Do some over-the-counter antimicrobials do more harm than good?

The Tale of Triclosan Toxicology

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The Graduate Students

- Lisa Weatherly, PhD GSBSE
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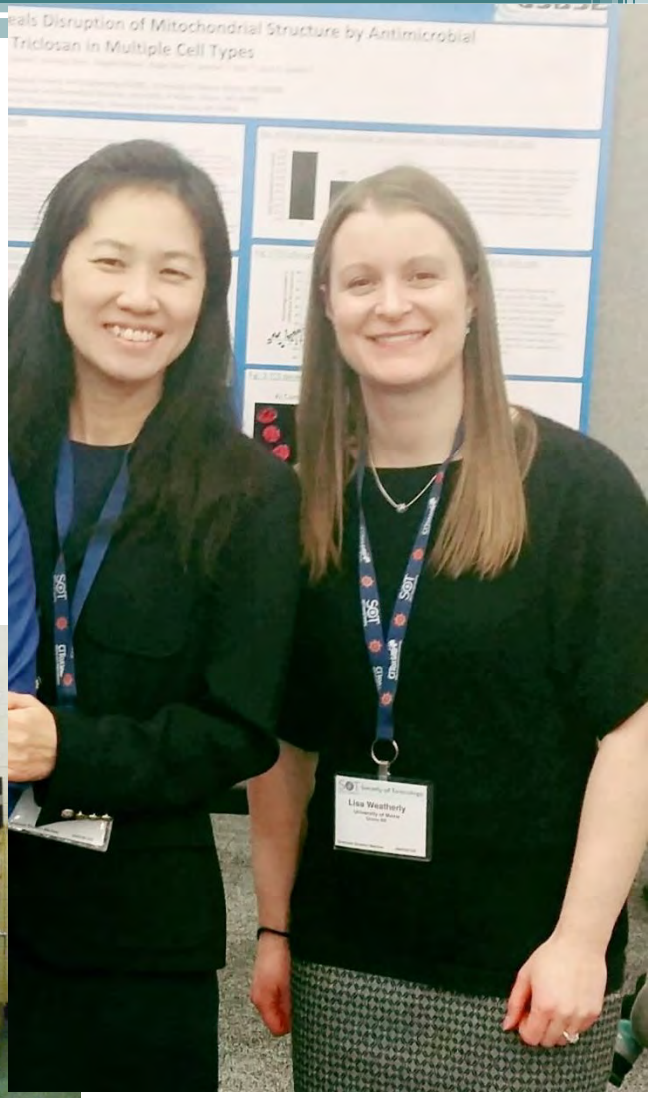
- Juyoung Shim, PhD
  - Assistant Professor, UMaine-Augusta
- Rachel Kennedy-Smith, PhD GSBSE
  - Postdoc Columbia University; medical science liaison
- Lee Hutchinson, MS
  - Biochemist/Manager at IDEXX



The Graduate Students

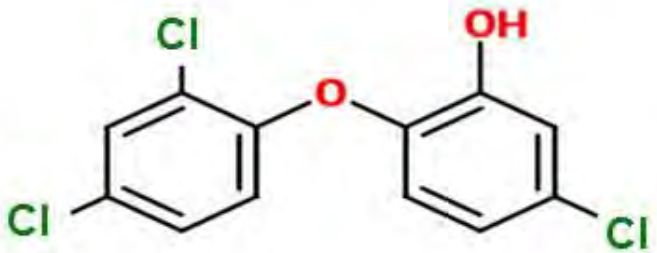
- Bright Obeng, PhD candidate
- Suraj Sangroula, MS
  - Integrated Project Services at National Institutes of Health
- Sasha Weller, MS
  - Amador Bioscience Business Development Manager
- Emily Ledue, MS student
- Hess lab graduate students integral to this work:
  - Brandon Aho, PhD candidate
  - Prakash Raut, PhD
  - Andrew Nelson, PhD
- Brandy Soos

Roadmap

- Many pharmaceutical agents in over-the-counter products are not subject to regulation and are present in high concentrations → into human bodies and the environment
- Numerous chemicals were “grandfathered in” when laws on toxicity evaluations such as TSCA were passed throughout the 20th century
- Example: the story of triclosan—a once-ubiquitous antibacterial agent
- And some of the toxicology research that detected adverse effects on eukaryotes:
  - Immune cell signaling
  - Mitochondria

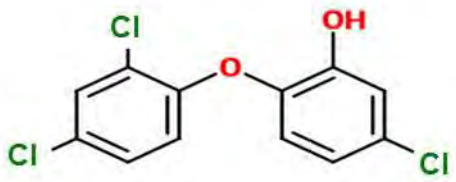
Triclosan (TCS): An antimicrobial agent

- An antibacterial agent that inhibits bacterial fatty acid synthase enzyme enoyl-acyl carrier protein reductase (FabI)
- From 1970s to 2010s, it became popular to add antibacterial agents to a wide variety of personal care and consumer products
  - ingredient in hand soaps, hospital soaps, mouthwashes, children's toys, cookware, etc.
- Despite…TCS-containing soap products did not provide any additional skin-sanitizing benefits compared to soap not containing TCS (Kim et al. 2015)
- And despite increasing bacterial resistance to TCS (affecting humans and environment) (Drury et al. 2013; Nietch et al. 2013; Chen et al. 2009; Suller and Russell 2000)
- So widespread that 75% of US population had significant TCS in their bodies 2003-2004 NHANES (Calafat et al.,*EHP*, 2008)
  - urine concentrations ranging from 7.9 nM to 13.1 µM



➢ **Positive clinical effects:**

- Before antibacterial resistance due to overuse, was an **effective anti-gingival agent** (Rover, *Am J Dent*. 2014) → Colgate Total toothpaste



Triclosan (TCS)

PCP (toothpaste, soap, mouthwash) production ~ 1 million pounds

Global annual consumption of 132 million liters, sales ~$886 million

1970

1995

2000

2010

2015

Antimicrobial agent TCS use in hospital soaps started in 1972

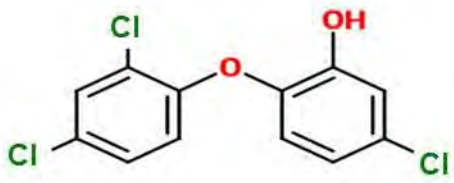
75% of liquid/foam soaps contained TCS

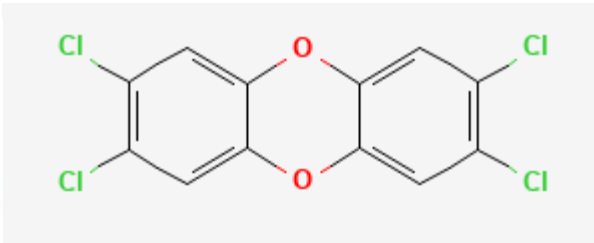
93% of liquid/foam soaps contained TCS

Global production ~10.5 million pounds

(U.S. EPA; Denmark EPA; Bloomberg; FDA 2013; Weatherly and Gosse *JTEHB*, 2017)

Status of Triclosan ~2010





- Wide exposure, in the top-selling toothpaste, many products
- 2010 Dr. Susan Richardson (at EPA) visited UMaine and discussed TCS as an emerging contaminant detected by her analytical chemistry laboratory
  - Looks like dioxin
  - detection in lakes, rivers (Zhang 2007 *Chemosphere*), and soils (Lapen 2008 *Sci Total Environ*)

- Large majority of Americans were exposed, but almost no information of eukaryotic, animal effects of TCS (no human health information)
  - Just a few studies published around that time—sheep, fish—showing endocrine disruption by TCS (Chen 2007 *Toxicol Appl Pharmacol*; Helbing 2011 *Tox Sci*)
  - We were studying the effects of endocrine disruptors (including arsenic) on immune cells so doctoral student Rachel Kennedy-Smith ran back to the lab and started experiments

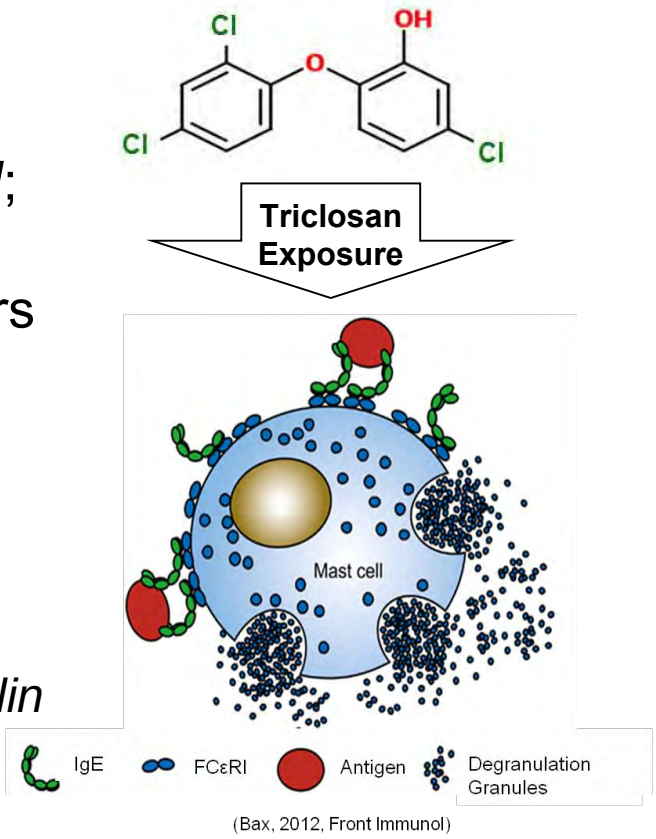
Triclosan Exposure

- TCS is readily absorbed into skin and oral mucosa (Queckenberg 2010 *Antimicrob Agents Chemother*; Lin 2000 *Am J Dent*; Gilbert 1987 *J Pharm Pharmacol*)
  - not metabolized for many hours (Moss, *Food Chem Toxicol.* 2000).
- TCS levels found in human tissues exposed to TCS products (**10mM**) for 1 hour is **0.4-64 nmol TCS/mg tissue protein.** (reviewed in Weatherly, *JTEHB,* 2017)
- Our studies used micromolar concentrations of TCS, equivalent to **~2.5 nmol TCS/mg protein** found in cell culture (Weatherly, *JTEHB,* 2017)
- *Do the benefits of (widespread) exposure outweigh any potential risk?*
  → Need toxicology data to inform

➢ **Dosages used in our experiments were relevant to actual human exposures to TCS products.**

Does TCS Affect Mast Cell Signaling?

- TCS causes endocrine disruption (Chen 2007 *Toxicol Appl Pharmacol*; Helbing 2011 *Tox Sci*)
  - Endocrine disruptors disturb mast cell signaling (Narita, *EHP*, 2007)
- Some studies showed clinical alleviation of eczema by TCS (Tan, *Clin Exp Dermatol*, 2010; Sporik,*J Allergy Clin Immunol,* 1997)
  - Could mast cells be involved?

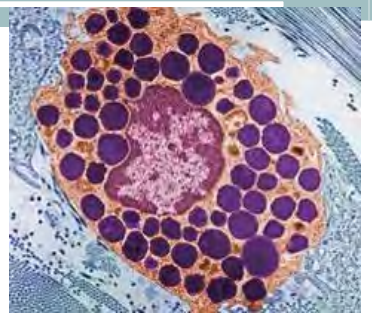


(Bax, 2012, Front Immunol)

Mast cells share many signaling elements with numerous other cell types including immune cells like T cells:

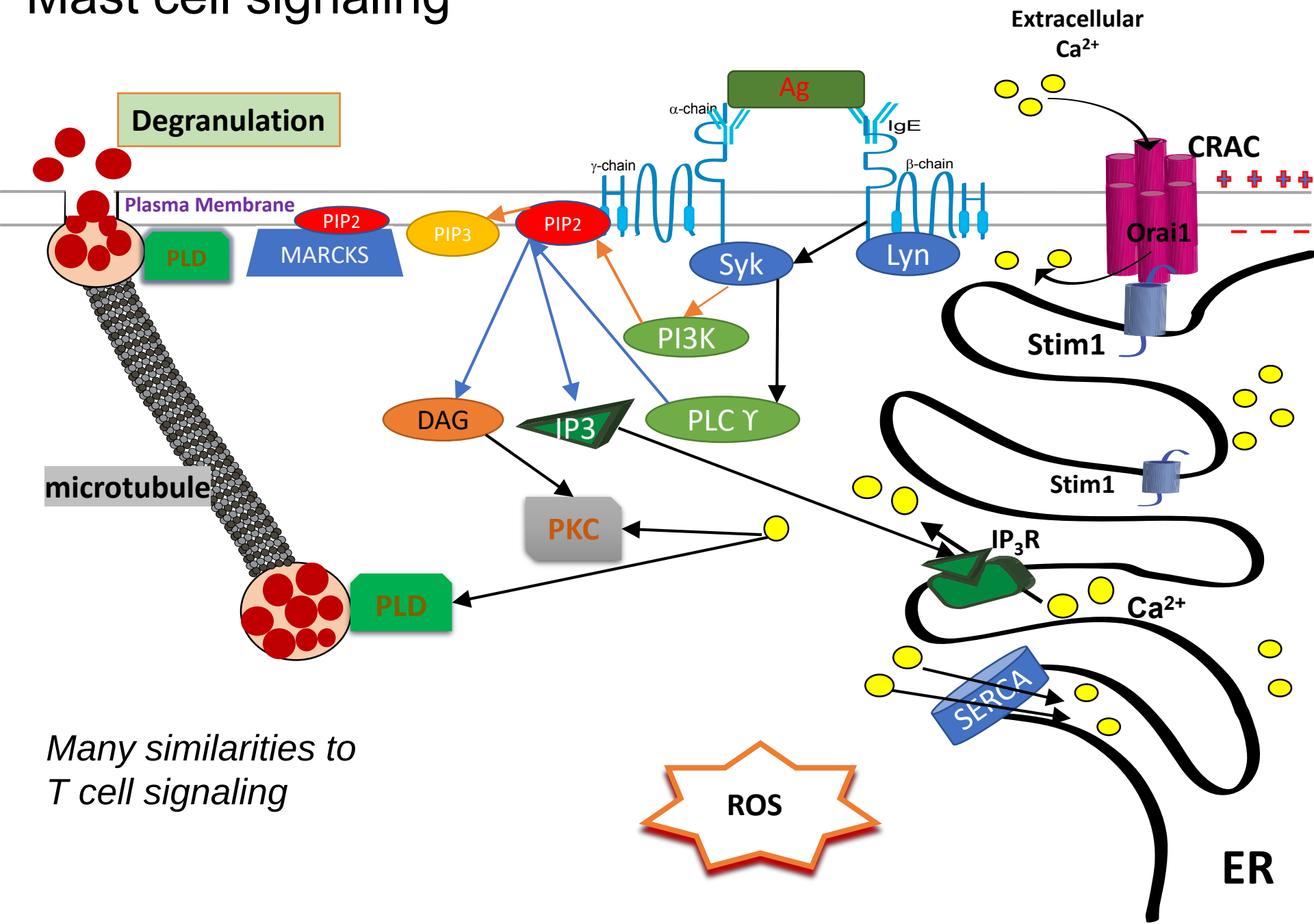
prediction of TCS effects in different cell types that share common signal transduction elements.

Mast cells



- Highly granulated immune cells
- Release granules upon stimulation: degranulation, a type of exocytosis
  - histamine, tryptase, serotonin, β-hexosaminidase, etc.(Schwartz, *J Investig Dermatol,* 1980)
- Found in most tissues and species
- Found at surface/borders: capillaries, nerve terminal connections, GI, respiratory mucosa, skin, etc
- Critical players in allergy, asthma, autoimmunity, infectious disease, cancer, and CNS disorders (autism, anxiety, MS) (Galli, *Nature*, 2008; Abraham, *Nat Rev Immunol,* 2010).

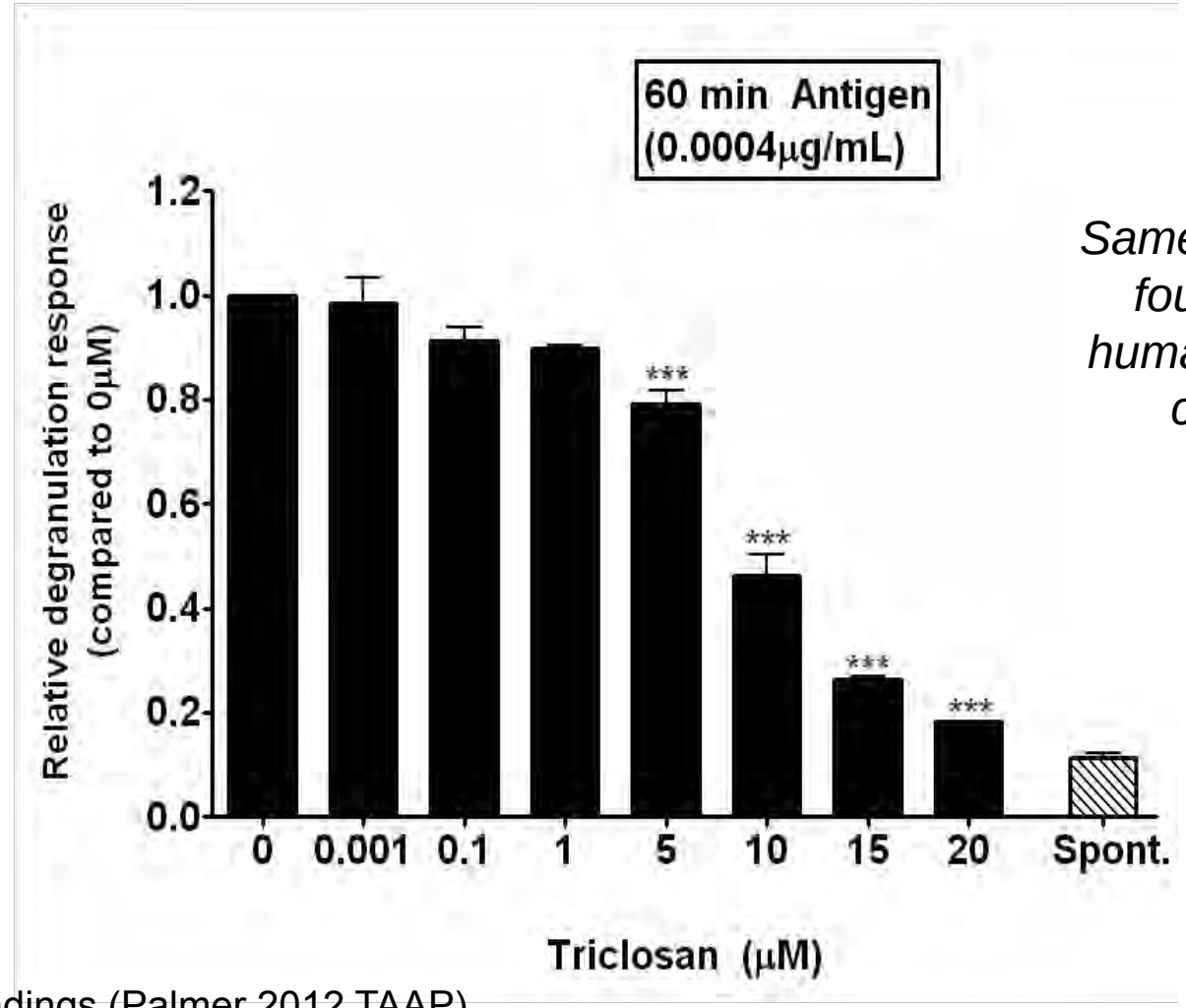
Mast cell signaling



Many similarities to T cell signaling

- Non-cytotoxic TCS doses used in all experiments
- Also, the doses used are ~1000-fold lower than those in personal care products

TCS inhibits mast cell degranulation of rat mast cells



Same results found in human mast cells

Follow up on earlier findings (Palmer 2012 TAAP)

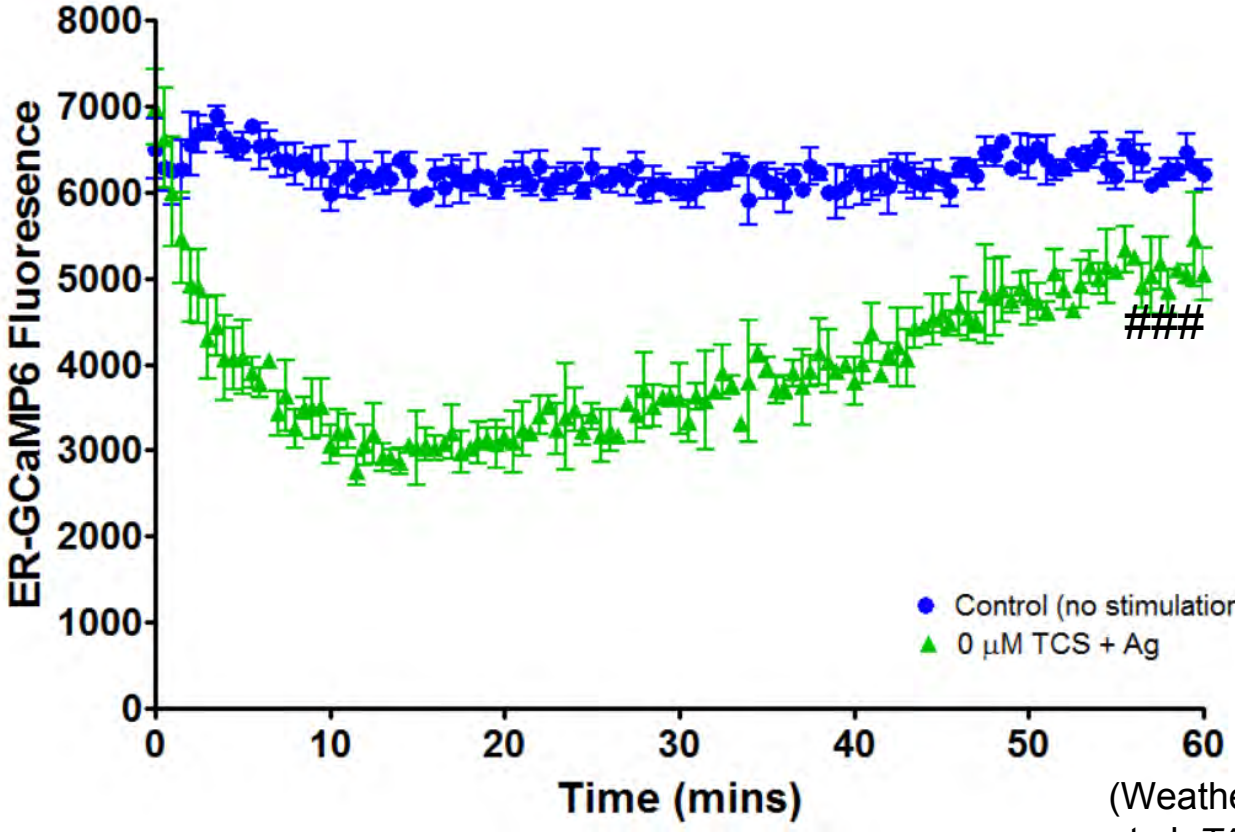
*** $p < 0.001$

(Weatherly *et al.*, *Journal of Visualized Experiments*, 2013)

What is the mechanism underlying triclosan's inhibition of mast cell degranulation?

Efflux of calcium from the ER due to antigen stimulation of mast cells

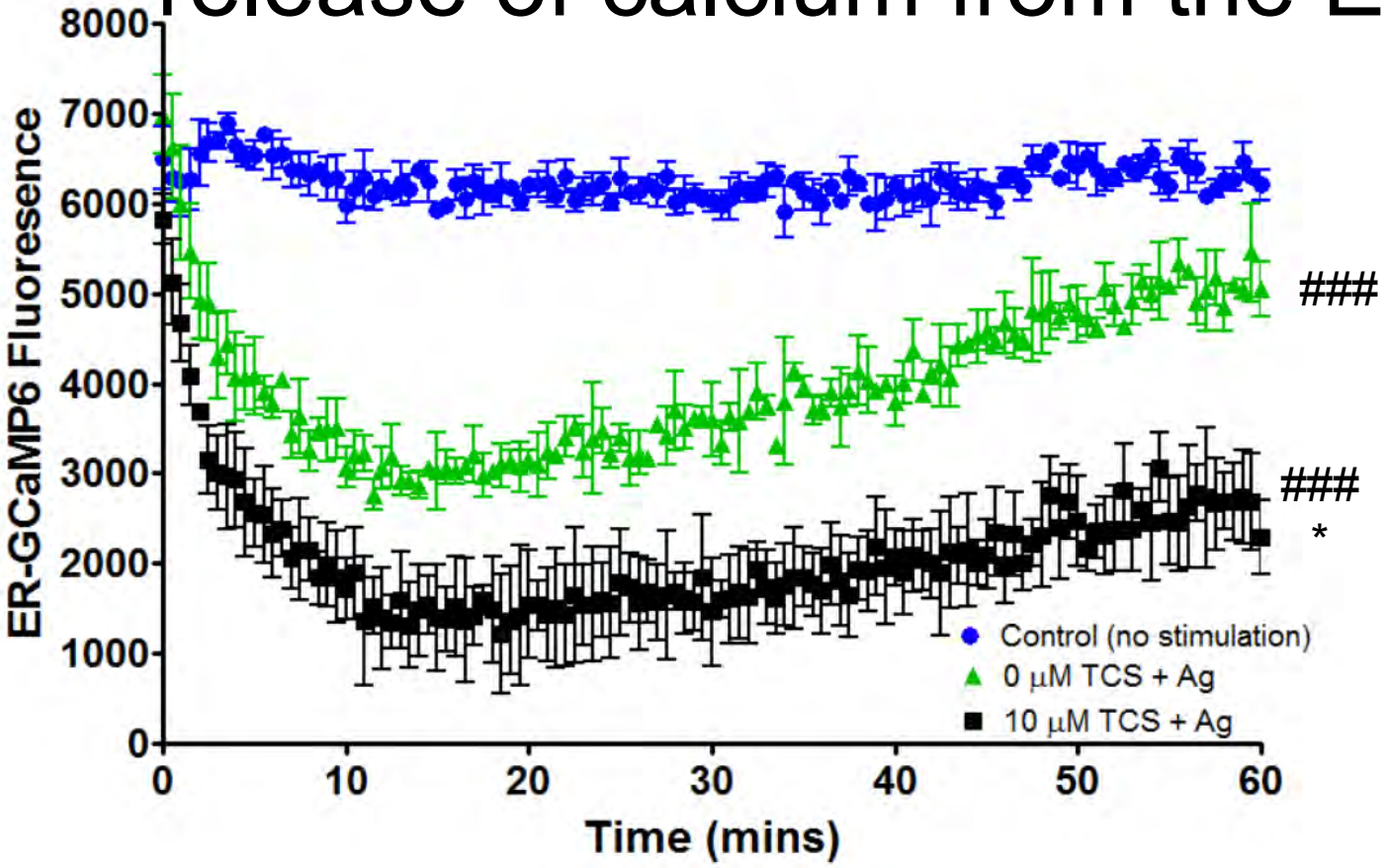
- Efflux of calcium from the ER activates STIM1/ORAI1 interaction
- Called store-operated calcium entry (SOCE)
- Then calcium into cytosol, through plasma membrane CRAC channels



p < 0.001 (compared to control)

(Weatherly LM and Nelson et al, *TAAP*, 2018)

TCS actually enhances antigen-stimulated release of calcium from the ER



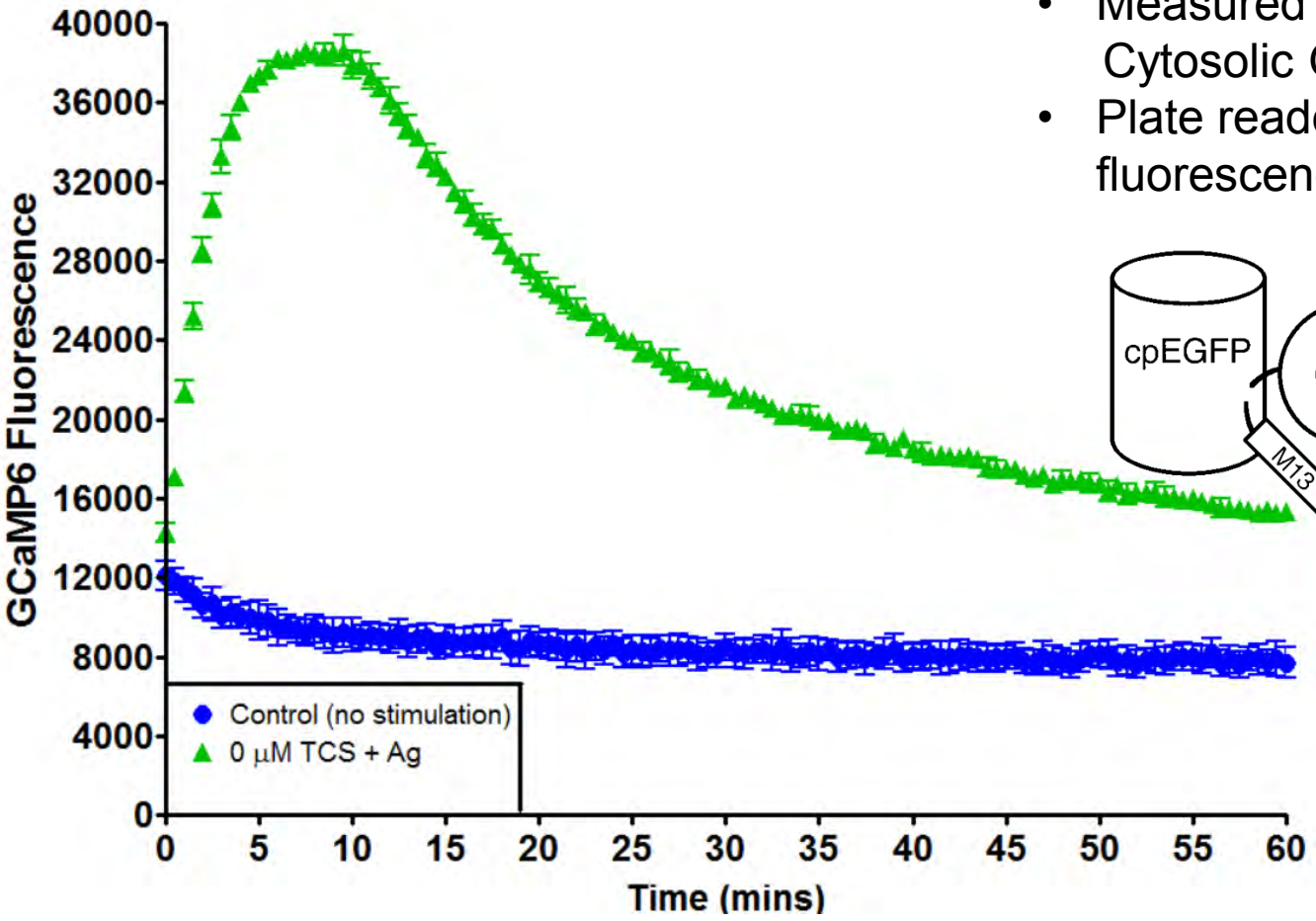
- TCS actually increases efflux of ER Ca^{2+}
  - So not a mechanism of degranulation inhibition

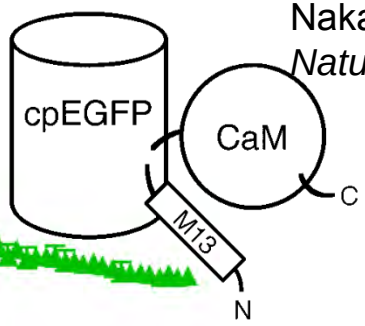
$p < 0.001$ (compared to control)
* $p < 0.05$ (compared to Ag)

(Weatherly LM and Nelson et al, *TAAP*, 2018)

But…TCS decreases cytosolic calcium levels of mast cells

- RBL-2H3 mast cells
- Measured using Cytosolic GCaMP6
- Plate reader-based fluorescence assay

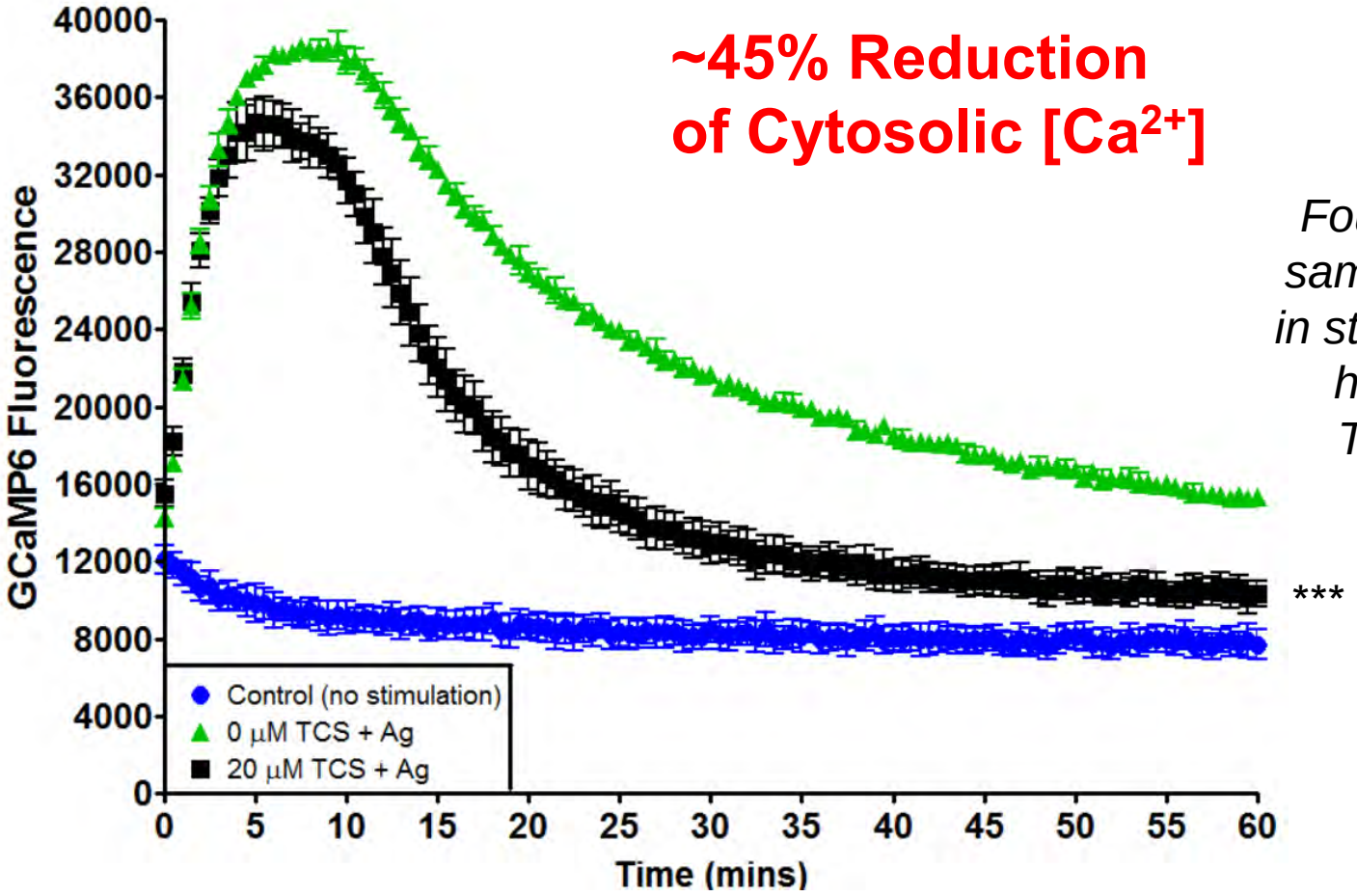




Nakai, et al., *Nature*, 2000

(Weatherly LM and Nelson et al, *TAAP*, 2018)

TCS decreases cytosolic calcium levels of rat mast cells



Found the same effect in stimulated human T cells

*** p < 0.001

(Weatherly LM and Nelson et al, *TAAP*, 2018)

What happens when Ca^{2+} influx fails due to TCS exposure?

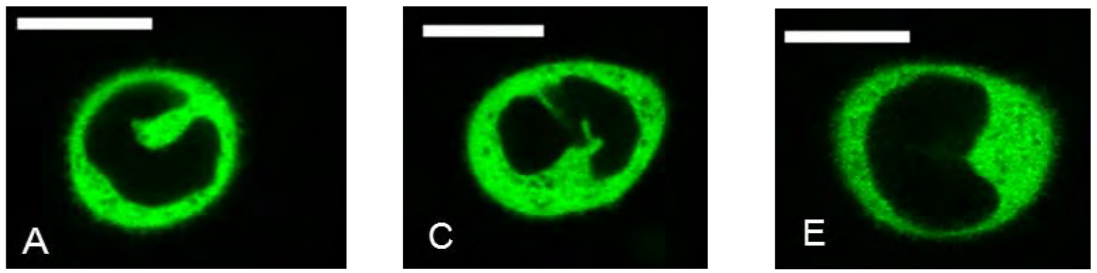
- Protein kinase C (PKC) translocation is delayed
- Phospholipase D (PLD) activity decreases
- Microtubules do not polymerize: the “railroad” system for moving granules to the plasma membrane for degranulation is shut down

Protein Kinase C (PKC)

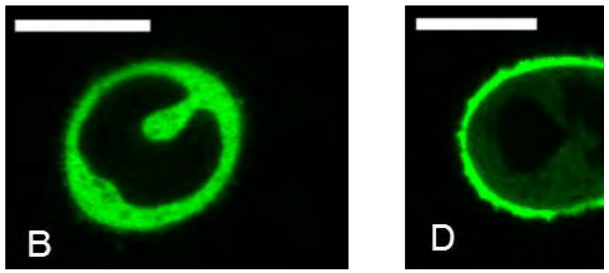
- **PKC β and δ** are particularly important in degranulation (Nechushtan, *Blood,* 2000; Cho, *J Allergy Clin Immunol*. 2004)
  - PKC β is activated by DAG and **Ca^{2+}**
  - PKC δ is activated by DAG and by **ROS** (Cho, *J Allergy Clin Immunol*. 2004)
- PKC translocation from cytoplasm to plasma membrane is a hallmark for PKC activation (Mochly-Rosen, *MBoC*. 1990)

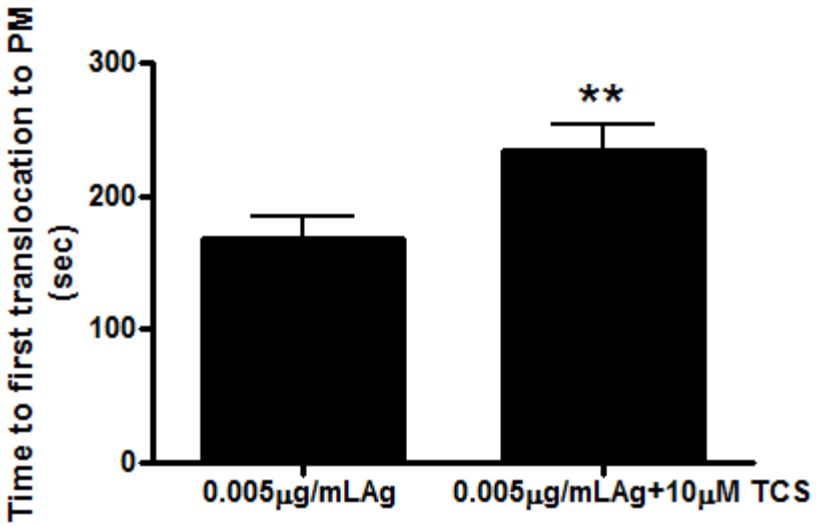
Triclosan *delays* PKC βII translocation

- YFP-PKCβII-YFP
- Confocal



Scale bar, 10 µm





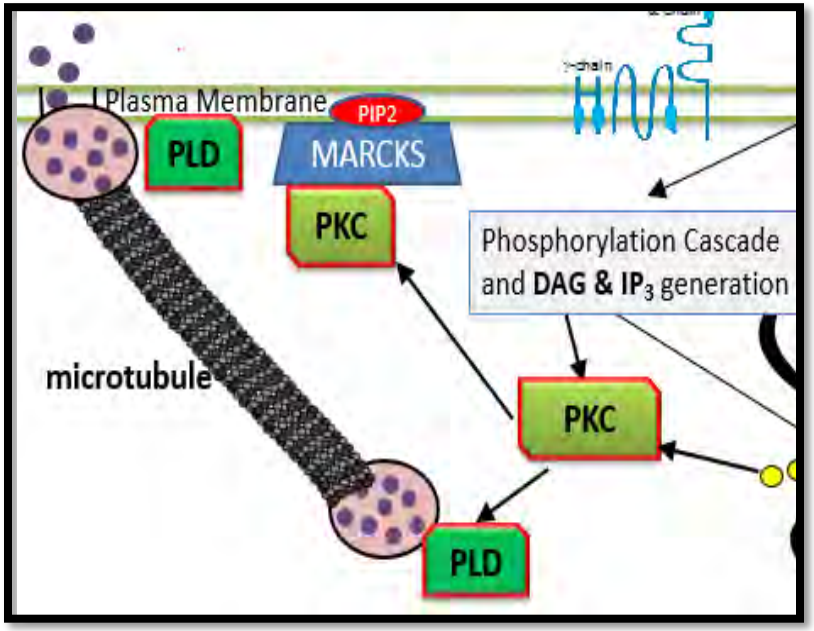
- Same results were found with **PKC δ**
- **Indicates a reduction in PKC activity upon TCS exposure**

(Shim *et al.*, *JAT*, 2019)

- N= 43 to 50 cells
- One-tailed t-test, ** $p<0.01$

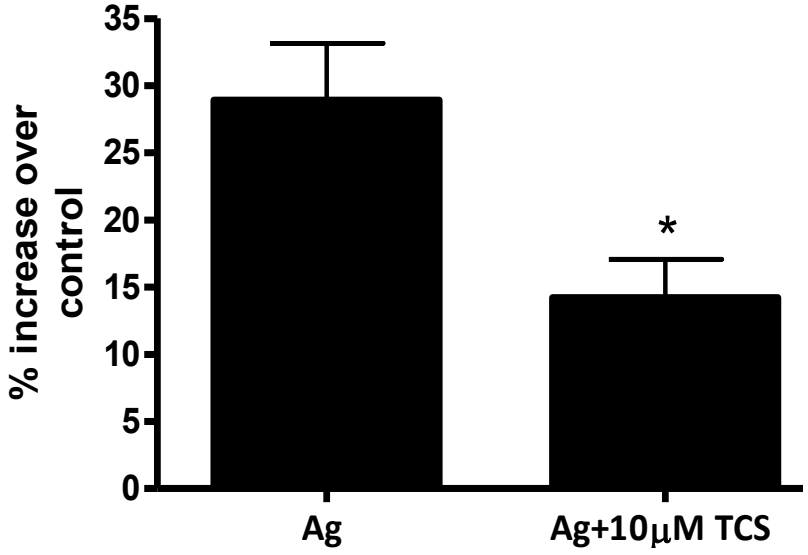
Phospholipase D (PLD)

- Increases in intracellular Ca^{2+} and PKC translocation work in tandem to activate PLD (Lin, *Eur J Biochem.,* 1992)
- PLD activity is necessary for degranulation (Choi, *J Immunol.* 2002)
- PLD produces phosphatidic acid, a negatively-charged phospholipid with a small headgroup that promotes negative membrane curvature, thought to facilitate membrane-vesicle fusion
- Two PLD isoforms:
  - PLD1 and PLD2



Triclosan inhibits PLD activity in Ag-stimulated mast cells

PLD1/2 activity ELISA:
Amplex® Red PLD Assay Kit



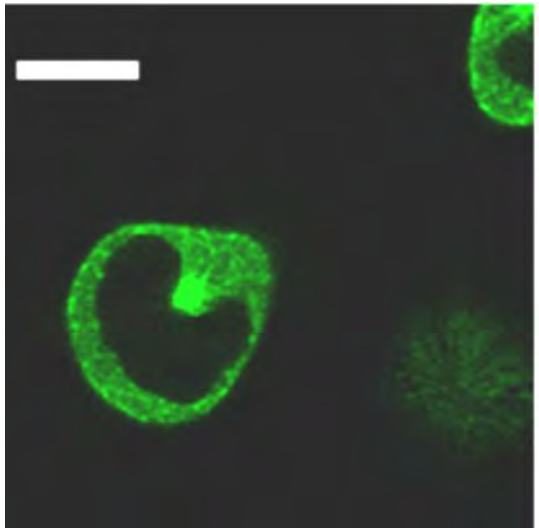
One-tailed t-test, *P<0.05

(Shim *et al.*, *JAT*, 2019)

- TCS inhibits Ag-activated PLD activity.

TCS inhibits microtubule polymerization in mast cells

Control



Microtubule polymerization assay

- EGFP-alpha-tubulin (Rusan *et al*., *MBoC*, 2001)
- Incubation with Ag + 10 µM TCS for 1 hour
- Confocal imaging

- Increased cytosolic Ca^{2+} stimulates association of the positive regulator protein Git1 with tubulin, in turn causing enhanced degranulation (Sulimenko V, *J. Immunol*., 2015)

(Weatherly LM and Nelson et al, *TAAP*, 2018)

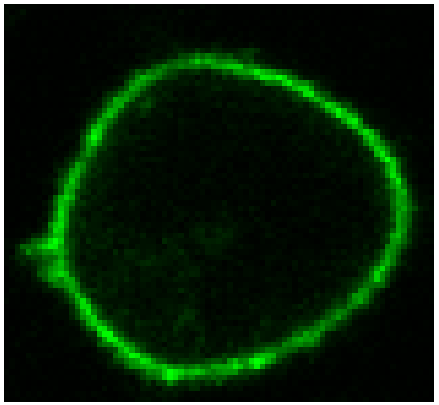
- **TCS suppresses the polymerization of microtubules, likely because cytosolic Ca^{2+} is required for polymerization.**

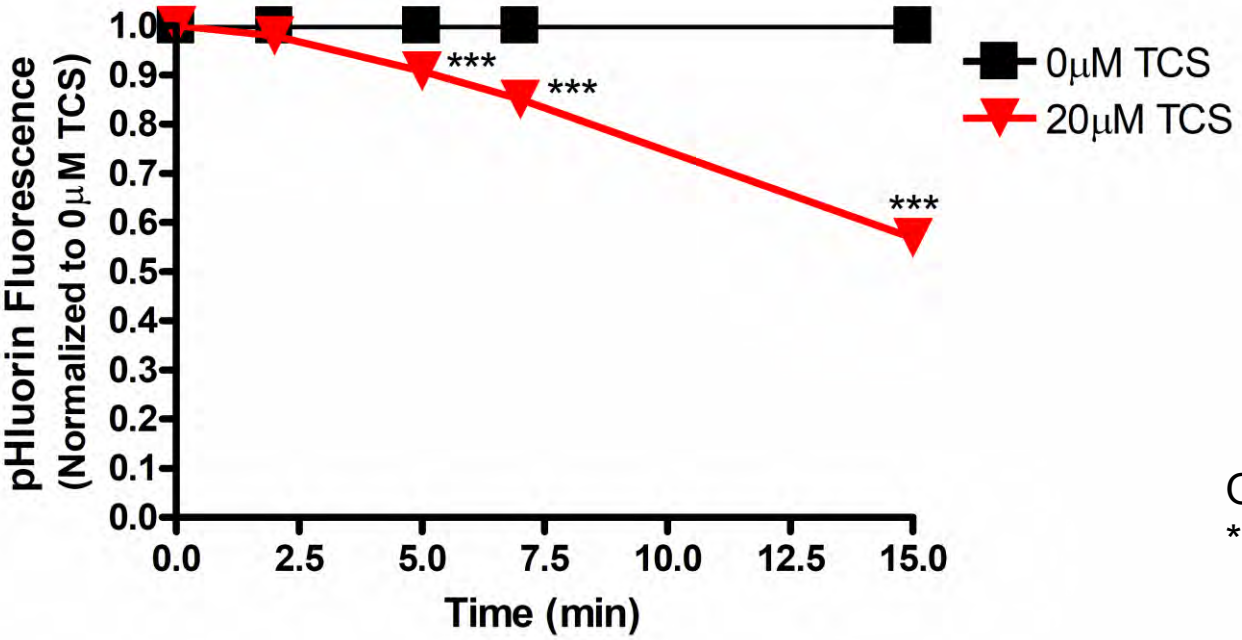
Scale bar = 10 µm

How does TCS inhibit store-operated calcium entry (SOCE) through the CRAC channel?

Triclosan decreases cytosolic pH as reported by mcherry-SEpHluorin

- Lyn-tailed mcherry-SEpHluorin is properly targeted to the plasma membrane in mast cells





- RBL-2H3 cells
- Lyn-tailed mcherry-SEpHluorin
- Amaxa transfection
- N=35 Control
- N=52 Triclosan
- Confocal imaging

One way Anova,
***p<0.001

(Sangroula *et al*, *TAAP*, 2020)

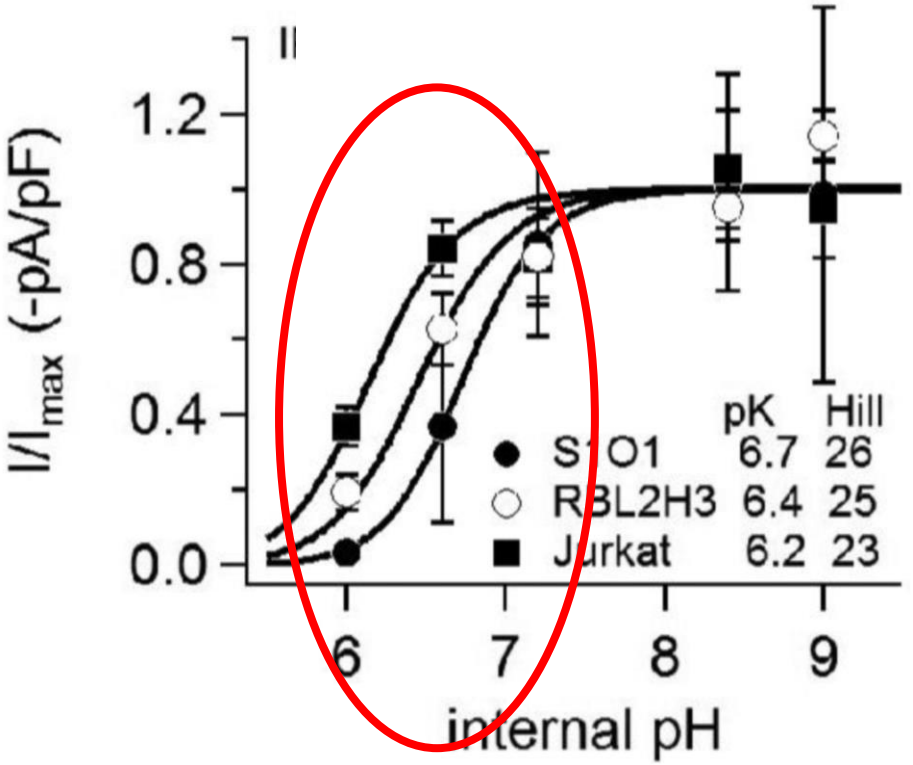
How much acidification is caused by triclosan?

Method	Magnitude of pH Change
Theoretical estimate	-0.3
ArcLight experiments	-0.23 ± 0.02
pHlourin experiments	-0.37± 0.02
Average	-0.3

(Sangroula *et al*, *TAAP*, 2020)

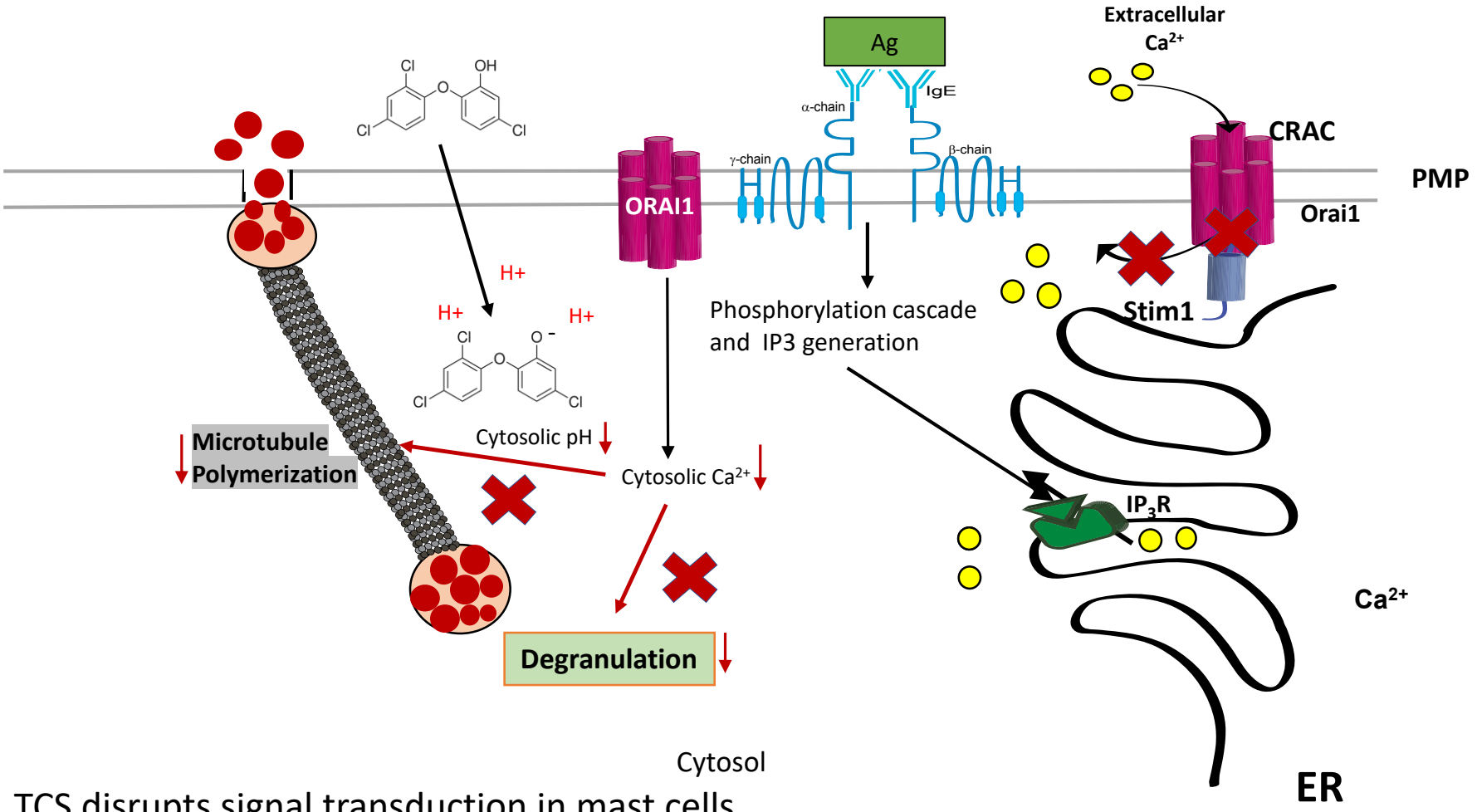
TCS-induced cytosolic acidification may reduce cytosolic Ca^{2+} influx in immune cells

- TCS-induced ΔpH = -0.3
  - Base 10 log scale.
- Acidification of the cytosol in RBL-2H3 mast cells and Jurkat T-cells decreases I_{CRAC} (Beck et al, *Cell Calcium*, 2014)
  - I_{CRAC} = current due to Ca^{2+} influx across CRAC channel.
- Most drastic effects in all cell types lies between pH 6 and 7.
- Modest -0.3 pH change in this range can thus potently alter Ca^{2+} influx.



Beck et al, *Cell Calcium*, 2014

Summary: Triclosan mechanism of action on immune cell signaling



TCS disrupts signal transduction in mast cells and T cells

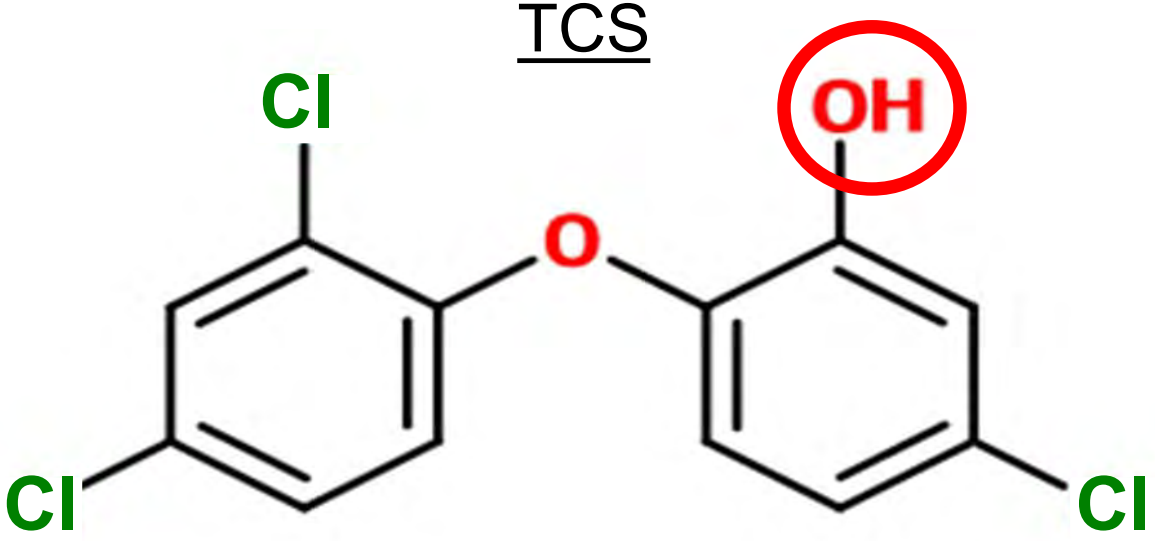
- Effects on common signaling components predict effects in disparate cell types

- Now onto triclosan and mitochondria
- A completely different toxic action of TCS

TCS has similar features to known mitochondrial uncouplers

- Ionizable proton
- Lipophilic when protonated
- pKa ~ 8 (Pubchem)
- Hypothesized TCS is affecting mitochondrial function

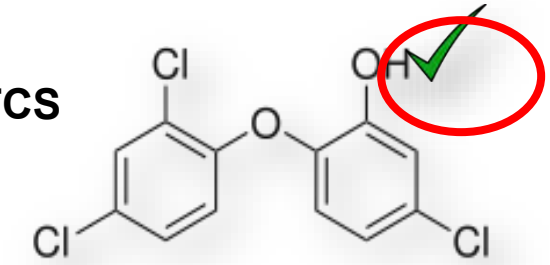
TCS

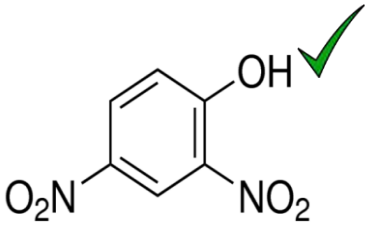


Mitochondrial Uncoupler (MU)

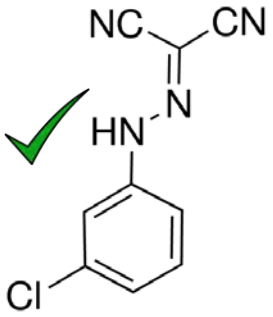
- MUs (e.g. DNP, CCCP, FCCP)
  - Small hydrophobic molecules with a ionizable proton
  - can cross the inner mitochondrial membrane.

TCS

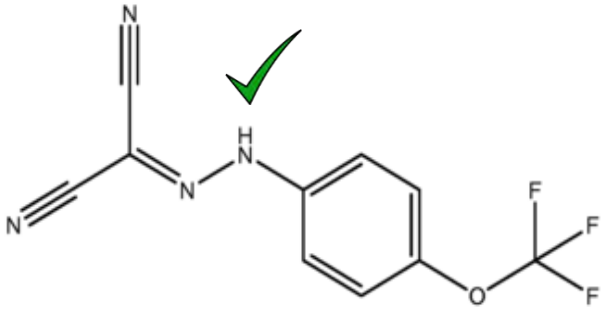




2, 4-Dinitrophenol (DNP)



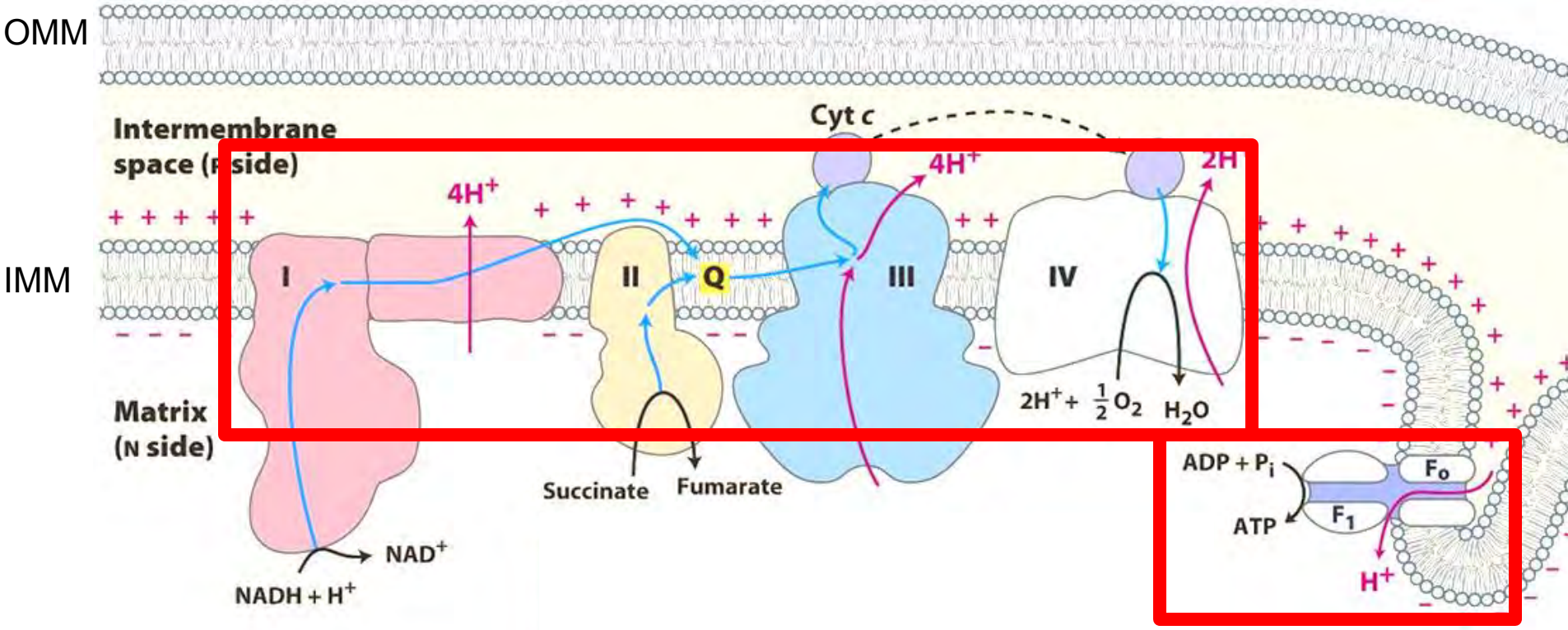
Carbonyl cyanide 3-chlorophenylhydrazone



Carbonyl cyanide p-trifluoromethoxyphenylhydrazone

FCCP

Mitochondrial Function

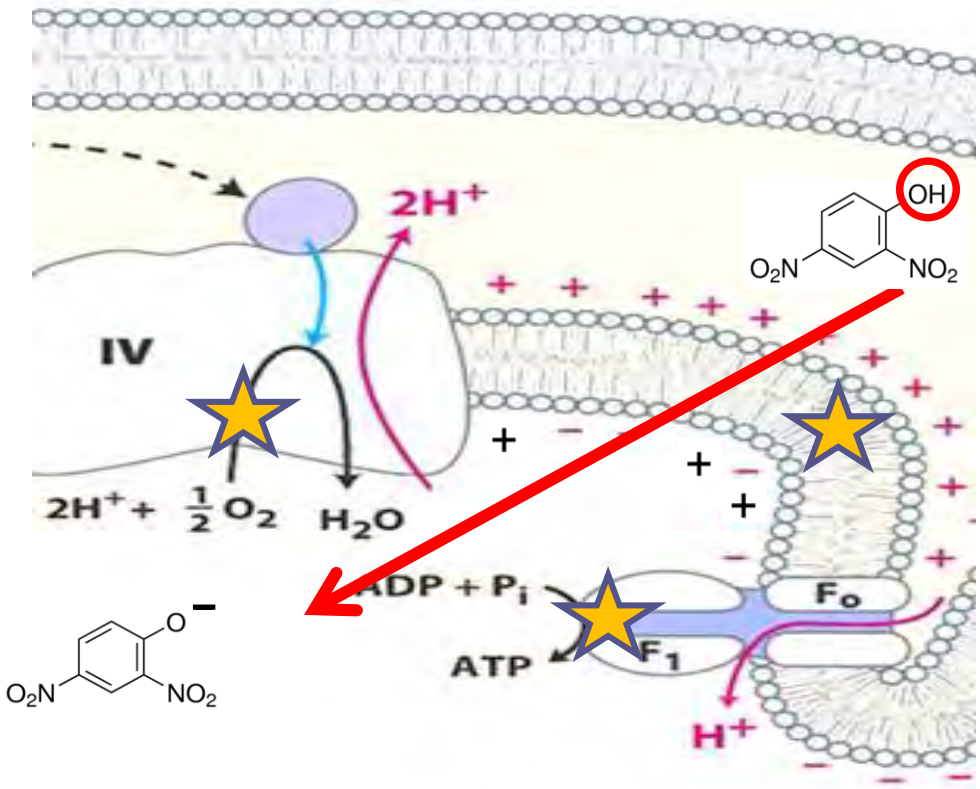


- Energy is conserved by pumping of H^+ for electrochemical gradient
- Electron transport to O_2 releases energy
- Proton motive force drives ATP formation

(Figure reproduced from Nelson et al., 2008).

How mitochondrial uncouplers disrupt mitochondrial function

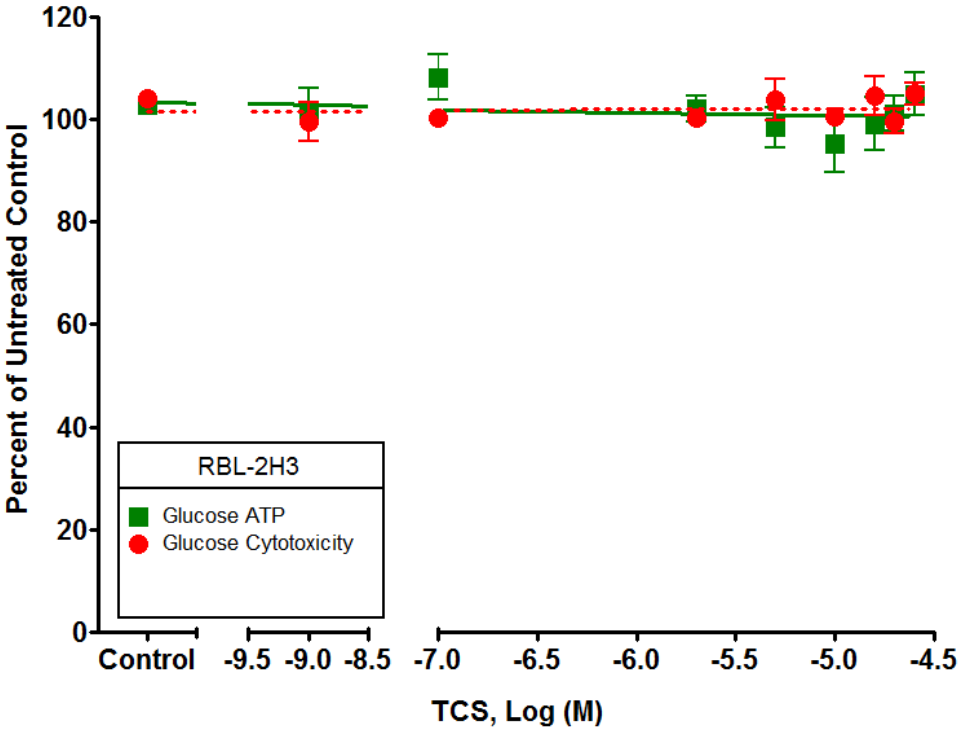
- Enable protons to flow across inner membrane



- 3 main features of uncouplers
  1. Decrease ATP
  2. Increase oxygen consumption rate
  3. Breakdown of mitochondrial membrane potential

(Figure adapted from Nelson et al., 2008).

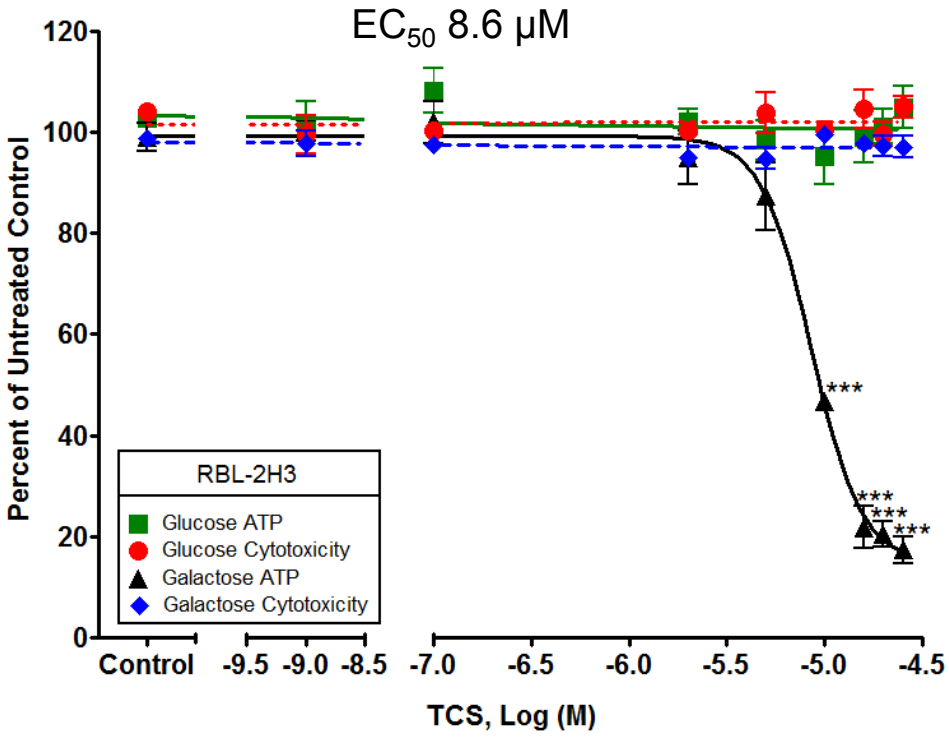
TCS decreases ATP production in multiple cell types

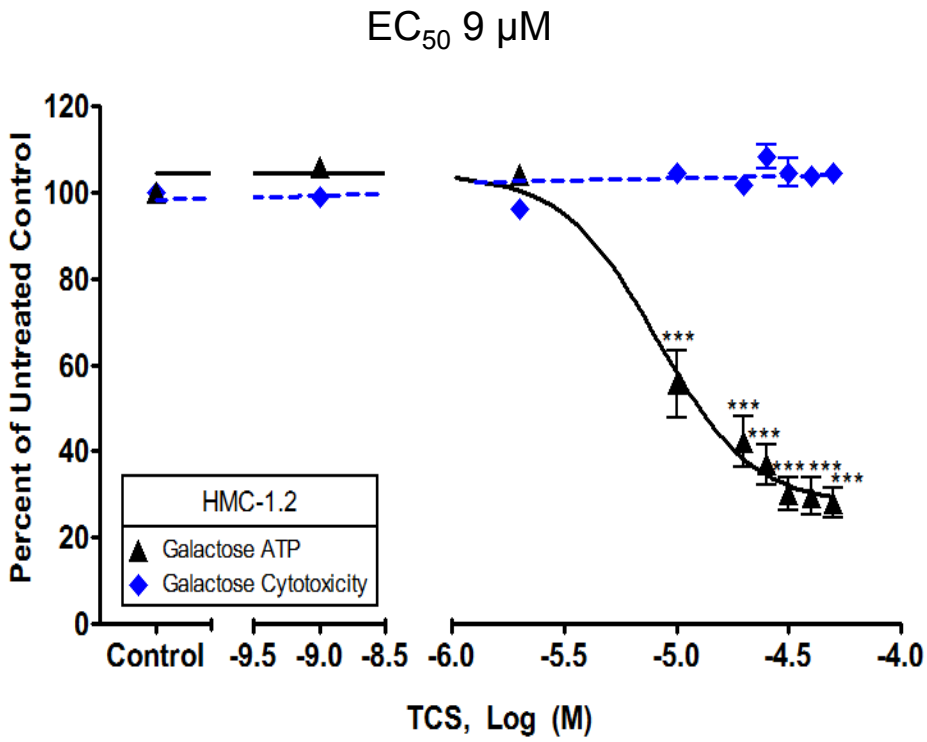


*** $p < 0.001$

(Weatherly, LM *et al.*, *Journal of Applied Toxicology*, 2015)

TCS decreases ATP production in multiple cell types



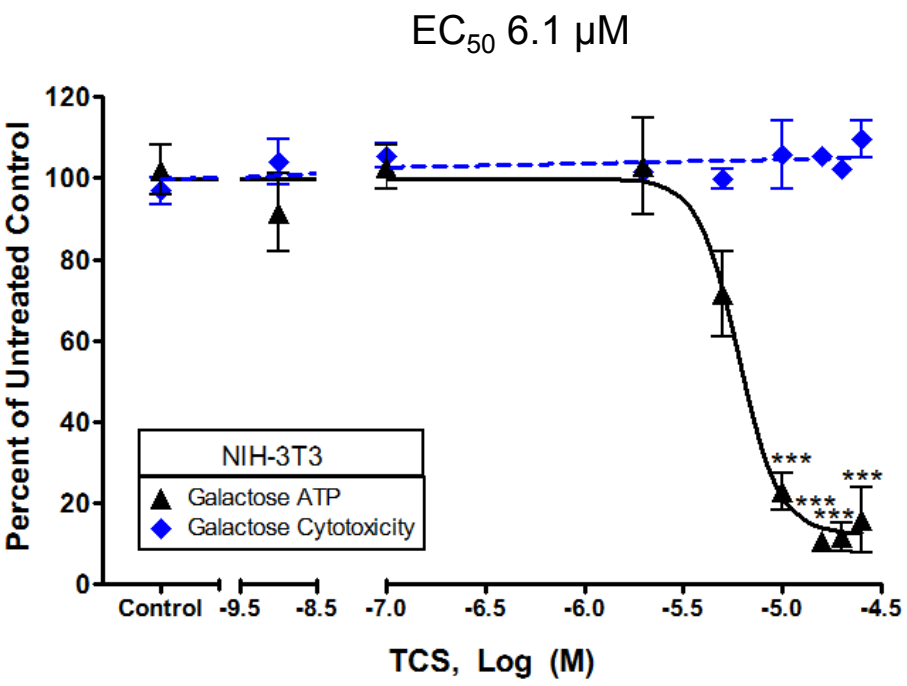


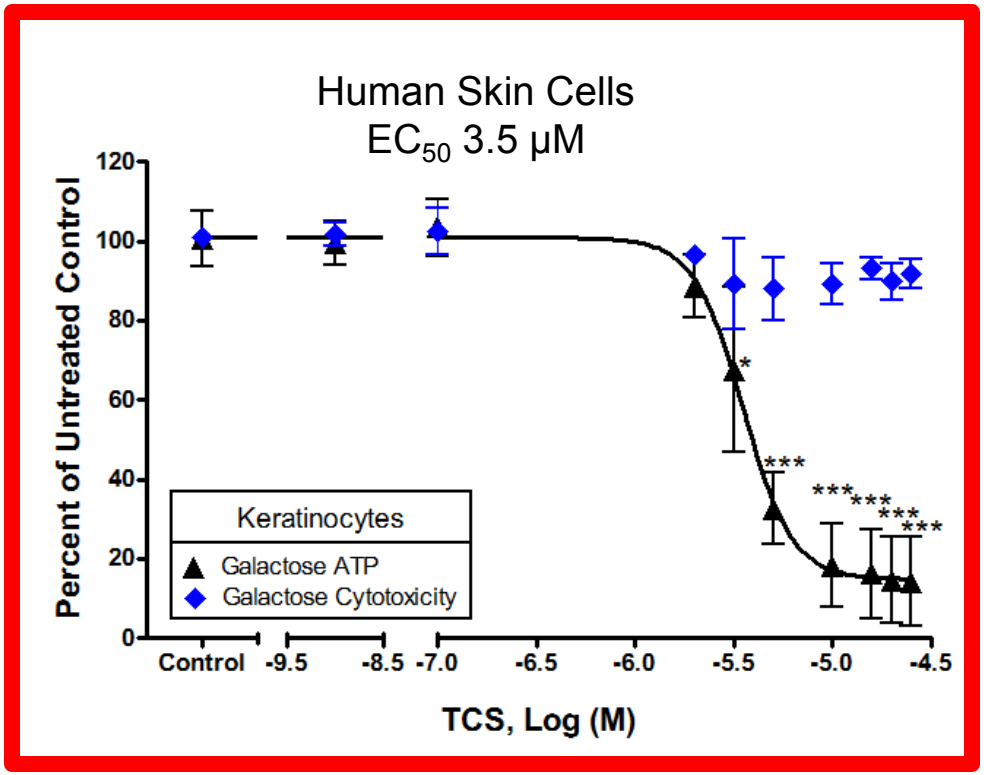
- One of the most potent known mitochondrial uncoupler, CCCP, only ~ 10-fold more potent than TCS
- EC_{50} 0.8 µM (RBL), 1.2 µM (HMC)

*** $p < 0.001$

(Weatherly, LM *et al.*, *Journal of Applied Toxicology*, 2015)

TCS decreases ATP production in multiple cell types





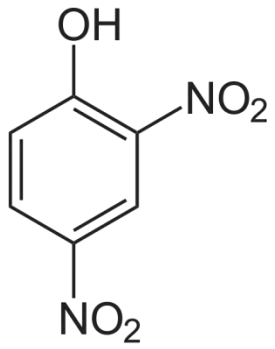
- One of the most potent known mitochondrial uncoupler, CCCP, only ~ 10-fold more potent than TCS
- EC_{50} 0.7 µM (mouse), 0.35 µM (human skin)

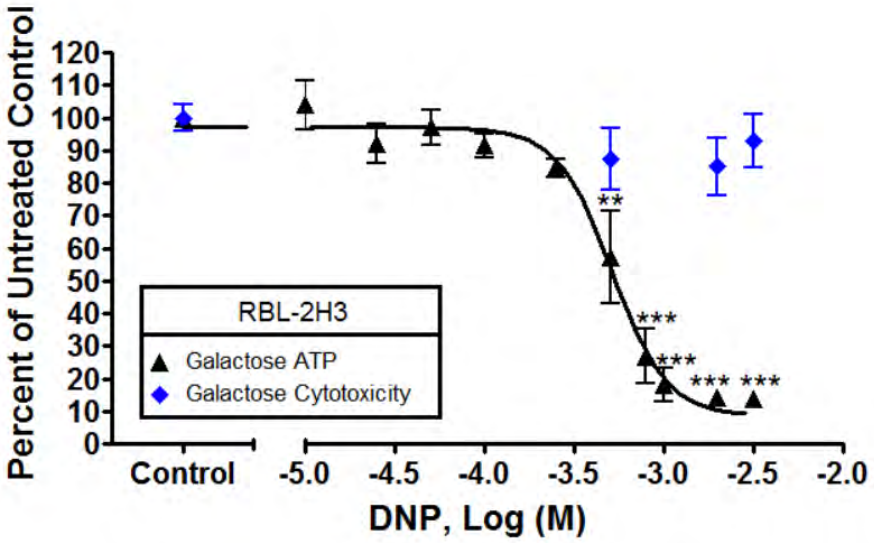
* $p < 0.05$, *** $p < 0.001$

(Weatherly, LM *et al., Journal of Applied Toxicology*, 2015)

TCS is 60X more toxic to mitochondria than DNP

- 2,4-Dinitrophenol
- Used as diet drug in 1930's
- Banned due to toxic effects 1938
  - Cataracts, Skin lesions, Cardiovascular system, Kidney, Death (Poole 1934 *mShpere*; Boardman 1935 *Cal West Med*; Tainter 1933 *Am J Public Health Nations Health*)





Cell Type	Toxicant	EC_{50}
Rat mast cells	TCS	8.6 µM
Rat mast cells	DNP	533 µM

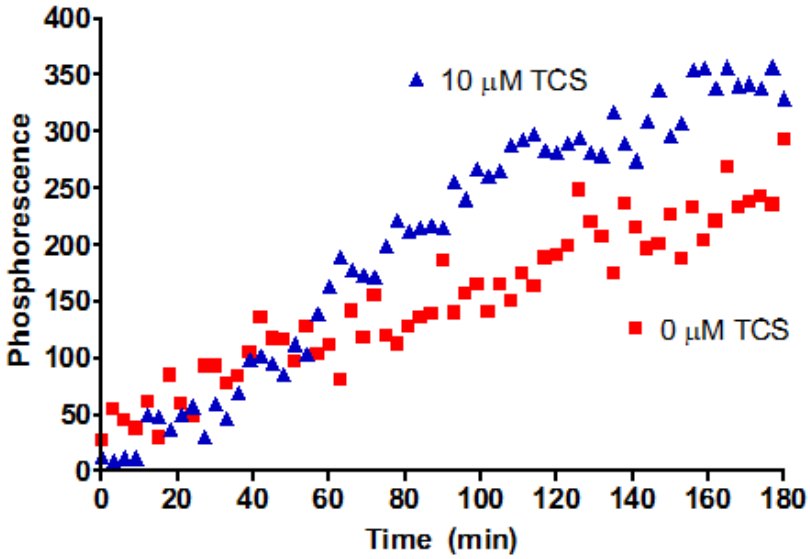
- Similar results found in primary human keratinocytes

** p < 0.01, *** p < 0.001

(Weatherly LM and Nelson, *et al.*, *TAAP*, 2018)

TCS increases oxygen consumption rate

A)

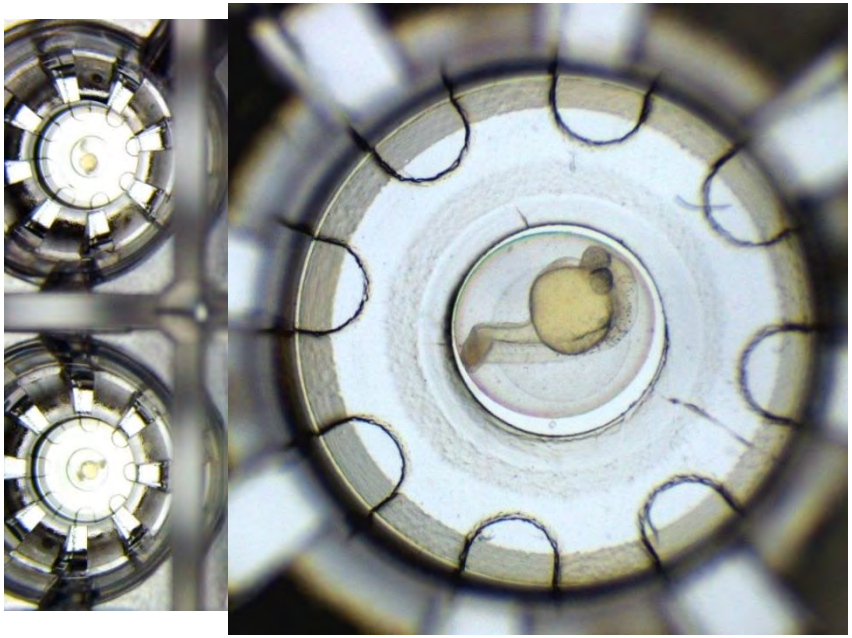


- CCCP increases OCR slope to ~ 1.5 at 1 μM

** $p < 0.01$

(Weatherly, LM *et al.*, *Journal of Applied Toxicology*, 2015)

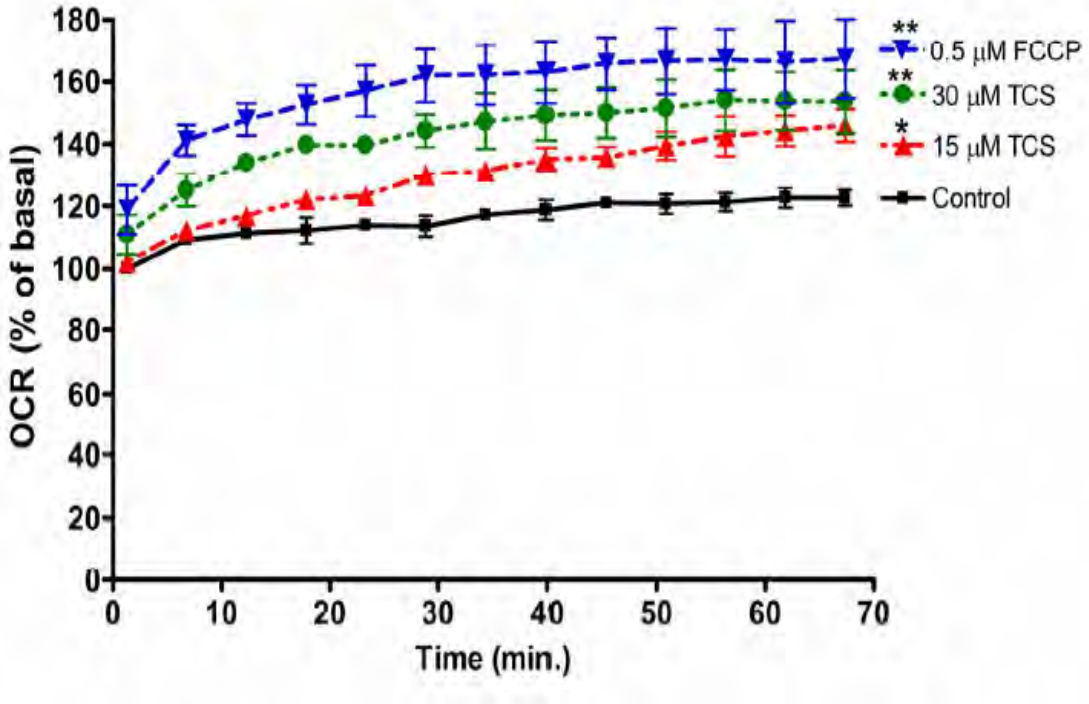
Is TCS Mitochondrial uncoupler *(in vivo)?*



1) Temperature control (28-29 °C)
2) 96 well format
   i. Seahorse XFe 96 Extracellular Flux Analyzer
   ii. 96 well spheroids plate
3) Single embryo/well

(Shim *et al.*, *JAT*, 2016)

TCS increases oxygen consumption *in vivo*

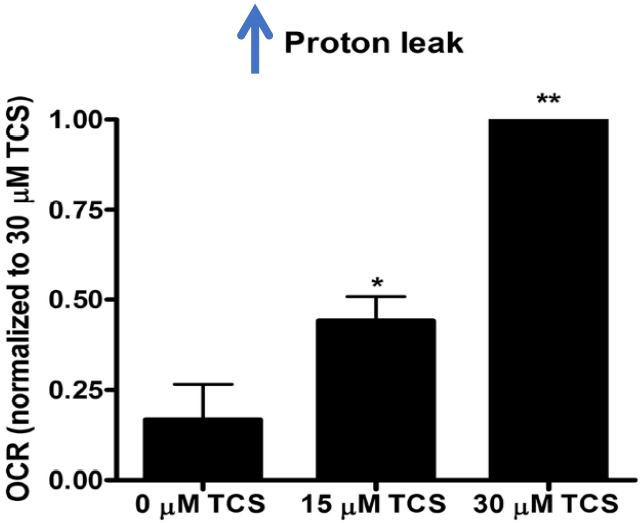


One-way ANOVA with Dunnett's post-tests; *p< 0.05, **p< 0.01.

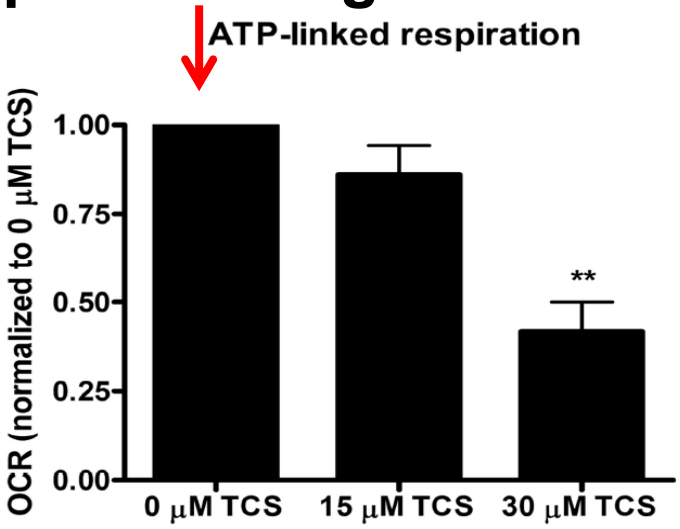
→ Utilized TCS doses did not cause mortality

(Shim *et al.*, *JAT*, 2016)

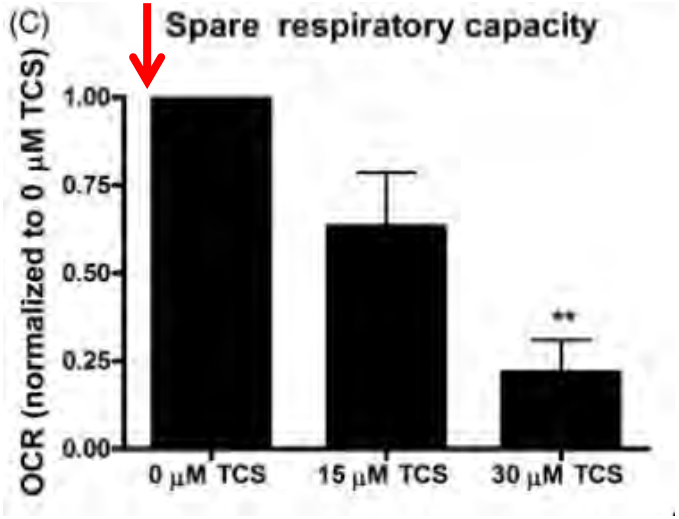
TCS as a mitochondrial uncoupler in living zebrafish



Mitochondrial uncoupling and inefficient ATP production



Respiration that is correlated with mitochondrial ATP production



Measurement of the organism's ability to respond to an increase in energy requirement, an indicator of cell fitness or flexibility.

- One-way ANOVA with Dunnett's post-tests; *p< 0.05, **p< 0.01

(Shim *et al.*, *JAT*, 2016)

Triclosan is a mitochondrial uncoupler

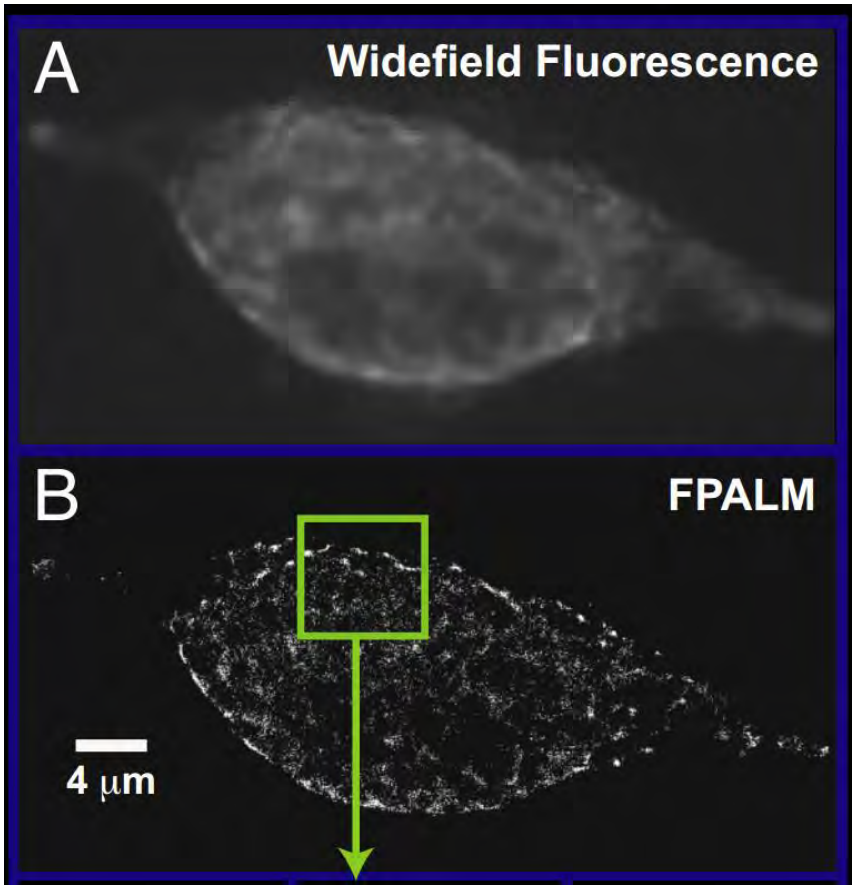
- ✓ Decrease ATP production
- ✓ Increase Oxygen Consumption Rate
- ✓ Decrease mitochondrial membrane potential

- ✓ Multiple cell types, species
- ✓ *In vivo*, in zebrafish
- ✓ Ionizable proton

Does TCS also distort mitochondrial shape?

(Shim *et al.*, *JAT*, 2016)

Super-resolution Microscopy – Fluorescence Photoactivation Localization Microscopy (FPALM)



(Hess 2007 *Proc Natl Acad Sci*)

Invented by Prof. Samuel Hess at UMaine

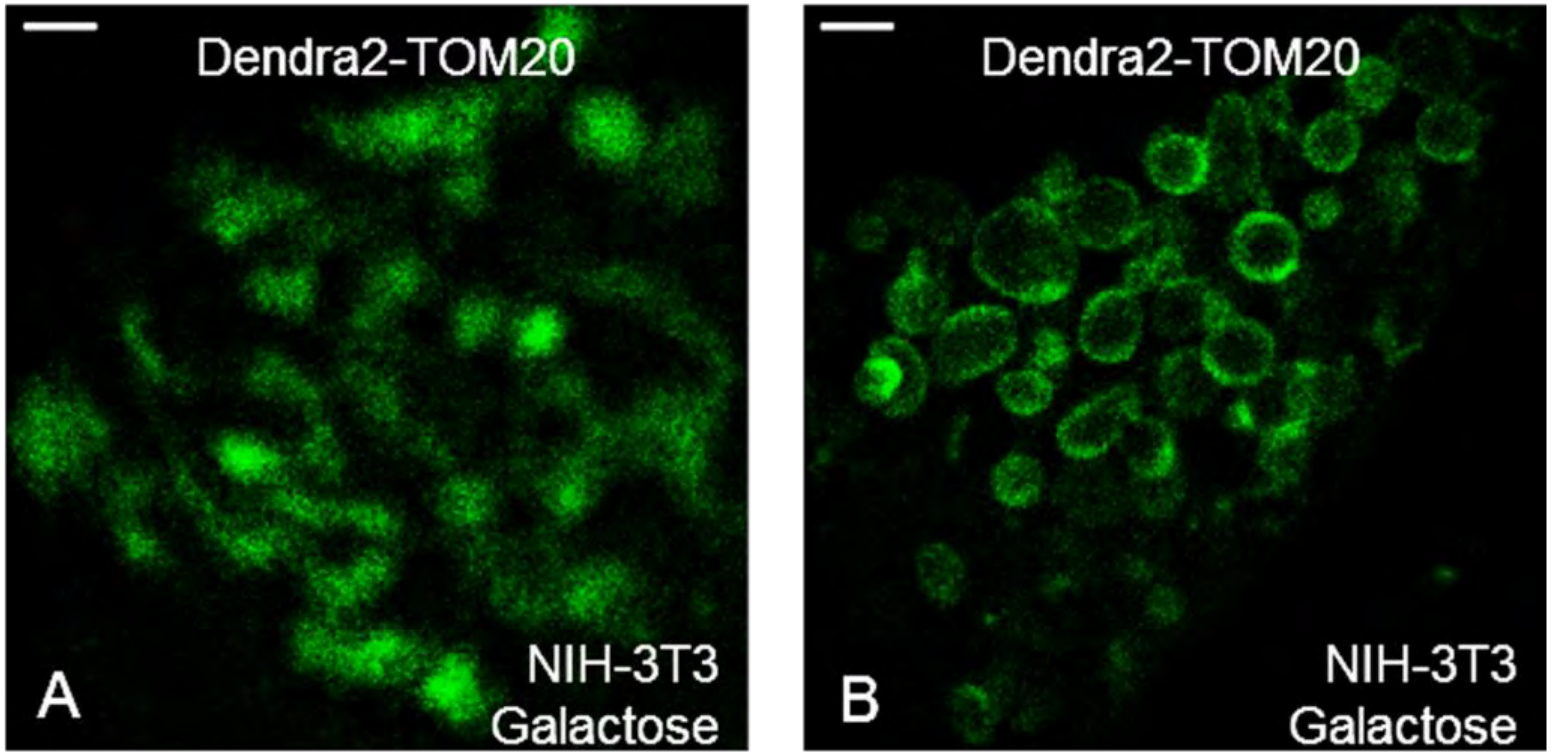
Confocal resolution 200-250 nm

FPALM resolution 20-40 nm

TCS distorts mitochondrial shape in mouse fibroblasts in galactose media

Control TCS

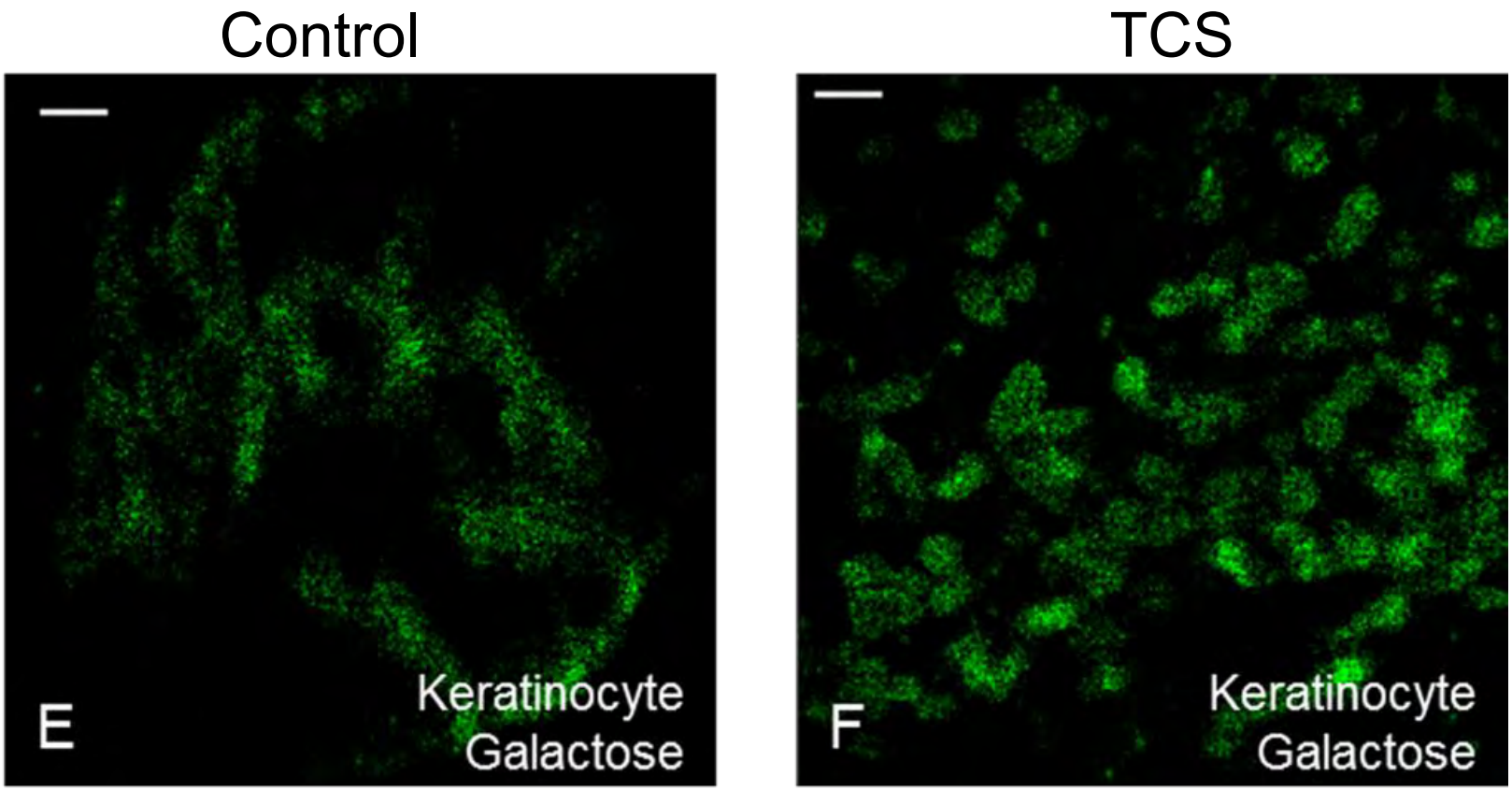
Scale bar 1 µm



- Known mitochondrial uncouplers also cause donut-shaped mitochondria

(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

TCS distorts mitochondrial shape in primary human skin cells in galactose media

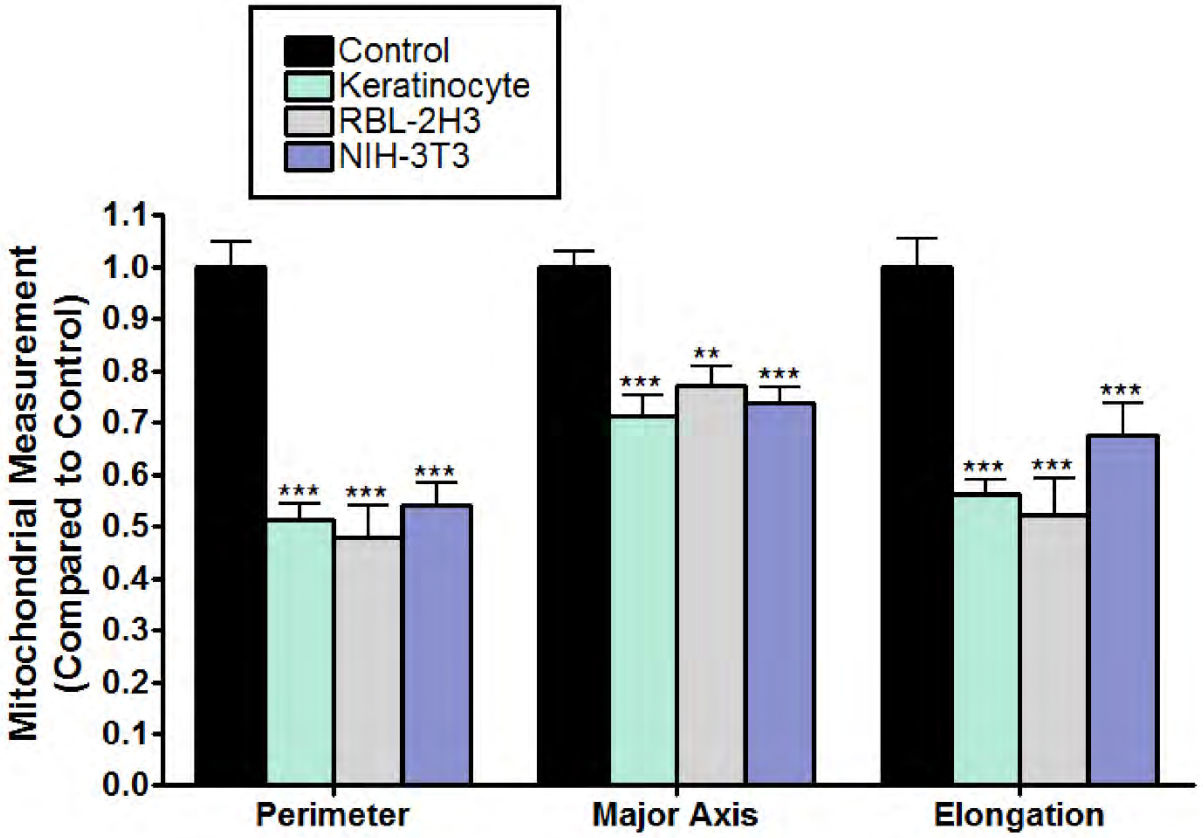


- Known mitochondrial uncouplers also cause mitochondrial fission

Scale bar = 1 μm

(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

TCS distorts mitochondrial shape in multiple cell types

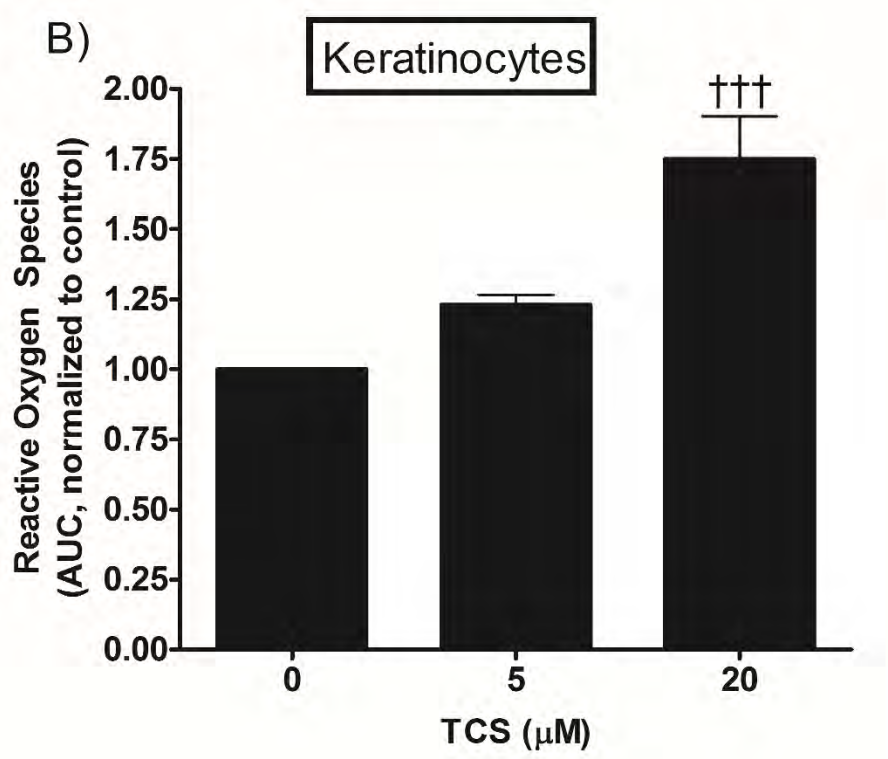


- data from cells imaged between 30 and 60 min after TCS exposure combined for each cell type.
- each parameter was first normalized to the average of the corresponding control
- normalized values were combined and presented as means ± SEM
- data from 4 to 22; at least 2 independent days of imaging
- 3D FPALM: TCS increases mitochondrial surface area and volume in mast cells

**p < 0.01 and ***p < 0.001, as determined by one-way ANOVA followed by Tukey's post-hoc test.

(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

TCS increases cytosolic reactive oxygen species production in primary human skin cells



- Similar results found with rat mast cells
- Biochemical mechanism in distortion of mitochondrial morphology

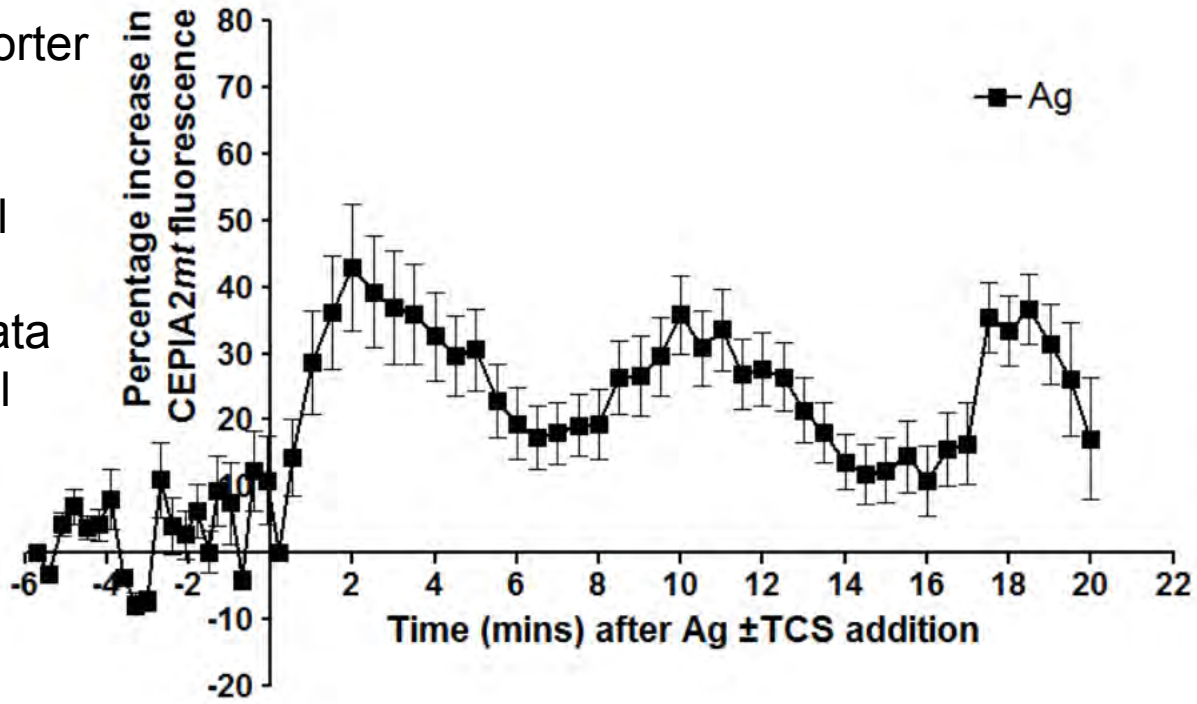
Increased ROS linked to mitochondrial fission (Fan 2010 *Free Radic Biol Med*; Deheshi 2015 *J Neurochem*)

*** $p < 0.001$

(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

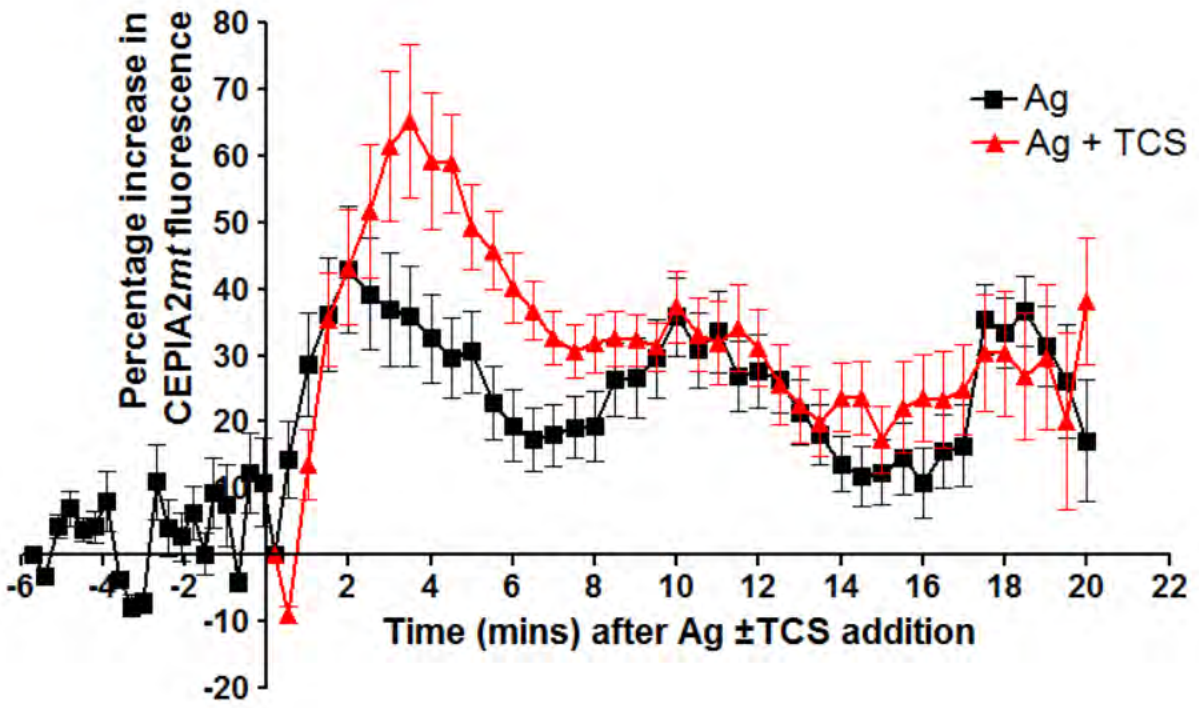
TCS alters mitochondrial calcium levels of rat mast cells

- Genetically encoded reporter construct specific for mitochondrial calcium
- Single-cell data from confocal microscopy



(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

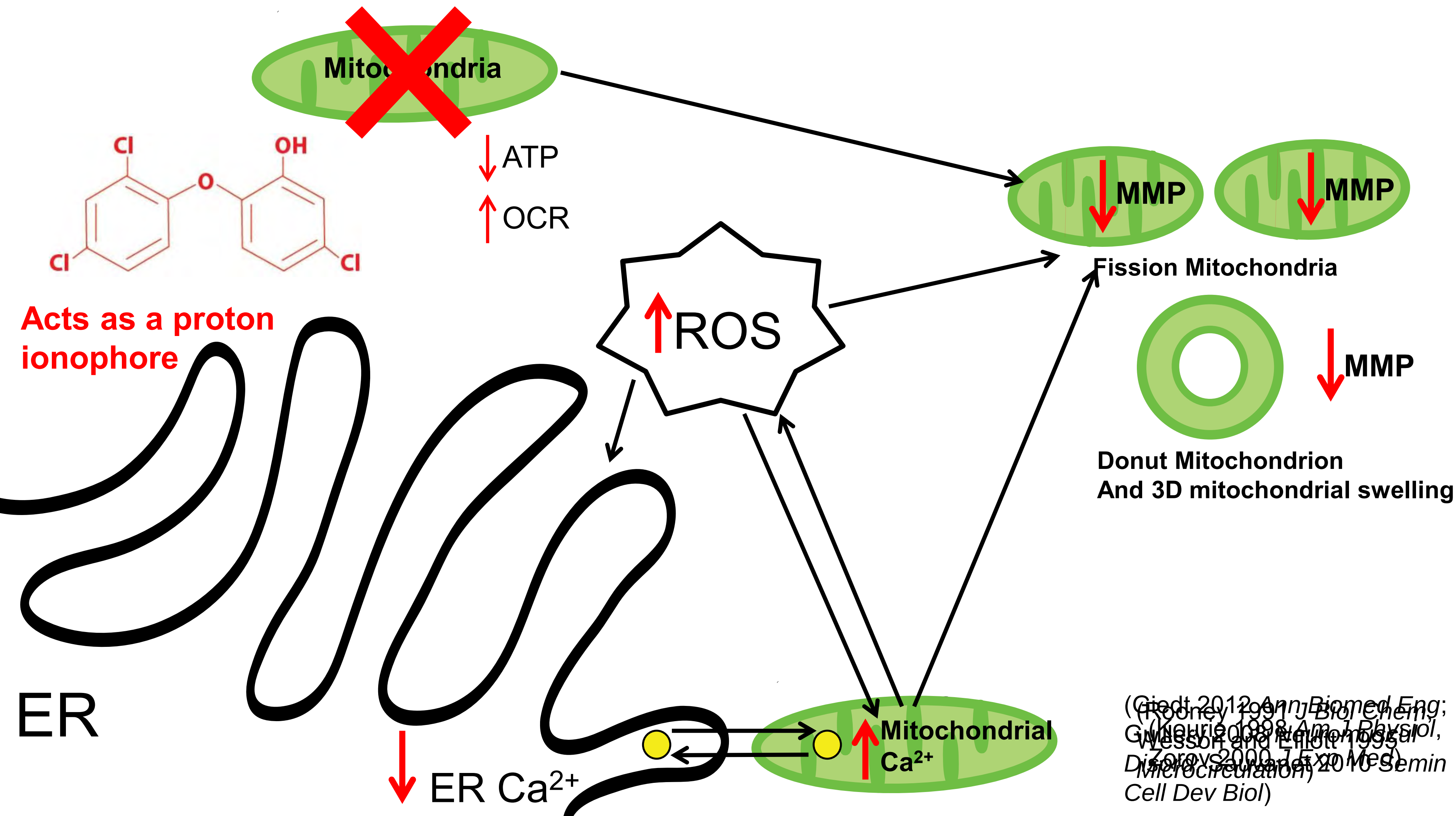
TCS alters mast cell mitochondrial Ca^{2+} levels



- Disruption of Ag-stimulated calcium oscillations
- Enhanced mitochondrial calcium related to mitochondrial fission (Ahmad 2013 *Cell Death Dis*; Kaddour-Djebbar 2010 *Int J Oncol*)

(Weatherly LM, Nelson AJ, *et al.*, *TAAP*, 2018)

Summary: TCS effects on mitochondria



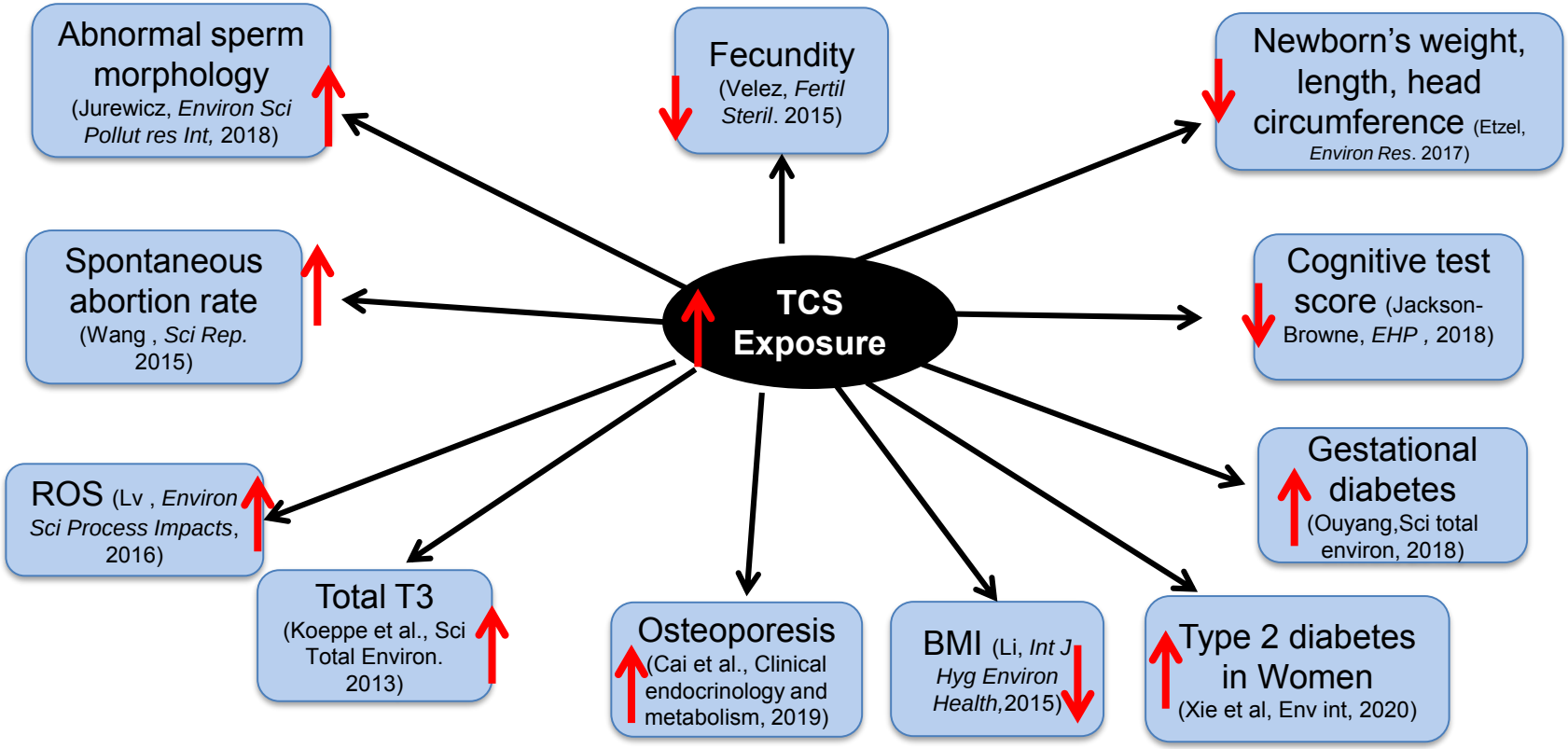
(Giedt 2012 *Ann Biomed Eng*; Guillery 2008 *Neuromuscul Disord*; Sauvanet 2010 *Semin Cell Dev Biol*)

(Rooney 1991 *J Biol Chem*, Wesson and Elliott 1995 *Microcirculation*)

(Kourie 1998 *Am J Physiol*, Zorov 2000 *J Exp Med*)

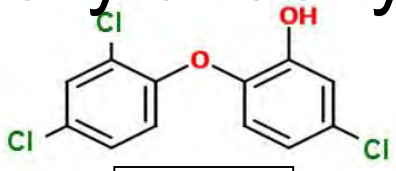
TCS Human Health Effects

documented ~2013-onward

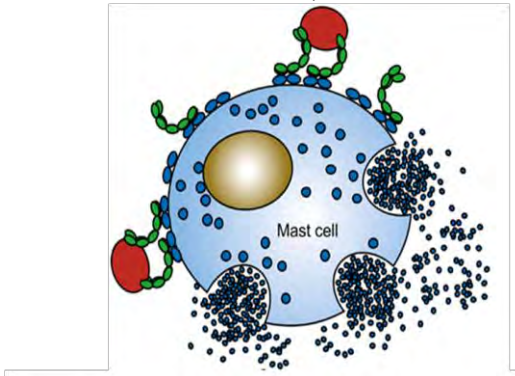


Adapted from Weatherly et al., *JTEHB*, 2017

TCS signaling toxicity underlying mechanisms



Triclosan Exposure

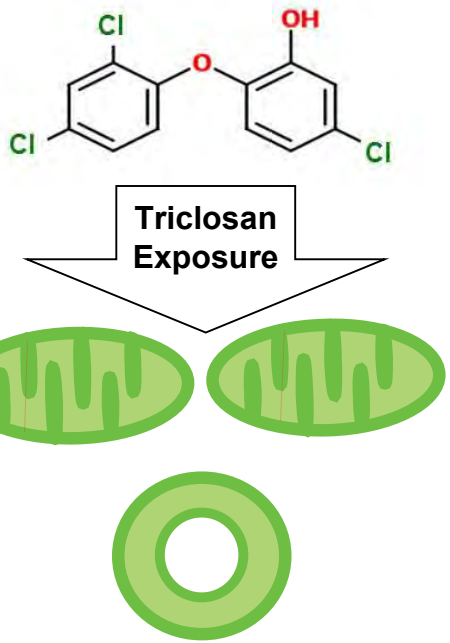


Mast Cell Dysfunction

Affect Biological Functions

Immune effects

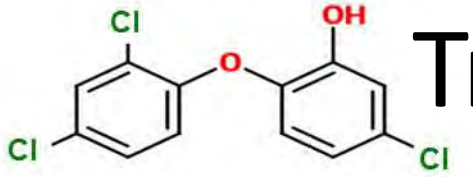
TCS mitochondrial toxicity & ROS generation underlying mechanisms



Mitochondrial Dysfunction

Affect Biological Functions

Reproductive/Developmental Defects
Change in Bioenergetic State



Triclosan (TCS) timeline

PCP (toothpaste, soap, mouthwash) production ~ 1 million pounds

FDA "bans" TCS from PCP soap products 2016; from hospital soaps 2017

1970

1995

2000

2015

Antimicrobial agent TCS use in hospital soaps started in 1972

75% of liquid/foam soaps contained TCS;
93% by 2010

Unfortunate that the positive use of TCS as an anti-gingival was negated by overuse? –> this benefit may have outweighed the risk

2019: FDA "bans" TCS from hand sanitizer; Colgate Total toothpaste relaunch without triclosan in Super Bowl ad blitz

(U.S. EPA; Denmark EPA; Bloomberg; FDA 2013; Weatherly and Gosse *JTEHB*, 2017)

The Undergraduate Students:

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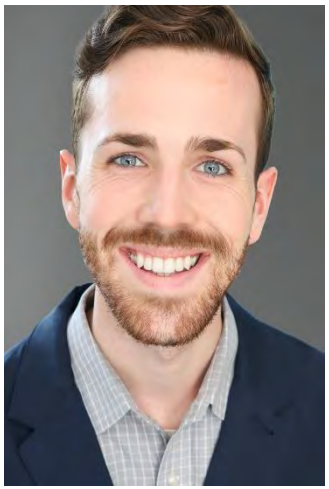
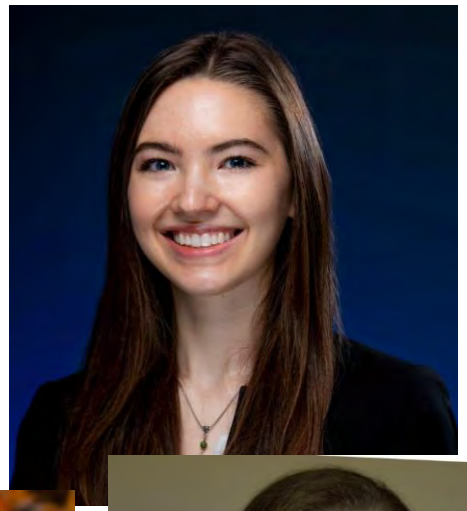



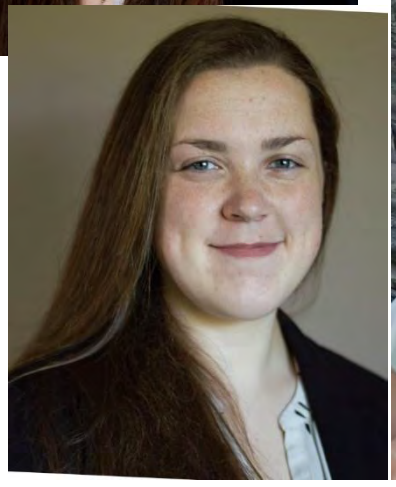

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