

Nanoelectropulse-induced electromechanical signaling and control of biological systems

Task 5:

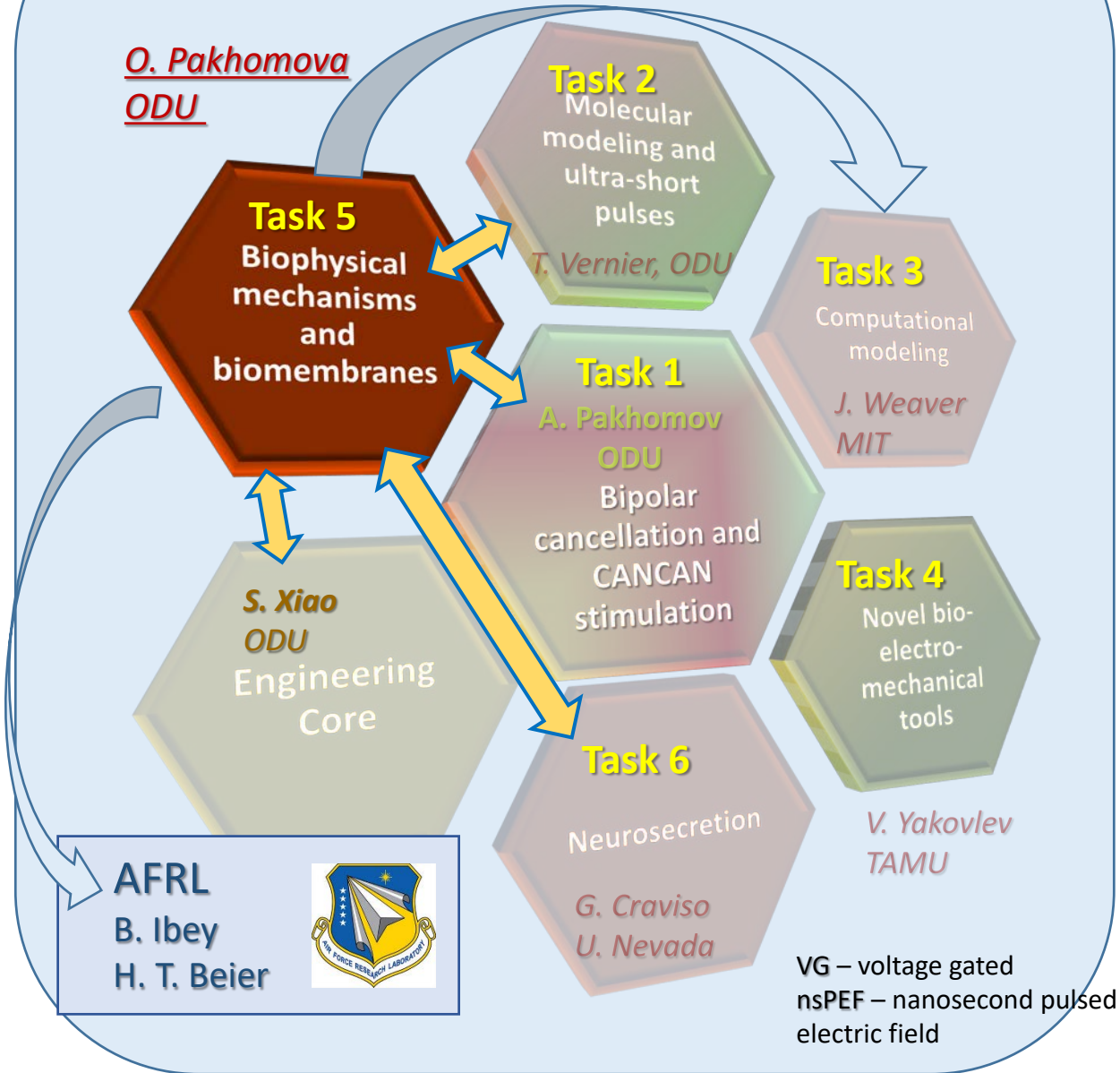
Primary effects of nsPEF in excitable membranes and the primary mechanism(s) of the cancellation phenomenon:

Uncovering mechanisms of activation of voltage gated Ca^{2+} channels



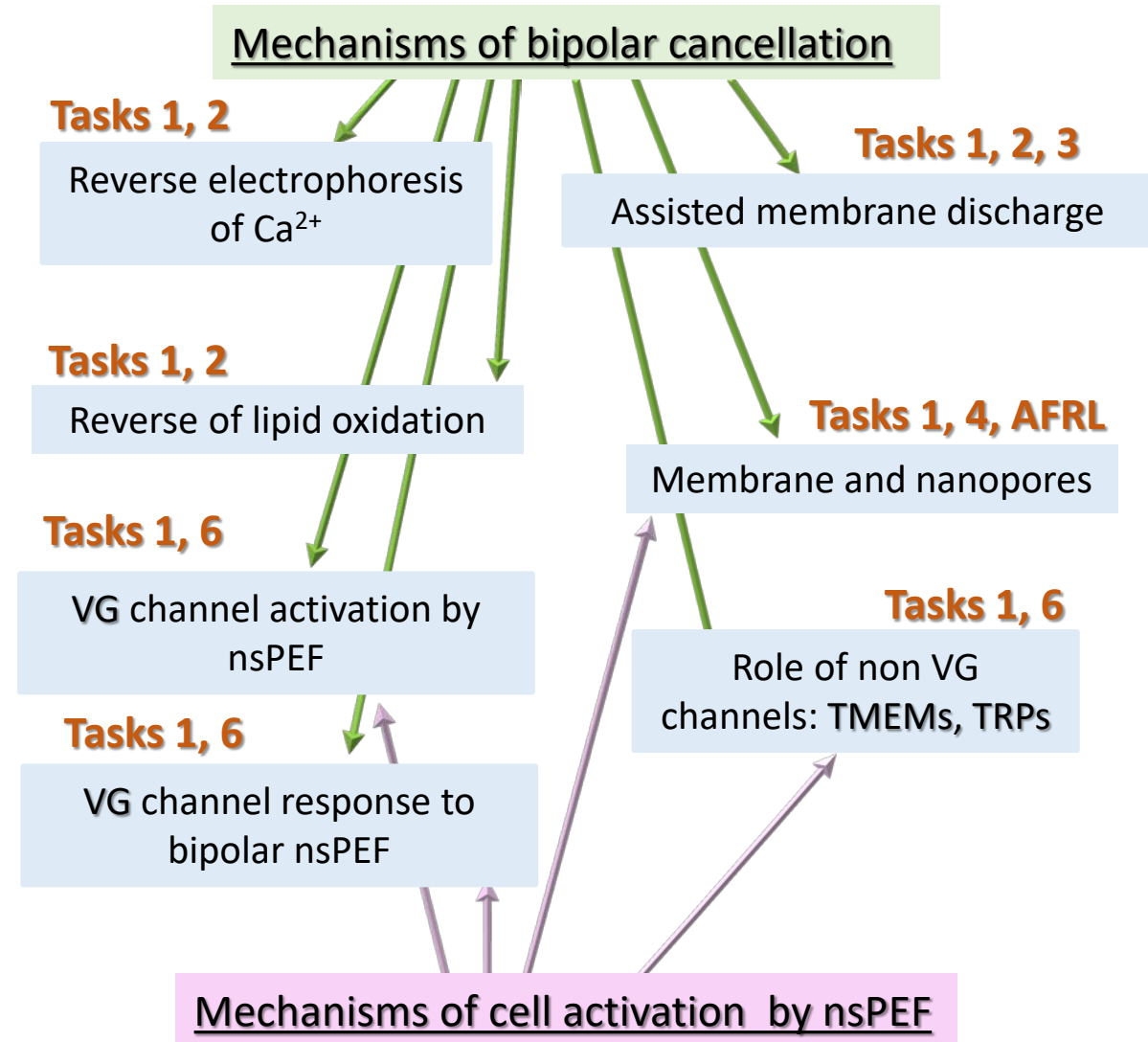
AFOSR MURI review, Arlington, VA, USA
April 18, 2018

MURI Team Structure

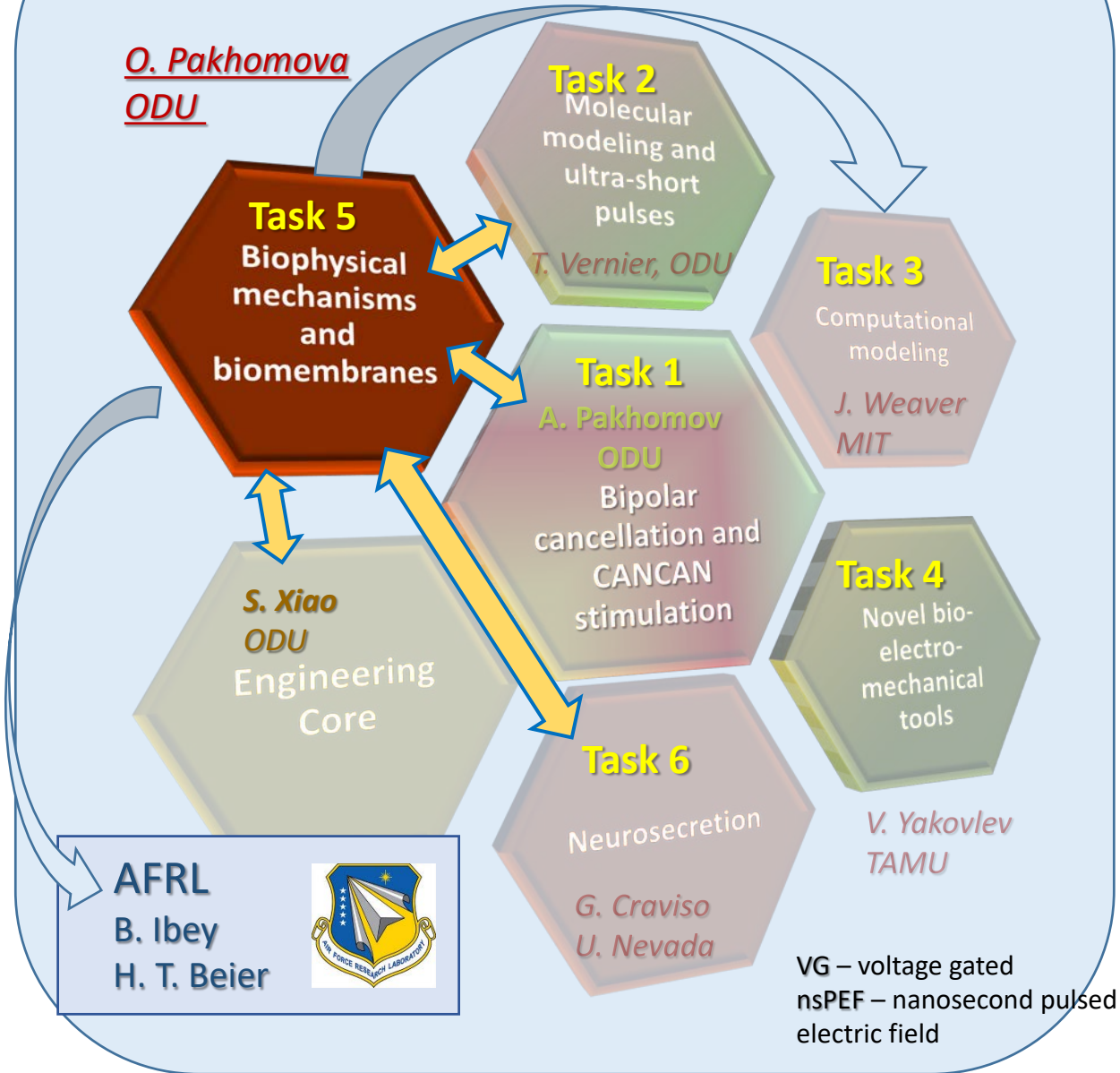


Task 5.

Biophysical mechanisms and biomembranes

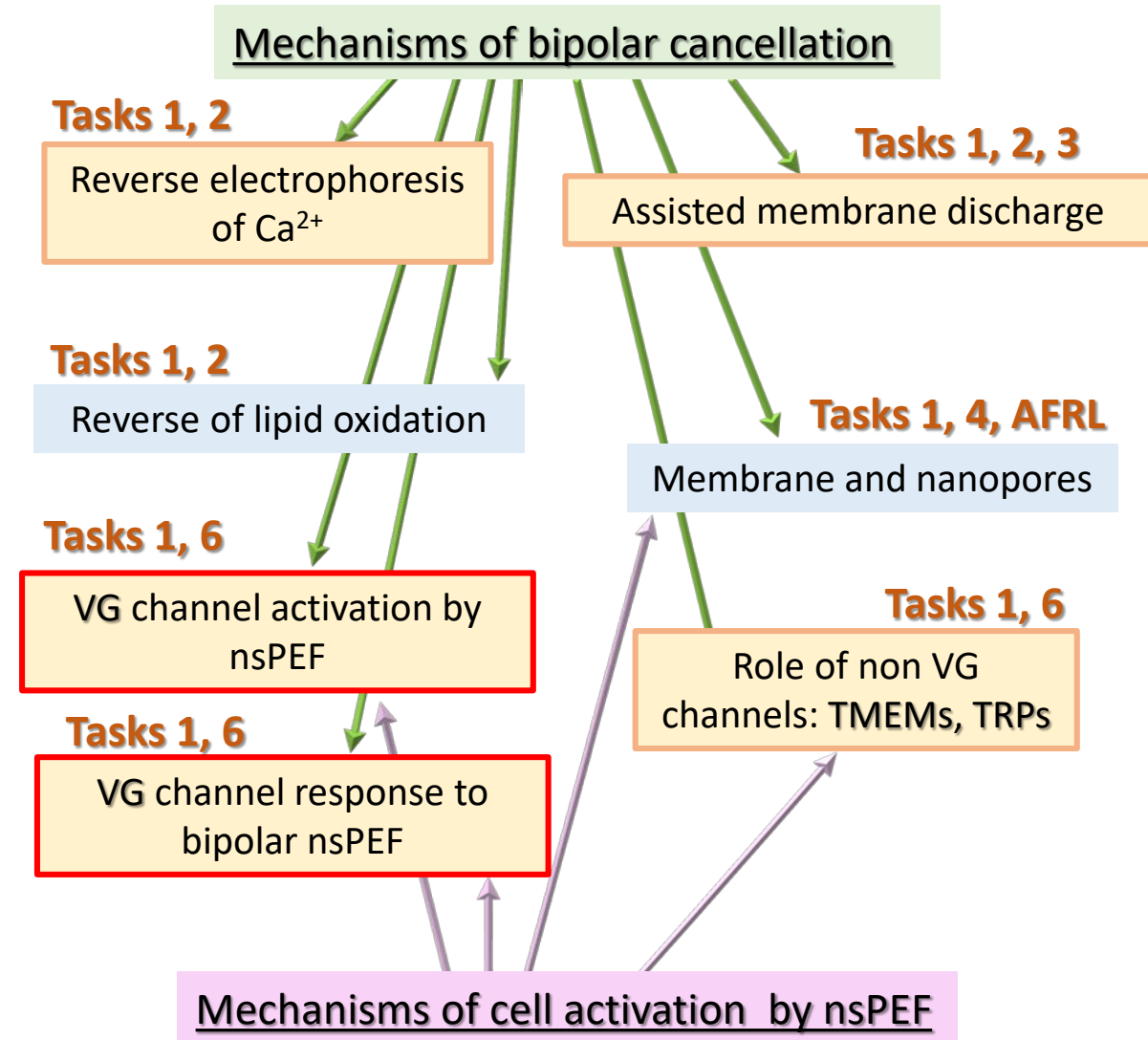


MURI Team Structure



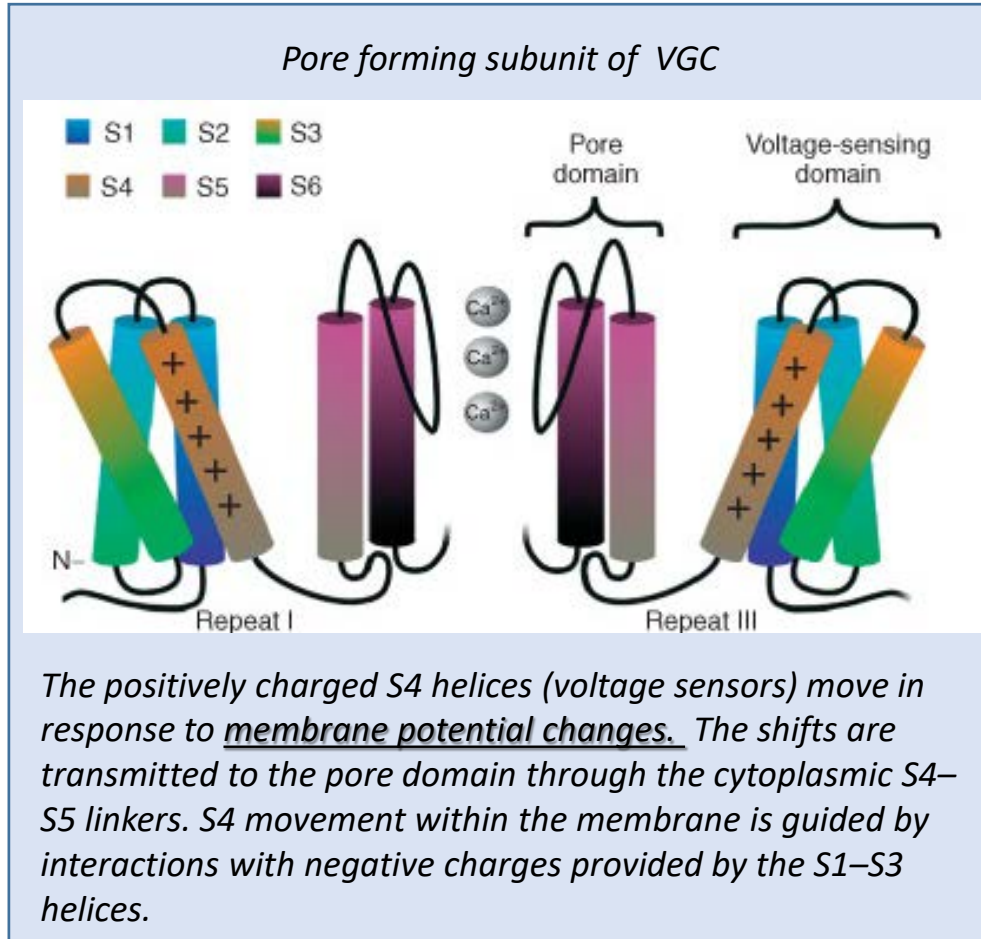
Task 5.

Biophysical mechanisms and biomembranes



1. Role of Voltage Gated Channels (VGC) in excitable cells activation by nsPEF

Collaboration with G. Craviso, N. LeBlanc (Univ Nevada), A. Pakhomov (ODU)



The figure is adapted from WIREs Membr. Transp. Signal. 2014

VG – voltage gated

VGC – voltage gated channels

VGCC - voltage gated calcium channels

nsPEF – nanosecond pulsed electric field

EP – electric pulses

- Depolarization of the transmembrane potential is needed to trigger VG channels opening by shifting the voltage sensor
- Time for sensor shift normally takes as long as 10-100 μ s
- Nanosecond pulsed electric fields (nsPEF) are too short for sensor shift
- Membrane discharging time after nsPEF application still may not long enough for VG opening
- Membrane electroporabilization (ME) may serve as an alternative mechanism for VG channel activation by nsPEF. ME occurs in a few nanoseconds after nsPEF application and is followed by a long-lasting depolarization, which is sufficient for the voltage sensor response and channel opening
- A critical question explored in this work was whether VG channels can be activated by nsPEF without electroporative damage to the cell membrane

Will explore

VGC activation threshold and mechanism

VGC activity reversal by bipolar nsPEF

VGC inactivation by nsPEF

CaV1.3, L-type voltage gated calcium channel (VGCC)

VGCCs

Responsible for regulation of membrane excitability and intracellular Ca^{2+} levels.

Open in response to membrane depolarizations and allow Ca^{2+} ions to enter cells along its 10,000-fold chemical gradient.

VGCC 1.3:

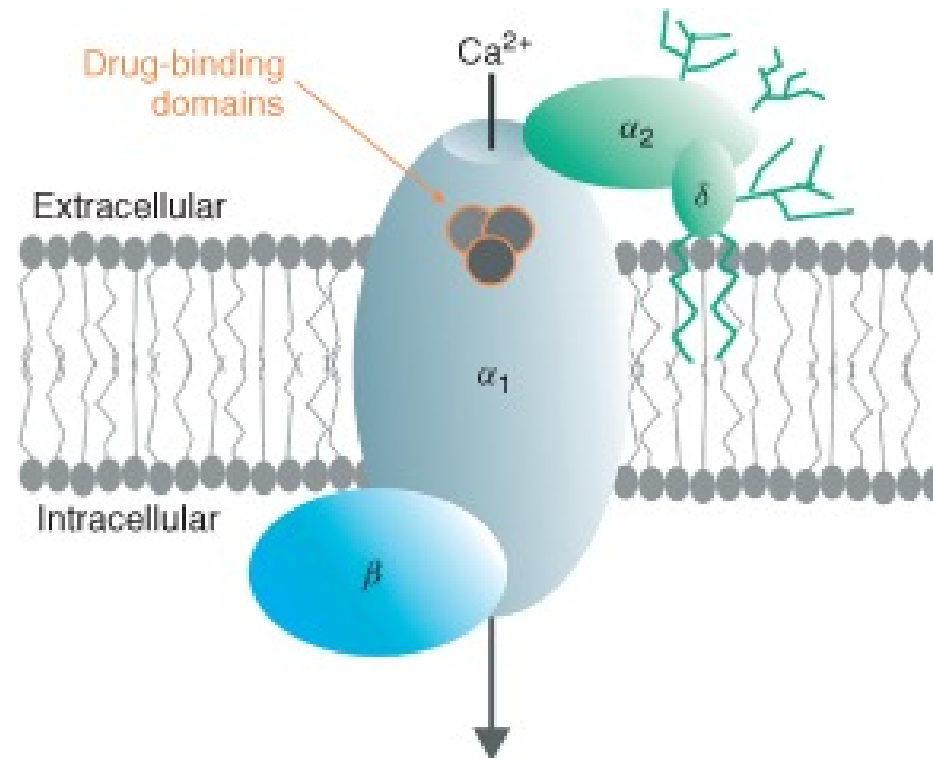
Expressed in heart, neurons, endocrine cells, and sensory cells

Play a role in neuronal excitability, synaptic plasticity, learning and memory, pacemaker activity

Unique in activation profile: can start activating at voltages as negative as -50 mV

Present in Chromaffin cells (Task 6)

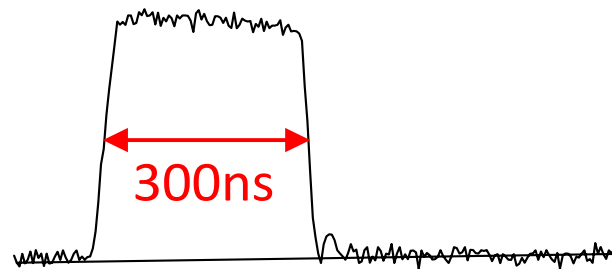
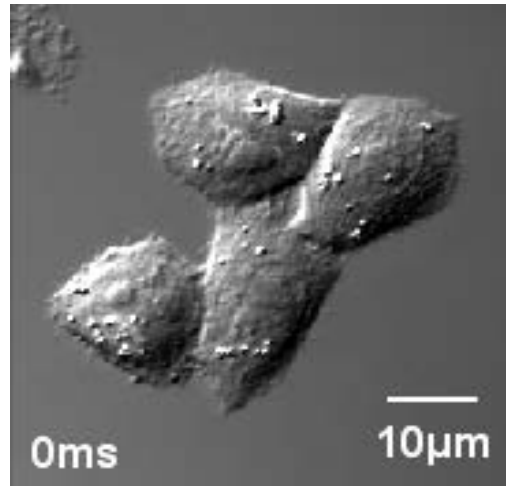
Predicted membrane topology for VGCC



CaV1.3, L-type voltage gated calcium channel (VGCC)

Approach

CaV1.3 assembled in HEK293 cells, which are free of VGC.
Compared Ca^{2+} influx into cells with VGCC (+) or without VGCC (-)



Endpoints

Confocal imaging to detect intracellular Ca^{2+} concentration using **Fluo4-AM** dye,
P-clamp recording for membrane conductance and VGCC currents

Exposure system geometry

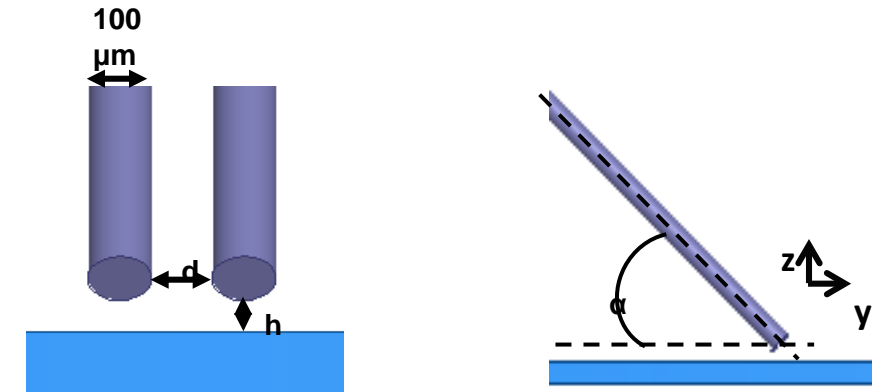
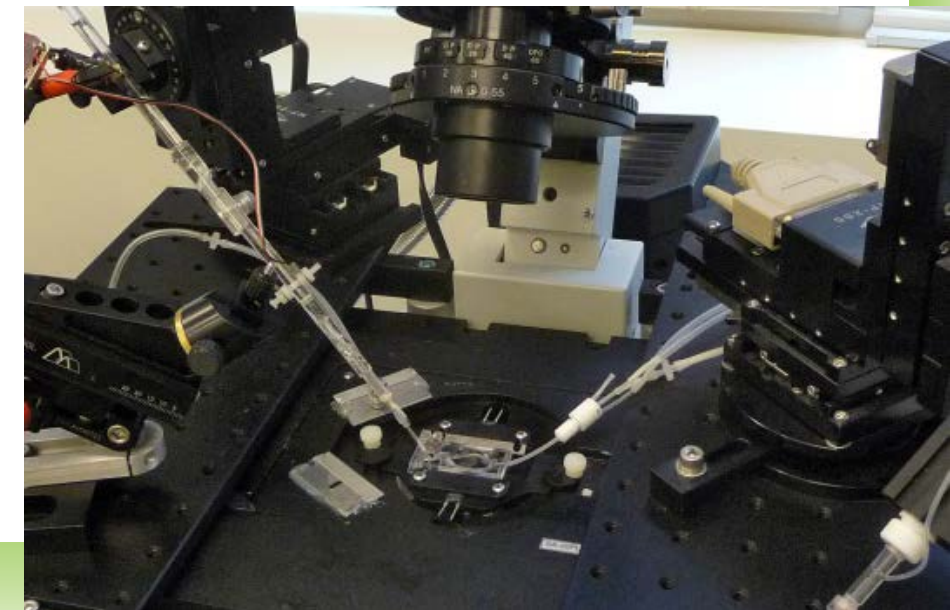


Figure courtesy of M. Casciola (Task 1)

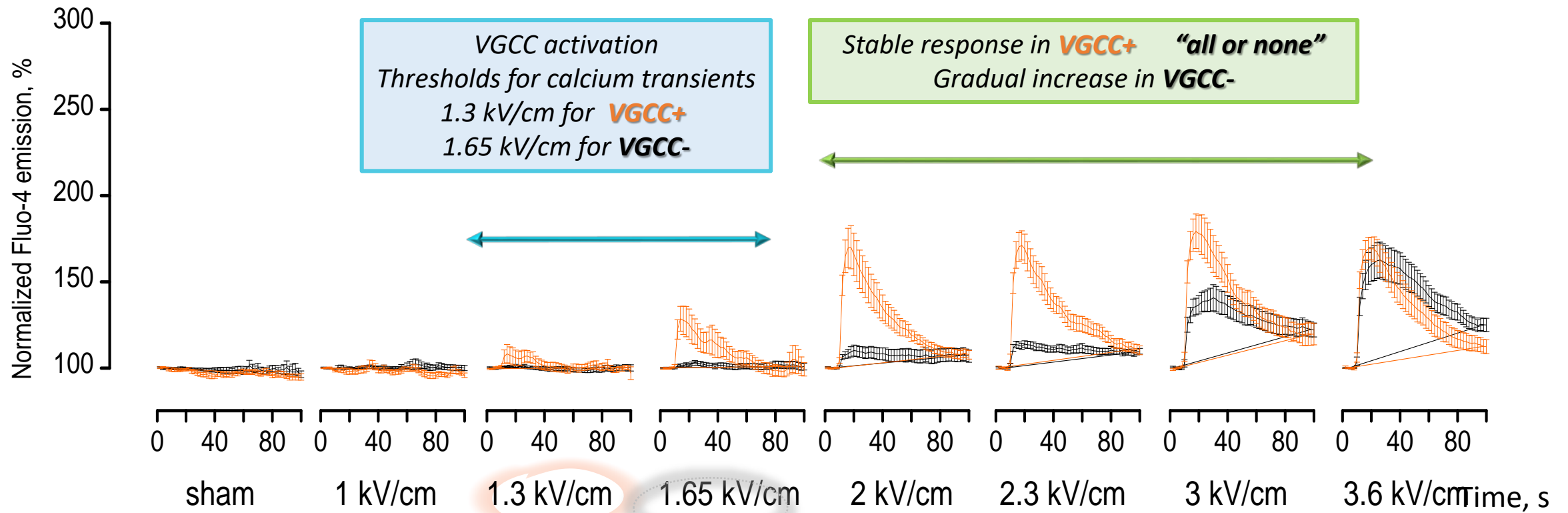


VGCC activation by unipolar nsPEF

VGCC+ cells with VGCCs

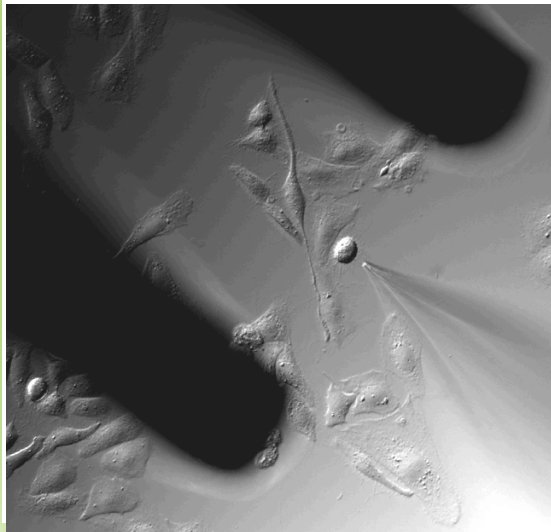
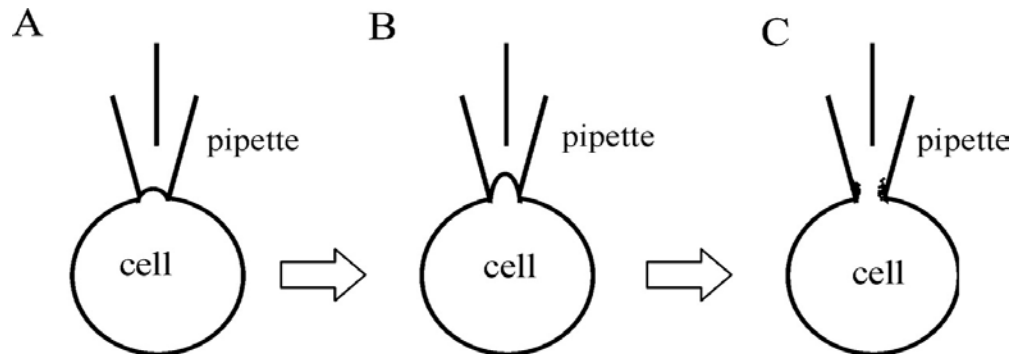
VGCC- cells without VGCCs

Ca^{2+} influx into HEK293 cells after sequential exposure to unipolar 300 ns pulses

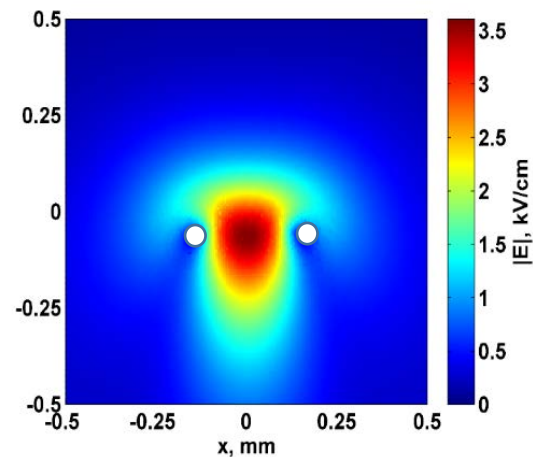


Membrane conductance in HEK293 cells after exposure to single unipolar 300 ns pulse

Whole cell patch clamp



E-field distribution, 2 mm above the coverslip

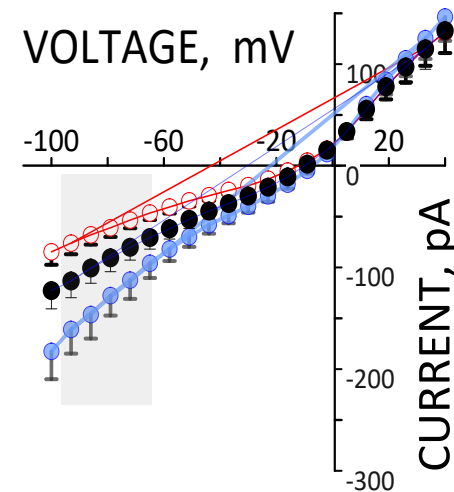


Map calculation by M. Casciola (Task 1)

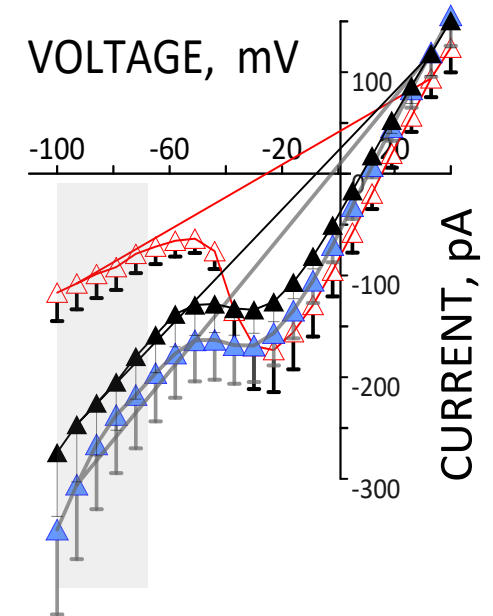
IV curves are recorded before and after exposure to nsEP at 1.8 kV/cm

Figure courtesy of K. Hristov (Task 1)

VGCC-



VGCC+



-10s (before exposure)

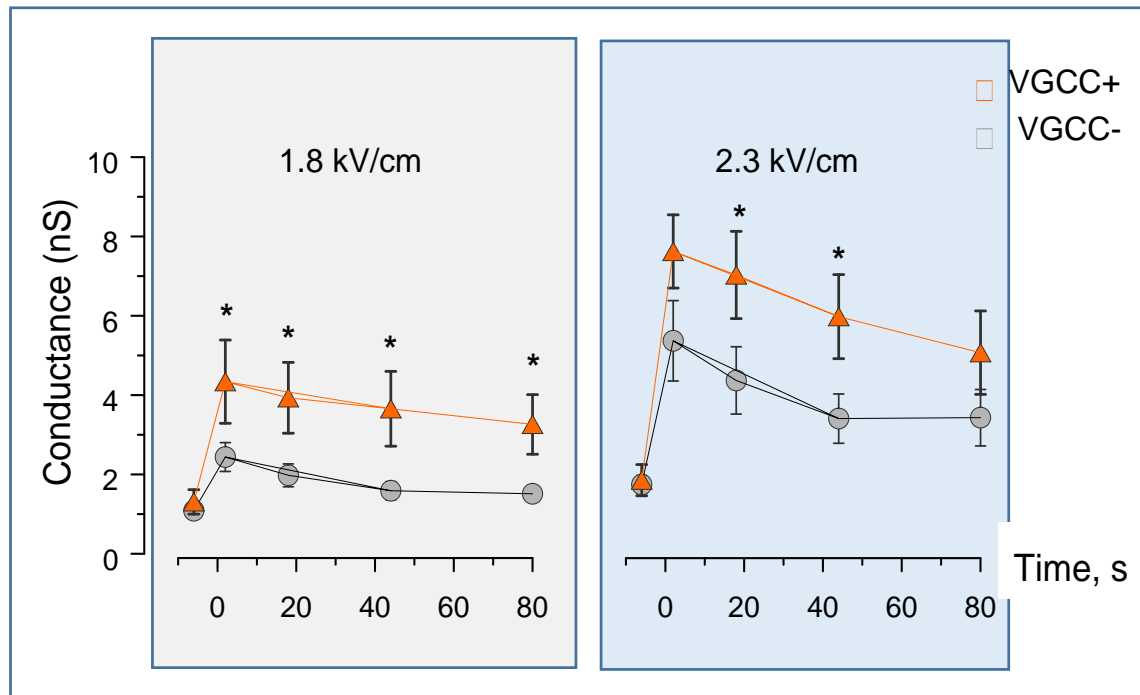
2s after exposure

80s after exposure

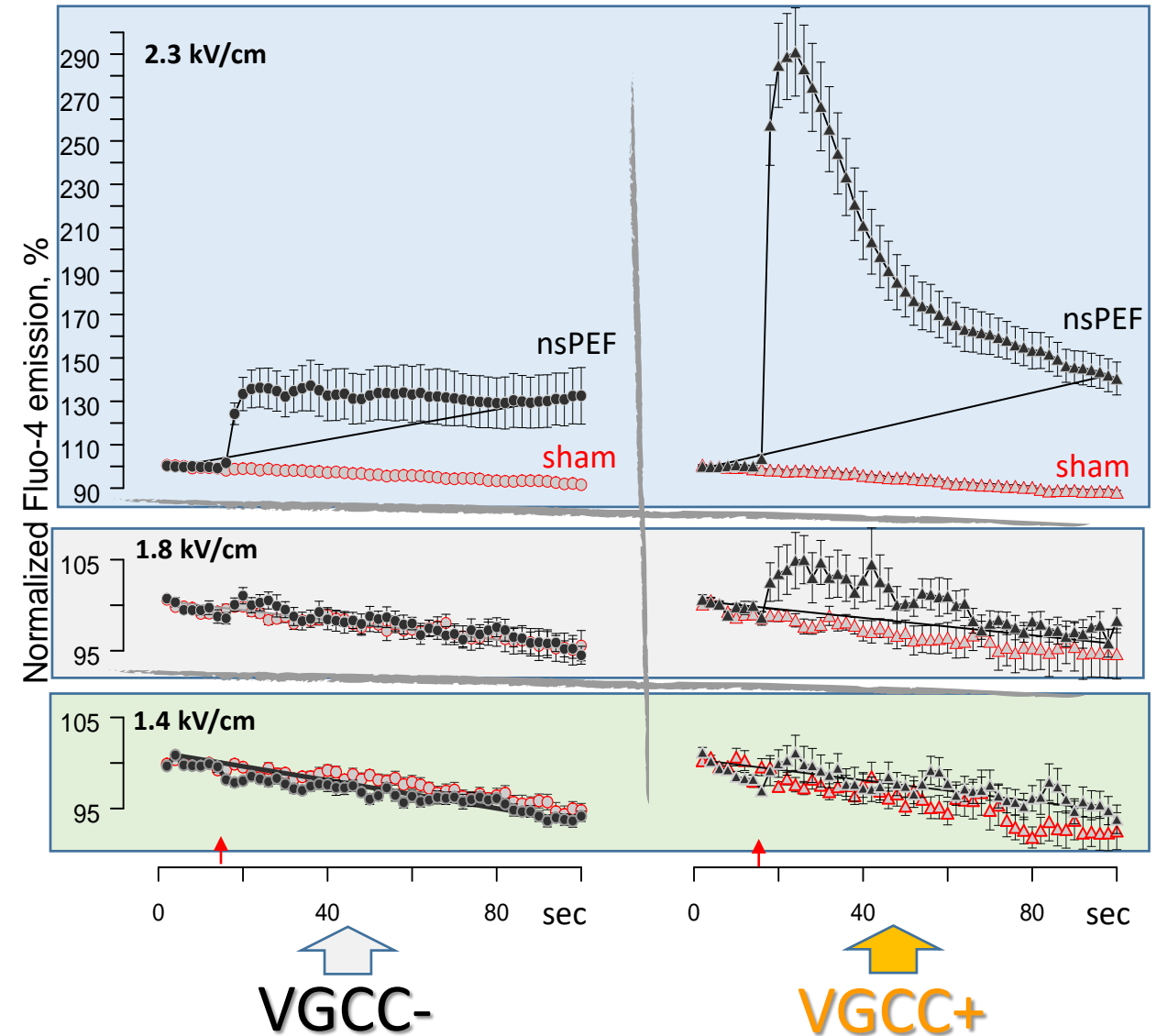
Membrane conductance and calcium transients after exposure to unipolar 300 ns

Membrane conductance measured before and after exposure to EP at 1.4, 1.8 and 2.3 kV/cm

Exposure to EP at 1.4 kV/cm did not affect membrane conductance.



Ca^{2+} transients in VGCC- and VGCC+ cells after exposure to nsPEF at 1.4, 1.8, and 2.3 kV/cm



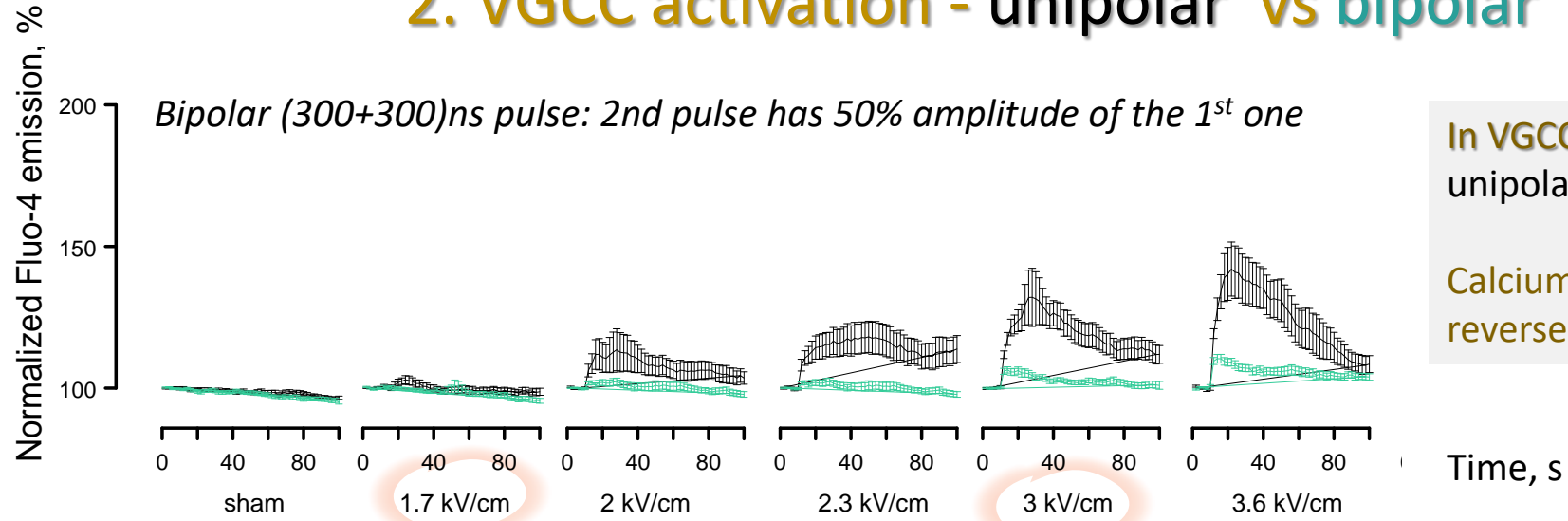
Findings:

- *Thresholds for calcium transients are very close in VGCC+ and VGCC- cells and are in a range of 1.3-1.7 kV/cm*
- *Membrane conductance is altered by nsPEF stimulation with intensity 1.8 kV/cm and 2.3 kV/cm in both type of cells*
- *The conductance raise is larger in VGCC+ cells than in VGCC- cells*
- *Membrane conductance is not recovered in 80s after exposure*
- *Rise of Ca^{2+} transients correlates with membrane conductance increase.*
- *Both the membrane conductance and calcium transients were not altered by nsPEF of 1.4 kV/cm*

Conclusions:

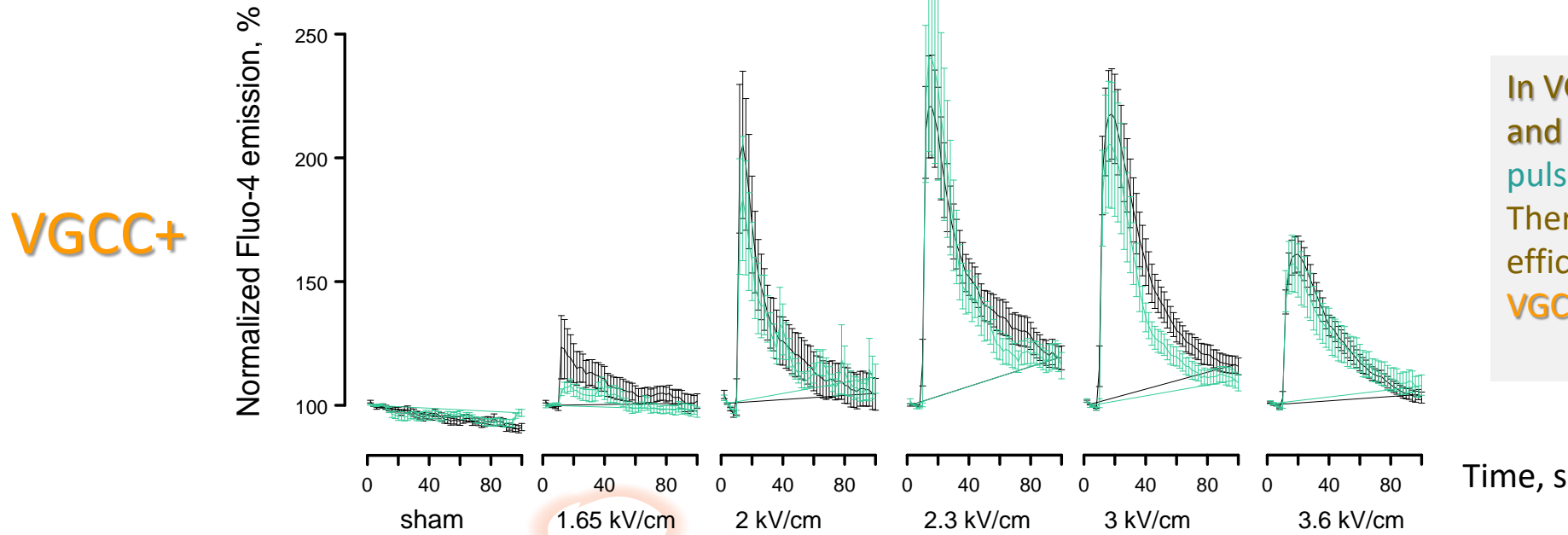
- *VGCCs can be activated by short 300 ns pulses.*
- *Electroporative membrane depolarization is likely mechanism for VGCC opening*
- *On the range of 2-3.6 kV/cm Ca^{2+} transients gradually increasing with dose in VGCC- cells and are stable in VGCC+ cells, thus pointing on “all or none” mechanism of response for VGCC.*

2. VGCC activation - unipolar vs bipolar



In VGCC- cells thresholds are 1.7 kV/cm unipolar and 3 kV/cm for bipolar pulses

Calcium transients are attenuated by reverse polarity pulse in VGCC- cells .

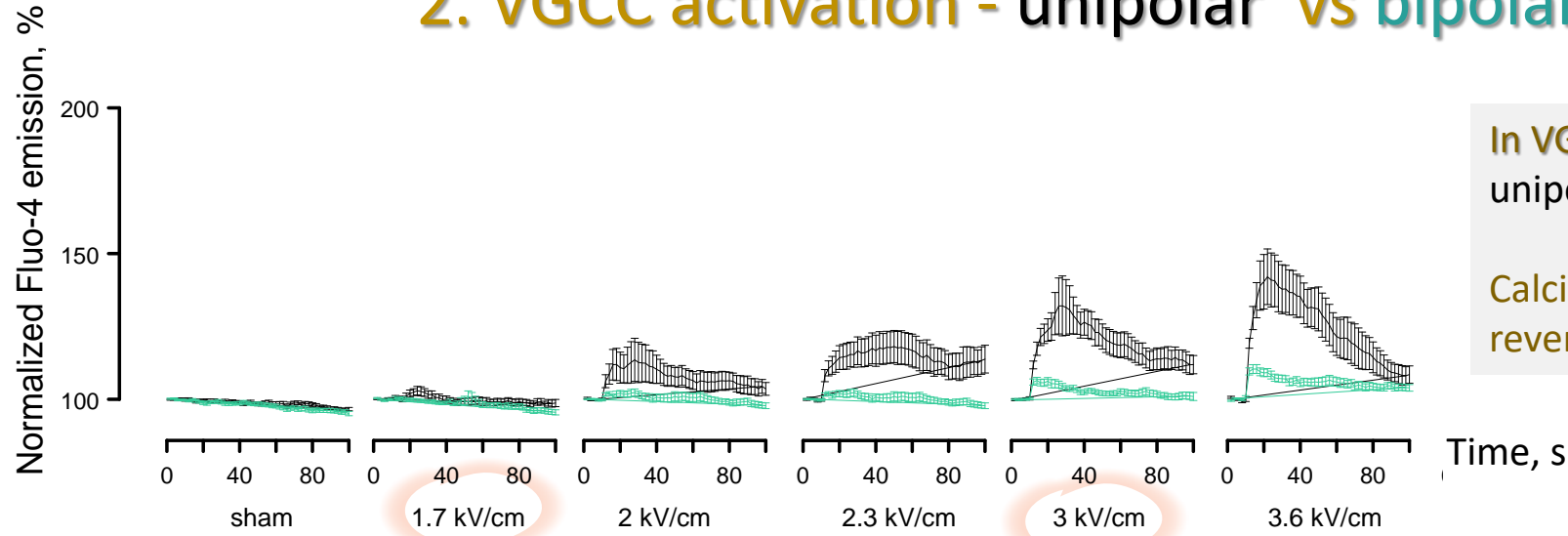


In VGCC+ cells threshold is 1.65 kV/cm and similar for unipolar and bipolar pulses.

There is little or no difference in efficiency of uni- and bipolar pulses in VGCC+ cells on the range 2-3.6 kV/cm “all-or-none” response

2. VGCC activation - unipolar vs bipolar

VGCC-

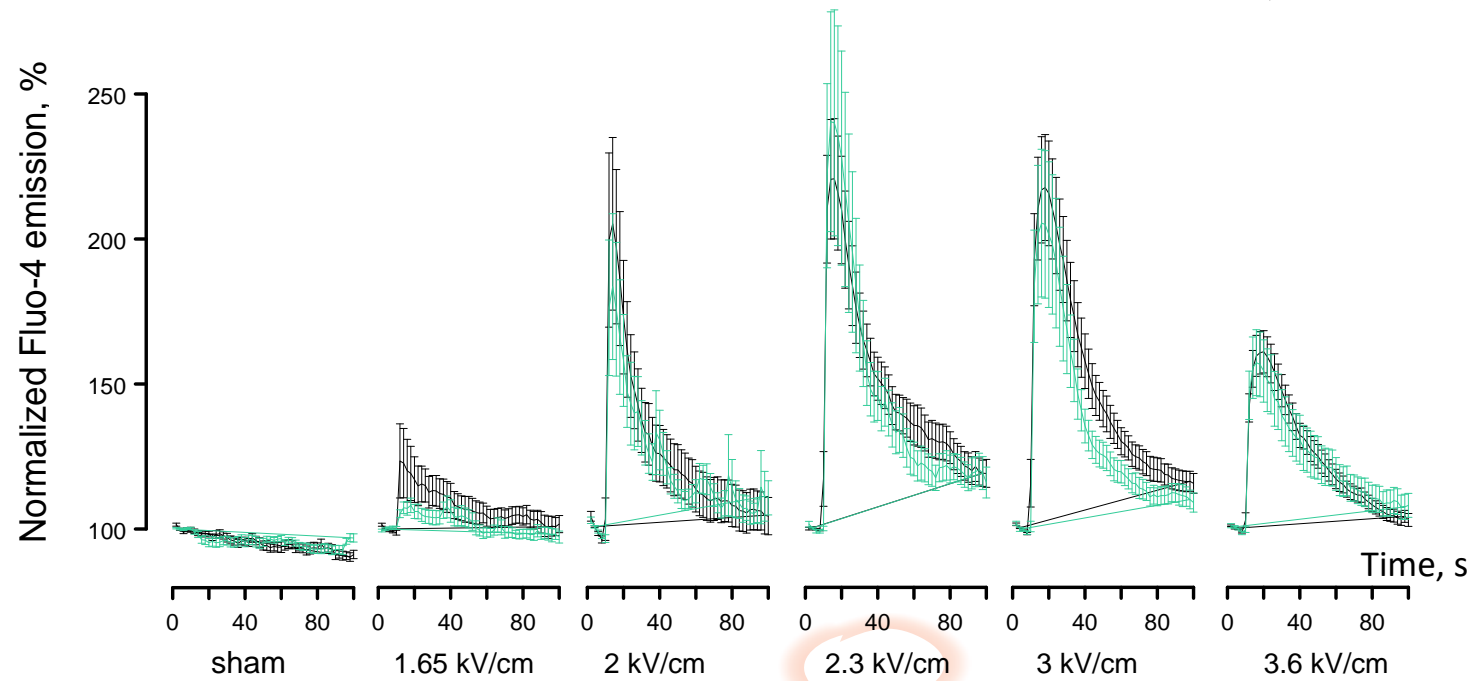


In VGCC- cells thresholds are 1.7 kV/cm unipolar and 3 kV/cm for bipolar pulses

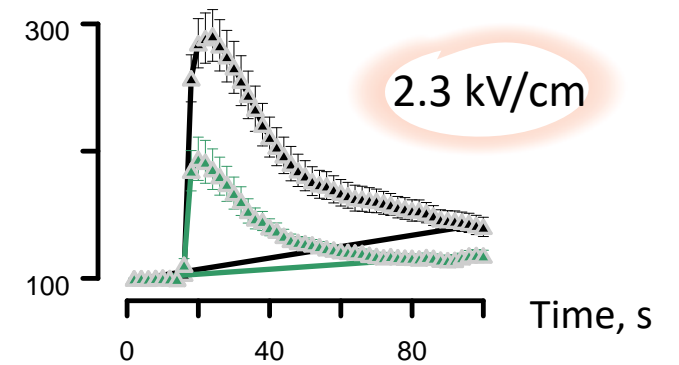
Calcium transients are attenuated by reverse polarity pulse in VGCC- cells .

☐ Unipolar
☐ Bipolar

VGCC+



Attenuation by bipolar pulses can be obtained after exposure to single nsPEF.



2. VGCC activation - unipolar vs bipolar

Findings and take-home messages

- *Ca²⁺ transients thresholds evoked by unipolar and bipolar pulses are the similar in VGCC+ cells (1.65 kV/cm). They are different in VGCC- cells: 1.7 kV/cm for unipolar and 3 kV/cm for bipolar exposures.*
- *VGCC activation can be attenuated by reverse polarity pulse. However, the strength of the attenuation has limitations (yet to be explored). For example attenuation is less efficient if multiple pulses are applied.*

VGCC future directions

- *Continue with bipolar pulses action on VGCC*
- *VGCC inhibitors will be used to prove that Ca²⁺ entry is due to VGCC activity*
- *TMP assessment and comparison with VGCC activity (new dyes or technique are needed)*
- *Short ~10 ns pulses action on VGCC (can be qualitatively different)*
- *Comparison VG Na⁺ channel in HEK293 model and in excitable cells, such as cardiomyocytes or neurons*

3. TMEM16F is a facilitator of immediate cell response to nsPEF

Collaboration with G. Craviso, N. LeBlanc (Univ Nevada), A. Pakhomov (ODU),

TMEM16F

chloride channel, activated by Ca^{2+}
cation channel (nonspecific)
Phosphatidylserine (PS) scramblase

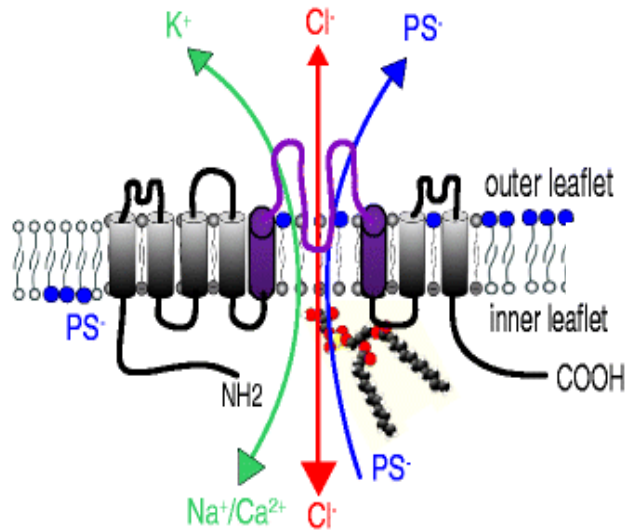
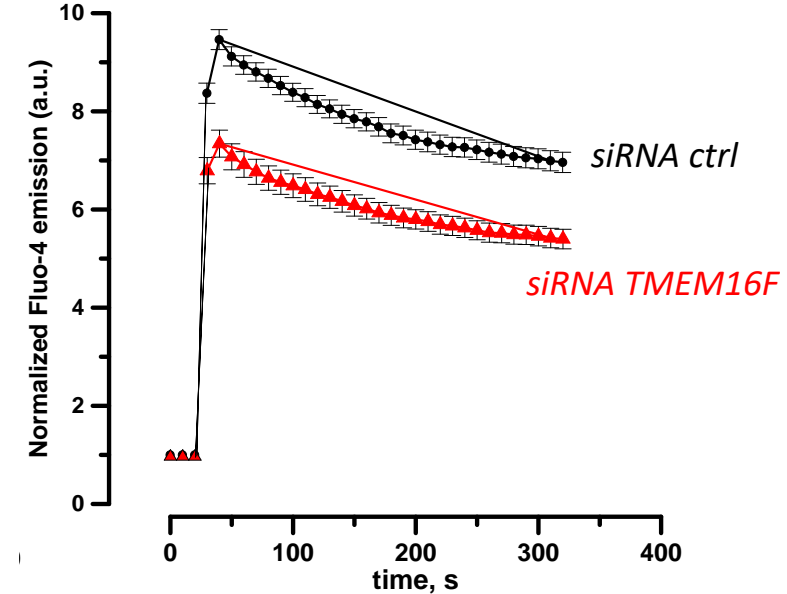
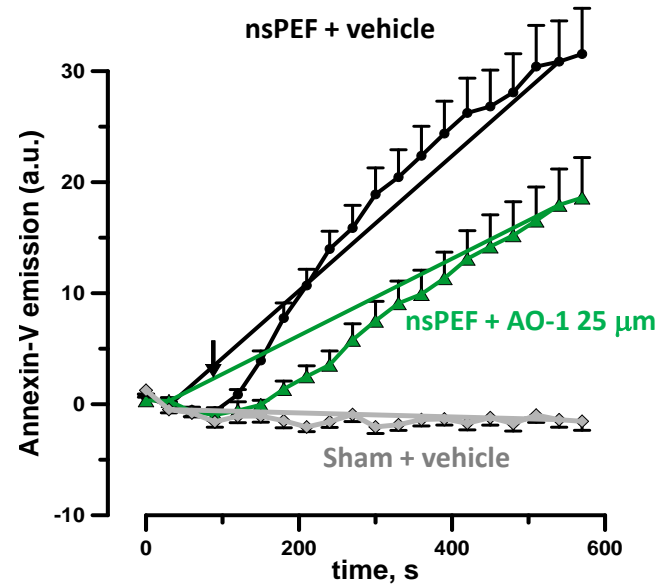


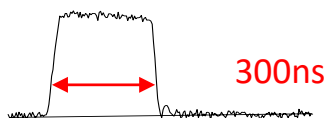
Figure is adapted from Kunzelmann et al., 2014

Experimental data: figures courtesy of C. Muratori (Task 5)

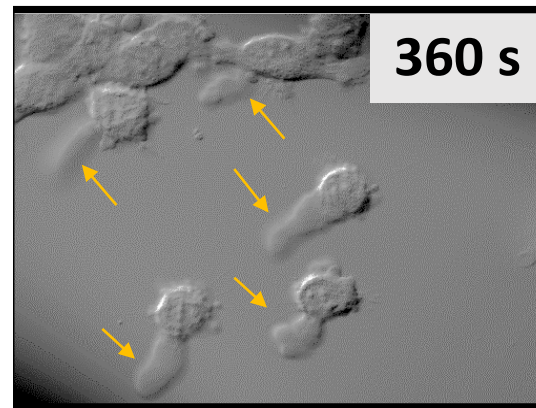
1 pulse, 300 ns, 25.5 kV/cm



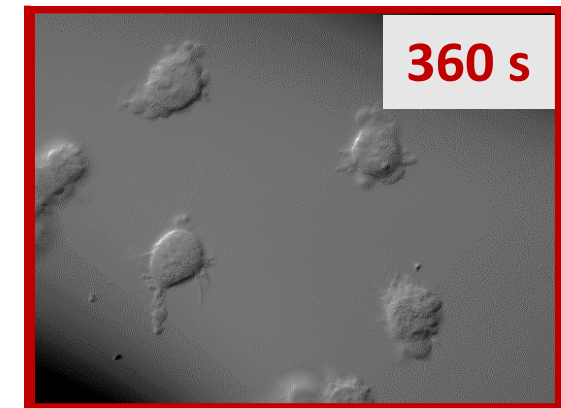
TMEM16F functions in HEK293 cells were
➤ inhibited by si-RNA or AO-1 inhibitor
➤ increased by overexpression



One 300-ns unipolar pulse
at 4.2 - 25.5 kV/cm



control siRNA



TMEM16F knockout

3. TMEM16F is a facilitator of immediate cell response to nsPEF

Findings:

TMEM16F overexpression increases

- cell sensitivity to nsPEF

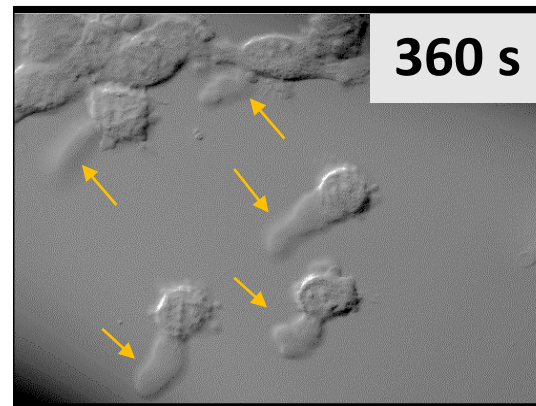
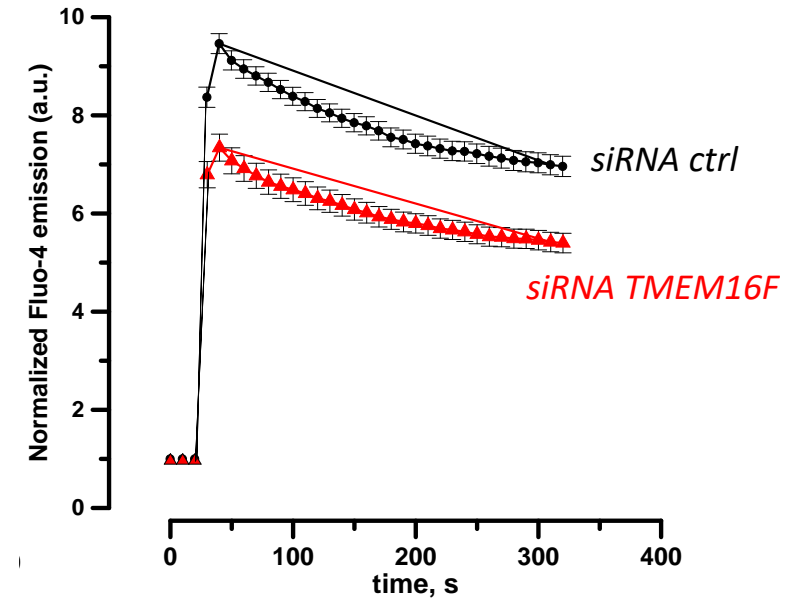
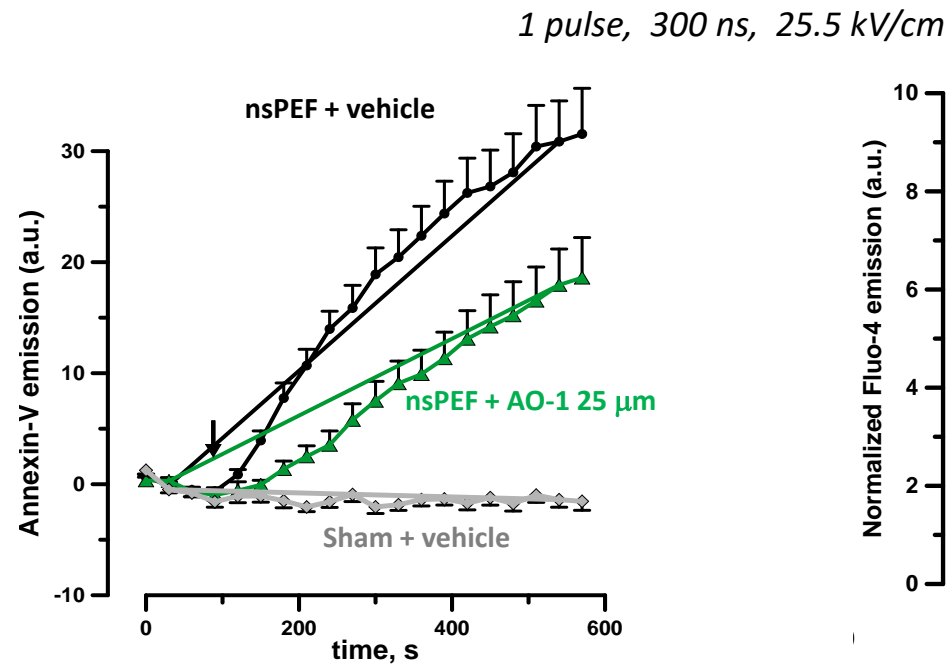
TMEM16F inhibition suppresses nsPEF-modulated

- PS externalization
- Ca^{2+} transients
- Cl^- currents at positive membrane potentials
- Anodotropic membrane blebbing

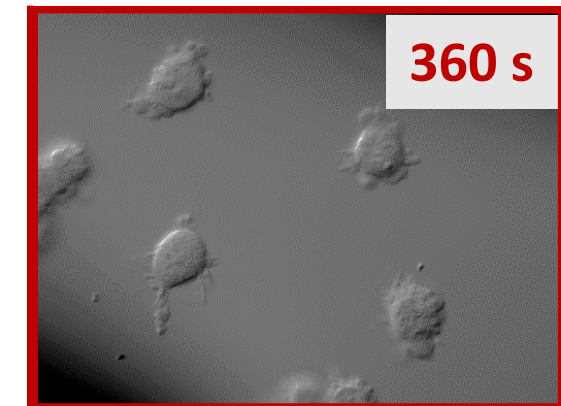
Conclusions:

- nsPEF triggers scramblase and Cl^- channel functions of TMEM16F
- nsPEF-induced membrane blebbing is controlled by TMEM16F

Experimental data: figures courtesy of C. Muratori (Task 5)



control siRNA



TMEM16F knockout

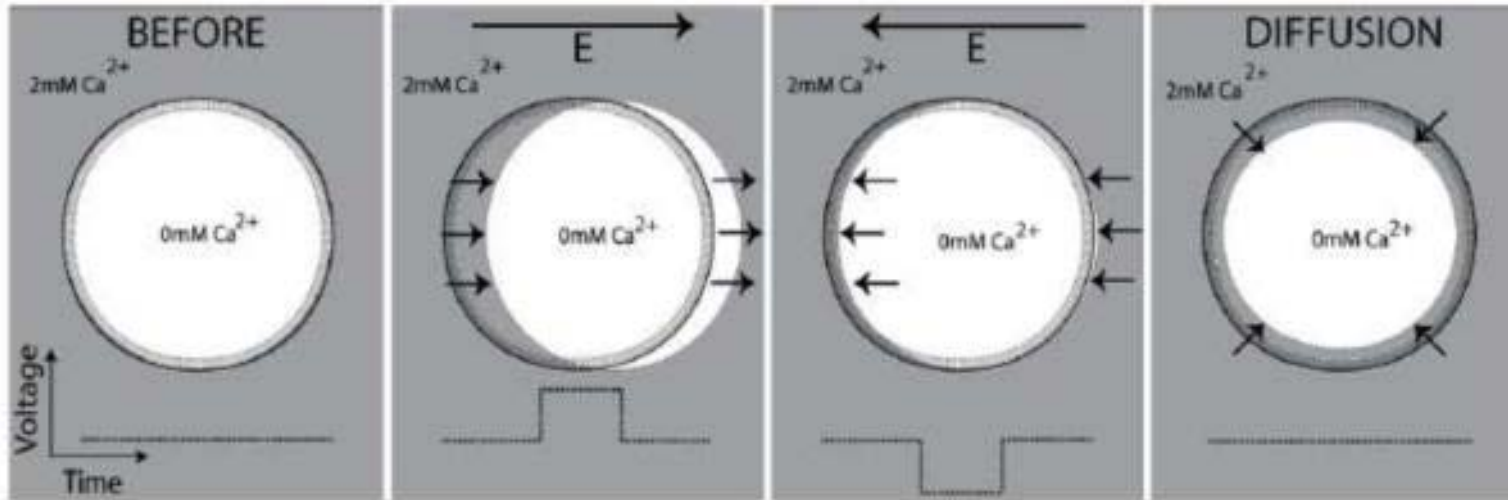
4. Role of Ca^{2+} in the cancellation by bipolar pulses

Collaboration with A. Pakhomov, K. Schoenbach, S. Xiao, (ODU), B. Ibey (FRL)

Experimental data

Gianulis et al. Scientific Reports. 2015;5:13818.

Hypothesis: cancellation is happening due to reversal of electrophoretic entry of Ca^{2+} when the electric field polarity is reversed



Schoenbach et al. Bioelectrochemistry 2015;103:44-51.

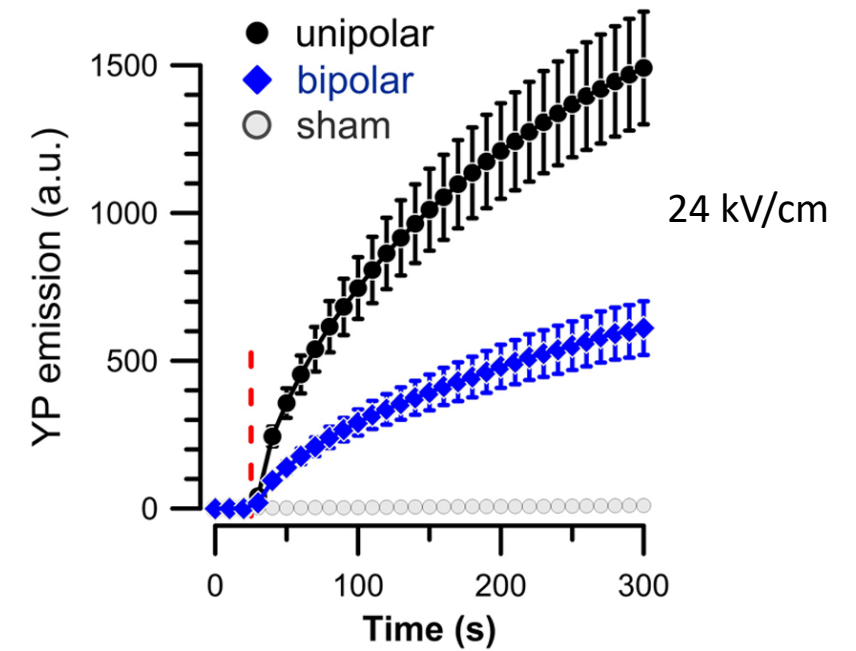
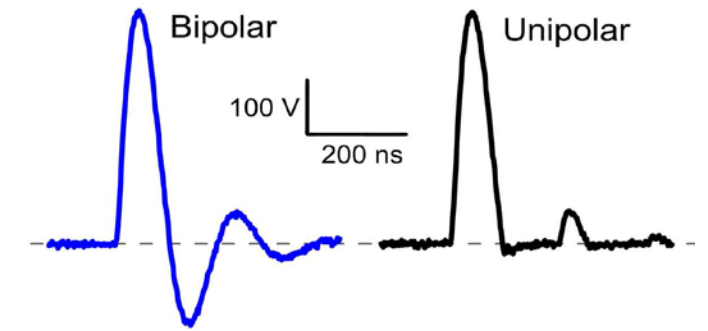
During the First pulse phase Ca^{2+} ions enter cells through nanopores
During second inverse polarity phase Ca^{2+} ions flow in opposite direction

Findings

Bipolar pulses are less efficient in a buffer free of Ca^{2+} ions.

Conclusions

Cancellation phenomenon CAN NOT be explained
by the reversal of the electrophoretic flows of small ions, such as Ca^{2+} .



Ca^{2+} is buffered with 2 mM EGTA in extracellular
and both intra- and extracellular solutions

5. Role of assisted membrane discharge in the cancellation by bipolar pulses

Collaboration with A. Pakhomov, T. Vernier, S. Xiao (ODU),
G. Saulis (Kaunas University, Lithuania)

Assisted Membrane Discharge Hypothesis

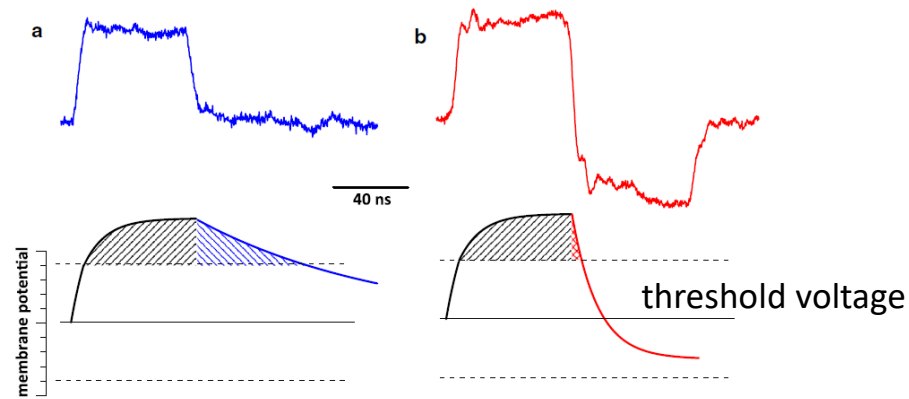
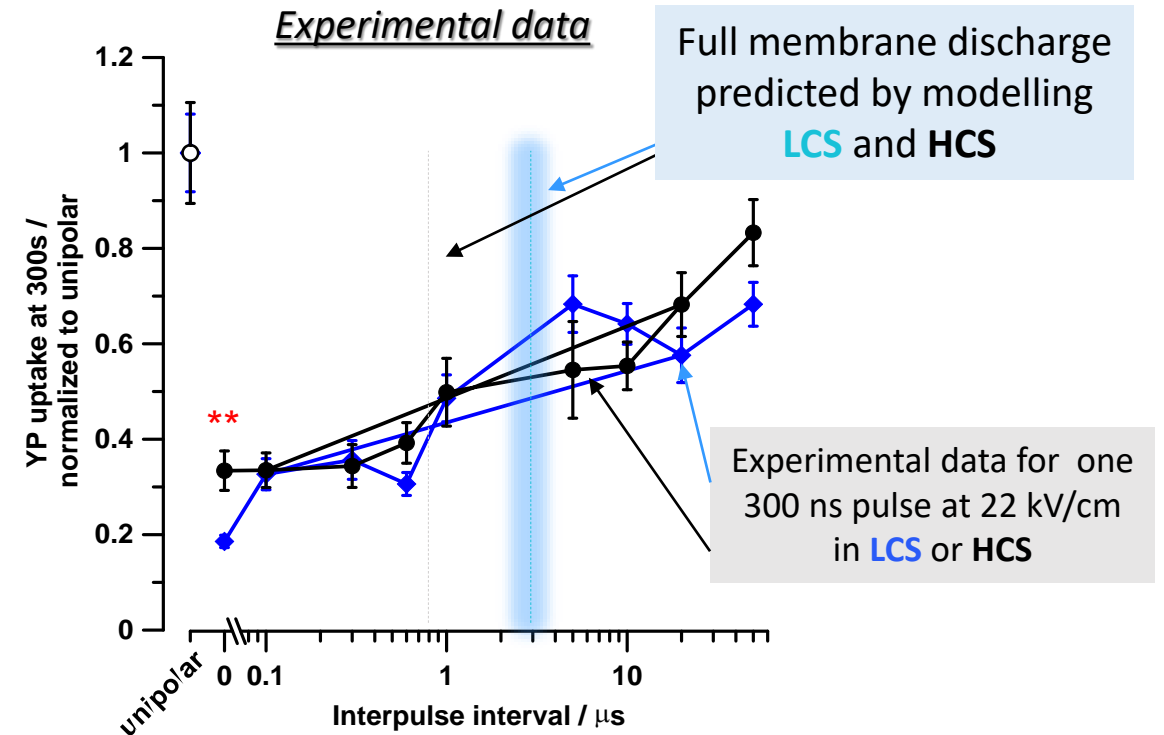
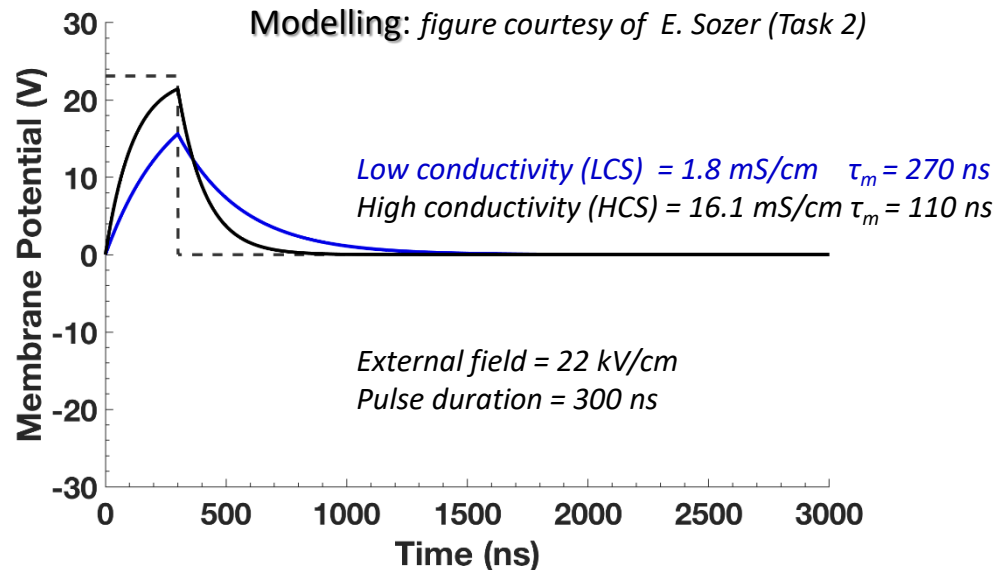


Figure courtesy of A. Pakhomov (Task 1)



Pakhomov, et al., Cell and Mol Life Sci, 2014: 71(22)4431
Gianulis et al., Bioelectrochemistry, 2018, v. 119: 10-19

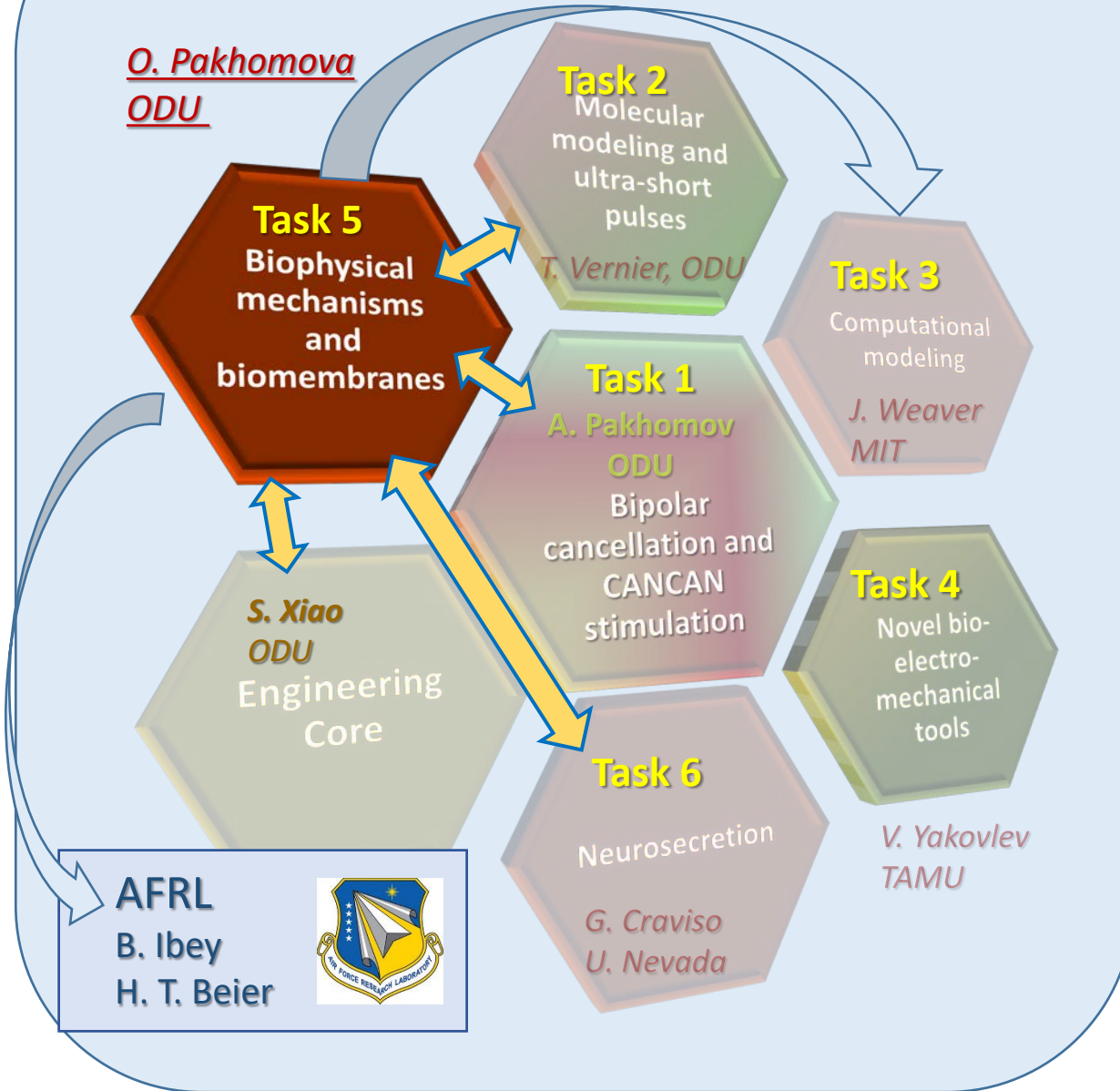
Findings:

Time-dependent decay of bipolar cancellation is independent of plasma membrane time charging constant
Bipolar cancellation still occurs much longer after the membrane is fully discharged.

Conclusions:

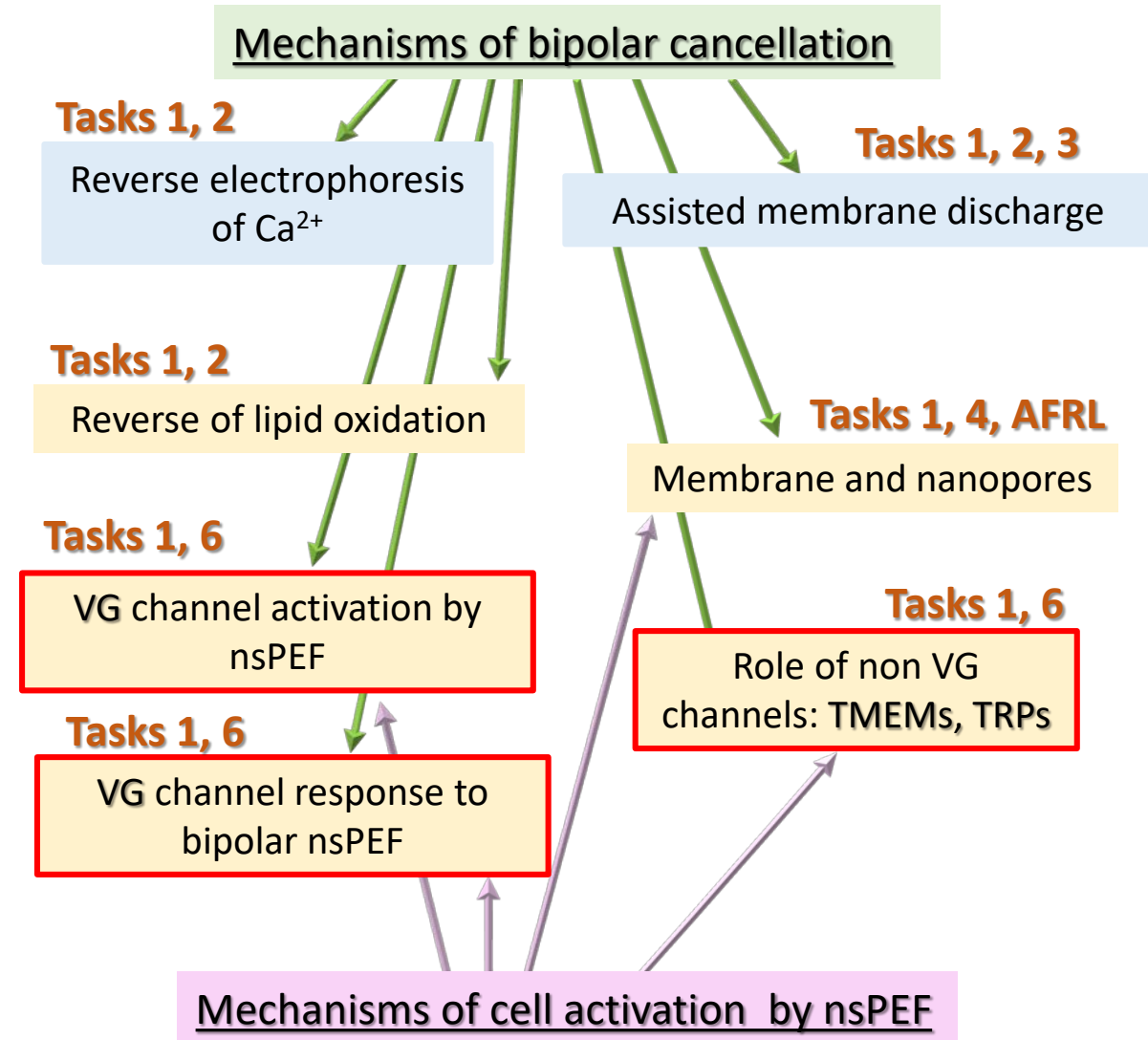
Bipolar cancellation by assisted membrane discharge was not supported in experiments, which suggests the involvement of a different mechanism

MURI Team Structure



Task 5.

Biophysical mechanisms and biomembranes



Funding: AFOSR-MURI



Acknowledgements:

Engineering core

Dr. S. Xiao and the team

ODU (Tasks 1, 3, 5)

M. Casciola
E. Gianulis
K. Hristov
U. Mangalanathan
C. Muratori
A. Pakhomov
E. Sozer
T. Vernier

AFRL

B. Ibey

University of Nevada (Tasks 6)

G. Craviso
N. LeBlanc

MIT (Task 3)

T.R. Gowrishankar
J. Stern
J. Weaver



Many thanks!

July 9-13, 2018

Workshop in Fundamental and Applied Bioelectrics

Norfolk, VA

<https://www.odu.edu/bioelectrics>

2018BioelectricsWorkshop.com

*Opened to anyone who is new at the area of
Bioelectrics.*

Norolk, Chesapeake Bay

Questions?