahr, Litvak et al. 2008). Regarding vestibular prostheses, electrical current spread
causes misalignment between the axis of the eye and head rotation as the three ampullary and two macular branches of the vestibular nerve are all close together (Della Santina, Migliaccio et al. 2007, Fridman, Davidovics et al. 2010). Recent research has focused on using photonics to manipulate and stimulate cells for therapeutic applications. Optical stimuli can be focused allowing higher spatial resolution without contact or stimulation artifact (Wells, Kao et al. 2005), and could result in a significant functional improvement in these neuroprostheses. These optical technologies include optogenetics, thermogenetics (Fenno, Yizhar et al. 2011, Bernstein, Garrity et al. 2012) and direct stimulation of cells using infrared radiation (IR, typically 1400-1600 nm and 1840-2100 nm) or near infrared radiation (NIR, typically 790-850 nm).

IR and NIR have been previously shown to consistently elicit in vivo and in vitro cell activation. More precisely, responses have been reported in peripheral and cranial nerves (Wells, Kao et al. 2005, Izzo, Suh et al. 2007, Teudt, Nevel et al. 2007, Fried, Lagoda et al. 2008), vestibular hair cells (Rajguru, Richter et al. 2011), cultured and stem cell derived neurons (Katz, Ilev et al. 2010, Bec, Albert et al. 2012, Bas, Van De Water et al. 2014), astrocytes (Zhao, Zhang et al. 2009), and cardiomyocytes (Smith, Kumamoto et al. 2008, Dittami, Rajguru et al. 2011). Potential therapeutic applications of IR and NIR under research include optical pacing of the heart (Jenkins, Duke et al. 2010) and neuroprostheses (Rajguru, Matic et al. 2010, Matic, Robinson et al. 2013). Previous results suggest that IR stimulation of the cochlea is spatially selective. Tuning curves obtained via masking experiments (Matic, Walsh et al. 2011) and inferior colliculus measurements in vivo (Richter, Rajguru et al. 2011) showed that the width of the tuning
curves obtained with IR stimulation was similar to those of acoustic stimuli. Additionally, IR and NIR stimulation do not require any genetic or pharmacological modification of the target tissue. However, the heating of tissue limits the rate and maximum radiant exposure of stimulation (Wells, Kao et al. 2007, Thompson, Wade et al. 2012, Thompson, Wade et al. 2013). This heating is caused primarily by water absorption of most of the incident energy (Shapiro, Homma et al. 2012).

Previous work suggests that optical thermal effects override effects of pressure, electric fields or photochemistry in pulsed IR stimulation (Wells, Kao et al. 2007). It has been shown that IR results in a capacitive photothermal membrane current (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014), which is likely a universal phenomenon. Still, 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\(^{2+}\)]\(_i\)) fluxes and that Ca\(^{2+}\) signaling is primarily responsible for somatic IR excitability (Smith, Fujita et al. 2001, Iwanaga, Kaneko et al. 2006, Smith, Iwanaga et al. 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 (mNCX) (Dittami, Rajguru 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 (λ = 1863 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 \([Ca^{2+}]_i\) transients 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.

2.2 MATERIALS AND METHODS

Spiral Ganglion and Vestibular 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 3-4 day postnatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) (Figure 4A). Tissue was dissociated by incubation in 50 g ml\(^{-1}\) trypsin (Life Technologies, Carlsbad, CA, USA) at 37°C for 10 minutes and by being passed through an 18G needle (BD, Franklin Lakes, NJ, USA), followed by centrifugation at 4°C. The neurons were cultured in glass bottom dishes coated with poly-D-lysine (MatTek Corporation, Ashland, MA, USA) with complete Dulbecco’s modified Eagle’s medium (DMEM) [supplemented with 1% N1 (Sigma-Aldrich, St. Louis, MO, USA), 500 units ml\(^{-1}\) penicillin and total 6 g l\(^{-1}\) glucose] and incubated at 37°C, 5% CO\(_2\) for 4 days.
Immunocytochemistry

The neurons were fixed with 100% methanol chilled at -20°C (VWR, Radnor, PA, USA) for 6 minutes, rinsed 3 times with 0.1 M Phosphate Buffered Saline (PBS, pH = 7.4) buffer, permeabilized with 1% Triton X-100 (Shelton Scientific Inc., Shelton, CT, USA), and blocked with 5% normal goat serum (Vector Labs, Burlingame, CA, USA) in PBS for 1 hour at room temperature. The slides were incubated with anti-β-Tubulin (TUJ1; Covance, Princeton, NJ, USA), in 5% normal goat serum and 0.25% Triton X-100 overnight at 4°C. After 3 rinses with PBS, the neurons were incubated with the secondary antibody, goat anti-mouse IgG Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA), in 5% normal goat serum and 0.25% Triton X-100 for 1 hour at 25°C. Staining control slides were incubated only with the secondary antibody. Finally, after 3 rinses with PBS, the neurons were incubated with 600 nM 4′,6-diamino-2-fenilindol (DAPI; Life Technologies, Carlsbad, CA, USA) solution, rinsed once more, cover-slipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63X oil immersion objective.

Ca 520 AM loading and Ca\(^{2+}\) imaging

The neurons were loaded and incubated at 37°C for 90 minutes with 5 µM Ca\(^{2+}\)-sensitive dye Ca 520 AM (Zhong, Han et al. 2012, Kodama and Togari 2013), (U-Pharm Laboratories LLC, Parsippany, NJ, USA) (Figure 4B). 10 µM Fluo-4 AM (Life Technologies, Carlsbad, CA, USA) was also used for loading in a subset of the cultures and comparing results with Ca 520 AM. Ca 520 AM showed improved signal to noise ratio. All fluorescence data presented here were collected using this dye. After
incubation, the dye was washed and replaced with artificial perilymph solution (125 mM NaCl; 3.5 mM KCl; 25 mM NaHCO$_3$; 1.2 mM MgCl$_2$; 1.3 mM CaCl$_2$; 0.75 mM NaH$_2$PO$_4$; 5 mM glucose), or Ca$^{2+}$-free Dulbecco’s Phosphate Buffered Saline (DPBS, pH = 7.4). Media temperature was measured with a thermometer (~25°C), and in specific experiments was cooled down (8, 18°C) or warmed to physiological condition (37°C) to study the effect of temperature on IR-evoked responses. In a subset of the cultures, ionomycin (Life Technologies, Carlsbad, CA, USA) at 1 µM in artificial perilymph, or a prepared 30 mM K$^+$ solution (KCl, Sigma-Aldrich, St. Louis, MO, USA) were added as a control. Ionomycin is a positive control of Ca$^{2+}$ AM, and the K$^+$ solution tests the neuronal nature of the cells in culture.

Image sequences measuring fluorescence were collected using a confocal microscope (SP5 upright, Leica, Wetzlar, Germany) with a resonant scanner and a 20X water immersion objective. Different 512X512 pixels frame$^{-1}$ sequences of the neurons exposed to pulsed IR stimuli were recorded for 1 minute (14-28 fps, 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, USA) connected to a Capella laser (Lockheed Martin Aculight, Bothell, WA, USA). The fiber was held and controlled with a micromanipulator allowing IR to be delivered ~300 mm away from the target cells (Figure 4C). A pilot light was used as a guide to position the laser beam. The laser source was configured to emit 4 ms pulses, $\lambda = 1863$ nm, with frequencies ranging from 0.25 to 5 Hz. IR-evoked responses
are wavelength dependent, and the selection of 1863 nm was done following previous experiments in cardiomyocytes (Dittami, Rajguru et al. 2011) and vestibular afferents (Rajguru, Richter 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 (FieldMax\(_{II}\), Coherent, Santa Clara, CA, USA). In these conditions, thermal but no stress confinement is expected. These stimulation parameters have been proved sufficient and safe for stimulation in previous work (Rajguru, Matic et al. 2010, Goyal, Rajguru et al. 2012).

**Pharmacological Array**

Pharmacological studies were conducted on 7 different groups of cultures. The neurons were incubated for 30 minutes at room temperature in 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 (TTX), ryanodine, 4-aminopyridine (4-AP), bepridil hydrochloride, or cyclopiazonic acid (all 50 mM, and from Tocris Bioscience, Ellisville, MO, USA) in artificial perilymph (with the exception of cyclopiazonic acid that was diluted in Ca\(^{2+}\)-free DPBS). IR stimulation was delivered to the neurons every 10 minutes 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 minutes following washout.
Figure 4. Experimental Outline. The neurons were cultured after dissociation of the spiral and vestibular ganglia isolated from 3-4 day postnatal rats (A). Before application of IR stimulation, the neurons were loaded with 5 μM Ca$^{2+}$ sensitive dye Ca 520 AM (B, Scale Bar = 10 μm). IR was delivered to the neurons with a multimodal 400 μm diameter optical fiber coupled to a Capella laser (λ = 1863 nm). Image sequences measuring Ca$^{2+}$ fluorescence changes under IR were recorded with a confocal microscope (C).
**Image Processing**

Image sequences were processed using ImageJ (NIH, Bethesda, MD, USA) to adjust brightness and contrast. Average values of the neurons in each frame were computed using Region of Interest (ROI) analysis. For comparison, several ROIs selecting the background fluorescence were added. These intensity values were imported in MATLAB (MathWorks, Natik, MA, USA) 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 ($\frac{\Delta F}{F_0}$, where $\Delta F = F - F_0$).

**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 transient) 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 $\frac{\Delta F}{F_0}$. 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, 716, 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 minutes of drug incubation and 10 minutes after washout of the drug).

Results of each model are reported as the regression coefficient $\beta$, SE ($\beta$), 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.
2.3 RESULTS

Immunocytochemical Analysis

On average, 99% of the cells stained positive for the neuronal marker TUJ1 (Figure 5). 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.

Figure 5. Immunocytochemical Analysis. The spiral (A) and vestibular (B) ganglion neurons stained positively for neuronal marker TUJ1. Cell nuclei were stained with DAPI to account for the number of cells in culture. Scale Bars = 10 µm.
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 (Figure 6A). 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 the recordings (Figure 6B).
Figure 6. Ca 520 AM and Culture Controls. The data are expressed as the 95% CI of the mean (N = 10 neurons). 1 µM ionomycin was used as a positive control of Ca 520 AM (A). The neurons responded with large transients lasting up to a few minutes. A 30 mM K+ solution was used to test the neuronal nature of the cells at the moment of imaging (B). The responses obtained were persistent till the end of recordings. \( \frac{\Delta F}{F_0} \), normalized fluorescence with respect to fluorescence intensity of the cell at rest.

**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 Ca\(^{2+}\) fluorescence matched to the low frequency IR pulses applied (0.25-1 pps). Ca\(^{2+}\) 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 time course of the transients (Figure 7). 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 (\( \beta = 0.83, \ SE = 0.96, P = 0.39 \)) or their magnitude (\( \beta = -0.06, \ SE = 1.56, P = 0.97 \)). Frequency
Figure 7. IR Stimulation of the Spiral and Vestibular Ganglion Neurons. IR stimulation (λ = 1863 nm) delivered for 1 minute to the neurons evoked controllable, pulse-by-pulse \([\text{Ca}^{2+}]_i\) responses. A, B, and C depict how varying the frequency of the IR pulses applied (1, 0.5 and 0.25 pps respectively) changes the time course of the IR-evoked \([\text{Ca}^{2+}]_i\) transients. Variations only affect the time course 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 ($\beta = 0.97$, SE = 0.74, $P = 0.19$) or their magnitude ($\beta = 1.18$, 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 ($\beta = 3.79$, SE = 0.62, $P < 0.001$) and their magnitude ($\beta = 4.01$, 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 (Figure 8A), and their magnitude linearly (Figure 8B).

Fluorescence variations of 208 neurons in 33 stimulation trials from 5 culture plates were analyzed to account for the effects of different types of medium and changes in temperature on the experimental settings. Comparisons between fluorescence recordings at 25°C and 8°C, 25°C and 18°C, and 25°C and 37°C showed no significant differences in the number of evoked transients ($\beta_{25-8} = -0.56$, SE = 0.51, $P_{25-8} = 0.27$; $\beta_{25-18} = -0.41$, SE = 0.51, $P_{25-18} = 0.42$; $\beta_{25-37} = 0.87$, SE = 0.71, $P_{25-37} = 0.22$) and their magnitude ($\beta_{25-8} = -0.27$, SE = 0.64, $P_{25-8} = 0.68$; $\beta_{25-18} = -0.75$, SE = 0.60, $P_{25-18} = 0.21$; $\beta_{25-37} = 0.15$, SE = 0.68, $P_{25-37} = 0.82$). The type of media, i.e. artificial perilymph or Ca$^{2+}$-free DPBS, had no impact on the experimental settings, as there were no significant differences either in the number of evoked transients ($\beta = 0.1$, SE = 0.50, $P = 0.84$) and their magnitude ($\beta = -0.15$, 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 when compared with the control group (n = 62, k = 3). Ryanodine is a blocker of ryanodine receptors (RyRs) inhibiting calcium-induced calcium release (CICR) from the sarcoplasmic/endoplasmic reticulum (Sutko, Airey et al. 1997). The effect of this drug on the magnitude could be observed after 30 minutes of incubation (β = -2.58, SE = 1.20, P = 0.03) (Figure 9A). Cyclopiazonic acid is a reversible inhibitor of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (Plenge-Tellechea, Soler et al. 1997). This drug was diluted in Ca\(^{2+}\)-free DPBS to further confirm involvement of Ca\(^{2+}\) extruded by the endoplasmic reticulum in the IR-evoked response. The effect of this drug on the magnitude could be observed after 30 minutes of incubation (β = -2.27, SE = 0.91, P = 0.01) (Figure 9B). 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 when compared with the control group. CGP-37157 is a selective antagonist of mNCX blocking mitochondrial Ca\(^{2+}\) extrusion (Baron and Thayer 1997). Ruthenium Red inhibits the activity of the mCU (Moore 1971), the rapid mode of mitochondrial Ca\(^{2+}\) uptake (RaM) (Sparagna, Gunter et al. 1995, Gunter, Buntinas et al. 2000), and Letm1 Ca\(^{2+}/H^+\) antiporter (Jiang, Zhao et al. 2009) blocking mitochondrial Ca\(^{2+}\) uptake. Effect of both these two drugs could be observed after 10 and 30 minutes of incubation (Figure 9C, D).
Figure 8. IR-evoked Response Depends on Radiant Exposure. The data are expressed as mean ± S.D.; Composite depicts dependence on radiant exposure (mJ cm\(^{-2}\)) of the number (A, sigmoidal) and the magnitude (B, linear) of the IR-evoked transients. IR stimulation was delivered for 1 minute to the neurons. With 637 mJ cm\(^{-2}\) of radiant exposure, the percentage of evoked transients given the time of stimulation was 94 ±
13.4%, and their magnitude $0.12 \pm 0.06 \frac{\Delta F}{F_0}$. With 398 mJ cm$^{-2}$, the percentage of evoked transients was $51.3 \pm 37.6\%$, and their magnitude $0.06 \pm 0.04 \frac{\Delta F}{F_0}$. When comparing low (398 mJ cm$^{-2}$) to high (557, 637, 716, 796 mJ cm$^{-2}$) radiant exposure settings, there is a highly significant change in the number and magnitude of the IR-evoked transients ($***) P < 0.001$.

However, at 30 minutes, the effects of Ruthenium Red on the number of evoked transients ($\beta = -2.45, \text{SE} = 0.62, P < 0.001$) and their magnitude ($\beta = -2.84, \text{SE} = 1.34, P = 0.03$) were the most significant of all drugs tested. CGP-37157 effects were not as potent ($\beta = -1.60, \text{SE} = 0.66, P = 0.02; \beta = -2.74, \text{SE} = 1.23, P = 0.03$). 2-APB (n = 99, k = 4), an IP$_3$ antagonist, TTX (n = 94, k = 3), a blocker of voltage-gated Na$^+$ channels, 4-AP (n = 52, k = 2), a blocker of voltage-gated K$^+$ channels, and bepridil hydrochloride (n = 54, k = 3), a blocker of membrane Ca$^{2+}$ channels, all failed to inhibit the IR response ($P > 0.05$ after 30 minutes; Figure 10).

2.4 DISCUSSION

**Pulsed IR evoked [Ca$^{2+}$]$_i$ transients**

This study shows that low frequency IR pulses (0.25-1 pps; $\lambda = 1863$ nm) entrain controllable, pulse-by-pulse intracellular [Ca$^{2+}$]$_i$ transients in cultured neonatal spiral and vestibular ganglion neurons. The results confirm that IR evoked Ca$^{2+}$ signaling is responsible for somatic IR excitability in these neurons. IR-evoked [Ca$^{2+}$]$_i$ events have
Figure 9. IR Modulates Mitochondrial Ca\textsuperscript{2+} Cycling. Treatment 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 minutes of drug incubation, and 10 minutes after washing the drug. Both blockers of Ca\textsuperscript{2+} cycling of the endoplasmic reticulum, ryanodine and cyclopiazonic acid, reduced the magnitude of the IR-evoked transients. Both blockers of mitochondrial Ca\textsuperscript{2+} cycling, CGP-37157 and Ruthenium Red, reversibly inhibited the IR-evoked response.
Figure 10. Pharmacological Analysis. The data are expressed as mean ± S.D.; Composite depicts the effect of each pharmacological agent on the number (A) and magnitude (B) of the IR-evoked transients. IR stimulation was delivered for 1 minute before loading the drug, after 30 minutes of drug incubation, and 10 minutes after washing the drug. Each pharmacological agent is compared to the control group without drug; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. 
been described before in HeLa cells (Smith, Fujita et al. 2001, Iwanaga, Kaneko et al. 2006) and cardiomyocytes (Smith, Kumamoto et al. 2008, Dittami, Rajguru et al. 2011).

Statistical analysis of the Ca\(^{2+}\) fluorescence recordings indicated that the number and magnitude of the IR-evoked Ca\(^{2+}\) events were not dependent upon the presence of [Ca\(^{2+}\)]\(_o\), as in HeLa cells and wild type oocytes (Smith, Fujita et al. 2001, Shapiro, Homma et al. 2012). 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, Rajguru 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, Baker 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 ~22.2°C for a 10 ms pulse (7.3 mJ, 5.8 J cm\(^{-2}\)) (Shapiro, Homma et al. 2012, Liljemalm, Nyberg et al. 2013). 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 TRPV channels (Albert, Bec et al. 2012) or changing the capacitance of the plasma membrane (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014). Nevertheless, cooling down the extracellular medium to 8°C did not have a significant effect on the IR-evoked transients. Similar results have been described in vestibular afferents in vivo (Rajguru, Richter 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, Bec et al. 2012). However, TRPV4 would be expected to remain under its heat activation threshold [>27°C] (Guler, Lee 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 (Shapiro, Homma et al. 2012, Liu, Frerck et al. 2014). 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).

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, Richter et al. 2006) and vestibular afferents (Rajguru, Richter 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 \([\text{Ca}^{2+}]_i\) transients are evoked with amplitudes
larger than $0.1 \frac{\Delta F}{F_0}$. Lowering the radiant exposure to $\sim 398$ 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 towards a Gaussian-shaped envelope (Norton, Bowler et al. 2013) and the radiant energy is highest at the center of the beam. Based on these results, it is unlikely that the $[\text{Ca}^{2+}]_i$ transients were a result of activation of mechanosensitive $\text{Ca}^{2+}$ influx led by an optoacoustic phenomenon (Teudt, Maier et al. 2011).

**Role of Mitochondrial $\text{Ca}^{2+}$ Cycling**

The results suggest an endogenous sensitivity of these neurons to IR radiation, with $[\text{Ca}^{2+}]_i$ 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: $\sim 7.6$ ms in vestibular afferents (Rajguru, Richter et al. 2011) and $\sim 2.5$ ms in the cochlea (Richter, Rajguru et al. 2013).

To confirm the possibility of an intracellular origin, we treated the neurons with pharmacological agents. Both blockers of mitochondrial $\text{Ca}^{2+}$ cycling Ruthenium Red and CGP-37157 reversibly inhibited the IR response, and ryanodine and cyclopiazonic acid reduced its magnitude (Figure 11). The results strongly suggest that mitochondria play a key role in the infrared-evoked intracellular $\text{Ca}^{2+}$ response of the neurons. The role of $\text{Ca}^{2+}$ signaling in controlling biological processes such as muscle contraction and
neurotransmission is well established (Berridge, Lipp et al. 2000, Berridge, Bootman et al. 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, Maechler 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 (Sasaki, Naka et al. 1992, Ma 1993, Chen and MacLennan 1994, Jiang, Zhao et al. 2009, Vriens, Appendino 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 rapid mode (RaM) of Ca\(^{2+}\) uptake that is kinetically distinct and is faster than the Ca\(^{2+}\) uptake by the mCU (Sparagna, Gunter et al. 1995, Gunter, Buntinas et al. 2000). 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 $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ 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, Barrett et al. 1998), so blocking $\text{Ca}^{2+}$ extrusion from intracellular stores could reduce $\text{Ca}^{2+}$ buffering in mitochondria. Since delivery of cyclopiazonic acid 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, IP$_3$-channel antagonist 2-APB also inhibited the IR response (Dittami, Rajguru et al. 2011). However, intramitochondrial $\text{Ca}^{2+}$ signaling following IP$_3$-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 (Karu 1999). IR ($\lambda = 700$-2000 nm) has been shown to induce cytochrome c release in isolated liver mitochondria (Frank, Oliver 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, Homma 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, Norman et al. 2009). For example, cochlear implants 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, Collins, Zwolan et al. 1997, Chatterjee and Shannon 1998, Shannon, Fu et al. 2004). Additionally, tissue-electrode interactions cause fibrosis with deleterious effects (O'Leary, Fayad et al. 1991, Gstoettner, Plenk et al. 1997, Eshraghi, Hoosien et al. 2010, Bas, Gupta et al. 2012). The loss of perceptual channels in cochlear implants results in a poor performance for music perception and word recognition in noisy environments (McDermott 2004, Drennan and Rubinstein 2008, Spahr, Litvak 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, Dai et al. 2013, Nie, Ling 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, Migliaccio et al. 2007, Fridman, Davidovics et al. 2010). This electrical current spread causes misalignment between the axis of the eye and head rotation. Encoding of otolith endorgan transduction using electrical stimulation has proven even more difficult (Goto, Meng et al. 2003, Goto, Meng et al. 2004) probably because axons representing different
Figure 11. The Ca\textsuperscript{2+} microdomain between the ER and mitochondria. Composite depicts ER-mitochondria Ca\textsuperscript{2+} transfer. Ruthenium Red and CGP-37157 block mitochondrial Ca\textsuperscript{2+} cycling. Ruthenium Red blocks the three different pathways of mitochondrial Ca\textsuperscript{2+} uptake, and RyRs (Calcium induced calcium released channels like IP\textsubscript{3}R) in the ER. CGP-37157 inhibits mitochondrial Ca\textsuperscript{2+} extrusion by blocking the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Ryanodine and cyclopiazonic acid block Ca\textsuperscript{2+} transport in the ER. Ryanodine blocks Ca\textsuperscript{2+} extrusion through RyRs, and cyclopiazonic acid blocks the ER Ca\textsuperscript{2+}-ATPase. Adapted (Shin and Muallem 2010).
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, Richter et al. 2011), so feasible and advantageous use of IR in vestibular prostheses requires further research into what determines one type of response or another. 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 pre-treatment.

Pressure wave generation and propagation has been reported to occur in thermal confinement (Teudt, Maier et al. 2011), and has been proposed as a primary mediator of the IR response relying on functional hair cells in the cochlea (Schultz, Baumhoff 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 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 Ca\(^{2+}\) dynamics, respiratory metabolism, and events controlling synaptic transmission in the inner ear. Changes in the elements of mitochondrial retrograde signaling (Ca\(^{2+}\), \(\Delta\Psi_m\), reactive oxygen species (ROS), nitric oxide (NO), 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 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.
CHAPTER 3: PULSED IR MODULATES MITOCHONDRIAL MEMBRANE POTENTIAL IN CULTURED NEURONS

3.1 INTRODUCTORY REMARKS

Infrared radiation (IR, typically 1400-1600 nm and 1840-2100 nm) and near infrared radiation (NIR, typically 790-850 nm) have been shown to excite cells without genetic or pharmacological modification. These non-contact, optical stimuli elicit reversible and controllable responses in a variety of cell types (Wells, Kao et al. 2005, Izzo, Richter et al. 2006, Smith, Kumamoto et al. 2008, Zhao, Zhang et al. 2009, Cayce, Friedman et al. 2011, Dittami, Rajguru et al. 2011, Rajguru, Richter et al. 2011), and may provide significant improvement in spatial selectivity over conventional electrical stimulation (Richter, Matic et al. 2011). The rapid rate of change of temperature during an IR pulse can trigger neurotransmitter release (Wells, Thomsen et al. 2007, Rajguru, Richter et al. 2011), but the molecular mechanism(s) underlying IR-evoked responses are not fully characterized. Pulsed IR induces a capacitive photothermal membrane current (Shapiro, Homma et al. 2012, Liu, Frerck et al. 2014). However, the relatively small amplitude of this depolarization would not be sufficient to trigger action potentials in most neurons. Pulsed IR is known to evoke changes in intracellular Ca$^{2+}$ in neurons in vivo and in vitro (Cayce, Bouchard et al. 2014, Lumbreras, Bas et al. 2014). This is in agreement with previous studies reporting that IR applied to the cell body modulates intracellular [Ca$^{2+}$], and that this signaling plays a major role in induced IR excitability (Iwanaga, Kaneko et al. 2006, Smith, Iwanaga et al. 2006). Pharmacological results show that IR likely
activate endoplasmic reticulum (ER) Ca\(^{2+}\) extrusion with strong dependence on mitochondrial Ca\(^{2+}\) cycling (Dittami, Rajguru et al. 2011, Lumbreras, Bas et al. 2014). Ca\(^{2+}\) stored in the ER can be extruded to the cytosol through ryanodine (RyRs) or IP\(_3\) receptors (IP\(_3\)Rs), with subsequent mitochondrial uptake followed by return to the ER (Berridge, Bootman et al. 1998, Szabadkai and Duchen 2008). Mitochondria close to endoplasmic or sarcoplasmic Ca\(^{2+}\) release sites are exposed to higher [Ca\(^{2+}\)] making them likely to have higher rates of Ca\(^{2+}\) uptake (David, Barrett et al. 1998). This Ca\(^{2+}\) uptake through the mitochondrial Ca\(^{2+}\) uniporter (MCU) depends on the electrochemical gradient defined by the mitochondrial transmembrane potential (ΔΨ\(_{\text{m}}\)) and the [Ca\(^{2+}\)] gradient between the cytosol and the mitochondrial matrix (Brookes, Yoon et al. 2004, Szabadkai and Duchen 2008). Mitochondrial Ca\(^{2+}\) transport can in turn affect ΔΨ\(_{\text{m}}\) (Szabadkai and Duchen 2008).

Taken together with previous work, the present study presents evidence that pulsed IR (λ = 1863 nm) hyperpolarizes mitochondria in cultured neurons via acceleration of the ETC. These changes in mitochondria are likely initiated by a mechanism involving intracellular Ca\(^{2+}\) released from the ER (Dittami, Rajguru et al. 2011, Lumbreras, Bas et al. 2014). This increase in [Ca\(^{2+}\)]\(_i\) also appears to be upstream of an acidic shift in cytoplasmic pH. This decrease in pH\(_i\) may be due to H\(^+\) extrusion from the mitochondria and H\(^+\) influx from the extracellular medium via the plasmalemmal Ca-ATPase, which is a Ca\(^{2+}\)/H\(^+\) exchanger (Wu, Chen et al. 1999).
3.2 MATERIALS AND METHODS

All procedures complied with and were approved by the University of Miami Institutional Animal Care and Use Committee. The spiral and vestibular ganglion neurons from 2-3 postnatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were cultured as previously described (Lumbreras, Bas et al. 2014).

Assessment of ΔΨ_m

Changes in ΔΨ_m were measured using three different sensors: rhodamine 123 (Rhod123; Sigma-Aldrich, St. Louis, MO, USA), tetramethylrhodamine ethyl ester (TMRE), and JC-1 (both from Enzo Life Sciences, Farmingdale, NY, USA) (Perry, Norman et al. 2011). The neurons were loaded and incubated at 37°C for 30 minutes in either 10 µM (quenching) Rhod123, 100 nM (non-quenching) TMRE or 1.5 µM JC-1. After incubation, the dye-containing loading medium was washed and replaced with artificial perilymph (125 mM NaCl; 3.5 mM KCl; 25mM NaHCO₃; 1.2 mM MgCl₂; 1.3 mM CaCl₂; 0.75 mM NaH₂PO₄; 5 mM glucose), and in specific experiments with Ca²⁺-free Dulbecco’s Phosphate Buffered Saline (DPBS, pH = 7.4). The neurons were further incubated for 15 minutes at room temperature to allow for the fluorescent signal to reach steady state. The temperature of extracellular media during imaging was measured with a thermometer (~25°C), and in specific experiments the media was cooled down (4°C) or warmed to physiological condition (37°C). 5 mg/ml ATP-synthase blocker oligomycin A and/or 10 µM protonophore uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; both from Sigma-Aldrich, St. Louis, MO,
USA) were used as controls to induce mitochondrial hyperpolarization and/or depolarization. A 140 mM potassium gluconate solution (D-gluconic acid, Sigma-Aldrich, St. Louis, MO, USA) was used as a control to confirm lack of plasma membrane potential (ΔΨₚ) contributions to the responses recorded in Rhod123 and TMRE.

**Assessment of pHᵢ**

The neurons were loaded and incubated at 37°C for 30 minutes in 10 µM pHrodo™ Green AM (Life Technologies, Carlsbad, CA, USA). After incubation, the dye-containing loading medium was washed and replaced with artificial perilymph. Cultures used for calibration were further incubated at 37°C for 5 minutes in a solution containing nigericin (Sigma-Aldrich, St. Louis, MO, USA) and valinomycin (Tocris Bioscience, Ellisville, MO, USA) at a 10 µM concentration each to clamp the intracellular pH with specific extracellular buffer pH.

**Pulsed Infrared Radiation**

IR stimulation (λ = 1863 nm) was delivered with a multimodal 400 µm diameter optical fiber (Ocean Optics, Dunedin, FL, USA) connected to a Capella laser (Lockheed Martin Aculight, Bothell, WA, USA) (Lumbreras, Bas et al. 2014). The fiber was held and controlled with a micromanipulator allowing IR to be delivered ~300 µm away from the target cells. In a set of experiments, a train of IR pulses (100 µs, 100 pps) was delivered to the neurons and fluorescence shifts in Rhod123, TMRE and pHrodo™ Green AM were measured. In these experimental settings, the radiant exposure varied from 178 to 374 mJ cm⁻². For pulse-by-pulse recordings (4 ms, 0.25-1 pps) in Rhod123
fluorescence, the radiant exposure varied from 398 to 809 mJ cm\(^{-2}\). Thermal, rather than stress confinement is expected in these experimental settings given the selected pulse widths. The energy output at the tip of the fiber was measured in air using a digital optical power/energy meter (FieldMax\(\text{II}\), Coherent, Santa Clara, CA, USA). Image sequences measuring fluorescence in these experiments were collected using a confocal microscope (SP5 upright, Leica, Wetzlar, Germany) with a resonant scanner and a 20X water immersion objective. Different 512X512 pixels per frame sequences of the neurons exposed to pulsed IR stimuli for 30 s were recorded (14-28 fps). In experiments analyzing long-term effects of IR, radiation over one third of the surface of each culture plate was carried out for 10 minutes (1 ms, 30 Hz, 661 mJ cm\(^{-2}\)) on a cold pad in a laminar flow hood. Culture plates containing both radiated and non-radiated neurons were cover-slipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63X oil immersion objective.

**Pharmacology**

The cultured neurons were incubated for 30 minutes at room temperature in presence of either ammoniated ruthenium oxychloride (Ruthenium Red, 50 \(\mu\)M), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157, 50 \(\mu\)M; both from Tocris Bioscience, Ellisville, MO, USA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM, 10 \(\mu\)M), Antimycin A (10 \(\mu\)M), Rotenone (100 nM), 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (4-Hydroxy-TEMPO, 50 \(\mu\)M) or Cyclosporin A (CsA, 1 \(\mu\)M; all remaining from
Sigma-Aldrich, St. Louis, MO, USA) in artificial perilymph. IR stimulation was delivered to the neurons prior to and at 10 and 30 minutes during incubation. The pulse-by-pulse fluorescence responses in Rhod123 were recorded. The neurons were then washed and fresh artificial perilymph was added to remove the pharmacological compound. IR stimulation was repeated ~10 minutes following the washout.

**Immunofluorescence Labeling**

Culture plates containing both radiated and non-radiated neurons were incubated at 37°C for 6 hours post-radiation before immunostaining for cytochrome c, and 24 hours for cleaved caspase-3. Neurons pre-treated with 1 µM FCCP were used as a positive control of cleaved caspase-3 and as a negative control of colocalization of cytochrome c and mitochondria. In the cases where only immunolabeling of β-Tubulin neuronal marker or cytochrome c was done, MitoTracker® Orange CM-H₂TMRos (500 nM; Life Technologies, Carlsbad, CA, USA) was added to each culture plate and incubated at 37°C for 30 minutes. The neurons were then fixed with 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS, pH = 7.4) for 24 hours at 4°C. After fixation, the neurons were rinsed three times in PBS, and subsequently permeabilized with 1% Triton X-100 (Shelton Scientific Inc., Shelton, CT, USA) and blocked with 5% normal goat serum (Vector Labs, Burlingame, CA, USA) in PBS for 1 hour at room temperature. The neurons were then separated into three staining groups, and incubated with either (1) anti-β-Tubulin mouse monoclonal antibody (TUJ1; Covance, Princeton, NJ, USA), or (2) anti-cytochrome c mouse monoclonal antibody (Life Technologies, Carlsbad, CA, USA),
or (3) TUJ1 and anti-cleaved caspase-3 (Asp$^{175}$) rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C in blocking media. After rinsing three times with PBS, staining groups (1) and (2) were incubated for 1 hour at room temperature with secondary antibody Alexa 488-labeled goat anti-mouse IgG, and group (3) with Alexa 488-labeled goat anti-mouse IgG and TRITC-labeled goat anti-rabbit IgG (both from Life Technologies, Carlsbad, CA, USA).

Control slides determining specific binding of secondary to primary antibodies were incubated only with secondary antibodies. Finally, after three rinses with PBS, all groups were incubated with 600 nM 4',6-diamino-2-fenilindol (DAPI; Life Technologies, Carlsbad, CA, USA), rinsed once more, cover-slipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63X oil immersion objective.

**Reactive Oxygen Species Detection**

Culture plates containing both radiated and non-radiated neurons were incubated at 37°C for 6 hours post-radiation before staining for reactive oxygen species. Neurons pre-treated with 1 µM FCCP were used as a positive control. CellROX® deep red reagent (5 µM; Life Technologies, Carlsbad, CA, USA) was added to each culture plate and incubated at 37°C for 30 minutes. The neurons were rinsed three times with PBS, and then fixed with 4% paraformaldehyde in 0.1 M PBS for 20 minutes at 4°C. The fixed neurons were rinsed three times with PBS, and then were stained with DAPI (600 nM) for 10 minutes at room temperature. The neurons were rinsed again three times with PBS,
cover-slipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63X oil immersion objective.

**Image Processing and Colocalization Analysis**

Image sequences measuring Rhod123, TMRE, and pHRodo™ Green AM fluorescence were processed using ImageJ (NIH, Bethesda, MD, USA). Average fluorescence values of the neurons in each frame were computed, and then imported in MATLAB (MathWorks, Natik, MA, USA) to plot the fluorescence variations of each neuron in every sequence. Normalization of Rhod123 and TMRE was done with respect to the average fluorescence value of the first frame, which represented fluorescence intensity of the cell at rest ($\frac{\Delta F}{F_0}$, where $\Delta F = F - F_0$). ROS (CellROX®) and cleaved caspase-3 signal intensities were measured and normalized with respect to DAPI fluorescence. JC-1 red/green ratio was computed by measuring red and green fluorescence independently. The size of the region of interest (ROI) was the same for all images. Colocalization analysis of cytochrome c and mitochondria (MitoTracker®) post-radiation was carried out using Volocity software (PerkinElmer, Waltham, MA, USA). Images were iteratively deconvolved, and then intensity based quantitative colocalization coefficients were computed. Coefficients reported are Pearson’s correlation and Manders’ Coefficients (M1, M2). Pearson’s correlation was computed as the ratio between the covariance of the channels and the product of their standard deviations, and describes whether there is a linear relation between channels (range from -1 to 1; 1 standing for complete positive correlation, -1 for negative correlation, and 0 for no correlation). M1 or
M2 coefficients provide the proportion of the intensity in one channel coincident with some signal in the other channel over its total intensity (range from 0 to 1; 0 standing for non-overlapping images, and 1 for total colocalization).

Statistics

The data presented in this manuscript are expressed as mean ± S.D. One-way analysis of variance (ANOVA) followed by Dunnett post hoc testing was used to compare IR-evoked responses in TMRE or Rhod123, mean fluorescence intensities of ROS or cleaved caspase-3, and JC-1 red/green signal ratios. Two-way ANOVA followed by Bonferroni post hoc testing was used in the pharmacological and colocalization analyses. A P value of <0.05 was considered significant. All calculations were performed on a computer equipped with GraphPad Prism v 5.00c software for Mac OS X® (GraphPad Software, La Jolla, CA, USA).

3.3 RESULTS

IR Transiently Modulates ΔΨm

Cultures of neonatal spiral and vestibular ganglion neurons were used for all the experiments. Both kinds of cultures were primarily composed of neurons that stained positively for neuronal maker TUJ1 and mitochondria (Figure 12). Two fluorescent probes, Rhod123 and TMRE, were used to analyze changes in ΔΨm during pulsed IR stimulation (λ = 1863 nm). Rhod123 was used in quenching mode and TMRE in non-
quenching (Figure 13). Mitochondrial hyperpolarization produced by oligomycin A and depolarization produced by FCCP were used to confirm quenching and non-quenching behaviors (Figures 14A and 14D). Trains of IR stimuli (100 µs, 100 pps, 178-374 mJ cm⁻²) delivered to the quiescent neurons for 30 s resulted in a decrease in Rhod123 fluorescence. The same stimuli produced an increase in TMRE fluorescence. These transient IR-evoked responses suggest mitochondrial hyperpolarization, and were dependent on laser radiant exposure (Figures 14B and 14E). The magnitude of the mitochondrial hyperpolarization increased linearly with radiant exposure (Rhod123, n = 13 neurons per group, k = 3 culture plates; TMRE, n = 15 per group, k = 3) (Figures 14C and 14F). Removal of extracellular Ca²⁺ did not have an effect on the IR-evoked response, with no significant changes observed in either case (Ca²⁺-free DPBS, Rhod123, n = 10, k = 2, P > 0.05; TMRE, n = 9, k = 2, P > 0.05). To study IR induced photothermal effects on the mitochondrial hyperpolarizations, the temperature of the extracellular media was changed in specific experiments. There were no significant differences between hyperpolarizations recorded at 25°C and 4°C (Rhod123, n = 8, k = 2, P > 0.05; TMRE, n = 12, k = 2, P > 0.05). The IR-evoked responses were also recorded before and after depolarizing ΔΨₚ, using a potassium gluconate solution. There was no significant difference in the magnitude of IR-evoked hyperpolarizations, indicating that the fluorescence results reported here are mitochondrial and not cytoplasmic (Rhod123, n = 10, k = 2, P > 0.05; TMRE, n = 12, from k = 2 culture plates, P > 0.05).
Figure 12. Immunocytochemical Analysis. The cultured spiral (SGN, A-D) and vestibular (VGN, E-H) ganglion neurons were characterized using immunostaining. Cell nuclei were stained with DAPI to account for the number of cells present, and on average, 99% stained positive for the neuronal marker TUJ1. 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. Mitochondria were stained using MitoTracker® Orange CM-H₂TMRos. Scale Bars = 10 μm.
Figure 13. Assessment of $\Delta\Psi_m$. The neurons were loaded and incubated at 37ºC for 30 minutes in either 10 µM Rhod123 (A) or 100 nM TMRE (B). Loaded at high concentrations (quenching mode), Rhod123 autoquenching property results in an inverse correlation between dye concentration and $\Delta\Psi_m$. A decrease in signal reflects higher retention of Rhod123 in the mitochondria and an increase in $\Delta\Psi_m$ (i.e. hyperpolarization). Conversely, an increase in the Rhod123 fluorescence implies an unloading or unquenching event, suggesting a decrease in $\Delta\Psi_m$ (i.e. depolarization). Loaded at low concentrations (non-quenching mode), TMRE intracellular fluorescent signal arises from polarized mitochondria correlating with changes in $\Delta\Psi_m$. A decrease in fluorescence indicates depolarization of $\Delta\Psi_m$, and an increase hyperpolarization. Scale Bars = 10 µm.
Figure 14. IR Modulates $\Delta \Psi_m$. IR evoked normalized fluorescence responses ($\Delta F/F_0$, where $\Delta F = F - F_0$) recorded with Rhod123 (10 µM, quenching, A-C) and TMRE (100 nM, non-quenching, D-E) in cultured neurons. A and D depict representative controls confirming quenching and non-quenching behaviors. In the presence of $F_0/F_1$-ATPase inhibitor oligomycin A, Rhod123 fluorescence decreased indicating a rise in $\Delta \Psi_m$ (i.e. hyperpolarization). Conversely, an increase in the Rhod123 signal after addition of protonophore FCCP suggested depolarization of $\Delta \Psi_m$. TMRE shows opposite correlation when compared to Rhod123. B and E depict averaged fluorescence changes recorded in Rhod123 and TMRE respectively during IR stimulation ($\lambda = 1863$ nm, 100 µs, 100 pps, 178-374 mJ cm$^{-2}$) delivered for 30 s. Both $\Delta \Psi_m$ sensors showed IR induced mitochondrial hyperpolarization dependent on the radiant exposure. C and F show the peak hyperpolarization recorded at different radiant exposures (mean ± S.D.). Significant differences were observed when comparing the lowest radiant exposure settings (178 mJ cm$^{-2}$) with rest of the groups (**P < 0.01, *P < 0.05).
**IR pulse-by-pulse Hyperpolarizations in \( \Delta \Psi_m \)**

It has previously been shown that IR stimulation phase-locks neurons up to frequency rates of 100 Hz (Littlefield, Vujanovic et al. 2010, Rajguru, Richter et al. 2011). In order to correlate neuronal depolarization with pulsed IR and the mitochondrial response observed here, it is important to characterize whether these changes in \( \Delta \Psi_m \) are elicited with each laser pulse. Rhod123 in quenching mode was used in these experimental settings to resolve the pulse-by-pulse responses (Perry, Norman et al. 2011). Low frequency (4 ms; 0.25-1 pps) IR stimuli delivered to the quiescent neurons evoked controllable, pulse-by-pulse increases in \( \Delta \Psi_m \) (Figure 15). With each IR pulse, the Rhod123 fluorescence in the targeted neurons reduced and quickly returned to baseline levels demonstrating transient mitochondrial hyperpolarizations. Frequency variations of the IR stimuli changed the time course of the IR-evoked transients (Figure 15A, 15B, 15C), yet there were no significant differences in the number and magnitude of the evoked transients (n = 52, k = 3, \( P > 0.05 \)). In contrast, the laser radiant exposure had a significant effect on the resulting responses. When comparing low (398 mJ cm\(^{-2}\)) to high (557, 637, 716, 796 mJ cm\(^{-2}\)) radiant exposures, there was a highly significant change in the number and magnitude of the IR-evoked transients (n = 52, k = 3, \( P < 0.001 \)). Within the range tested, the number of IR-evoked transients increased with radiant exposure in a sigmoidal fashion (Figure 15D), and their magnitude linearly (Figure 15E). It is likely that smaller events were missed for lower strength stimuli. Removal of \([\text{Ca}^{2+}]_o\) did not impact the number or magnitude of IR-evoked transients (n = 28, k = 2, \( P > 0.05 \)). Additionally, there was no significant difference in the mitochondrial hyperpolarizations.
with extracellular media maintained at various temperatures. The magnitude of the response did not differ for neurons placed in media at 4°C, 25°C, and 37°C (n = 42 per group, k = 3, P > 0.05). Finally, the pulse-by-pulse responses were not altered by ΔΨ_p depolarization induced by incubating the neurons in a potassium gluconate solution. No significant difference was noted in the IR-evoked changes in ΔΨ_m (n = 40, k = 2, P > 0.05).

**Pharmacological Analysis**

Pharmacological studies were conducted to gain insight into the mechanism underlying the pulse-by-pulse IR-evoked changes in ΔΨ_m. Three compounds, BAPTA-AM (n = 19 neurons, k = 4), CGP-37157 (n = 13, k = 4), and Ruthenium Red (n = 20, k = 4) reversibly inhibited the IR-evoked response. BAPTA-AM is a [Ca^{2+}]_i chelator, while CGP-37157 blocks mitochondrial Ca^{2+} extrusion (Baron and Thayer 1997), and Ruthenium Red inhibits the three different pathways of mitochondrial Ca^{2+} uptake (Moore 1971, Gunter, Buntinas et al. 2000, Jiang, Zhao et al. 2009). Additionally, Antimycin A (n = 29, k = 4) and rotenone (n = 30, k = 4), both mitochondrial ETC inhibitors, also significantly diminished the number and magnitude of the IR-evoked transient hyperpolarizations. Antimycin A interferes with electron flow from cytochrome b_H in complex III (Q-cytochrome c oxidoreductase) (Alexandre and Lehninger 1984), and rotenone reversibly blocks complex I (NADH-CoQ reductase) (Li, Ragheb et al. 2003). The effect of these compounds on the number (Figure 16A) and magnitude (Figure 16B) of the transients could be observed following 10 minutes of incubation, and was
Figure 15. IR Elicits Pulse-by-Pulse Hyperpolarizations in $\Delta \Psi_m$. IR stimulation delivered for 30 seconds to the neurons evoked controllable, pulse-by-pulse decreases in Rhod123 fluorescent signal. A, B, and C depict responses to different frequency of the IR pulses applied (1, 0.5 and 0.25 pps respectively) and the evoked increases in $\Delta \Psi_m$. D and E depict dependence on laser radiant exposure (398-796 mJ cm$^{-2}$) of the number (sigmoidal) and magnitude (linear) of the IR-evoked response. The data are expressed as mean ± S.D. With 796 mJ cm$^{-2}$ of radiant exposure, the percentage of evoked transients during 30 s of stimulation was 96.9 ± 10.3%, and their magnitude $0.14 ± 0.05 \frac{AF}{F_0}$. With 398 mJ cm$^{-2}$, the percentage of evoked transients was 53.1 ± 43.6%, and their magnitude $0.04 ± 0.03 \frac{AF}{F_0}$. When comparing low (398 mJ cm$^{-2}$) to medium and high (557, 637, 716, 796 mJ cm$^{-2}$) radiant exposure settings, there was a significant increase in the number of
the IR-evoked transients (***$P < 0.001$). When comparing high (796 mJ cm$^2$) to medium and low (398, 477, 557, 637 mJ cm$^2$) radiant exposure settings, the magnitude of the IR-evoked transients was significantly smaller (***$P < 0.001$).

compared to the control group without drug ($n = 19$, $k = 4$). Cyclosporin A (CsA; $n = 22$, $k = 4$), a blocker of mPTP, and 4-Hydroxy-TEMPO (TEMPOL; $n = 22$, $k = 4$), a superoxide dismutase (SOD) mimic, both had no measurable effect on the IR-evoked transients.

**IR Transiently Decreases pH$_i$**

Mitochondrial activation is known to result in redox state change in the neurons, with mitochondrial hyperpolarization linked to proton gradient out of mitochondrial matrix. A fluorescent intracellular pH indicator (pHrodo™ Green AM, Figure 17A) was used to analyze whether IR induces a change in the pH$_i$ of the neurons. Prior to stimulation experiments, a subset of cultures was used for calibration so as to obtain a pH standard curve (Figure 17B). To rule out thermal sensitivity of the indicator, baseline fluorescence levels were measured in extracellular media at 25°C and 37°C. No temperature effects on pHrodo™ Green AM fluorescence were observed. IR stimuli (100 µs, 100 pps, 178-374 mJ cm$^2$) delivered to the neurons for 30 s increased intracellular fluorescence transiently (i.e. decreased pH$_i$). As the level of IR radiant exposure increased, a corresponding decrease in the pH$_i$ was observed ($n = 30$ per group, $k = 2$, Figure 17C). At a higher
radiant exposure (374 mJ cm\(^{-2}\)), the \(\Delta pH_i\) measured with IR stimulation was \(-0.268 \pm 0.107\), while at a lower radiant exposure (178 mJ cm\(^{-2}\)), the \(\Delta pH_i\) was \(-0.002 \pm 0.090\).

Figure 16. Pharmacological Analysis. Composite depicts the effect of various pharmacological compounds on the number (A) and magnitude (B) of the pulse-by-pulse IR-evoked mitochondrial hyperpolarizations. The data are expressed as mean ± S.D. IR stimulation (4 ms, 1 pps, 796 mJ cm\(^{-2}\)) was delivered for 30 s before loading the compounds (no drug), after 10 and 30 minutes of incubation with the compound, and 10 minutes post washout of the compound. Each pharmacological compound is compared to the control group without drug at the times indicated (**\(P < 0.01\), *\(P < 0.05\)). [Ca\(^{2+}\)] chelator BAPTA-AM, mitochondrial Ca\(^{2+}\) cycling blockers CGP-37157 and
Ruthenium Red, and ETC inhibitors Antimycin A and rotenone significantly diminished the IR-evoked response.

Figure 17. IR Induced Acidic Transients. The neurons were loaded and incubated at 37°C for 30 minutes in 10 µM pHrodo™ Green AM (A). Scale Bar = 10 µm. The neurons were further incubated at 37°C for 5 minutes in a solution containing nigericin and valinomycin at a 10 µM concentration each to clamp the intracellular pH with extracellular buffer pH at 4.75, 6.36, and 8.08. Relative fluorescence was measured in single neurons (n = 30 per group) to fit a linear relation among data points and obtain a pH standard curve (B). The data are expressed as mean ± S.D. IR stimulation (100 µs, 100 pps, 178-374 mJ cm⁻²) delivered for 30 s to the cultured neurons decreased pHᵢ transiently (C). When comparing low (178 mJ cm⁻²) to medium and high radiant
exposures (217, 259, 298, 336, 374 mJ cm$^{-2}$), there is a significant difference in the IR-evoked decrease in pH$_i$ (***$P < 0.001$, **$P < 0.01$).

**Long-term Effects of IR Stimulation of Neurons**

A collapse in the electrochemical gradient across the mitochondrial membrane, which can be reflected by $\Delta \Psi_m$, may be an early indication of the initiation of cellular death mechanisms such as apoptosis. To analyze the long-term effects of IR, we carried out measurements studying $\Delta \Psi_m$, reactive oxygen species (ROS) levels, colocalization between cytochrome c and mitochondria, and levels of cleaved caspase-3. Approximately a third of the total surface of each culture used in these experiments was radiated with pulsed IR for 10 minutes (1 ms, 30 Hz, 661 mJ cm$^{-2}$). Neurons radiated with pulsed IR were compared directly to non-radiated neurons from the same culture plate. Additionally, FCCP was used as a control for each experiment. An increase of total ROS levels (CellROX®) was observed in the radiated neurons 6 hours post-radiation (Figures 18A and 18B). Figure 19A shows that the normalized mean fluorescence (NMF) values in the non-radiated neurons remained low ($n = 17$, 0.046 ± 0.029 NMF), while in the radiated ones ($n = 41$, 0.173 ± 0.101 NMF, $P < 0.01$) and those treated with FCCP, there was a significant increase in levels of ROS ($n = 33$, 0.427 ± 0.182 NMF, $P < 0.001$). However, 6 hours post-radiation, cytochrome c remained colocalized with mitochondria in the radiated neurons (Figure 20). There were no significant differences between the colocalization coefficients computed in non-radiated (Figure 19B, $n = 15$, Pearson’s: 0.892 ± 0.016, M1: 0.901 ± 0.042, M2: 0.838 ± 0.045) and radiated neurons ($n = 28$,
Pearson’s: 0.855 ± 0.040, \( P > 0.05 \), M1: 0.860 ± 0.063, \( P > 0.05 \), M2: 0.888 ± 0.054, \( P > 0.05 \). In comparison, the neurons treated with FCCP did not show complete colocalization between cytochrome c and the mitochondria (n = 10, Pearson’s: 0.559 ± 0.078, \( P < 0.001 \), M1: 0.683 ± 0.108, \( P < 0.001 \), M2: 0.745 ± 0.067, \( P < 0.001 \)). JC-1 fluorescence was also measured at 24 hours post-radiation, and indicated normal pre-radiation levels of \( \Delta \Psi_m \) in the radiated neurons (Figures 18C and 18D). As shown in Figure 19C, there were no significant differences between the JC-1 red/green signal ratios computed in non-radiated (n = 24, 1.224 ± 0.317) or radiated neurons (n = 41, 1.208 ± 0.4643, \( P > 0.05 \)). As a control, FCCP treated neurons showed permanent mitochondrial depolarization (n = 15, 0.1760 ± 0.1751, \( P < 0.001 \)). Also, at 24 hours the levels of active caspase-3 in the radiated neurons were similar to non-radiated ones (Figures 18E and 18F). Figure 19D shows that the NMF values remained low in non-radiated (n = 19, 0.040 ± 0.026 NMF) and radiated neurons (n = 40, 0.040 ± 0.031 NMF, \( P > 0.05 \)), but significantly increased in the neurons treated with FCCP (n = 16, 0.251 ± 0.120 NMF, \( P < 0.001 \)).
Figure 18. ROS Detection, JC-1 Fluorescence, and Cleaved Caspase-3. ROS (CellROX®) production was analyzed 6 hours post IR radiation (1ms, 30 pps, 661 mJ cm⁻²) for 10 minutes. JC-1 fluorescence and cleaved caspase-3 (anti-cleaved caspase-3)
were analyzed at 24 hours. ROS levels increased in radiated neurons (B) when compared to non-radiated ones (A). However, JC-1 red aggregates in both radiated (C) and non-radiated neurons (D) indicating normal resting values of $\Delta \Psi_m$. Permanent loss of $\Delta \Psi_m$ did not occurred in the radiated cells. Lack of apoptosis in the neurons was further confirmed by lack of both active caspase-3 and nuclear fragmentation in radiated cells (F) as in non-radiated ones (G). Scale Bars = 10 µm.

3.4 DISCUSSION

The results obtained using fluorescence $\Delta \Psi_m$ sensors in the present experiments indicated that the inner mitochondrial membrane hyperpolarizes transiently during IR stimulation. These changes in $\Delta \Psi_m$ support the hypothesis that IR activates mitochondria-influenced intracellular Ca$^{2+}$ cycling. IR induced photothermal effect did not compromise the photostability of these $\Delta \Psi_m$ sensors. The significant change in $\Delta \Psi_m$ observed as an increased quenching of Rhod123 in response to the IR stimuli, was consistent with the results obtained using non-quenching TMRE. The increase in $\Delta \Psi_m$ was dependent on laser radiant exposure (178-374 and 398-809 mJ cm$^{-2}$), and could be observed pulse-by-pulse with low frequency IR stimuli delivered to the neurons (4 ms, 0.25-1 pps). Mitochondrial hyperpolarization has been reported in neurons in the presence of certain neurotoxins (Krohn, Wahlbrink et al. 1999, Norman, Perry et al. 2007), and has also been related to mitochondrial Ca$^{2+}$ uptake in detrimental processes leading to mPTP opening and permanent loss of $\Delta \Psi_m$ (Zorov, Filburn et al. 2000, Esterberg, Hailey et al. 2014).
Figure 19. Long-term Effects of IR Stimulation. Composite depicts the effect of IR on the production of ROS (A), the colocalization of cytochrome c and mitochondria (B), the state of $\Delta \Psi_m$ reflected on the JC-1 red/green signal ratio (C), and the levels of cleaved caspase-3 (D). ROS production and colocalization of cytochrome c and mitochondria were measured 6 hours post IR radiation (1ms, 30 pps, 661 mJ cm$^{-2}$) for 10 minutes. ROS (CellROX®) levels increased in radiated neurons (**$P < 0.01$) and the control group treated with $\Delta \Psi_m$ depolarizer FCCP (****$P < 0.001$) when compared to non-radiated neurons. Cytochrome c and mitochondria colocalized in both radiated and non-radiated neurons. Partial release of cytochrome c from the mitochondria was only observed in the cells treated with FCCP (Pearson’s Correlation coefficient (PCC) and the Mander’s Coefficients (M1, M2) (****$P < 0.001$)). JC-1 red/green signal ratio and levels of cleaved caspase-3 were measured 24 hours post IR radiation. The JC-1 red/green signal ratio
measured in non-radiated and radiated cells indicated normal resting values of $\Delta \Psi_m$. The control group treated with FCCP showed permanent depolarization of $\Delta \Psi_m$ reflected on the lower ratio values ($^{***}P < 0.001$). Cleaved caspase-3 measured in non-radiated and radiated cells was minimal, in contrast to the higher levels detected in FCCP treated neurons ($^{***}P < 0.001$).

In our experimental settings, IR stimulation for 10 minutes (1 ms, 30 Hz, 661 mJ cm$^{-2}$) did not result in irreversible neuronal damage, even as there was an increase in the levels of ROS at 6 hours post-radiation. One of the main regulators of mitochondrial ROS is $\Delta \Psi_m$ (Brookes, Yoon et al. 2004). ROS production in hyperpolarized mitochondria has been previously described as a ROS-induced ROS-released (RIRR) process (Zorov, Juhaszova et al. 2006). When the mPTP opens due to Ca$^{2+}$ overload and/or excessive ROS production, cytochrome c and other pro-apoptotic molecules (such as Smac/DIABLO, apoptosis inducing factor (AIF), and Endo G) are released into the cytosol (Bayir and Kagan 2008). Cytochrome c then associates with Apaf-1 to activate caspase-9, which in turn activates caspase-3. Colocalization of cytochrome c and mitochondria 6 hours post-radiation as observed here suggests that permanent mPTP opening did not occur in IR stimulated mitochondria. Additionally, JC-1 red/green ratios measured after 24 hours confirmed that $\Delta \Psi_m$ had returned to normal, pre-radiation levels. Cleaved caspase-3 levels measured at 24 hours were also minimal, and the lack of nuclear fragmentation suggests that even caspase-independent apoptosis is unlikely.
Figure 20. Colocalization of Cytochrome C and Mitochondria. Colocalization of cytochrome c (mouse anti-cytochrome c) and mitochondria (MitoTracker® Orange CM-H₂TMRos) were analyzed 6 hours post IR stimulation (1ms, 30 pps, 661 mJ cm⁻²) for 10 minutes. Composite depicts colocalization of cytochrome c and mitochondria in representative non-radiated (A-D) and radiated neurons (E-H). Yellow in the overlay images (D-H) appears where the green fluorescence of cytochrome c colocalizes with the red fluorescence of MitoTracker®. Scale Bars = 10 µm.

These results do not discard the possibility that higher IR doses may produce some persisting mitochondrial damage in the neurons.
Compounds targeting either mitochondrial Ca\(^{2+}\) cycling (Ruthenium Red and CGP-37157), \([\text{Ca}^{2+}]_i\) (BAPTA-AM) or mitochondrial ETC complexes (Antimycin A and rotenone) inhibited the IR-evoked increases in ΔΨ\(_m\). Since Ca\(^{2+}\) transport in the mitochondria can in turn affect ΔΨ\(_m\) (Szabadkai and Duchen 2008), the diminished IR-evoked response observed following blockage of mitochondrial Ca\(^{2+}\) cycling supports the hypothesis that mitochondria sequester Ca\(^{2+}\) during IR stimulation (Dittami, Rajguru et al. 2011, Lumbreras, Bas et al. 2014). The fact that the IR-evoked response was inhibited following \([\text{Ca}^{2+}]_i\) chelation also supports the hypothesis that intracellular Ca\(^{2+}\) stores like the ER may contribute the Ca\(^{2+}\) sequestered by the mitochondria. Blocking RyRs and the ER Ca\(^{2+}\)-ATPase in neurons has been shown to decrease the IR-evoked \([\text{Ca}^{2+}]_i\) response (Lumbreras, Bas et al. 2014). Ca\(^{2+}\) communication between ER and mitochondria is facilitated by microdomains, where RyRs and IP\(_3\)Rs in the ER are juxtaposed to mitochondrial voltage-dependent channels in the mitochondria-associated membranes (MAMs) (Berridge, Bootman et al. 1998, Szabadkai and Duchen 2008, Bononi, Missiroli et al. 2012). The main role of mitochondrial Ca\(^{2+}\) is the activation of oxidative phosphorylation (Brookes, Yoon et al. 2004). More precisely, it activates TCA cycle enzymes (McCormack and Denton 1993), ATP synthase (complex V) (Das and Harris 1990), and carriers like citrin (Contreras, Gomez-Puertas et al. 2007). As a whole, a rise in \([\text{Ca}^{2+}]_m\) results in an increase in respiratory rate, ATP production, and H\(^+\) extrusion from the mitochondrion (Santo-Domingo and Demaurex 2010). This correlation between mitochondrial Ca\(^{2+}\) and oxidative phosphorylation may explain the inhibition in the IR-evoked response when incubating the neurons with Antimycin A (blocker of complex III
of the ETC, Q-cytochrome c oxidoreductase) or rotenone (blocker of complex I, NADH-CoQ reductase). The results suggest that IR induces a rise in \([\text{Ca}^{2+}]_m\) which increases respiration. Slowing down the ETC appears to result in an overall downregulation of the IR-evoked cellular response observed here.

Another hypothesis that may explain the present results is that IR has a direct effect on certain component(s) of the ETC leading to its acceleration (Arvanitaki and Chalazonites 1961). Indeed, mitochondria are known to have several photoacceptors: cytochrome c oxidase (red light, NIR), the iron protoporphyrin IX (PpIX) of cytochromes b, c1, and c (green light), and NADH-dehydrogenase (blue light) (Gao and Xing 2009). Cytochrome c oxidase (complex IV) could be a potential candidate, since it is sensitive to NIR wavelengths (Karu, Pyatibrat et al. 2005).

CsA and TEMPOL had no detectable effect on the IR-evoked transients. CsA is known to block the mPTP, so the lack of inhibition observed suggests that mPTP likely does not flicker during the IR-evoked mitochondrial hyperpolarizations. This is supported by previous experiments showing that calcein does not enter in hyperpolarized mitochondria (Zorov, Juhaszova et al. 2006). It is possible that signaling via ROS (Brookes, Yoon et al. 2004) could produce or influence the IR-evoked responses, but ROS scavenger TEMPOL acting as a SOD mimic had no effect. Thus, the observed increase in ROS is more likely to be a correlate or consequence of the \(\Delta \Psi_m\) than a causative factor.

The IR induced decrease in \(\text{pH}_i\) may be explained as a result of increased \(\text{CO}_2\) in the cytosol as a byproduct of respiratory metabolism. Carbonic anhydrase enzymes catalyze
the production or consumption of $H^+$ depending on prevailing substrate concentrations; when CO$_2$ levels rise, CO$_2$ and H$_2$O are converted to H$_2$CO$_3$, which in turn dissociates into $HCO_3^-$ and $H^+$ (Casey, Grinstein et al. 2010). Another possible source of $H^+$ might be influx from the extracellular medium via the plasmalemmal Ca-ATPase, a known $Ca^{2+}/H^+$ exchanger (Wu, Chen et al. 1999).

IR has also been reported to result in a capacitive photothermal membrane current (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014). However, the relatively small amplitude depolarization described would not be sufficient to trigger action potentials in most neurons. This capacitive change is unlikely to be primarily responsible for the IR-evoked electrical response (Peterson and Tyler 2012). Pulsed IR stimuli induces a transient increase in temperature up to ~22.2°C for a 10 ms pulse (7.3 mJ, 5.8 J cm$^{-2}$) (Shapiro, Homma et al. 2012, Liljemalm, Nyberg et al. 2013). With radiant exposures in the order of mJ cm$^{-2}$, such temperature rise would be much smaller. The fast time course of the IR-evoked fluorescence changes in Rhod123 indicates that the major part of the mitochondrial hyperpolarizations is not due to a temperature change. Since most of this temperature increase is caused by water absorbing IR (Wells, Kao et al. 2007, Shapiro, Homma et al. 2012), thermal effects would be expected to have a much lower decay in fluorescence after the end of the IR pulses. This contrasts with the rapid change in Rhod123 signal following the end of stimulation. There were no significant differences in recordings done in media at 4, 25, and 37°C. The mitochondrial hyperpolarizations were also not dependent upon the presence of [Ca$^{2+}$]$_o$ suggesting that the responses are not driven by acute Ca$^{2+}$ influx from the medium.
An unexpected finding was that IR depolarized $\Delta \Psi_m$ in some neurons at low temperatures (4°C, data not shown). This effect reversed as the media was naturally allowed to warm up to the room temperature. Studies on plant mitochondria describe that net mitochondrial Ca$^{2+}$ uptake is higher at low temperatures because Ca$^{2+}$ extrusion slows down (Ferguson, Reid et al. 1985), and that respiration is a temperature-sensitive process (Armstrong, Badger et al. 2008). Previous recordings of IR-evoked [Ca$^{2+}$]$_i$ transients in media at 8°C were not significantly different to those recorded at 25°C (Lumbreras, Bas et al. 2014). Large Ca$^{2+}$ influx into the mitochondria at low temperatures could overwhelm a slower ETC and explain the unquenching of Rhod123 observed. Alternatively, lower ETC activity would be paralleled by a less negative $\Delta \Psi_m$, which ultimately could favor unquenching of Rhod123 under IR stimuli.

Further studies are needed to determine the correlations between IR-evoked changes in plasma-membrane depolarization $\Delta \Psi_p$ and the mitochondrial mechanism observed here. Since the transient increases in $\Delta \Psi_m$ could be elicited with each laser pulse applied, the IR-evoked intracellular Ca$^{2+}$ transients may mediate $\Delta \Psi_p$ depolarizations. The onset of an intracellular mechanism after IR exposure is supported by previous reports showing latencies between applied IR stimuli and consequent neurotransmitter release: ~7.6 ms in vestibular afferents (Rajguru, Richter et al. 2011) and ~2.5 ms in the cochlea (Richter, Rajguru et al. 2013). The mechanism(s) underlying the IR excitation of mitochondria are likely general across species and cell types. The role of Ca$^{2+}$ supports the hypothesis that IR-evoked intracellular Ca$^{2+}$ release can lead to neurotransmitter release from the vestibular hair cells or neurons as well as elicit contractile responses from the
cardiomyocytes (Jenkins, Wang et al. 2013). IR stimulation may also find significant applications in the study of subcellular pathways controlled by mitochondria. Results suggest that IR modulates known elements of mitochondrial signaling (i.e. Ca$^{2+}$, $\Delta \Psi_m$, ROS, and pH$_i$), and it is possible that IR may have other biological effects on cells (Toyokawa, Matsui et al. 2003).
CHAPTER 4: PULSED IR MODULATES PLASMA MEMBRANE POTENTIAL IN CULTURED NEURONS

4.1 INTRODUCTORY REMARKS

Optical control of neural activity with cell-specific activation or silencing (Boyden, Zhang et al. 2005, Bernstein, Garrity et al. 2012, Deisseroth and Schnitzer 2013, Richter and Tan 2014) shows promise both as a basic research tool and for development of prosthetic devices. Optical stimuli that are spatially confined may provide significant functional improvement over conventional electrical stimulation (Richter, Matic et al. 2011). Infrared radiation (IR, typically 1400-1600 nm and 1840-2100 nm) and near infrared radiation (NIR, typically 790-850 nm) elicit reversible and controllable responses in different cell types without genetic or pharmacological modification (Wells, Kao et al. 2005, Izzo, Richter et al. 2006, Smith, Kumamoto et al. 2008, Zhao, Zhang et al. 2009, Cayce, Friedman et al. 2011, Dittami, Rajguru et al. 2011, Rajguru, Richter et al. 2011).

Pulsed IR evokes intracellular Ca\(^{2+}\) transients (Cayce, Bouchard et al. 2014, Lumbrreras, Bas et al. 2014) and electrical responses in neurons (Littlefield, Vujanovic et al. 2010, Rajguru, Richter et al. 2011), but whether there is a direct correlation between Ca\(^{2+}\) and neuronal depolarization during IR stimulation has not been completely determined. Ascertaining such correlation is the focus of the present study. Pharmacological evidence indicates that IR likely activates Ca\(^{2+}\) transfer between the endoplasmic reticulum (ER) and mitochondria (Dittami, Rajguru et al. 2011, Lumbrreras, Bas et al. 2014). Mitochondria localized in high [Ca\(^{2+}\)] microdomains, like endoplasmic
Ca²⁺ release sites, have higher rates of Ca²⁺ uptake (David, Barrett et al. 1998). The ER can extrude Ca²⁺ to the cytosol through ryanodine (RyRs) or IP₃ receptors (IP₃Rs), with subsequent mitochondrial Ca²⁺ sequestration followed by return to the ER (Berridge, Bootman et al. 1998, Szabadkai and Duchen 2008). Changes in mitochondrial transmembrane potential (ΔΨₘ) may prove as a sensitive measurement of Ca²⁺ transport into mitochondria (Szabadkai and Duchen 2008), and indeed mitochondria hyperpolarize transiently under pulsed IR stimuli (Lumbreras and Rajguru 2014). Since IR-evoked responses in [Ca²⁺]ᵢ and ΔΨₘ could be elicited with each laser pulse applied, IR induced intracellular Ca²⁺ release may lead to transient plasma membrane depolarization as well.

Taken together with previous work, the present study presents evidence that pulsed IR (λ = 1863 nm) depolarizes the plasma membrane in cultured neurons following an increase in [Ca²⁺]ᵢ. Both the ER and mitochondria seem to be primarily involved in cycling intracellular Ca²⁺ contributing to depolarization of ΔΨₚ.

4.2 MATERIALS AND METHODS

**Neuronal Culture**

The spiral and vestibular ganglion neurons from 2-3 postnatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were cultured as previously described (Lumbreras, Bas et al. 2014). Housing conditions and experimental procedures used in this study were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publications No. 80-23,
Immunofluorescence Labeling Studies

Three independent experiments were carried out. The spiral and vestibular ganglion neurons were fixed with 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS, pH = 7.4) for 24 hours at 4°C. After fixation, the neurons were rinsed three times in PBS, and subsequently permeabilized (1% Triton X-100; Shelton Scientific Inc., Shelton, CT, USA) and blocked (5% normal goat serum; Vector Labs, Burlingame, CA, USA) in PBS for 1 hour at room temperature. The neurons were then incubated with 1:200 anti-β-Tubulin mouse monoclonal antibody (TUJ1; Covance, Princeton, NJ, USA) overnight at 4°C. After rinsing three times with PBS, the neurons were incubated for 1 hour at room temperature with 1:200 secondary antibody Alexa 488-labeled goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA). Control slides determining specific binding of secondary to primary antibodies were incubated only with the secondary antibody. Finally, after three rinses with PBS, the neurons were incubated with 600 nM 4’,6-diamino-2-fenilindol (DAPI; Life Technologies, Carlsbad, CA, USA), rinsed once more, cover-slipped and observed under a confocal microscope (LSM 700 inverted, Carl Zeiss AG, Oberkochen, Germany) with a 63X oil immersion objective.

Assessment of ΔΨ₂

Changes in ΔΨ₂ were measured using FluoVolt™ Membrane Potential Kit (Life Technologies, Carlsbad, CA, USA) (Moshtagh-Khorasani, Miller et al. 2013). The neurons were loaded and incubated at 37°C for 30 minutes in FluoVolt™ membrane
potential dye. After incubation, the dye-containing loading medium was washed and replaced with artificial perilymph (125 mM NaCl; 3.5 mM KCl; 25mM NaHCO$_3$; 1.2 mM MgCl$_2$; 1.3 mM CaCl$_2$; 0.75 mM NaH$_2$PO$_4$; 5 mM glucose) or with Ca$^{2+}$-free Dulbecco’s Phosphate Buffered Saline (DPBS, pH = 7.4). Media temperature during imaging was measured with a thermometer (~25°C), and in specific experiments was cooled down to 4°C. A 140 mM potassium gluconate solution (D-gluconic acid, Sigma-Aldrich, St. Louis, MO, USA) was added as a positive control to neurons pre-treated for 30 minutes with 10 µM K$^+$ ionophore valinomycin (Tocris Bioscience, Ellisville, MO, USA).

**Pulsed Infrared Radiation**

IR stimulation (λ = 1863 nm) was delivered with a multimodal 400 µm diameter optical fiber (Ocean Optics, Dunedin, FL, USA) connected to a Capella laser (Lockheed Martin Aculight, Bothell, WA, USA) (Lumbreras, Bas et al. 2014). The fiber was held and controlled with a micromanipulator allowing IR to be delivered ~300 µm away from the target cells. Trains of IR pulses (100 µs, 100 pps) were delivered to the quiescent neurons for 30 s while measuring changes in FluoVolt™ fluorescence. The laser radiant exposure varied from 178 to 374 mJ cm$^{-2}$, and was measured in air using a digital optical power/energy meter (FieldMax3H, Coherent, Santa Clara, CA, USA). Thermal, rather than stress confinement is expected in these experimental settings given the selected pulse widths. Image sequences measuring fluorescence were collected using a confocal microscope (SP5 upright, Leica, Wetzlar, Germany) with a resonant scanner and a 20X
water immersion objective. Different 512X512 pixels per frame sequences of the neurons exposed to pulsed IR stimuli were recorded (14-28 fps).

**Pharmacology**

The neurons were incubated for 30 minutes 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), ryanodine, cyclopiazonic acid (CPA) (all 50 µM, and from Tocris Bioscience, Ellisville, MO, USA) or BAPTA-AM (10 µM, Sigma-Aldrich, St. Louis, MO, USA) in artificial perilymph (except CPA that was diluted in Ca^{2+}-free DPBS). Pulsed IR was delivered to the neurons at 10 and 30 minutes during drug incubation, and IR-evoked fluorescence changes in FluoVolt™ were recorded. After 30 minutes, the neurons were washed with fresh artificial perilymph to remove the pharmacological compound. IR stimulation was repeated ~10 minutes following washout.

**Image Processing**

Image sequences measuring FluoVolt™ fluorescence were processed using ImageJ (NIH, Bethesda, MD, USA). Average fluorescence values of the neurons in each frame were computed, and then analyzed in MATLAB (MathWorks, Natik, MA, USA). Normalization was done with respect to the average fluorescence value of the first frame, which represented fluorescence intensity of the cell at rest ($\frac{\Delta F}{F_0}$, where $\Delta F = F - F_0$).
Statistics

One-way analysis of variance (ANOVA) followed by Dunnett post hoc testing was used to compare IR-evoked responses in FluoVolt™. Two-way ANOVA followed by Bonferroni post hoc testing was used in the pharmacological analysis. A $P$ value of $<0.05$ was considered significant. All calculations were performed on a computer equipped with GraphPad Prism v 5.00c software for Mac OS X® (GraphPad Software, La Jolla, CA, USA).

4.3 RESULTS

Immunocytochemical Analysis

Cultures of rat neonatal spiral and vestibular ganglion neurons were used for all the experiments. Both kinds of cultures were primarily composed of neurons that stained positively for neuronal marker TUJ1 (Figure 21). DAPI nuclear fluorescence served to quantify the cells in culture, and on average, 99% stained positive for TUJ1. Control slides (not shown) confirmed that the labeling observed was due only to specific binding of the secondary to the primary antibody.

IR Stimulation Transiently Modulates $\Delta \Psi_p$

FluoVolt™ Membrane Potential Kit was used to analyze changes in $\Delta \Psi_p$ during pulsed IR stimulation ($\lambda = 1863$ nm) (Figure 22A). Positive control responses could be elicited by adding potassium gluconate to neurons pre-treated with valinomycin (n = 10 neurons, k = 1 culture plate, Figure 22B). Trains of IR stimuli (100 $\mu$s, 100 pps, 178-374
Figure 21. Immunocytochemical Analysis. Composite depicts representative immunocytochemical characterization of the neuronal cultures used in all experiments. The cultures of spiral (A) and vestibular (B) ganglion neurons stained positively for the neuronal marker TUJ1. DAPI nuclear fluorescence served to quantify the number of cells in culture, to then determine the percentage of neurons. Scale Bars = 10 µm.

mJ cm<sup>-2</sup>) delivered to the quiescent neurons for 30 s resulted in an increase in FluoVolt fluorescence. These transient IR-evoked responses suggest ΔΨ<sub>p</sub> depolarization, and were dependent on laser radiant exposure (Figure 23A). The magnitude of the plasma membrane depolarization increased linearly with laser radiant exposure (n = 20 per group, k = 3) (Figures 23B). Removal of extracellular Ca<sup>2+</sup> did not have an effect on the IR-evoked response, with no significant changes observed in FluoVolt™ recordings.
(Ca\textsuperscript{2+}-free DPBS, n = 17, k = 2, \( P > 0.05 \)). To study IR induced photothermal effects on the plasma membrane, the temperature of the extracellular media was cooled down to 4°C in specific experiments. There were no significant differences between \( \Delta \Psi_p \) depolarization recorded at room temperature (\(~25°C\)) and 4°C (n = 8, k = 1, \( P > 0.05 \)).

Figure 22. Assessment of \( \Delta \Psi_p \). The neurons were loaded and incubated at 37°C for 30 minutes in fluorescent \( \Delta \Psi_p \) sensor FluoVolt\textsuperscript{TM} (A). A 140 mM potassium gluconate solution was used as a control to elicit depolarization in neurons pre-treated with 10 \( \mu \)M K\textsuperscript+ ionophore valinomycin (B). The data are expressed as the 95% CI of the mean (N = 10 neurons). The neurons responded with an increase in FluoVolt\textsuperscript{TM} fluorescence suggesting \( \Delta \Psi_p \) depolarization.
Figure 23. IR Modulates $\Delta \Psi_p$. Composite depicts IR evoked responses in $\Delta \Psi_p$ recorded using FluoVolt™ fluorescent probe. Fluorescence values were normalized with respect to that of the first frame in the sequence ($\Delta F/F_0$, where $\Delta F = F - F_0$). A depicts averaged fluorescence changes ($N = 20$) recorded in FluoVolt™ during IR stimulation ($\lambda = 1863$ nm, $100 \mu$s, 100 pps, 178-374 mJ cm$^{-2}$) delivered for 30 s. The $\Delta \Psi_p$ sensor showed IR induced plasma membrane depolarization dependent on the radiant exposure. B depicts the peak depolarization recorded at different radiant exposure settings (mean ± S.D.). Significant differences were observed when comparing the highest radiant exposure settings (374 mJ cm$^{-2}$) with the rest of groups ($*** P < 0.001$, **$P < 0.01$).
Pharmacological Analysis

Pharmacological studies were conducted to gain insight whether intracellular Ca\textsuperscript{2+} cycling contributes to the IR-evoked $\Delta \Psi_p$ depolarization. Ruthenium Red, CGP-37157, CPA, and ryanodine have previously proven effective in inhibiting or reducing the IR induced intracellular Ca\textsuperscript{2+} release (Lumbreras, Bas et al. 2014). In these experimental settings, BAPTA-AM (n = 18, k = 3), CGP-37157 (n = 22, k = 2), Ruthenium Red (n = 19, k = 3), CPA (n = 14, k = 2), and ryanodine (n = 21, k = 4) reversibly decreased the IR-evoked $\Delta \Psi_p$ depolarization (Figure 24). BAPTA-AM is a [Ca\textsuperscript{2+}]\textsubscript{i} chelator, CGP-37157 blocks mitochondrial Ca\textsuperscript{2+} extrusion (Baron and Thayer 1997), Ruthenium Red inhibits the three different pathways of mitochondrial Ca\textsuperscript{2+} uptake (Moore 1971, Gunter, Buntinas et al. 2000, Jiang, Zhao et al. 2009), CPA is a reversible inhibitor of the ER Ca\textsuperscript{2+}-ATPase (Plenge-Tellechea, Soler et al. 1997), and ryanodine blocks ER Ca\textsuperscript{2+} extrusion through RyRs (Sutko, Airey et al. 1997). The effect of all these compounds on the IR-evoked $\Delta \Psi_p$ depolarization could be observed after 10 minutes of incubation when compared to the control group (n = 23, k = 4) without drug (Figure 25).

4.4 DISCUSSION

Pulsed IR Depolarized $\Delta \Psi_p$

The increases in FluoVolt\textsuperscript{TM} fluorescence suggest that pulsed IR stimuli ($\lambda$ = 1863 nm, 100 $\mu$s, 100 pps) transiently depolarized $\Delta \Psi_p$ in the neurons. The observed depolarization increased linearly with radiant exposure (178-374 mJ cm\textsuperscript{-2}), and removal
Figure 24. IR Induced Intracellular Ca\(^{2+}\) Release Contributes to ΔΨ\(_p\) Depolarization.

Treatment with no drug (CONTROL; A), 10 μM BAPTA-AM (B), 50 μM Ruthenium Red (RR; C), 50 μM CGP-37157 (D), 50 μM cyclopiazonic acid (CPA; E), and 50 μM ryanodine (RYN; F). RR, CGP-37157, CPA, and RYN have previously proven effective in inhibiting or reducing the IR induced intracellular Ca\(^{2+}\) release. Each graph is a composite that depicts the averaged IR-evoked (100 μs, 100 pps, 374 mJ cm\(^{-2}\)) ΔΨ\(_p\) depolarization in neurons for 30 s before loading the compound, after 10 and 30 minutes of incubation with the compound, and 10 minutes post washout of the compound.
Figure 25. Pharmacological Analysis. Figure depicts the effect of various pharmacological compounds on the peak magnitude of the IR-evoked depolarization in $\Delta \Psi_p$. The data are expressed as mean ± S.D. IR stimulation (100 µs, 100 pps, 374 mJ cm$^{-2}$) was delivered for 30 s before loading the compounds (no drug), after 10 and 30 minutes of incubation with the compound, and 10 minutes post washout of the compound. Each pharmacological compound is compared to the control group without drug at the times indicated (**$P < 0.001$, *$P < 0.01$, *$P < 0.05$). BAPTA-AM is a $[\text{Ca}^{2+}]_i$ chelator, CGP-37157 blocks mitochondrial $\text{Ca}^{2+}$ extrusion, Ruthenium Red inhibits the three different pathways of mitochondrial $\text{Ca}^{2+}$ uptake, CPA is a reversible inhibitor of the ER $\text{Ca}^{2+}$-ATPase, and ryanodine blocks ER $\text{Ca}^{2+}$ extrusion through RyRs.
of extracellular Ca$^{2+}$ or cold media temperatures had no detectable effect on it. The results suggest that acute extracellular Ca$^{2+}$ influx or thermal effects on the plasma membrane are not primary mediators of the observed IR-evoked increases in $\Delta \Psi_p$. Pulsed IR induces transient increases in temperature caused by water absorbing most of the incident energy (Shapiro, Homma et al. 2012). Temperature goes up to $\sim$22.2°C for a 10 ms pulse (7.3 mJ, 5.8 J cm$^{-2}$) (Shapiro, Homma et al. 2012, Liljemalm, Nyberg et al. 2013), and such temperature rise is expected to be much smaller with radiant exposures in the order of mJ cm$^{-2}$. Research experiments suggest that the IR photothermal effect generates a capacitive membrane current (Shapiro, Homma et al. 2012, Okunade and Santos-Sacchi 2013, Liu, Frerck et al. 2014) depolarizing $\Delta \Psi_p$ up to 9 mV in artificial bilayers. This capacitance change might contribute to the IR-evoked depolarization in $\Delta \Psi_p$ observed here, whereas it cannot be entirely responsible for it as there were no significant differences between responses at 4°C and 25°C. In postsynaptic recordings in vestibular afferents in vivo (Rajguru, Richter et al. 2011), IR-evoked responses were also qualitatively similar at low temperatures (6-7°C) and whole organ temperature increases did not elicit persistent responses on their own.

A previous research study suggests that single laser pulses ($\lambda = 1450$ nm, 3-5 ns) elicited inward currents in spiral ganglion neurons held at their resting potential (Rettenmaier, Lenarz et al. 2014). These inward currents were dependent on radiant exposure, and led to $\Delta \Psi_p$ depolarization ($\sim$1.2 mV, 289 mJ cm$^{-2}$). In our experimental settings, the limitation in temporal resolution of the confocal microscope prevented resolving responses elicited by single low frequency laser pulses. The increase in
FluoVolt™ fluorescence suggesting depolarization in $\Delta \Psi_p$ stabilized a few seconds following the onset of stimulation, indicating slow resolving kinetics of the IR-evoked response. This slow increase in fluorescence could indicate that a train of IR pulses rather than a single stimulus is required to achieve maximal single cell depolarization. Regarding the magnitude of the IR-evoked depolarization observed here, no calibration of FluoVolt™ could be performed preceding stimulation experiments, to then turn relative fluorescence values into voltage. As a reference, the depolarization elicited by adding potassium gluconate was not much greater than those elicited under pulsed IR stimuli.

**IR Induced Intracellular Ca$^{2+}$ release contributes to $\Delta \Psi_m$ depolarization**

IR-evoked $[\text{Ca}^{2+}]_i$ events have been described before in HeLa cells (Smith, Fujita et al. 2001, Iwanaga, Kaneko et al. 2006), cardiomyocytes (Smith, Kumamoto et al. 2008, Dittami, Rajguru et al. 2011), and neurons (Cayce, Bouchard et al. 2014, Lumbreras, Bas et al. 2014). Previous pharmacological evidence indicates that IR likely activates Ca$^{2+}$ transfer between the ER and mitochondria (Dittami, Rajguru et al. 2011, Lumbreras, Bas et al. 2014). In this process, $\Delta \Psi_m$ hyperpolarizes via acceleration of the electron transport chain and there is an acidic shift in cytoplasmic pH (Lumbreras and Rajguru 2014).

Compounds targeting either mitochondrial Ca$^{2+}$ cycling (RR and CGP-37157), $[\text{Ca}^{2+}]_i$ (BAPTA-AM) or ER Ca$^{2+}$ extrusion (Ryanodine and CPA) reversibly decreased the IR-evoked depolarization in $\Delta \Psi_p$. Ruthenium Red, CGP-37157, CPA, and ryanodine have previously proven effective in inhibiting or reducing IR-evoked $[\text{Ca}^{2+}]_i$ transients (Lumbreras, Bas et al. 2014). These results suggest that the IR induced intracellular Ca$^{2+}$ release contributes to $\Delta \Psi_p$ depolarization. It is likely that ER Ca$^{2+}$ extruded to the cytosol
through RyRs and IP₃Rs activates plasma membrane channels. Previous research studies have observed that changes in \([\text{Ca}^{2+}]_i\) regulate certain ion channels activity, such as L-type and T-type \(\text{Ca}^{2+}\) channels (Charles, Piros et al. 1999, Hughes, Cope et al. 2002), calcium-activated non-selective (CAN) cation currents (Roe, Worley et al. 1998, Torihashi, Fujimoto et al. 2002), and \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channels (Brenner, Perez et al. 2000, Patterson, Henrie-Olson et al. 2002, Kawano, Otsu et al. 2003, Bond, Maylie et al. 2005). CAN channels are directly activated by elevations in \([\text{Ca}^{2+}]_i\), and carry primarily inward \(\text{Na}^+\) currents depolarizing \(\Delta \Psi_p\) (Partridge, Muller et al. 1994, Launay, Fleig et al. 2002). \([\text{Ca}^{2+}]_i\) activating a channel of these characteristics could explain the pharmacological results of the present study.

**Future Directions**

Further electrophysiological characterization of the \(\text{Ca}^{2+}\)-activated plasma membrane channel(s) introduced here is needed to completely understand the cellular mechanism underlying infrared neural stimulation. The ion carried, the type of channel, and its selectivity are open questions to be answered. IR may provide a novel tool to study the role of \([\text{Ca}^{2+}]_i\) in neural excitability and synaptic transmission. Postsynaptic vestibular afferent responses during IR stimulation have been shown to be excitatory, inhibitory or mixed (Rajguru, Richter et al. 2011), and it is still unknown what determines one type of response or another. The IR induced rise in \([\text{Ca}^{2+}]_i\) may modulate neurotransmitter release, and characterization of this process could lead to new research applications of IR in neuroscience.
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The present study investigated the cellular mechanism(s) underlying infrared neural stimulation (INS). Optical control of neural activity with cell-specific activation or silencing (Boyden, Zhang et al. 2005, Bernstein, Garrity et al. 2012, Deisseroth and Schnitzer 2013, Richter and Tan 2014) shows promise both as a basic research tool and for development of prosthetic devices. Pulsed infrared radiation (IR) can stimulate cells without requiring pharmacological or genetic manipulation, but the cellular mechanism(s) underlying IR-evoked responses remain elusive. Ascertaining such molecular mechanism(s) may lead to novel experimental and therapeutic applications.

The primary hypotheses tested in the present study were: 1) IR modulates intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in neurons based on previous pharmacological and Ca\(^{2+}\) imaging evidence on cardiomyocytes, 2) IR modulates mitochondrial transmembrane potential (ΔΨ\(_m\)) since mitochondrial Ca\(^{2+}\) transport can in turn affect ΔΨ\(_m\), and 3) IR increases plasma membrane potential (ΔΨ\(_p\)).

Cultured spiral and vestibular ganglion neurons responded with [Ca\(^{2+}\)]\(_i\) transients and electrical responses under pulsed IR stimuli. Pharmacological analysis revealed that IR activates Ca\(^{2+}\) signaling between the endoplasmic reticulum (ER) and mitochondria. In the process, ΔΨ\(_m\) hyperpolarizes via acceleration of the electron transport chain (ETC). This increase in [Ca\(^{2+}\)]\(_i\) also appears to be upstream of an acidic shift in cytoplasmic pH, and contributes to ΔΨ\(_p\) depolarization. The decrease in pH\(_i\) may be due to H\(^+\) extrusion from the mitochondria and H\(^+\) influx from the extracellular medium via the
plasmalemmal Ca-ATPase, which is a Ca\(^{2+}/H^+\) exchanger (Wu, Chen et al. 1999). The increase in \(\Delta \Psi_p\) may be partially due to \([Ca^{2+}]_i\) activation of a plasma membrane ion channel. Long-term radiation of the neurons proved to be safe without causing irreversible neuronal damage in our experimental settings.

Cultures of neonatal spiral and vestibular ganglion neurons were used in all experiments. This in vitro model has its experimental limitations, but the consistency of our results suggests that it is reliable to project adult and in vivo models. IR has been shown to elicit \([Ca^{2+}]_i\) transients in neurons in vivo; however, whether mitochondria also hyperpolarizes under pulsed IR in vivo stimuli needs to be confirmed.

The results of this study suggest that selective IR stimulation of spiral and vestibular ganglion neurons might be feasible. One of the primary challenges in the design of neuroprosthetic devices includes improving spatial selectivity (Grill, Norman et al. 2009). For example, cochlear implants 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, Collins, Zwolan et al. 1997, Chatterjee and Shannon 1998, Shannon, Fu et al. 2004). Additionally, tissue-electrode interactions cause fibrosis with deleterious effects (Bas, Gupta et al. 2012). The loss of perceptual channels in cochlear implants results in a poor performance for music perception and word recognition in noisy environments (McDermott 2004, Drennan and Rubinstein 2008, Spahr, Litvak 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, Dai et al. 2013, Nie, Ling 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, Migliaccio et al. 2007, Fridman, Davidovics et al. 2010). This electrical current spread causes misalignment between the axis of the eye and head rotation. Encoding of otolith endorgan transduction using electrical stimulation has proven even more difficult (Goto, Meng et al. 2003, Goto, Meng et al. 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, Richter et al. 2011), so feasible and advantageous use of IR in vestibular prostheses requires further research into what determines one type of response or another. The goal of this research is to develop optical neuroprostheses providing better frequency resolution and dynamic range than conventional stimuli. Future studies should determine the feasibility of an implantable INS device in the inner ear. The light source of this device ought to deliver trains of IR pulses at 200 pps with precise temporal control to provide sense of sound (Richter and Tan 2014). Electrical stimulation requires 100 times less energy than INS, so the increase in the number of independent channels should outweigh the additional energy required for stimulation. Research into the inflammatory process following implantation of this hypothetical INS device is needed to resolve whether surgical implantation compromises the selectivity of optical stimulation.

The results of this study also suggest that IR induced intracellular Ca\(^{2+}\) release may activate certain plasma membrane ion channels. Further electrophysiological characterization of the Ca\(^{2+}\)-activated plasma membrane channel(s) introduced here is needed to completely understand the cellular mechanism underlying infrared neural stimulation. The ion carried, the type of channel, and its selectivity are open questions not
answered in this study. Previous research studies have observed that changes in $[Ca^{2+}]_i$ regulate certain ion channels activity, such as L-type and T-type Ca$^{2+}$ channels (Charles, Piros et al. 1999, Hughes, Cope et al. 2002), calcium-activated non-selective (CAN) cation currents (Roe, Worley et al. 1998, Torihashi, Fujimoto et al. 2002), and Ca$^{2+}$-activated K$^+$ channels (Brenner, Perez et al. 2000, Patterson, Henrie-Olson et al. 2002, Kawano, Otsu et al. 2003, Bond, Maylie et al. 2005). It could be possible that IR may activate these different channel types depending on the stimulated cell. Indeed, postsynaptic vestibular afferent responses during IR stimulation have been shown to be excitatory, inhibitory or mixed (Rajguru, Richter et al. 2011), and it is still unknown what determines one type of response or another. Different channel types activated following IR induced intracellular Ca$^{2+}$ release could explain these results.

The results of this study also suggest that IR stimulation may also be utilized as a tool to study the role of mitochondria in Ca$^{2+}$ dynamics, respiratory metabolism, and events controlling synaptic transmission in the inner ear. Changes in the elements of mitochondrial retrograde signaling ($Ca^{2+}$, $\Delta \Psi_m$, reactive oxygen species (ROS), nitric oxide (NO), and fission-fusion of mitochondria) activate or suppress signal molecules in the cytoplasm and subsequent changes of downstream cascades (Gao and Xing 2009). Since this study shows that IR modulates $\Delta \Psi_m$ and mitochondrial Ca$^{2+}$, future studies should analyze whether IR activates other cellular pathways controlled by mitochondria depending on cell type. IR has been shown to promote wound healing (Toyokawa, Matsui et al. 2003), and this effect might be a consequence of stimulation of mitochondria under IR.
REFERENCES


