Toward the Application of CAN-CAN Technology – Attenuation of Ca^{2+} Signaling by Bipolar nsPEFs in a Neurosecretory Cell Type Involved in the "Flight or Fight" Response

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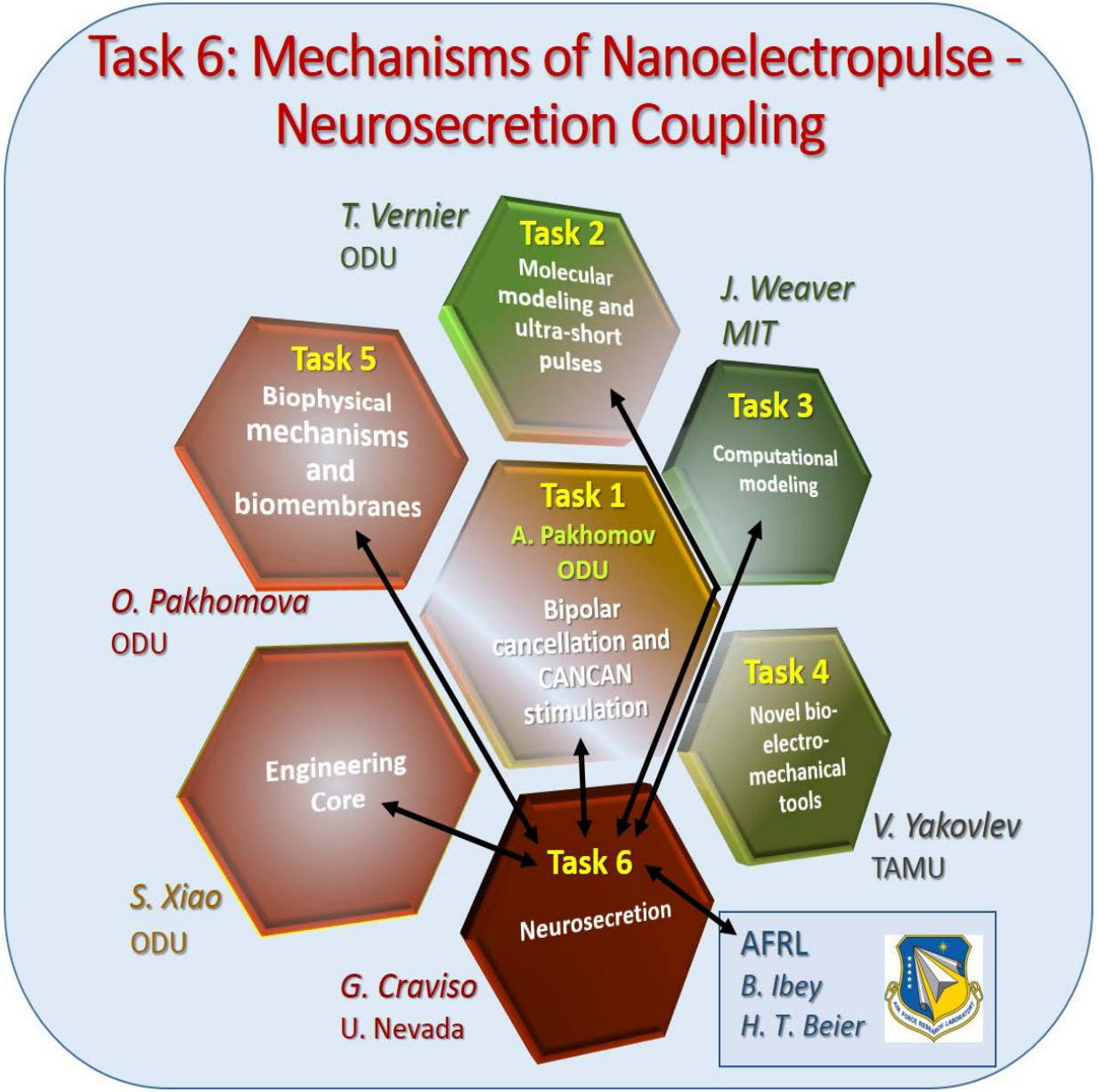
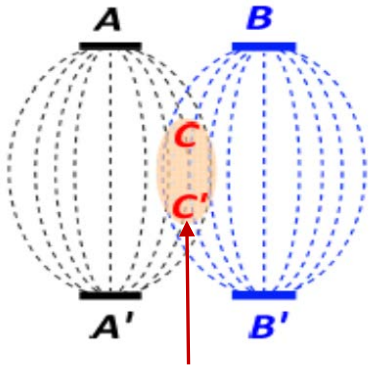
Task 6: Mechanisms of Nanoelectropulse - Neurosecretion Coupling





Goal: targeted stimulation of neurosecretion - CAN-CAN ES of neural tissue

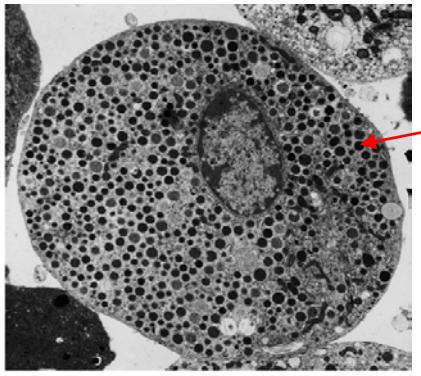
Specific Aims:

- Elucidate cell membrane effects of nsPEF
- Establish how the effects are linked to and impact Ca^{2+} signaling and neurosecretion
- Establish the basis for, and extent to which, nsPEF effects can be cancelled by bipolar pulses

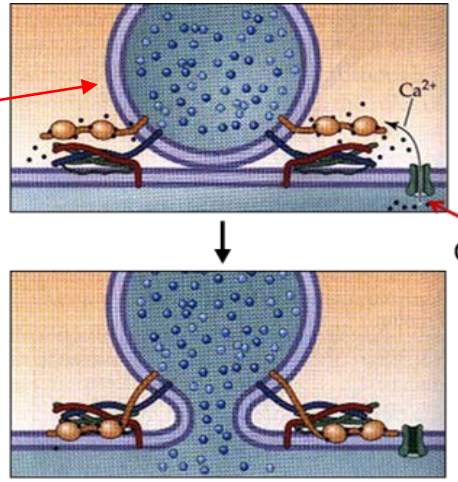
Cell Model to Study Neurosecretion: Isolated Neuroendocrine Adrenal Chromaffin Cells

Chromaffin cells are derived from the *neural crest* (neural origin)

- Localize to the *medulla of adrenal glands*
- Closely related to sympathetic neurons – i.e., are functionally equivalent to postganglionic sympathetic neurons (but do not have axons and dendrites)
- Synthesize, store and release the catecholamines epinephrine and norepinephrine into the blood stream (*"**flight or fight**" response*)
- Catecholamine release occurs by exocytosis, the same Ca^{2+}- dependent mechanism used by neurons to release neurotransmitters at nerve terminals



Electron micrograph of an adrenal chromaffin cell

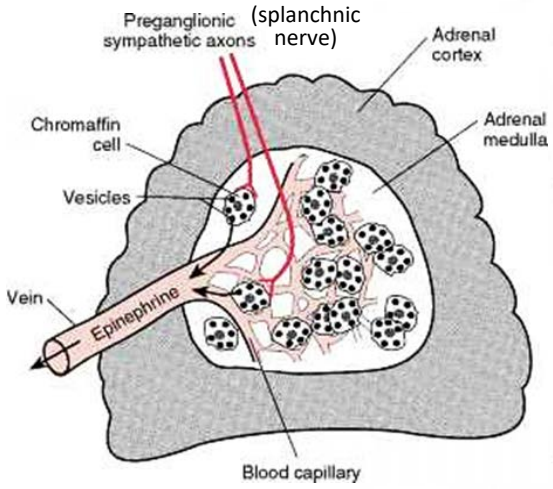


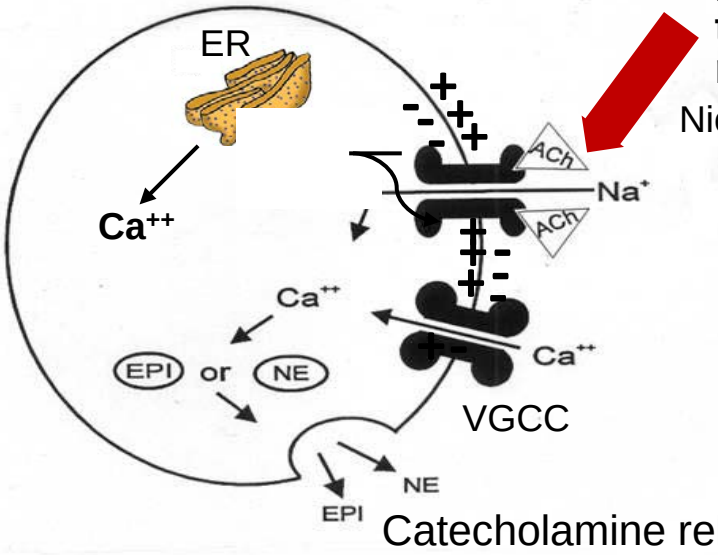
Ca^{2+} entry occurs via voltage-gated Ca^{2+} channels (VGCC) following membrane depolarization

Source: adrenal chromaffin cells isolated from the bovine species (most widely studied)

Physiological Stimulation of Catecholamine Release - *In vivo*

- Acetylcholine (ACh) released from splanchnic nerve terminals binds to and activates nicotinic cholinergic receptors that are ligand-gated Na^+ channels.
- Na^+ influx causes membrane depolarization and Ca^{2+} influx via voltage-gated Ca^{2+} channels that in turn stimulates catecholamine release





Primary stimulus that evokes neurosecretion:

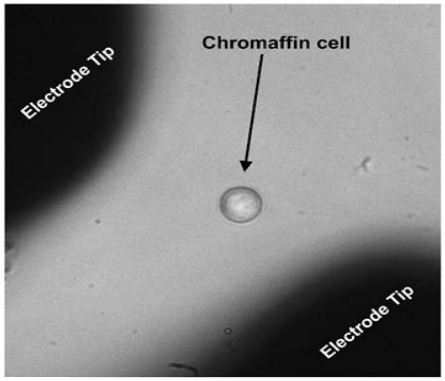
Nicotinic Receptor Activation

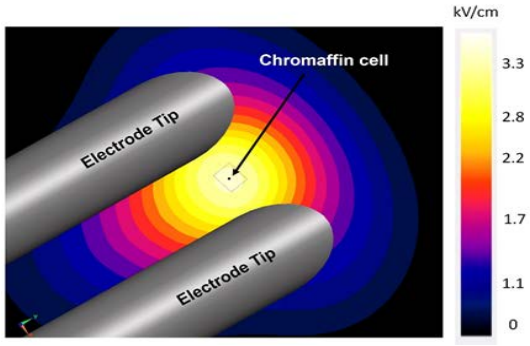
ACh: Acetylcholine
NE: Norepinephrine
EPI: Epinephrine
VGCC: Voltage-Gated Ca^{2+} Channel
ER: Endoplasmic Reticulum

Question: Can nsPEF mimic the physiological stimulus to evoke neurosecretion?

EXPERIMENTAL APPROACH

nsPEF Stimulus

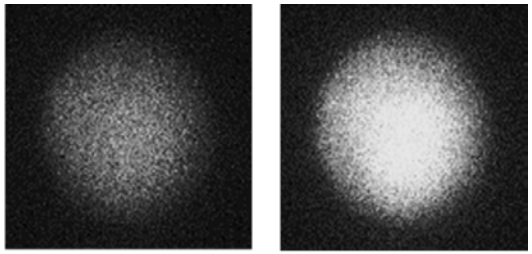




- 5 ns pulse generator
- 150 – 1000 ns bipolar pulse generator **(Engineering Core)**

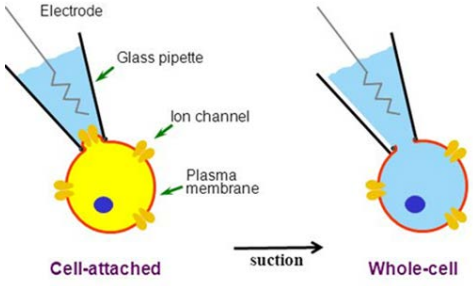
Monitoring $[Ca^{2+}]_i$

Cells loaded with Calcium Green-1 AM, a fluorescent Ca^{2+} indicator

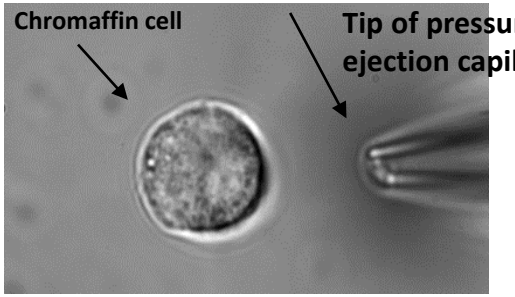


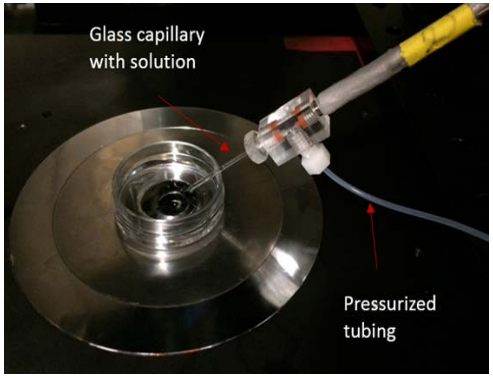
Before Stimulus After Stimulus

Patch clamp whole-cell recording



Physiological Stimulus

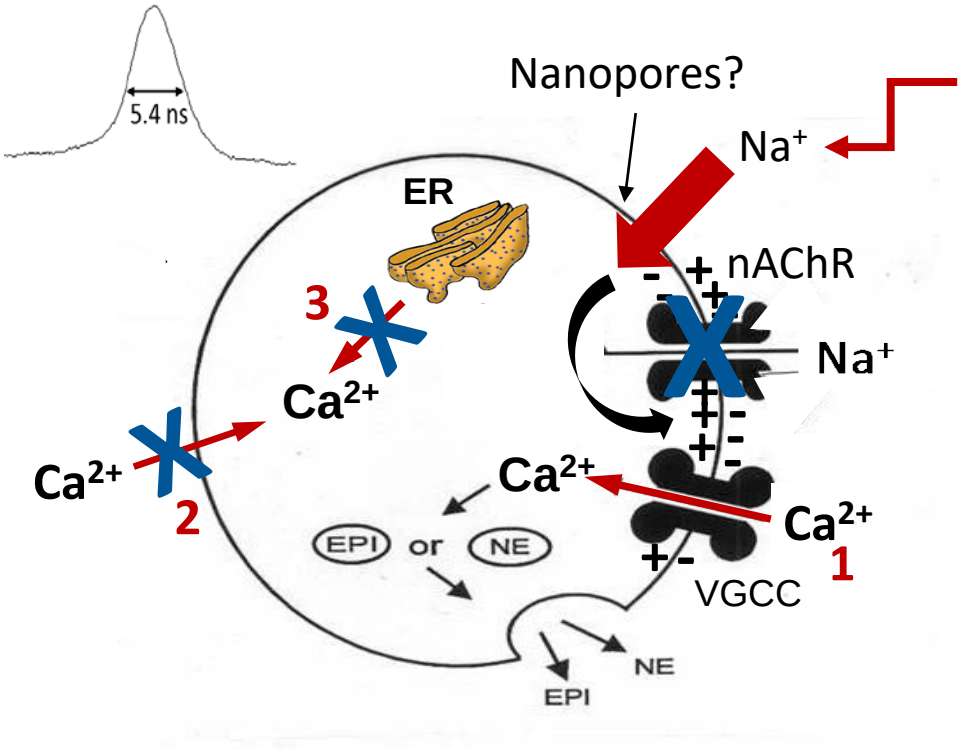




- Apply the nicotinic receptor agonist 1,1-dimethyl-4-phenyl piperazinium (DMPP) to mimic a physiological stimulus
- 5 ms per application

A Single 5 ns Pulse

Mimics Nicotinic Receptor Activation to Cause Ca^{2+} Influx via Voltage-Gated Ca^{2+} Channels and Stimulate Catecholamine Release



Na^+ influx due to plasma membrane permeabilization (nanopores) – causes membrane depolarization to a level that is sufficient to cause activation of VGCCs

1. Ca^{2+} influx solely through VGCCs

2. No Ca^{2+} influx directly across the plasma membrane

3. No Ca^{2+} release from internal stores

Nanoelectropermeabilization - Driven Membrane Depolarization

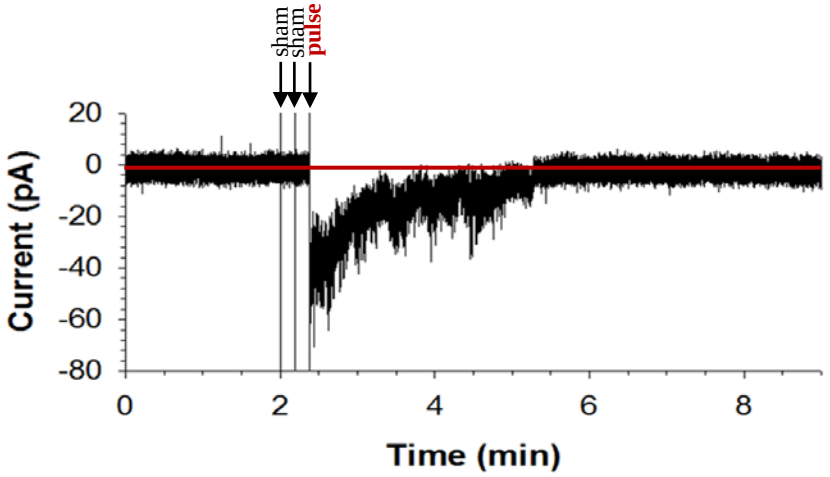
Supported by ion transport observed in molecular simulations – Task 2

<u>Significance</u>: plasma membrane subserving a function ascribed to voltage-gated Na^+ channels

Experimental Evidence for Nanoelectropermeabilization - Driven Membrane Depolarization and Voltage-Gated Ca^{2+} Channel Activation

A 5 ns pulse evokes an instantaneous inward current carried mostly by Na^+ (Yoon et al., 2016)

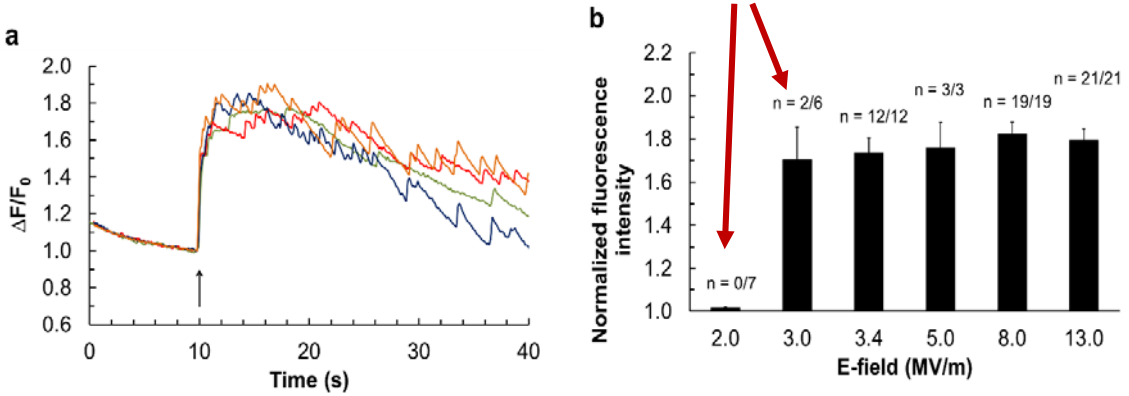
Whole-cell monitoring of membrane currents by patch clamp



Cell held at -70 mV

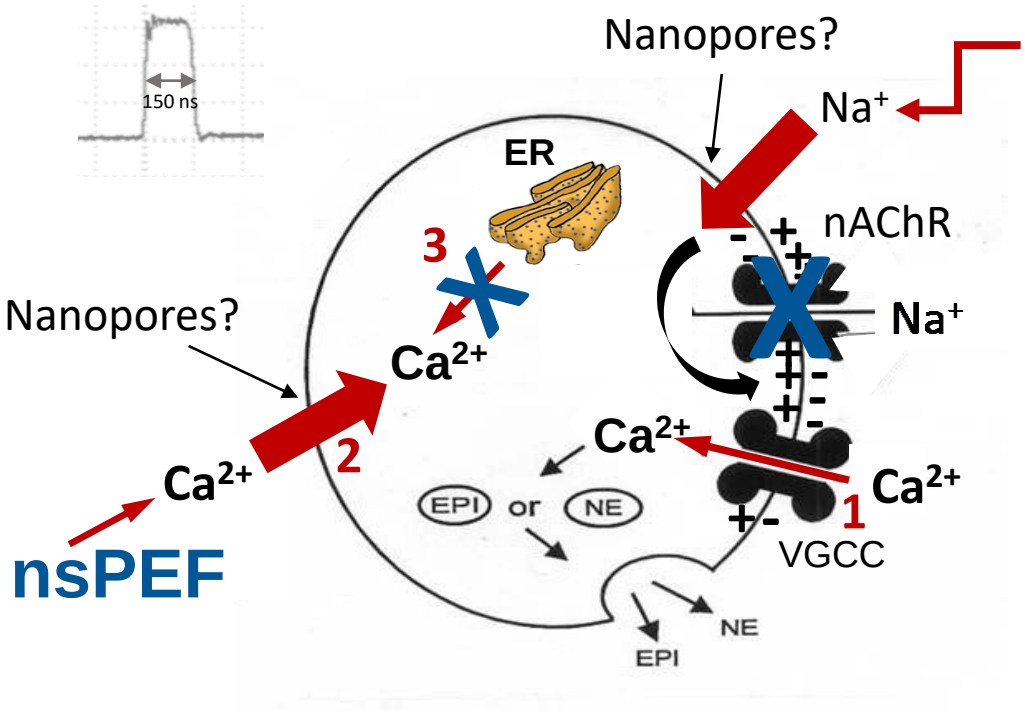
Membrane permeabilization and Ca^{2+} influx via VGCCs have the same E-field threshold (Zaklit et al., 2017): VGCC activation is all-or-none

Fluorescence monitoring of $[Ca^{2+}]_i$



Longer Duration nsPEFs (150 ns)

Cause Ca^{2+} Influx via Voltage-Gated Ca^{2+} Channels as well as nsPEF-Evoked Membrane Electropermeabilization



Na^+ influx via nanopores – causes membrane depolarization to a level sufficient to activate VGCCs

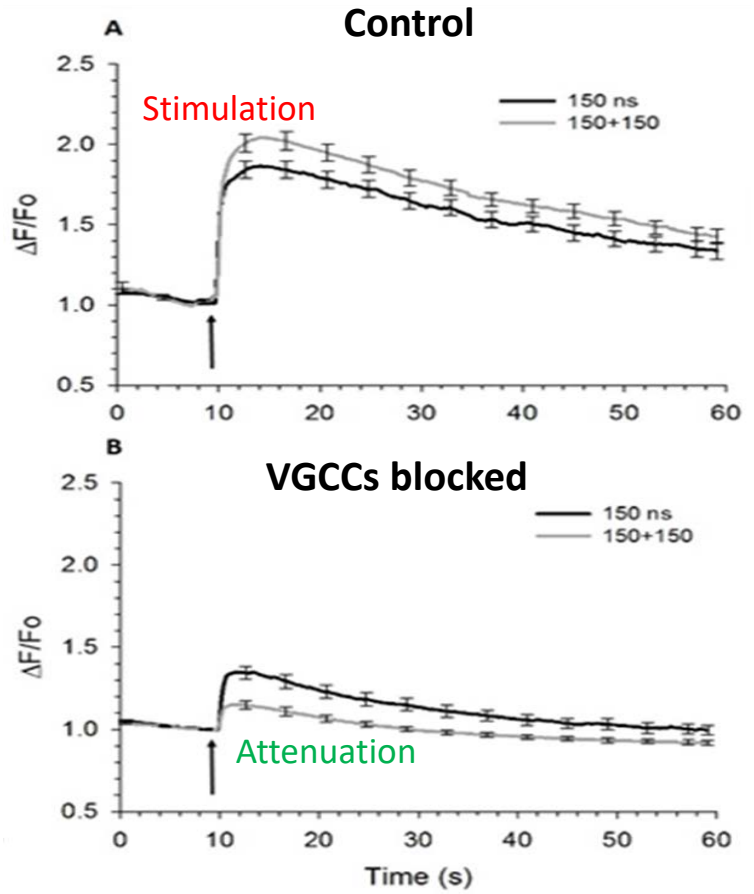
1. Ca^{2+} influx through VGCCs (60-70% of the response; all-or-none)
2. Ca^{2+} influx due to plasma membrane permeabilization (30-40% of the response; E-field sensitive)
3. No Ca^{2+} release from internal stores

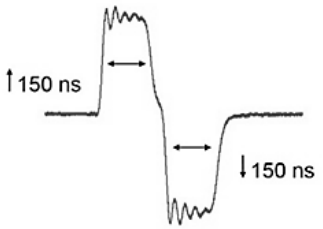
<u>Significance</u>: Depending on pulse duration (5 ns vs 150 ns), the nature of the permeabilization of the plasma membrane has changed with respect to the ionic species crossing the membrane (Bagalkot et al., 2018)

To confirm: whole-cell monitoring of membrane current underway

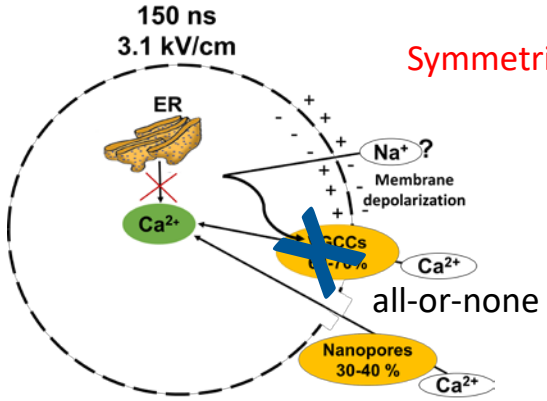
Two Ca^{2+} Entry Pathways Evoked by 150 nsPEF
How is Each Attenuated/Cancelled by a Bipolar Pulse?

1. *Symmetrical bipolar pulses* attenuate Ca^{2+} influx due to membrane permeabilization





Symmetrical bipolar pulse

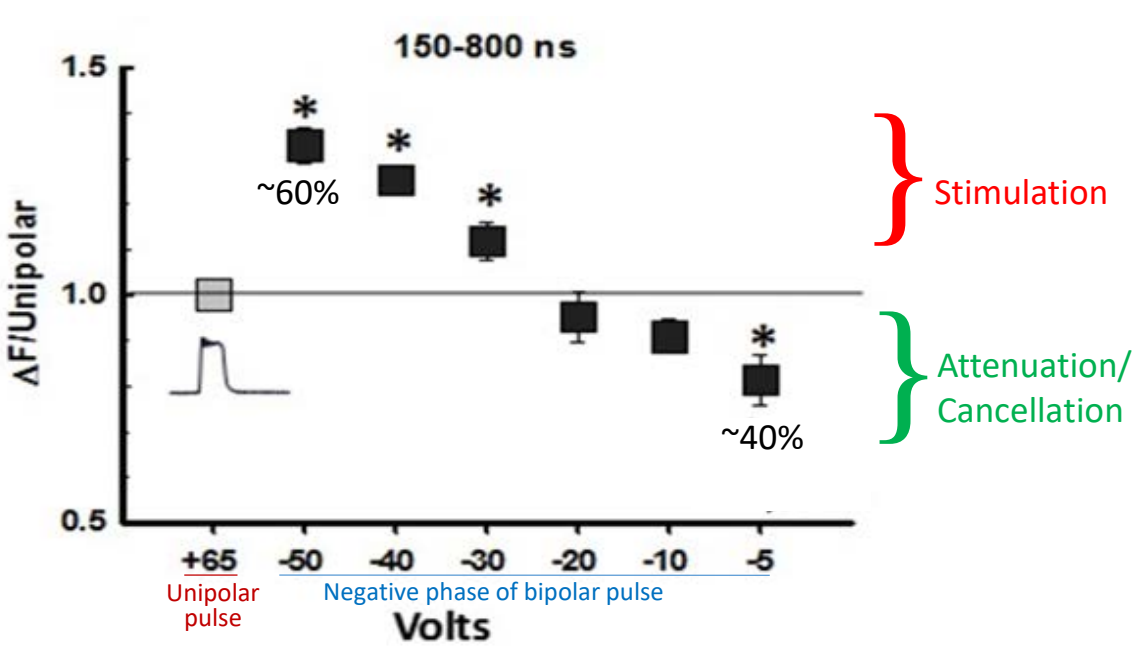


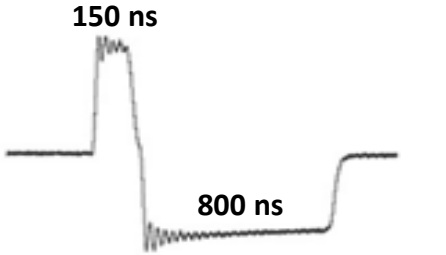
E-field sensitive component – responsible for evoking a greater response under control conditions, and showing attenuation when VGCCs are blocked

Bagalkot et. al., in preparation

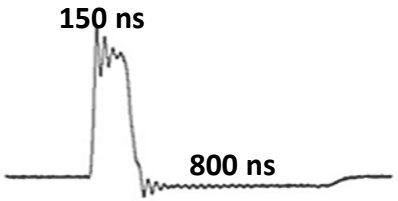
Two Ca^{2+} Entry Pathways Evoked by 150 nsPEF
How is Each Attenuated/Cancelled by a Bipolar Pulse?

2. *Asymmetrical bipolar pulses* (low amplitude, long duration negative phase) – do not have to block VGCCs to observe attenuation





Stimulating bipolar pulse



Attenuating bipolar pulse

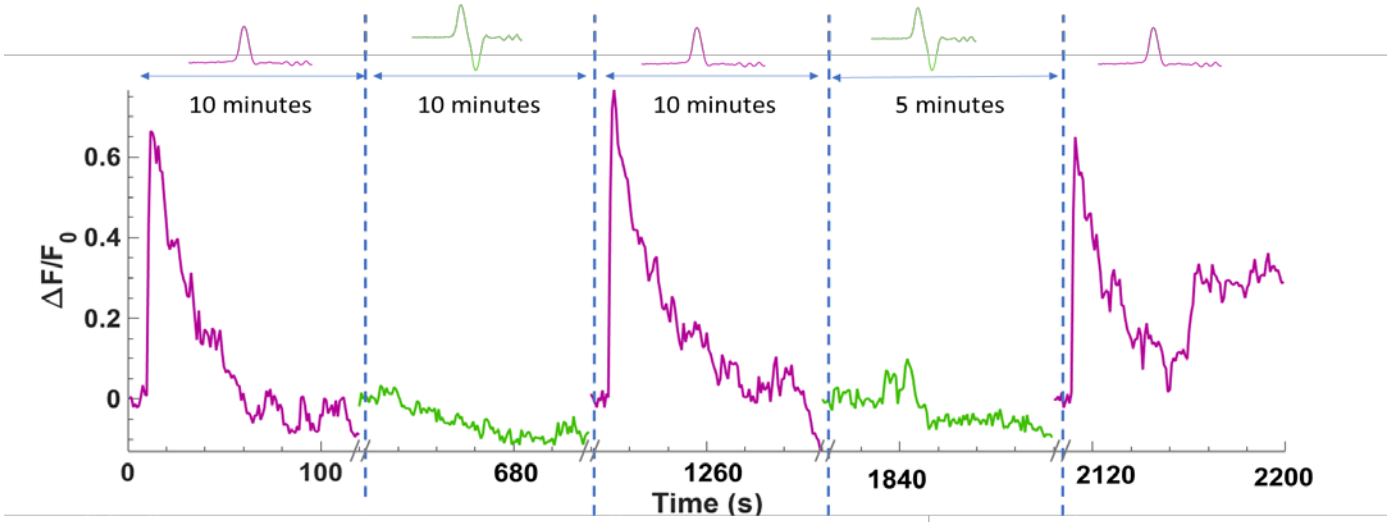
Bagalkot et. al., in preparation

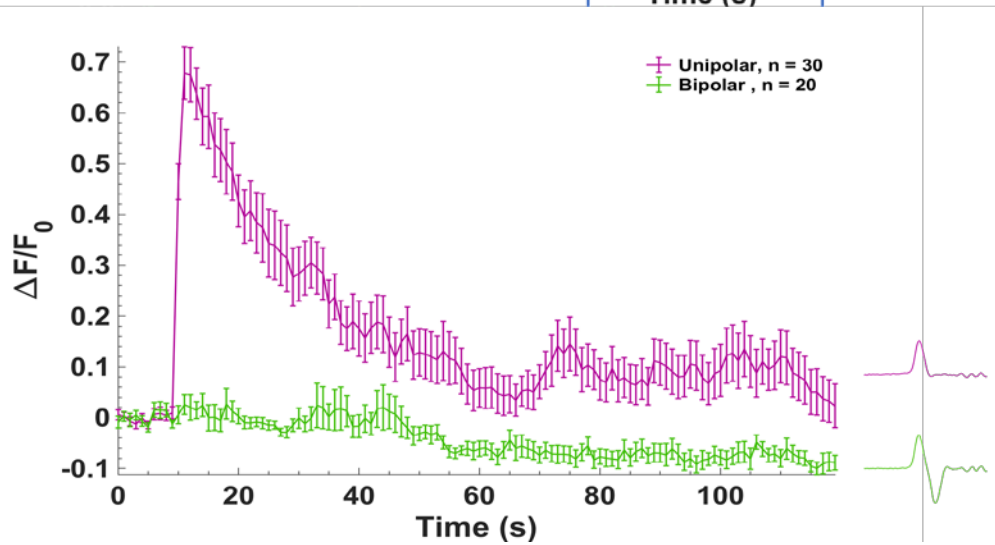
Significance: the only pathway of Ca^{2+} influx that can be attenuated is that due to membrane permeabilization – still get Ca^{2+} influx via VGCCs

Are ultrashort pulses the key?

Repetitive Cancellation of nsPEF-Evoked Ca^{2+} Responses in Chromaffin Cells Exposed to Ultrashort (2 ns) Bipolar Pulses

Esin Sozer, Ph.D. and Tom Vernier, Ph.D. – Task 2





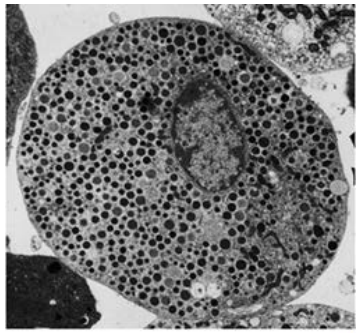
Pulse duration matters!

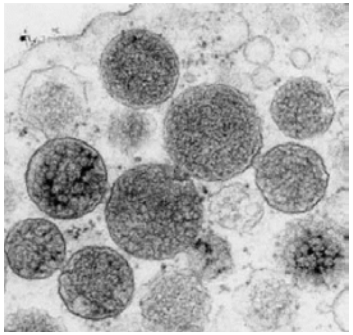
With ultrashort pulses:

- **Membrane does not become permeable to Ca^{2+}**
- **Influx of Ca^{2+} via VGCCs completely cancelled**

Efficacy of Ultrashort nsPEF for Evoking Ca^{2+}-Dependent Neurosecretion

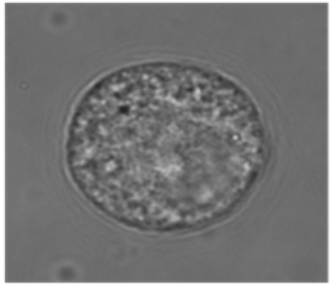
~ 20,000 secretory granules per cell that occupy 20 -30% of the cytoplasm



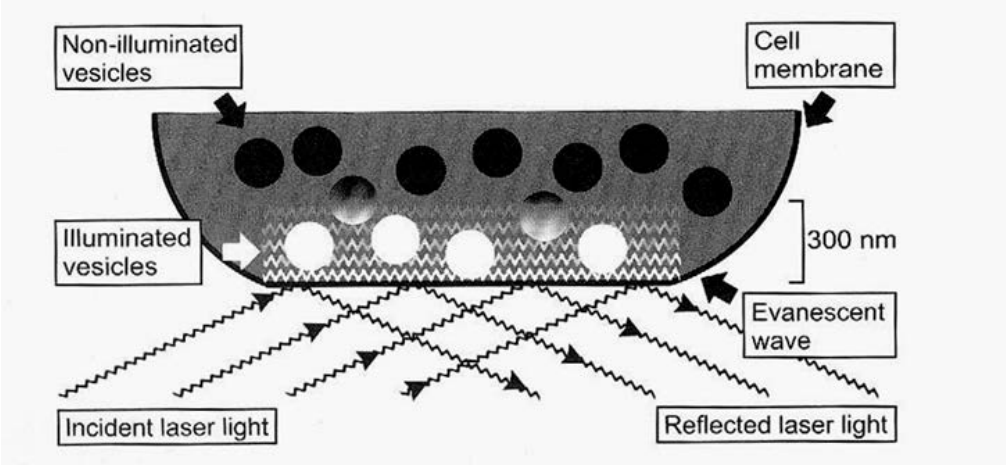


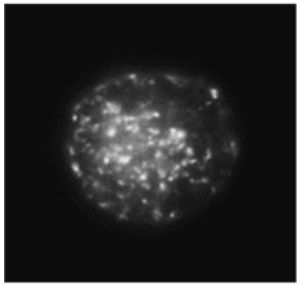
Individual granules stained with the fluorescent dye acridine organge

Monitor Exocytosis In Real-time by Total Internal Reflection Fluorescence (TIRF) Microscopy (Evanescent Wave Microscopy)



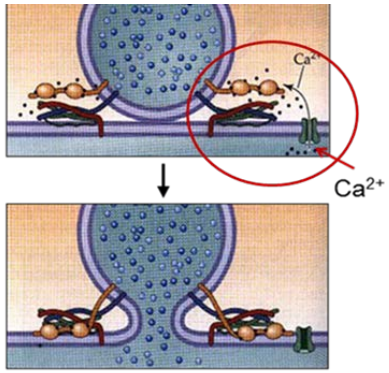
Bright Field

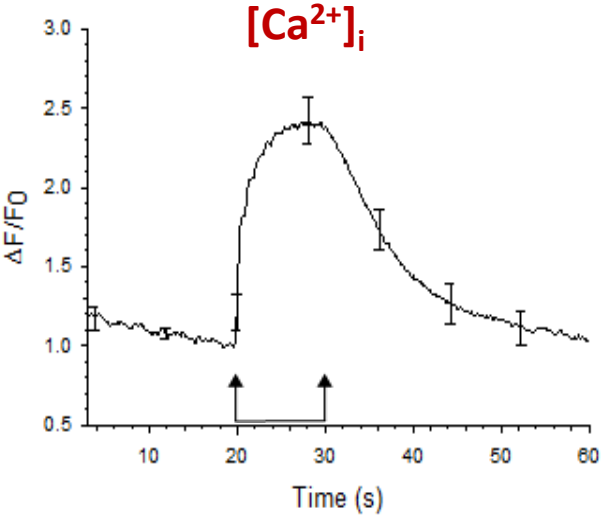


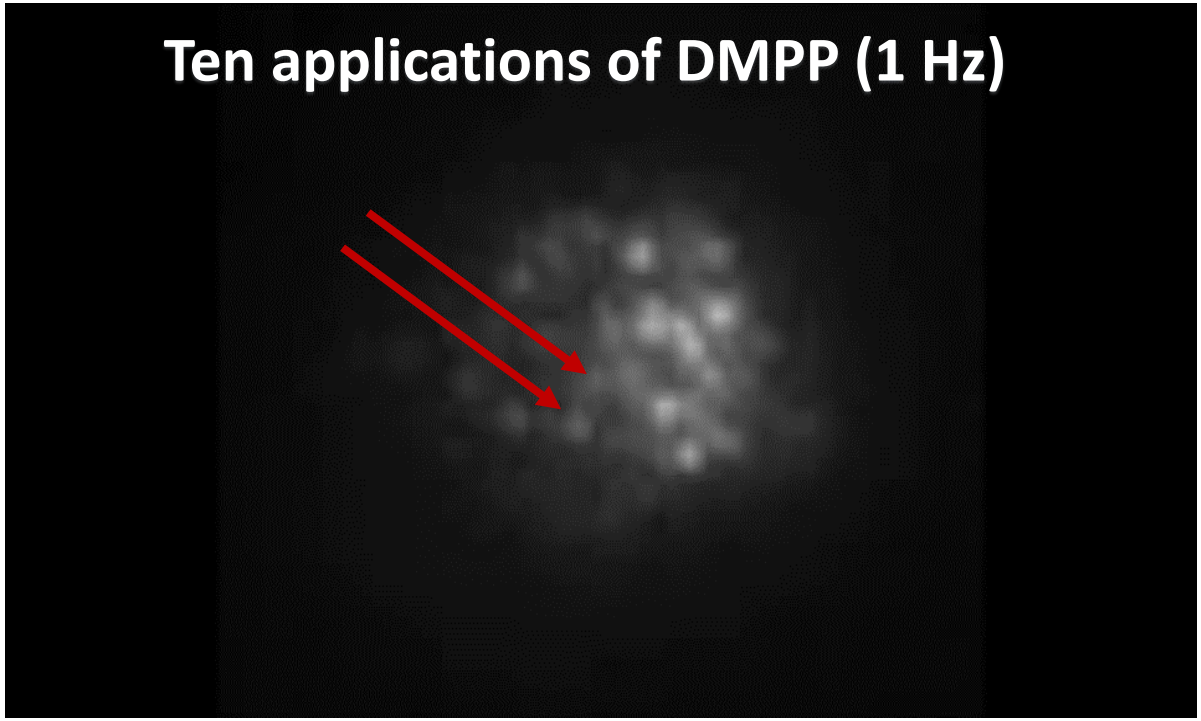


TIRF

Ca^{2+} - Dependent Exocytosis is Seen as "Bursts" of Fluorescence When Acridine Orange is Released from Secretory Granules

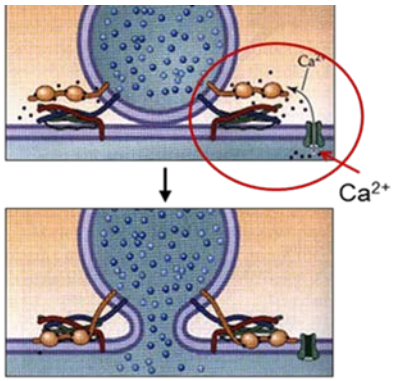


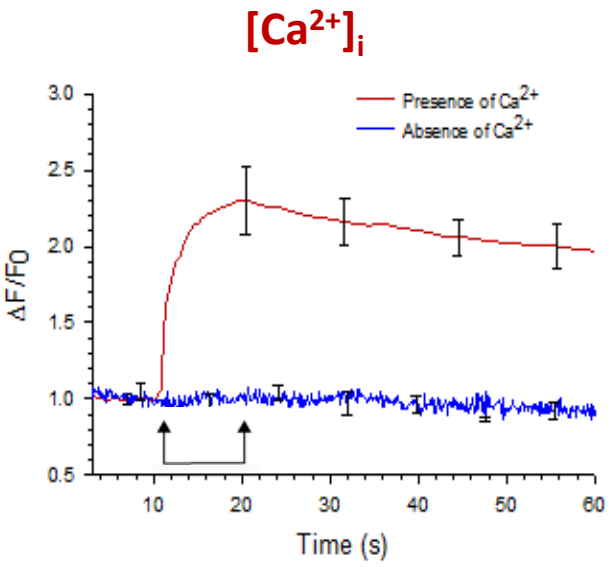


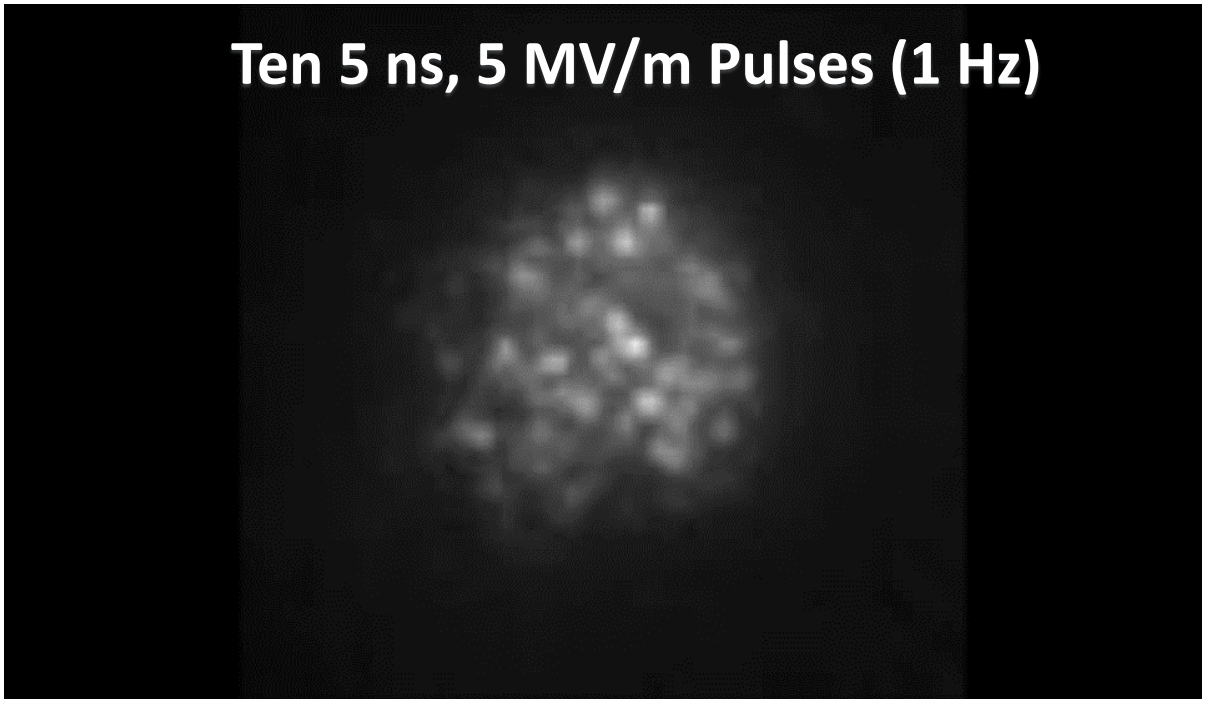


Exocytotic events are few in number and do not persist with time (correlate with the length of time $[Ca^{2+}]_i$ is elevated)

Ca^{2+} - Dependent Exocytosis is Seen as "Bursts" of Fluorescence When Acridine Orange is Released from Secretory Granules

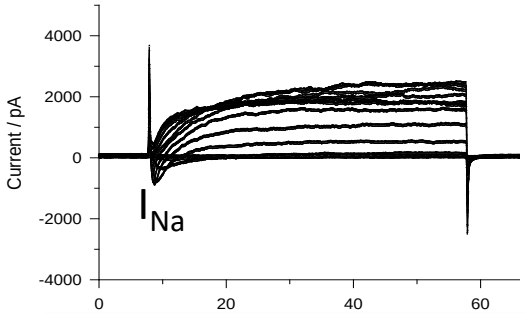


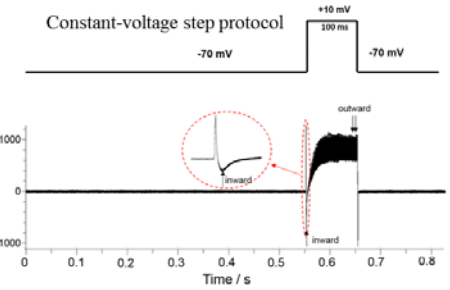


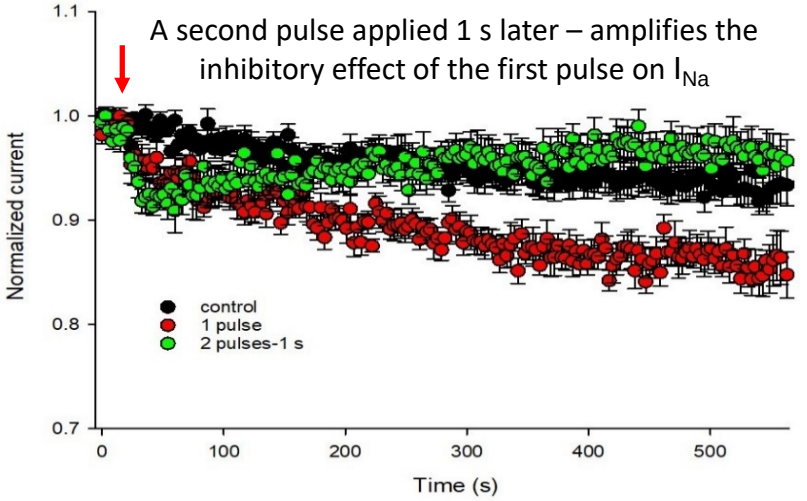


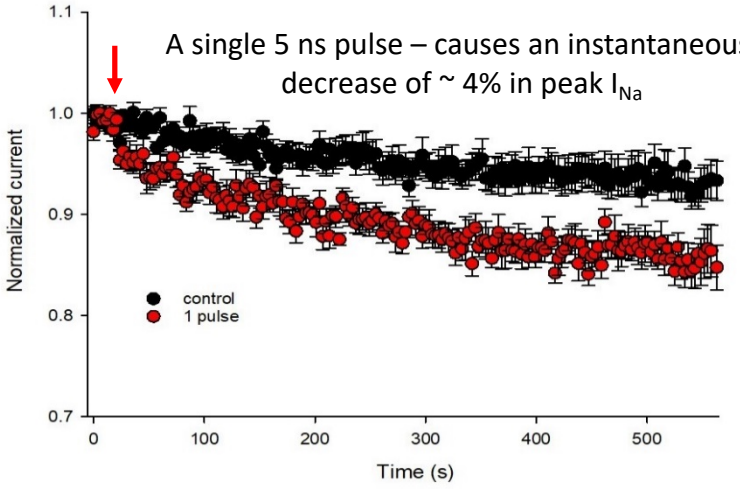
Exocytotic events are many in number and persist with time (correlate with the sustained increase in $[Ca^{2+}]_i$)

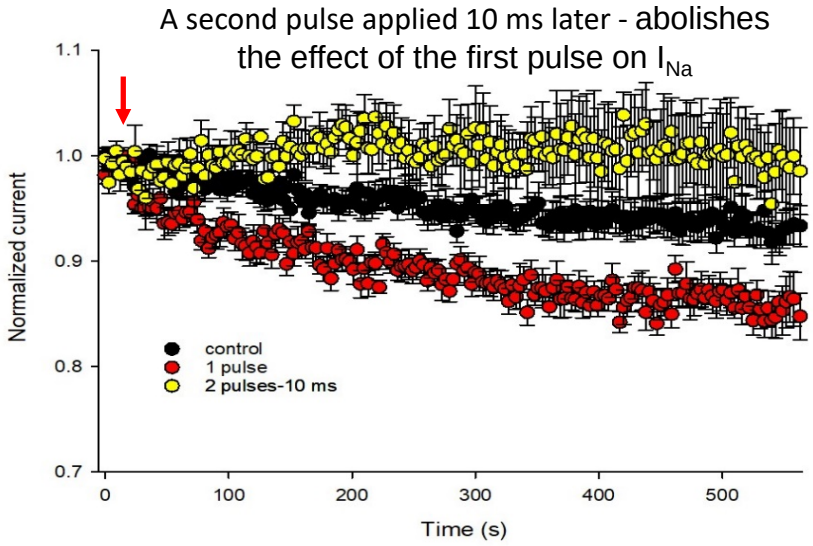
Cell Excitability Also Altered by nsPEF Effects on Voltage-Gated Na^+ Channels (I_{Na}) (Tasks 1, 2, 3 and 5)





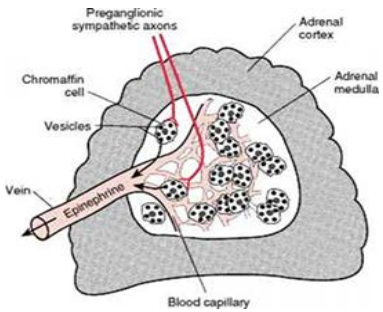




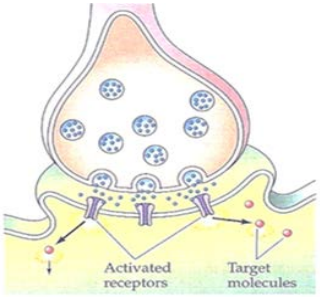


Future Directions

Work toward the potential application of CAN-CAN ES for neuromodulation – use of intact adrenal tissue from transgenic mice in which chromaffin cells express functional GCaMP3 in *Wnt1-GCaMP3* mice that responds to Ca^{2+} influx

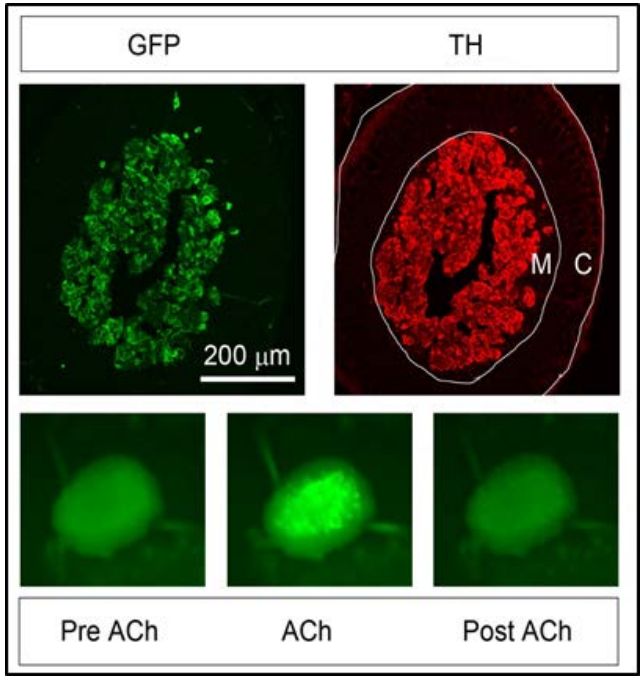


Synaptic transmission



Green fluorescent protein

Tyrosine hydroxylase



Immuno-stained adrenal slice

(M) Medullary chromaffin cells derived from the neural crest

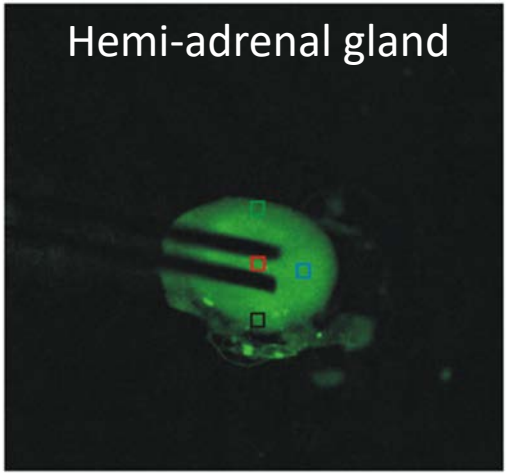
(C) Mesoderm derived adrenal cortex

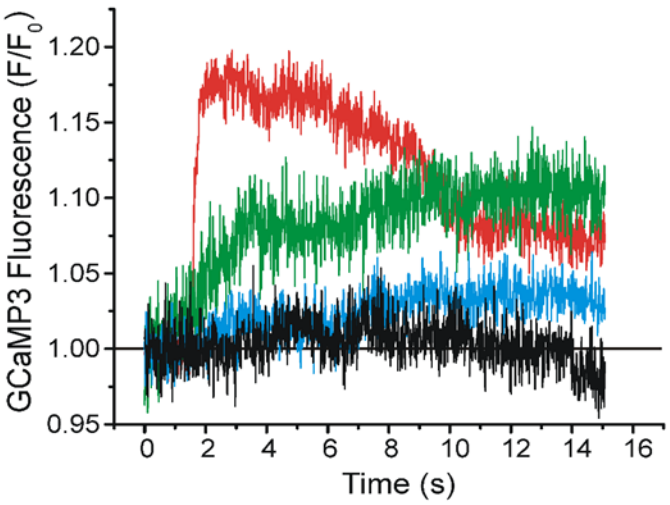
Hemi-adrenal gland before, during and after bath application of an acetylcholine receptor agonist – note the pronounced increase in fluorescence only in the medulla

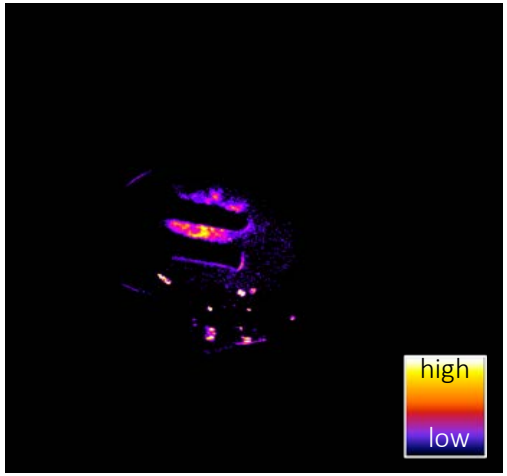
Transgenic mice generated by Dr. Tom Gould, Department of Physiology and Cell Biology, University of Nevada, Reno School of Medicine

CAN-CAN ES For REMOTE STIMULATION To Modulate the "Fight or Flight" Reflex *in vivo*

Response of adrenal medullary chromaffin cells to a single 400 ns pulse – note the response in the area of the medulla mainly between the electrodes







In collaboration with the AFRL (Bennett Ibey, Ph.D. and Caleb Roth, Ph.D.)
Goal: work toward the potential of carrying out whole body exposures targeting the adrenal gland ("adrenaline burst") – enhanced warfighter performance

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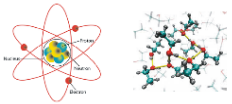
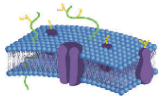
Toward the Application of CAN-CAN Technology

Atomic and Molecular (Tasks 2,3)



Membranes Lipids and Ion Channel Proteins (Tasks 2,3,4)



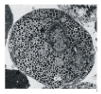
Biological Cells

Mammalian Cell Lines (e.g. HEK-293)

Expression of Ion Channels (Ca_V1, Na_V1, ANO6) (Tasks 1,5,6)

Excitable Cells (Tasks 1,6)

Chromaffin Cells (Task 6)



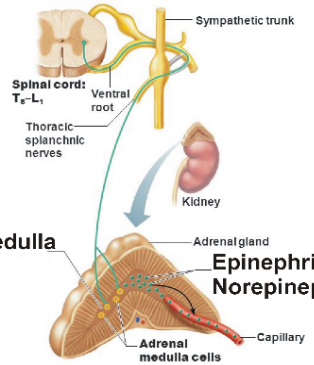
Neurosecretion

Neurons

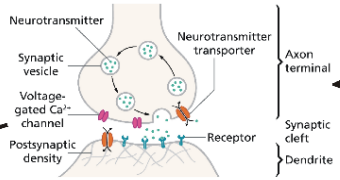
Excitability (Tasks 1,6 in progress)

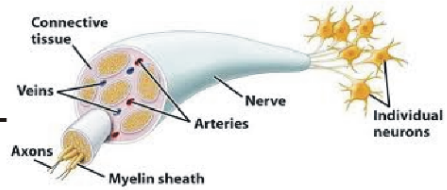
Nerve Excitability (Task 1)

Tissues and Organs



Synaptic Transmission (Task 6, in progress)





Thank you for your attention!

Questions?