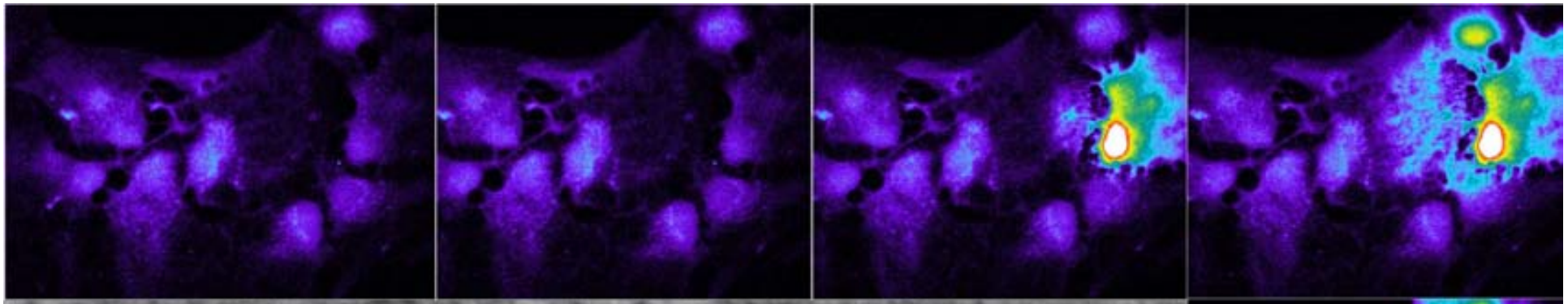

Implicació dels lisosomes en la mort cel·lular per inhibició de la síntesi de fosfatidilcolina i en la senyalització de calci per NAADP

Miquel Barceló Torns

TESI DOCTORAL

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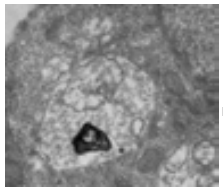
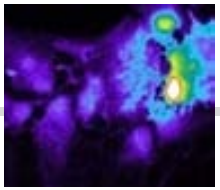
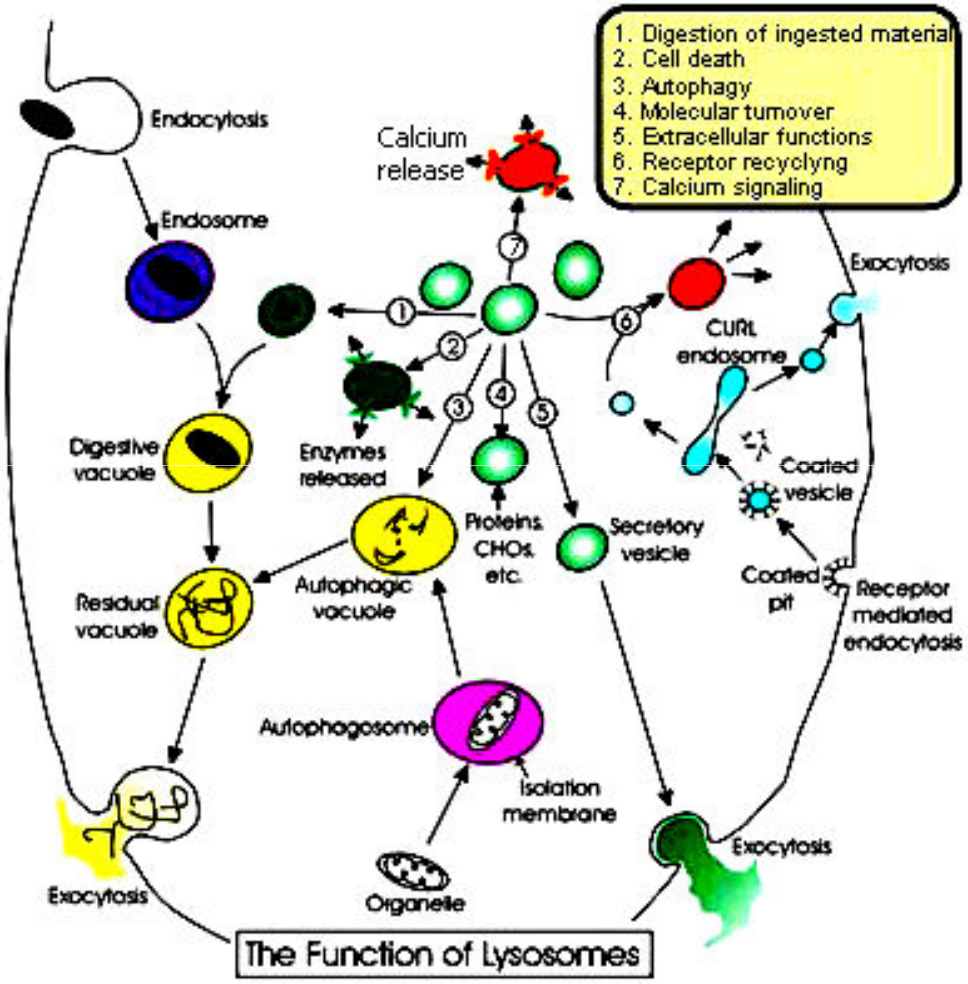
INc
Institut de
Neurociències



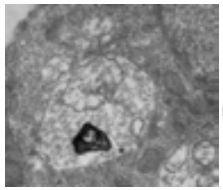


Functions of Lysosomes

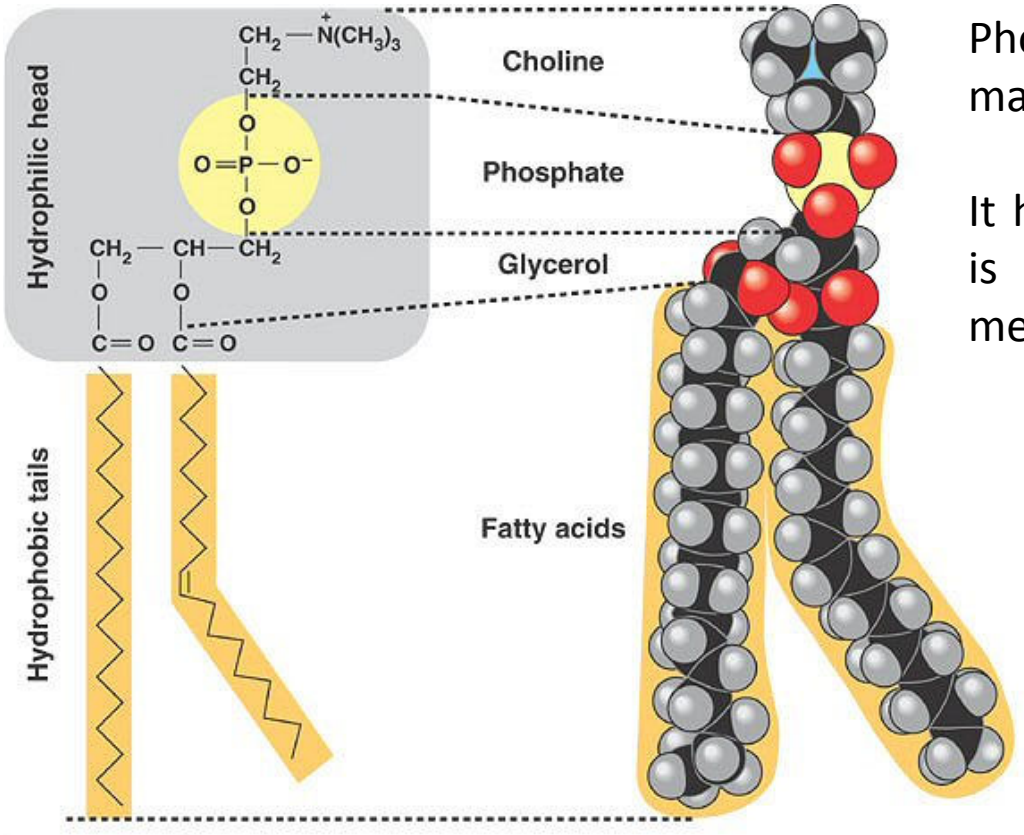
- Degradation of cell components
  - Cell death — 1st
  - Autophagy
- Exocytosis / Secretory pathway
  - Calcium signalling — 2nd



O'Bay DH., (2009)

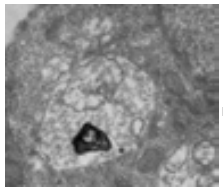


Phosphatidylcholine

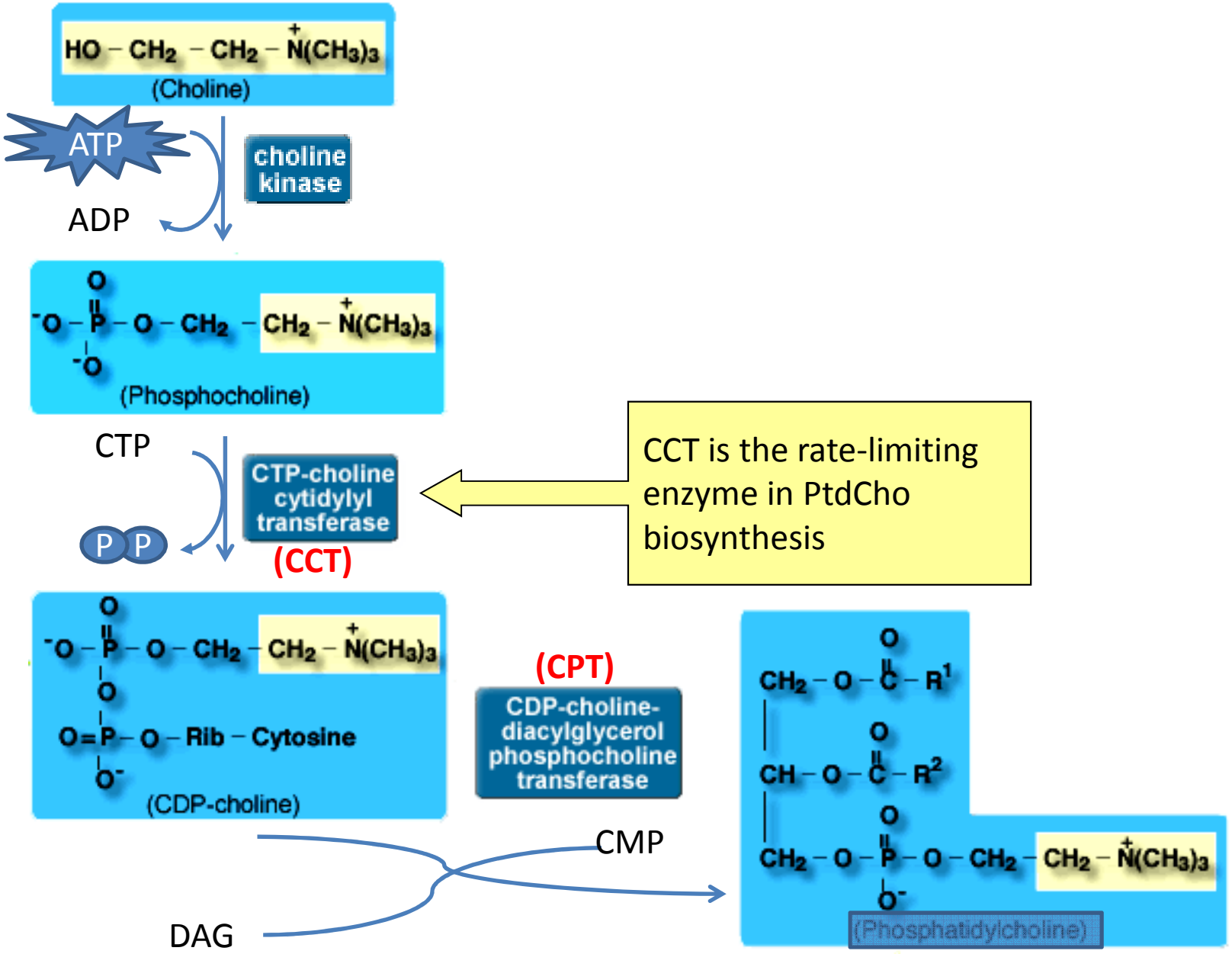


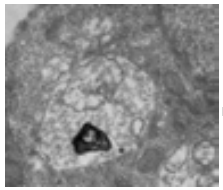
Phosphatidylcholine (PtdCho) is the major membrane phospholipid.

It has a prominent structural role, but is also a source of lipid second messengers.



The Kennedy pathway for PtdCho synthesis

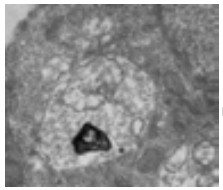




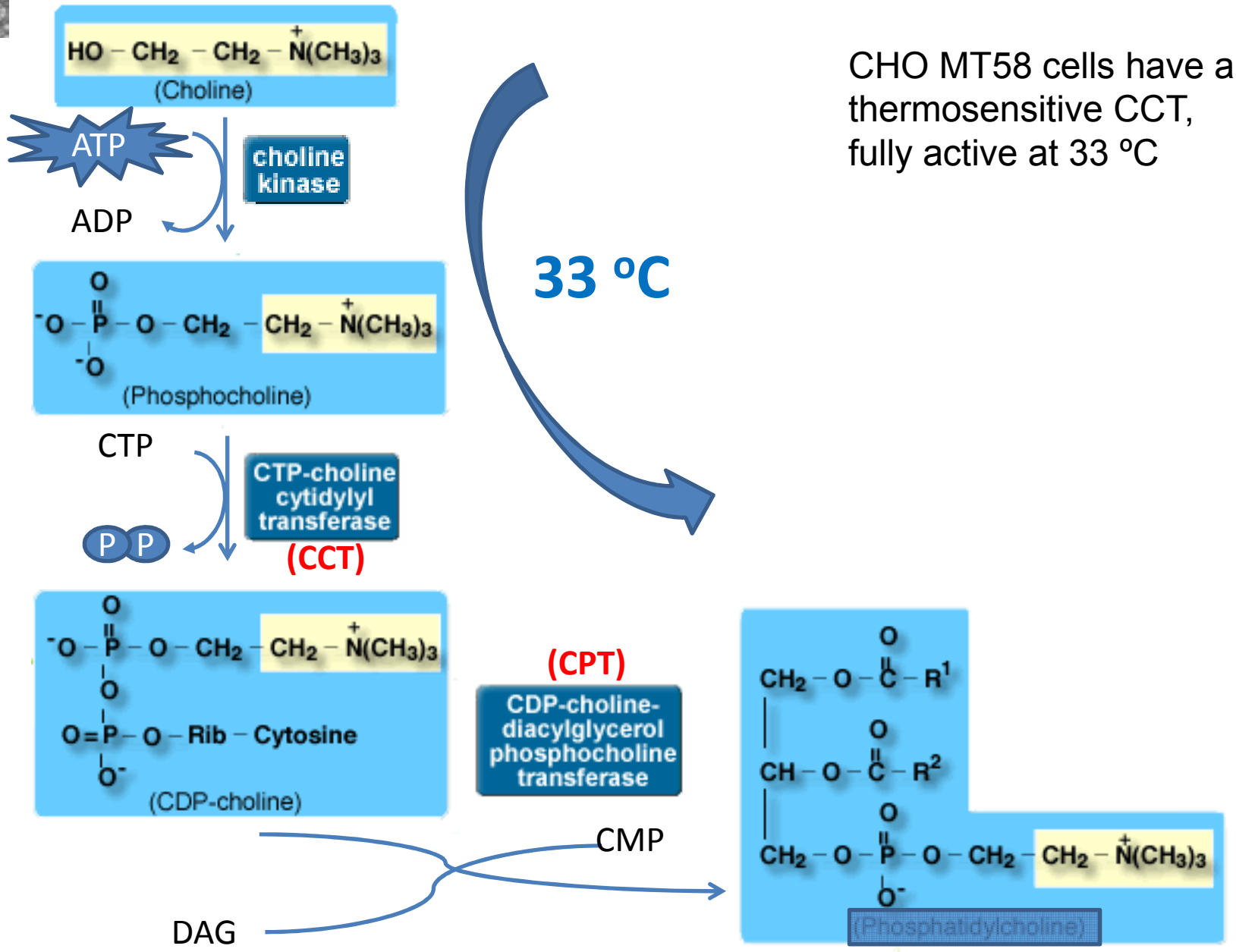
Disrupting PtdCho synthesis induces cell death

What type of cell death does occur?

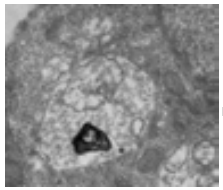
Choline deficiency		Yen et al. (1999) Shin et al.,(1997)
***Streptococcus pneumoniae* infection**		Zweigner et al., (2004)
Drugs (CCT inhibitors)	Hexadecylphosphocholine	Baburina and Jackowski (1998) Van der Sanden et al., (2004)
	ET-18-OCH_3	Boggs et al., (1995)
	C_2-Ceramide	Ramos et al., (2000; 2002; 2003)
CHO-MT58 cell line		Cui et al., (1996)



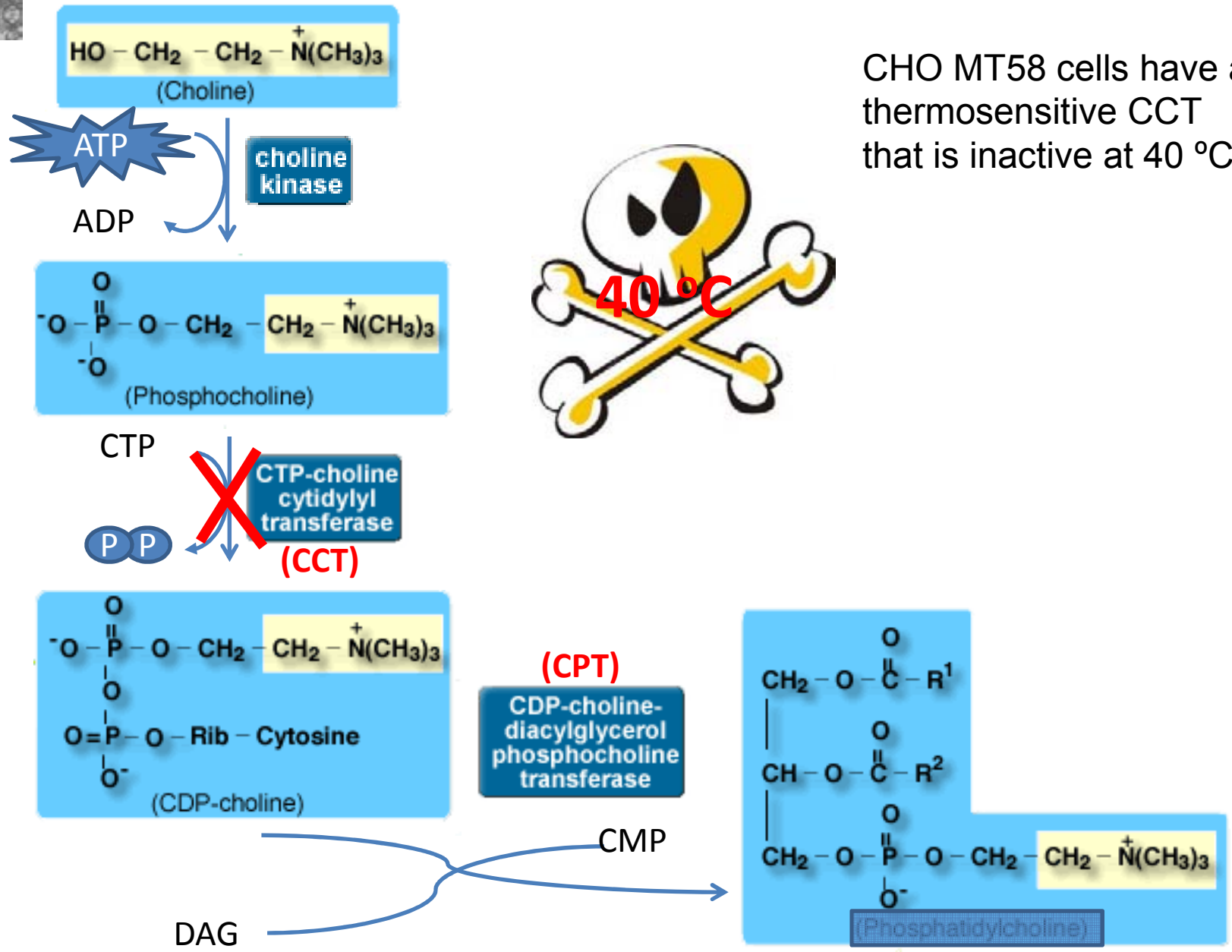
The CHO MT58 cell model



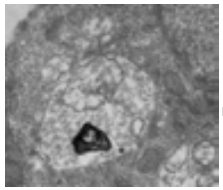
CHO MT58 cells have a thermosensitive CCT, fully active at 33 ºC



The CHO MT58 cell model

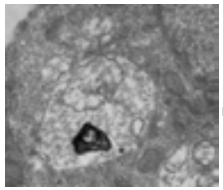


CHO MT58 cells have a thermosensitive CCT that is inactive at 40 ºC

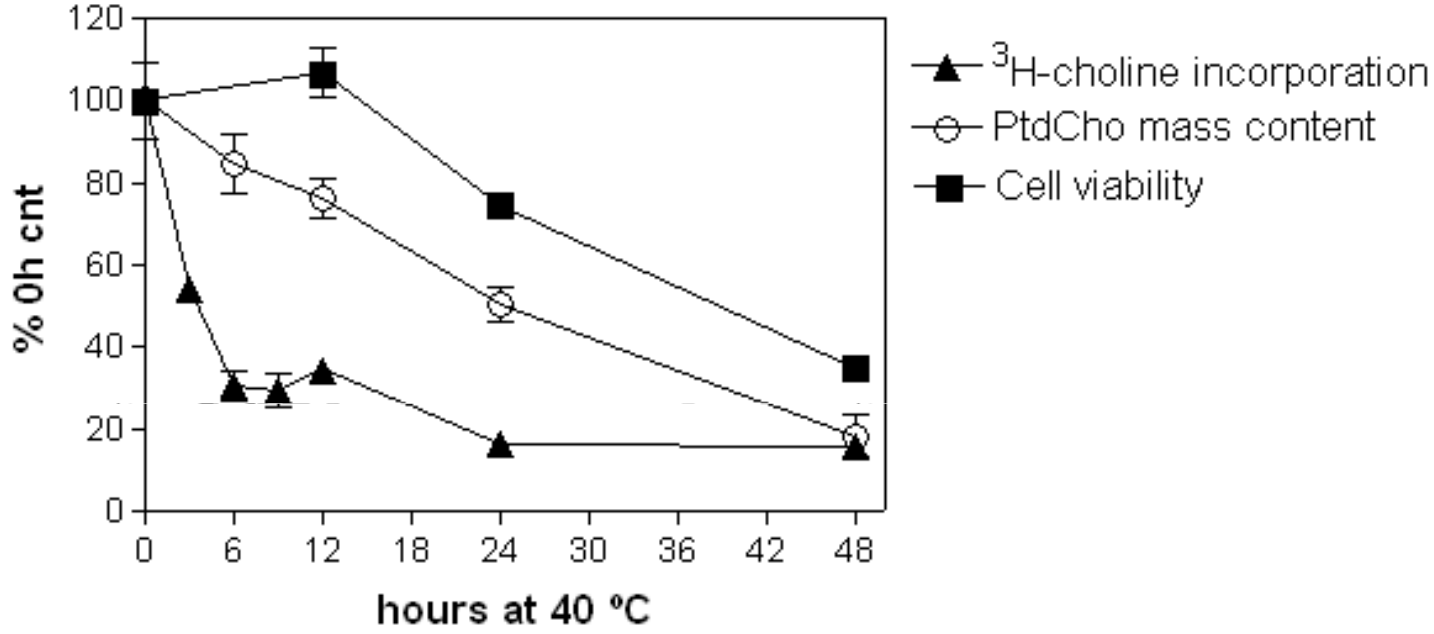


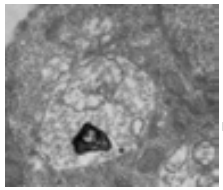
To characterize the cell death induced by inhibition of PtdCho synthesis in CHO-MT58 cells and to compare it to the canonical apoptosis induced by Actinomycin D.

To elucidate whether lysosomes and autophagy play a role in cell death caused by inhibition of PtdCho synthesis.



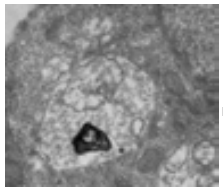
Cell death after thermal inhibition of CCT



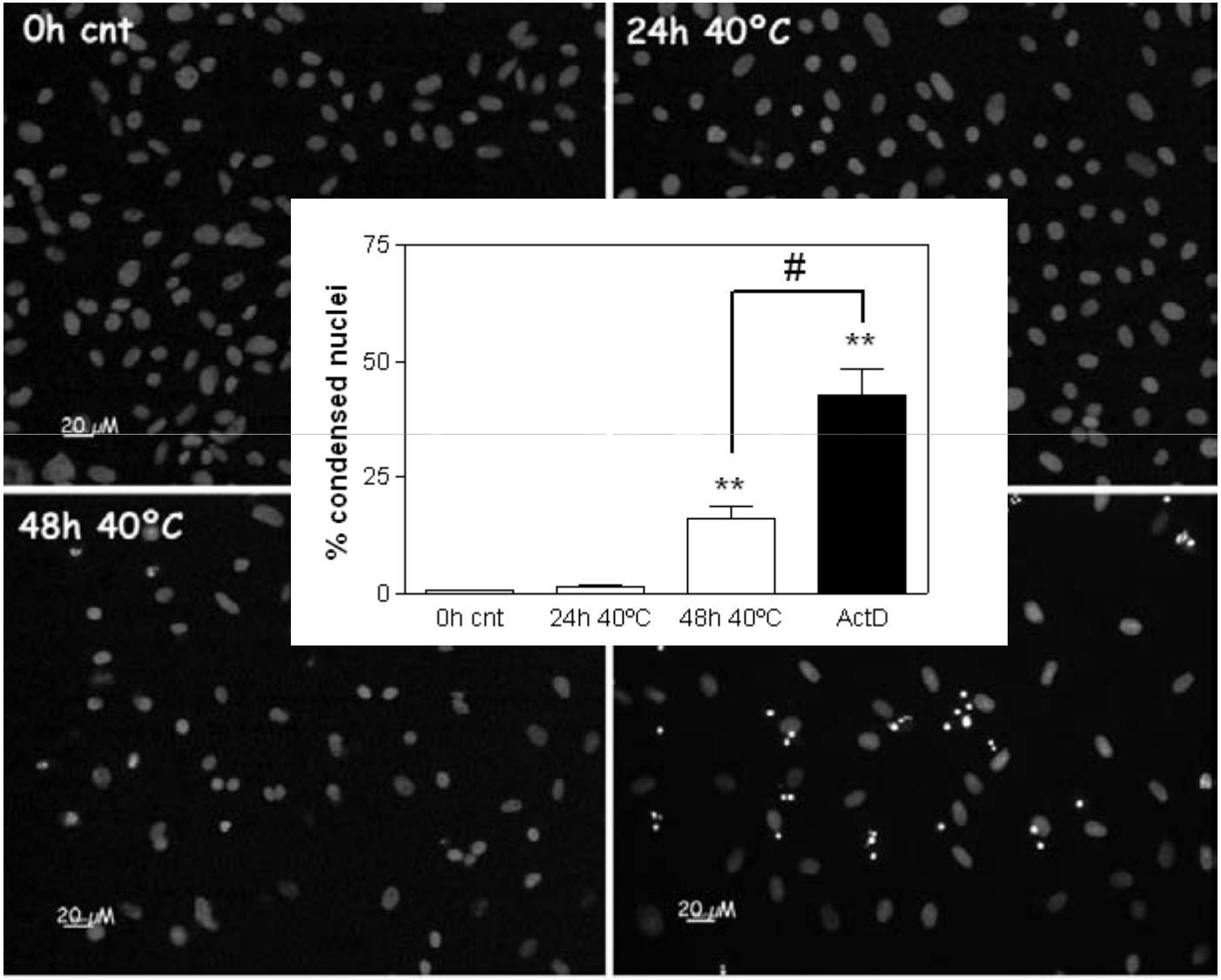


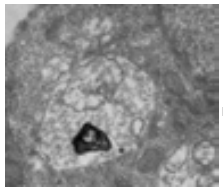
What is our apoptotic control?

Actimomycin D is a transcriptional inhibitor that induces a **canonical apoptotic death**

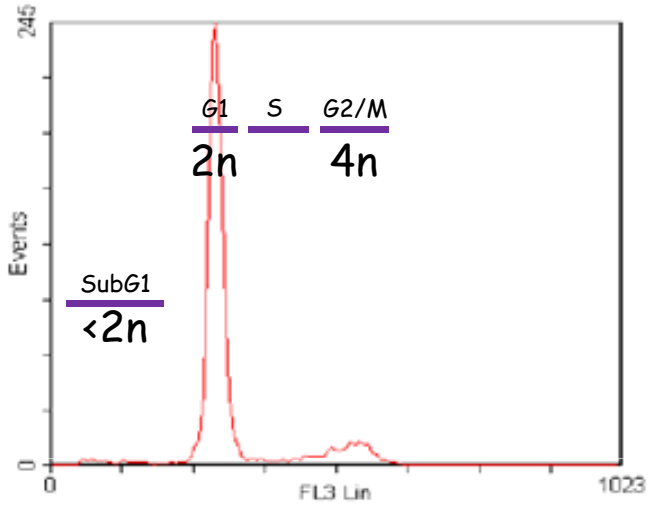


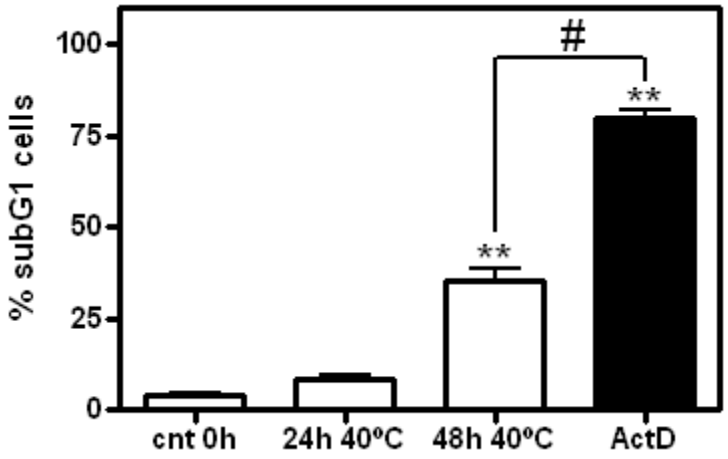
Little nuclear condensation

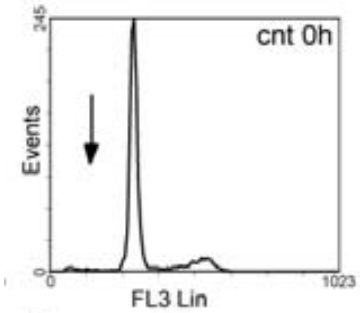


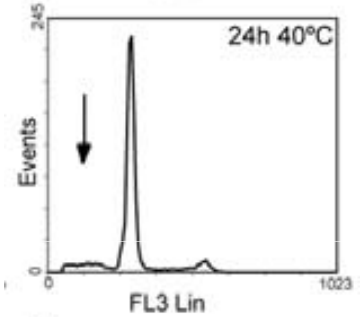


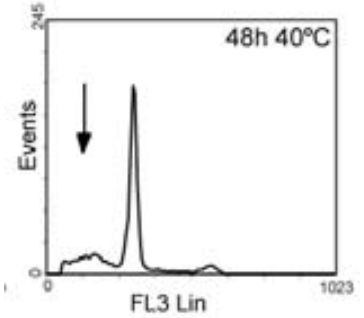
Little DNA fragmentation

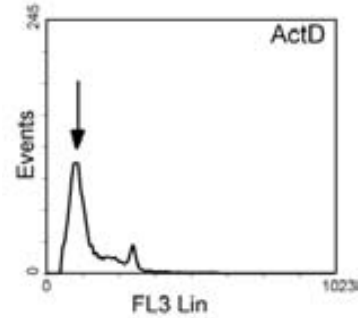


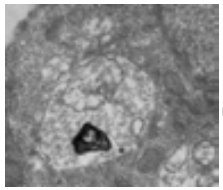




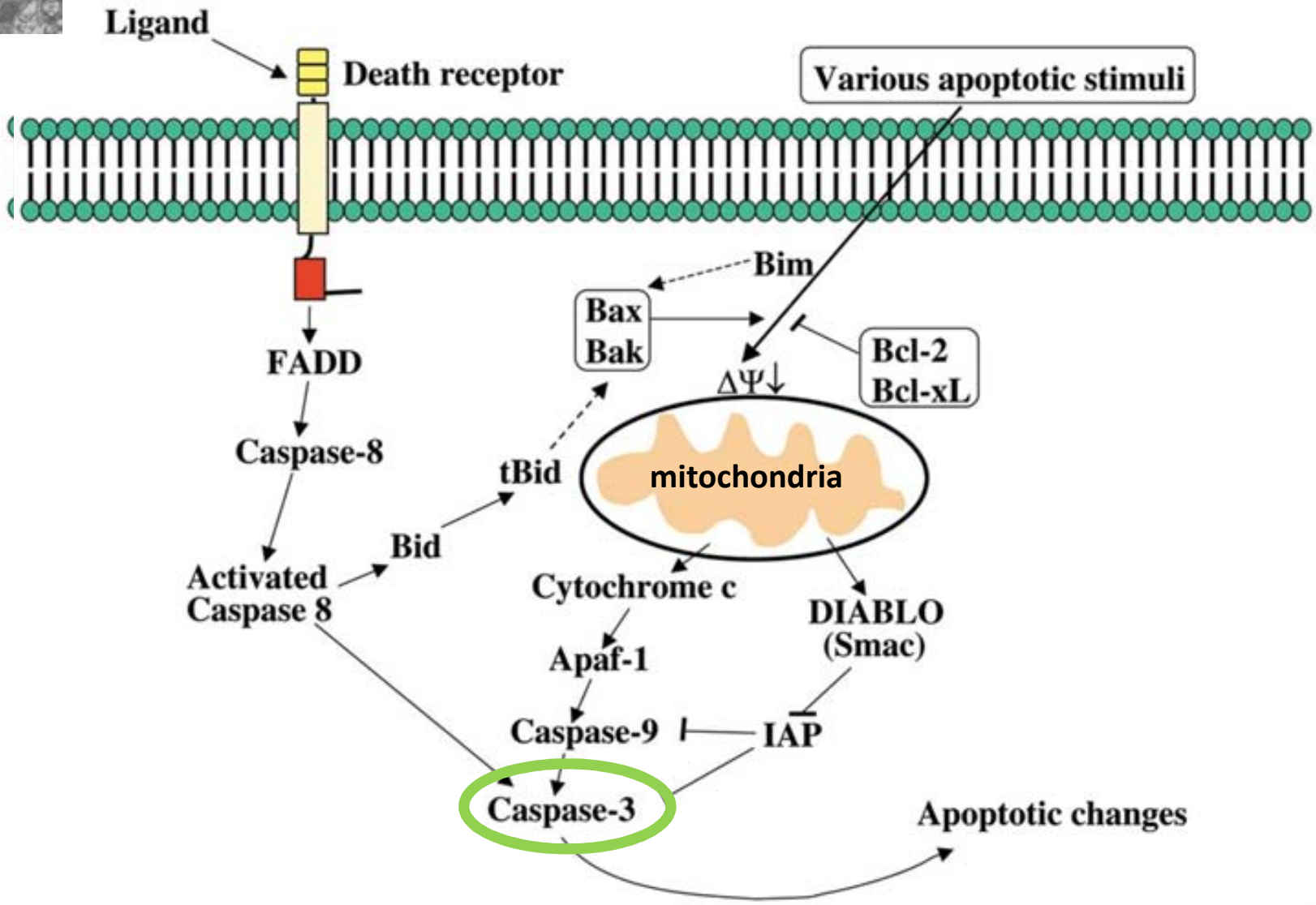




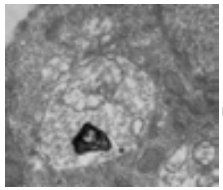




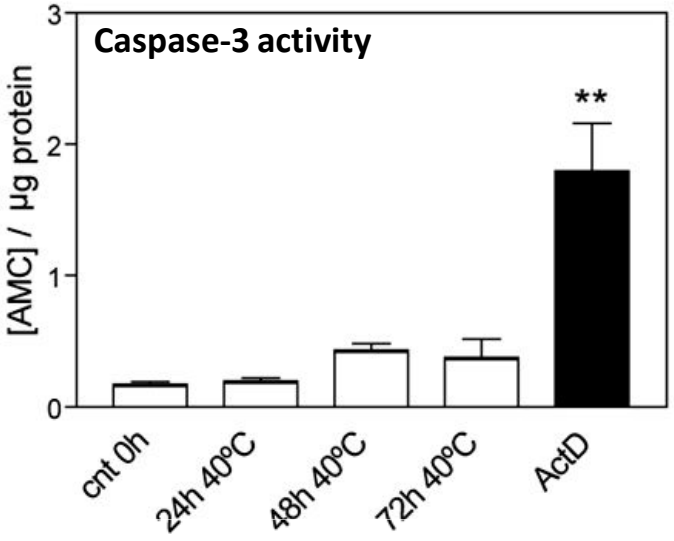
The role of caspase-3 in apoptosis

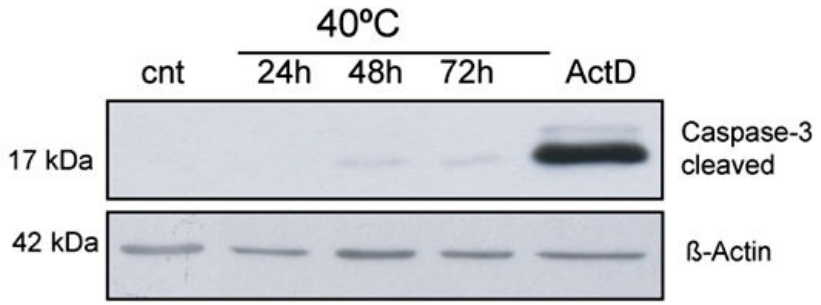


Mak and Yeh (2002)

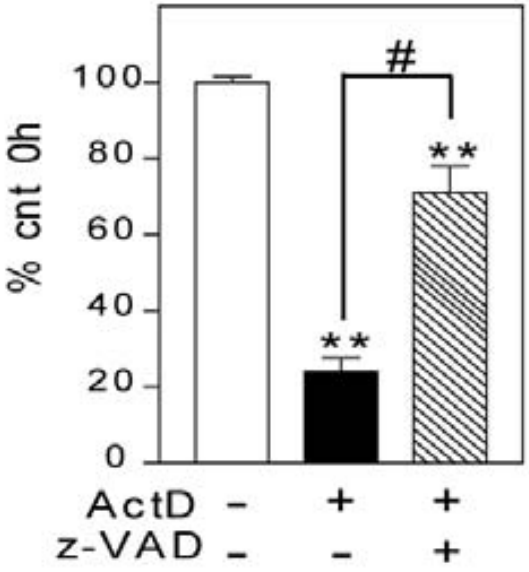


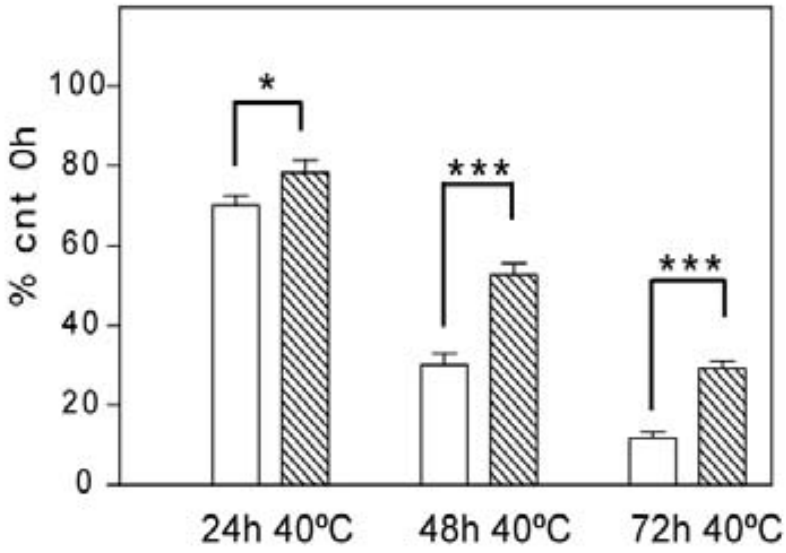
Caspase-3 is not activated

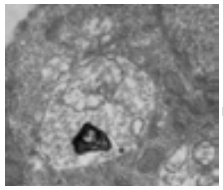




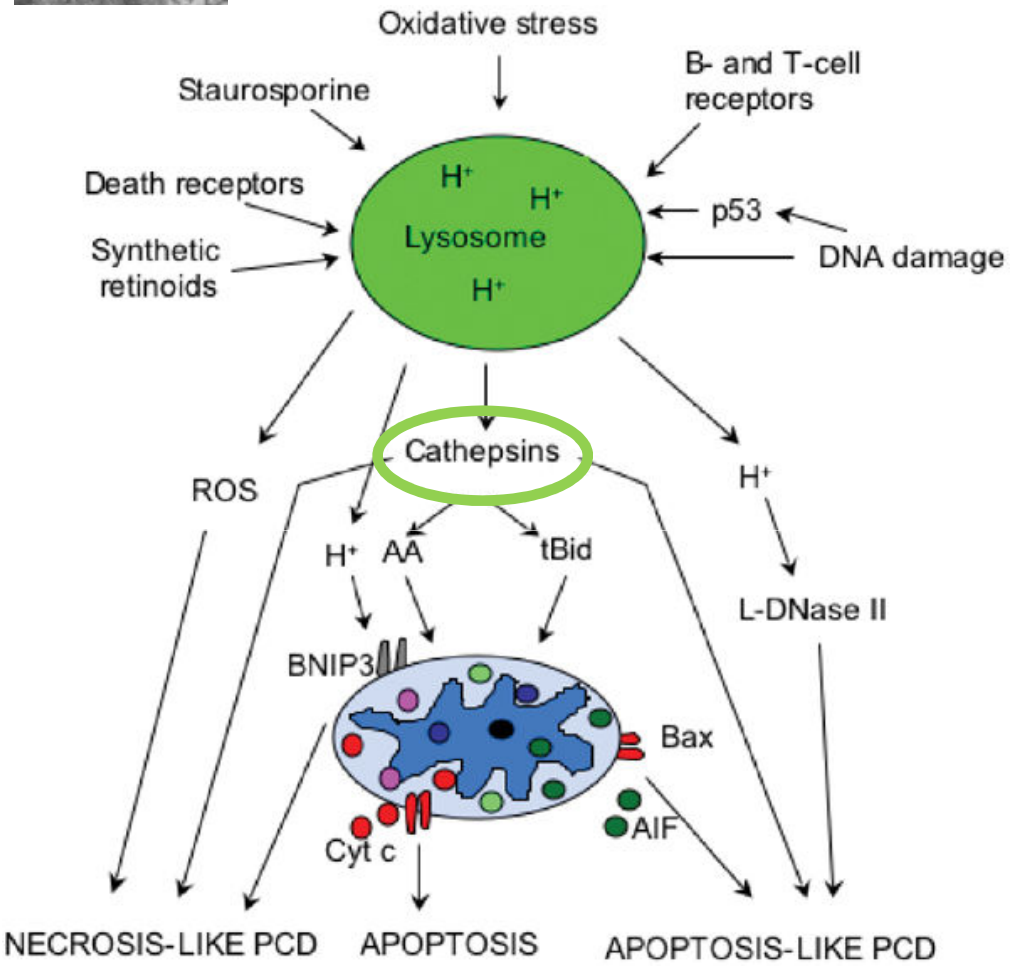
Cell viability

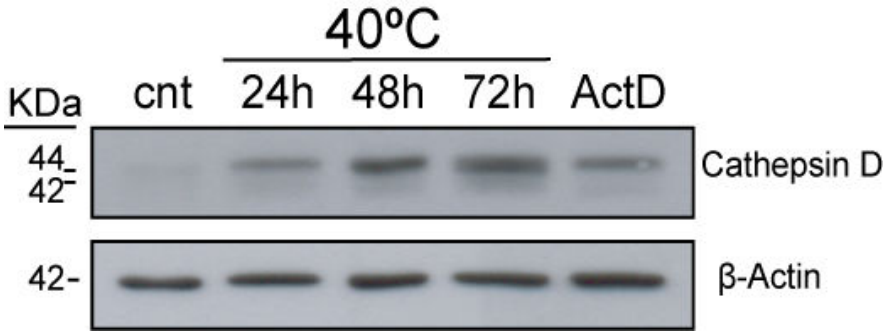






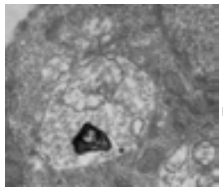
The role of cathepsins in cell death



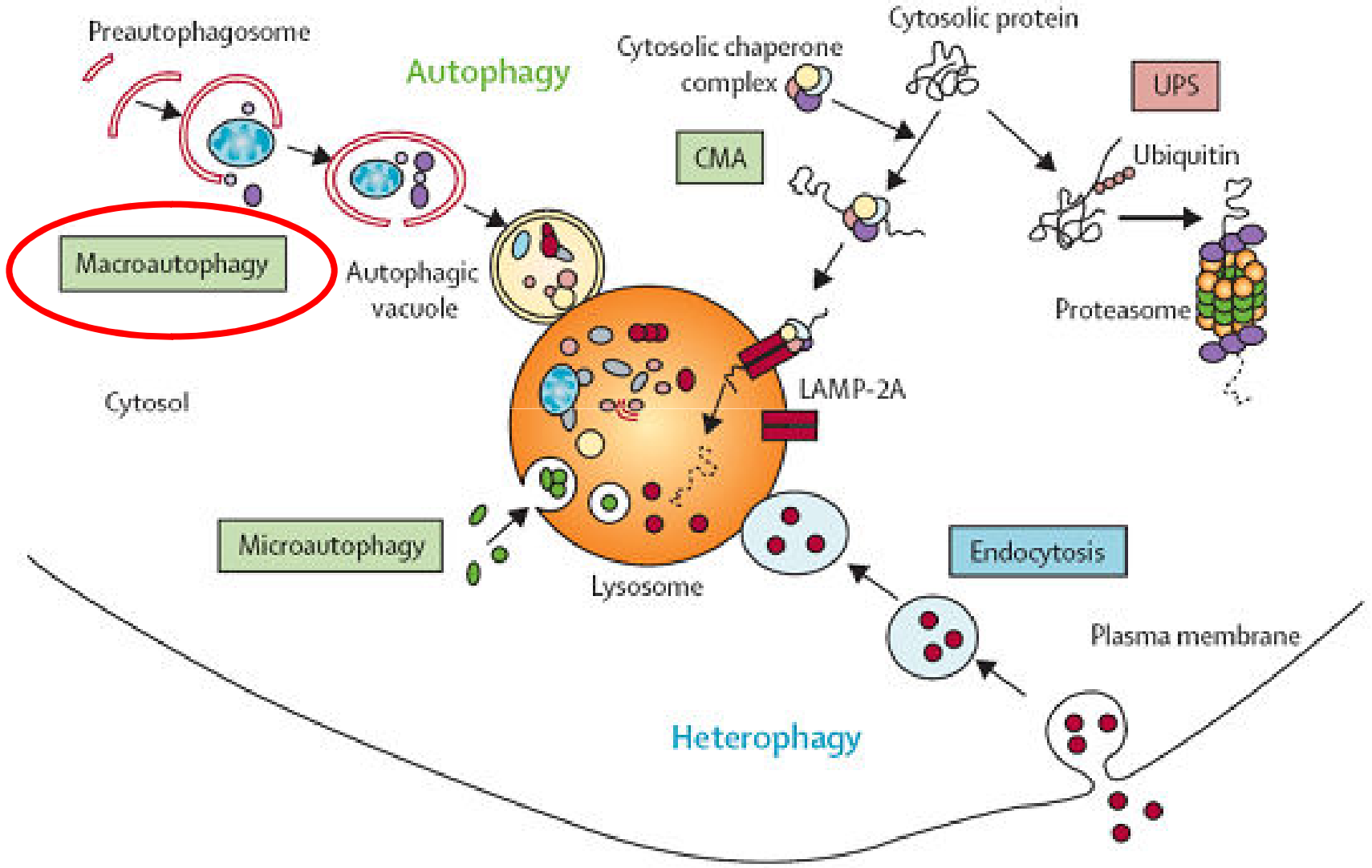


Increased Cathepsin D is a marker of autophagy

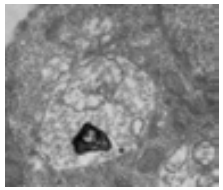
Jäättelä (2004)



Lysosomes are auto- and heterodigesters

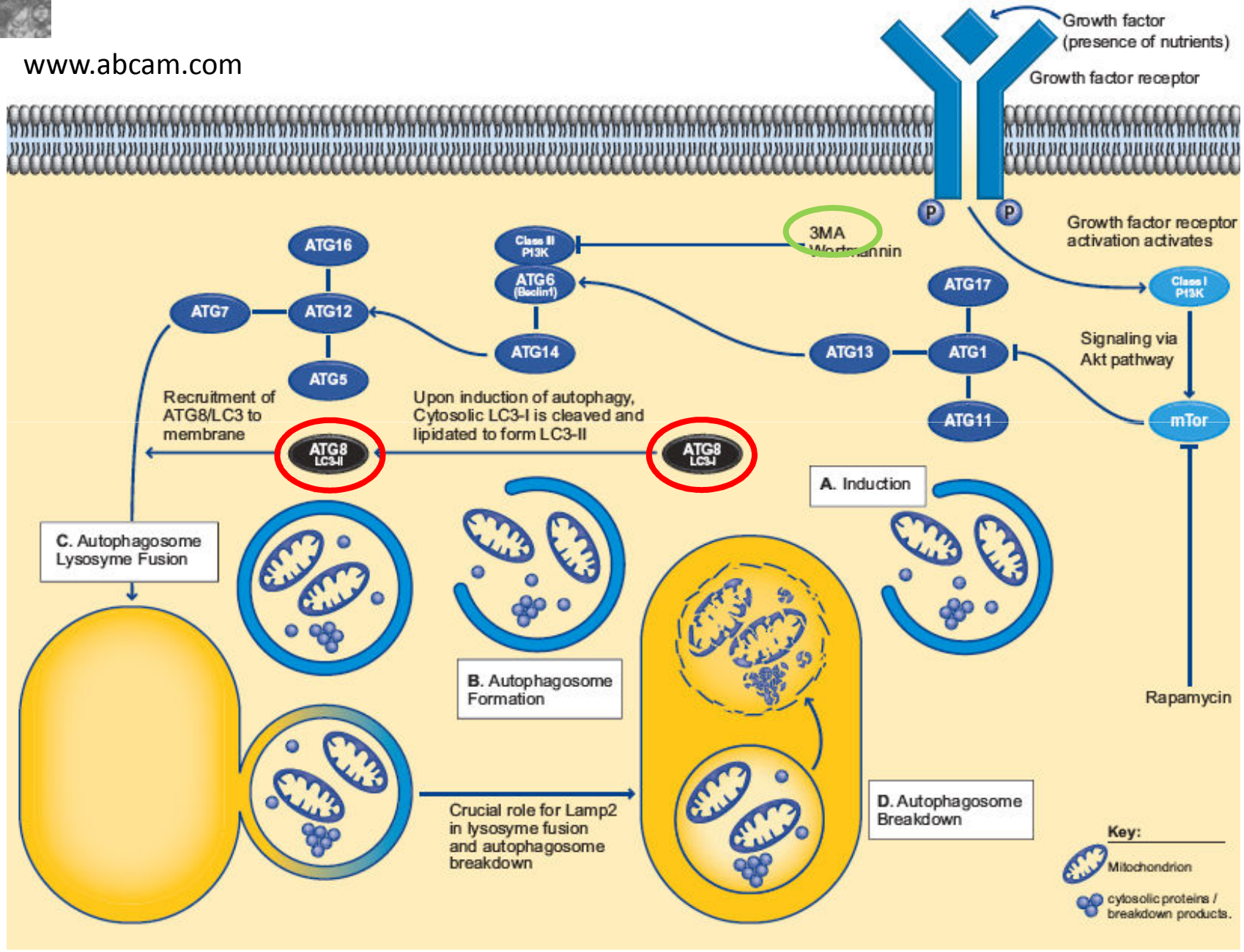


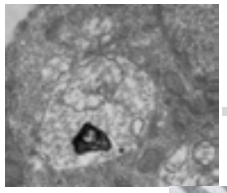
Wanker E., website



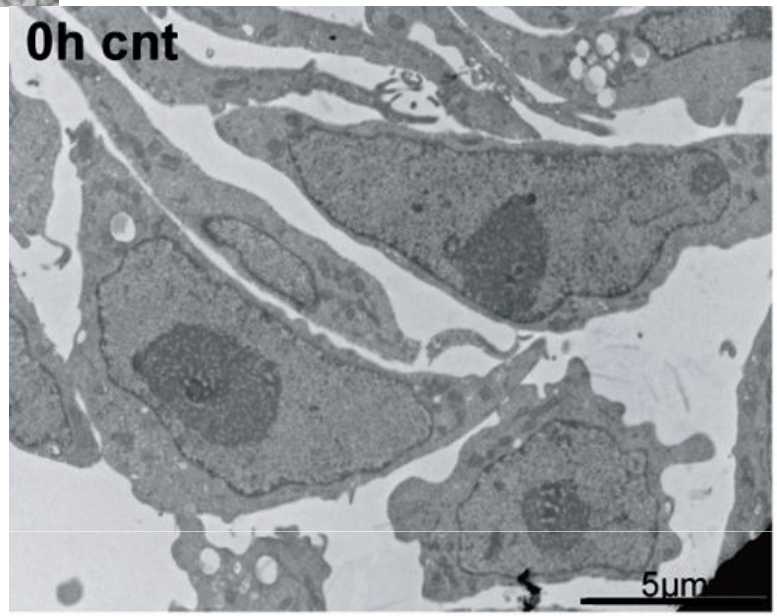
The mechanism of (macro)autophagy

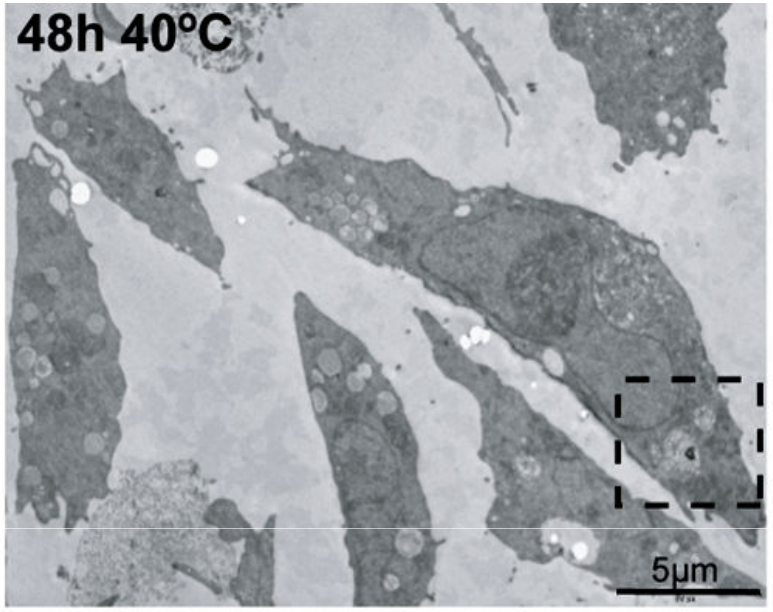
www.abcam.com

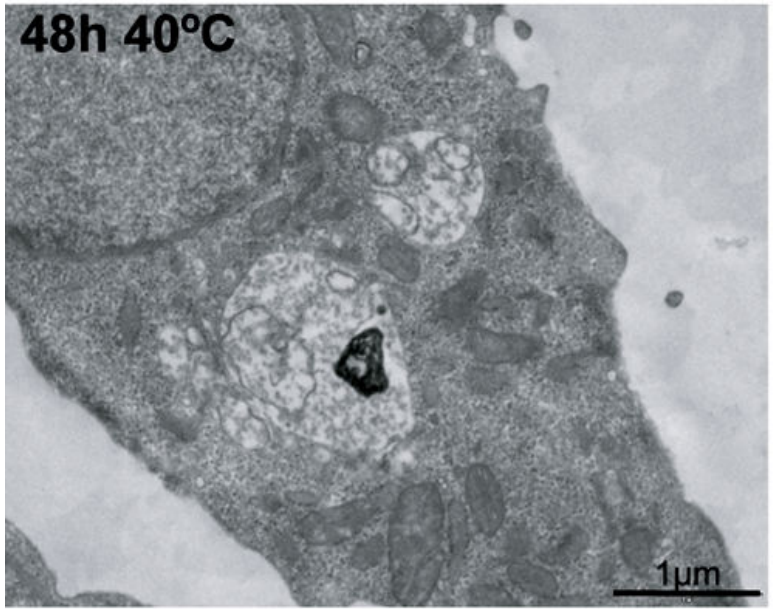


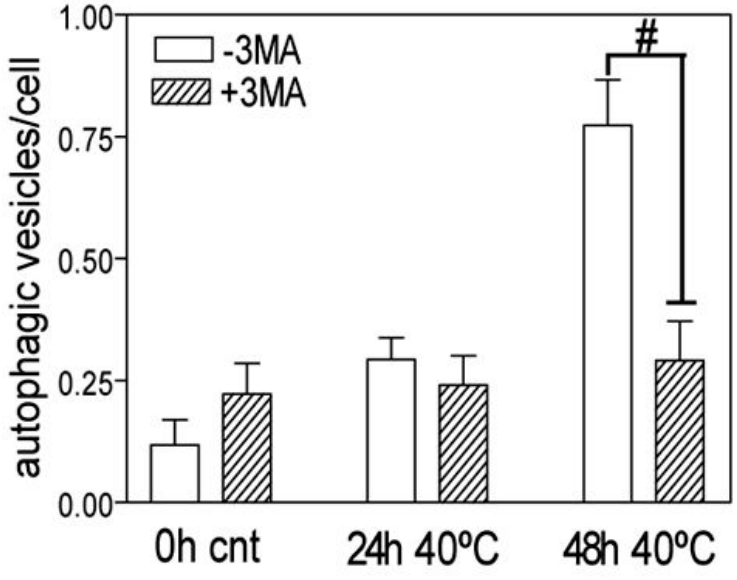


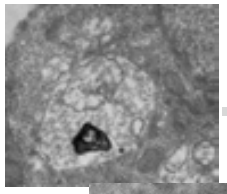
Autophagic vacuoles appear after CCT inhibition



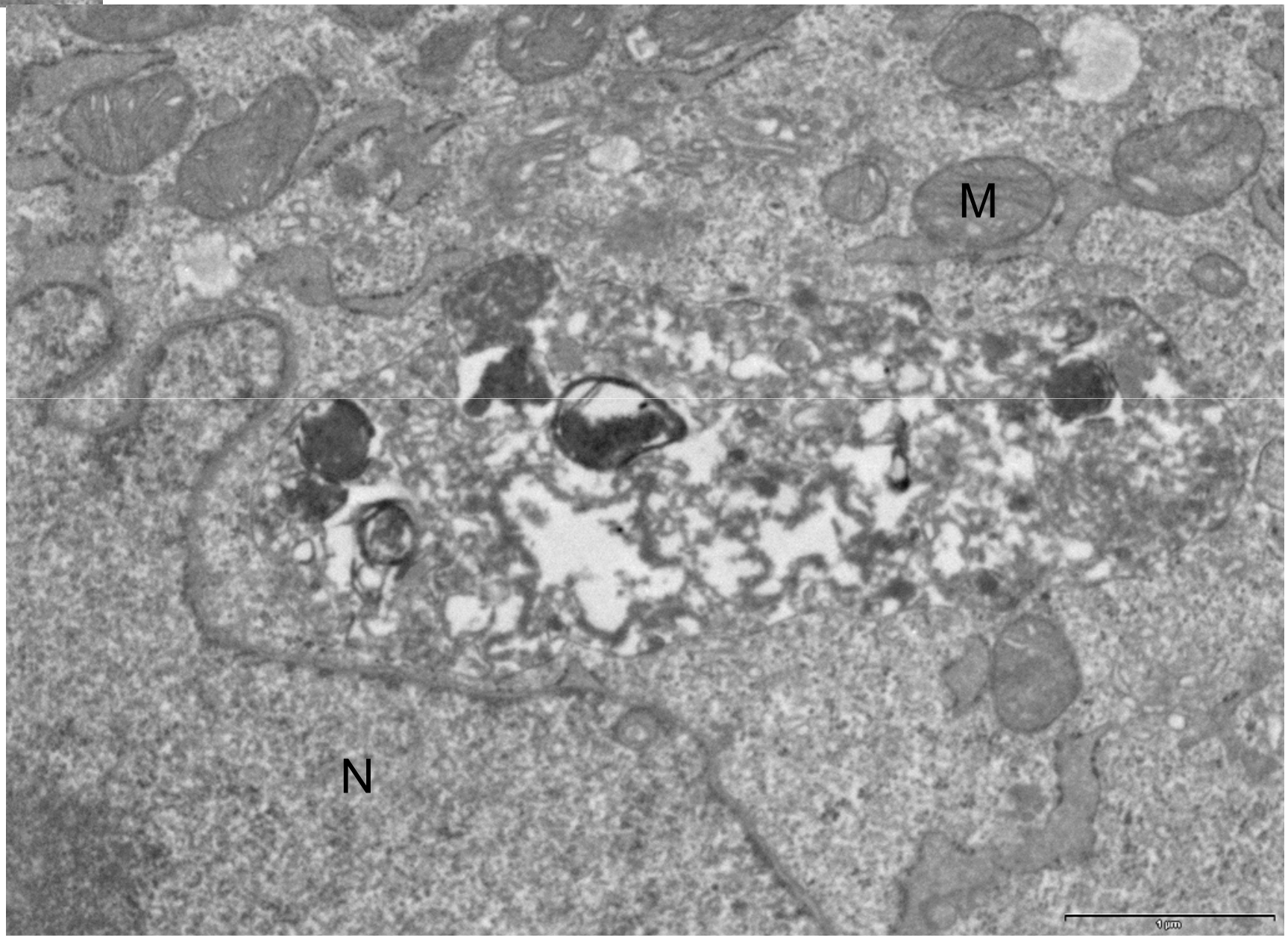


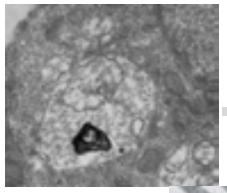




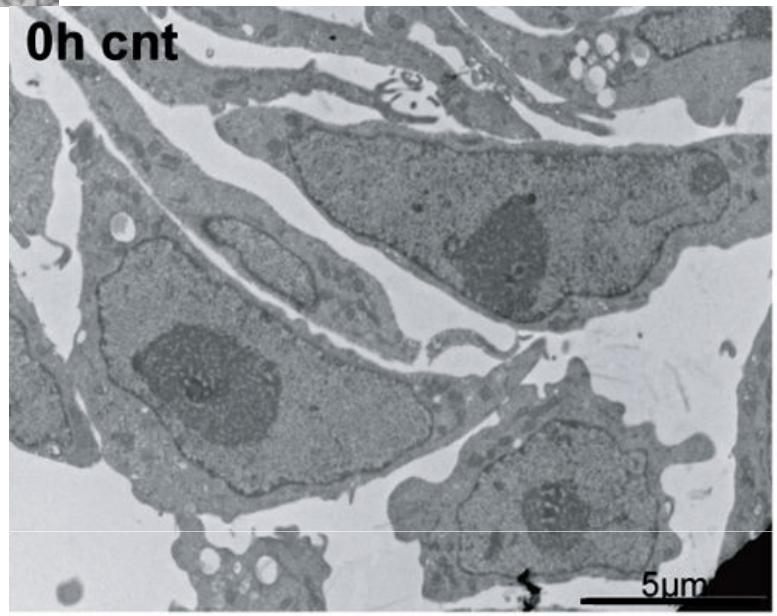


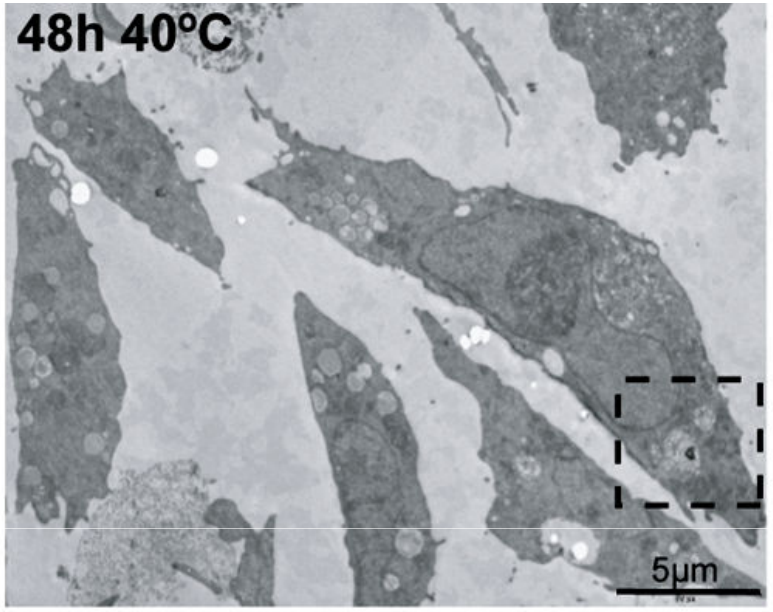
Some of them are huge

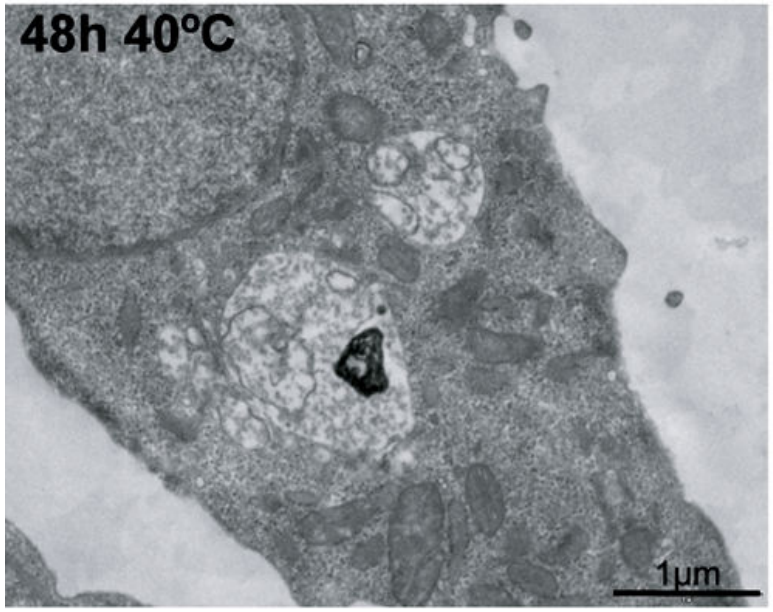


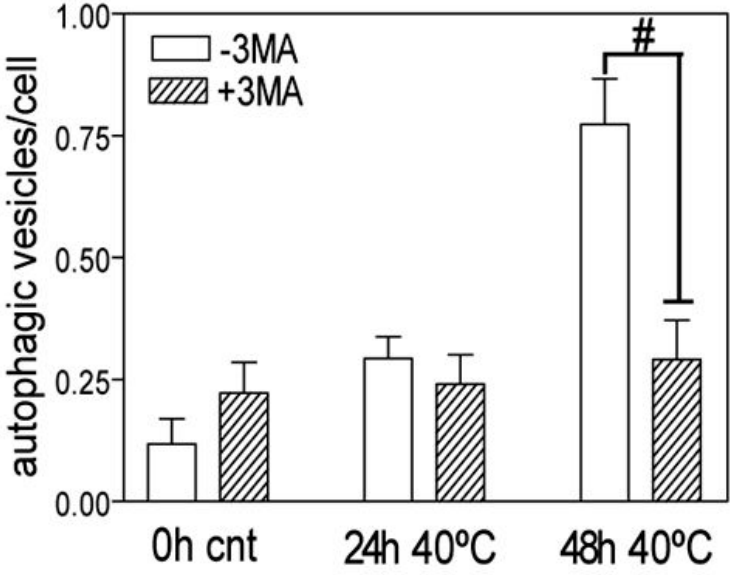


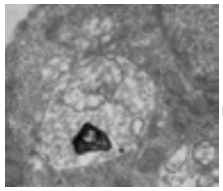
Autophagic vacuoles appear after CCT inhibition



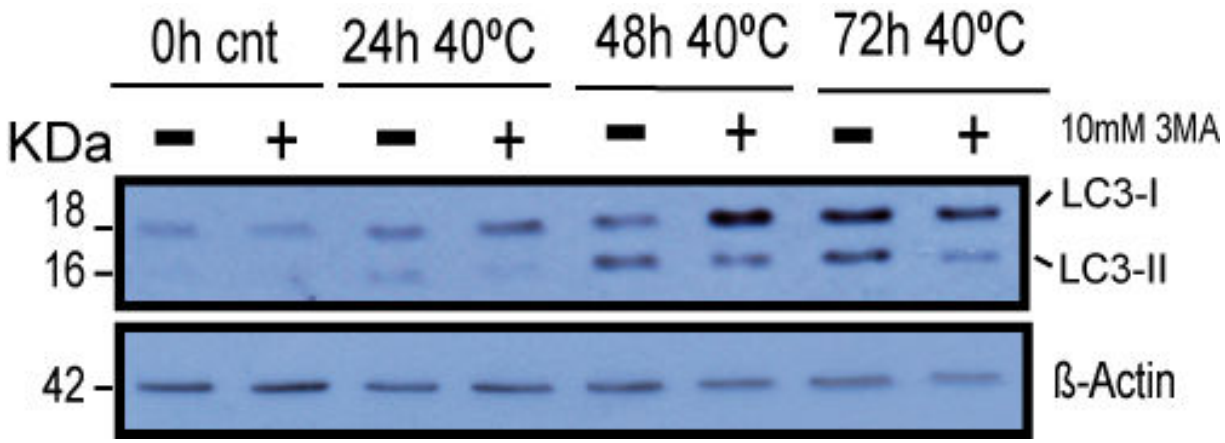


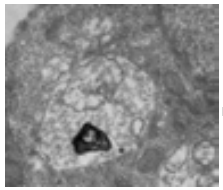




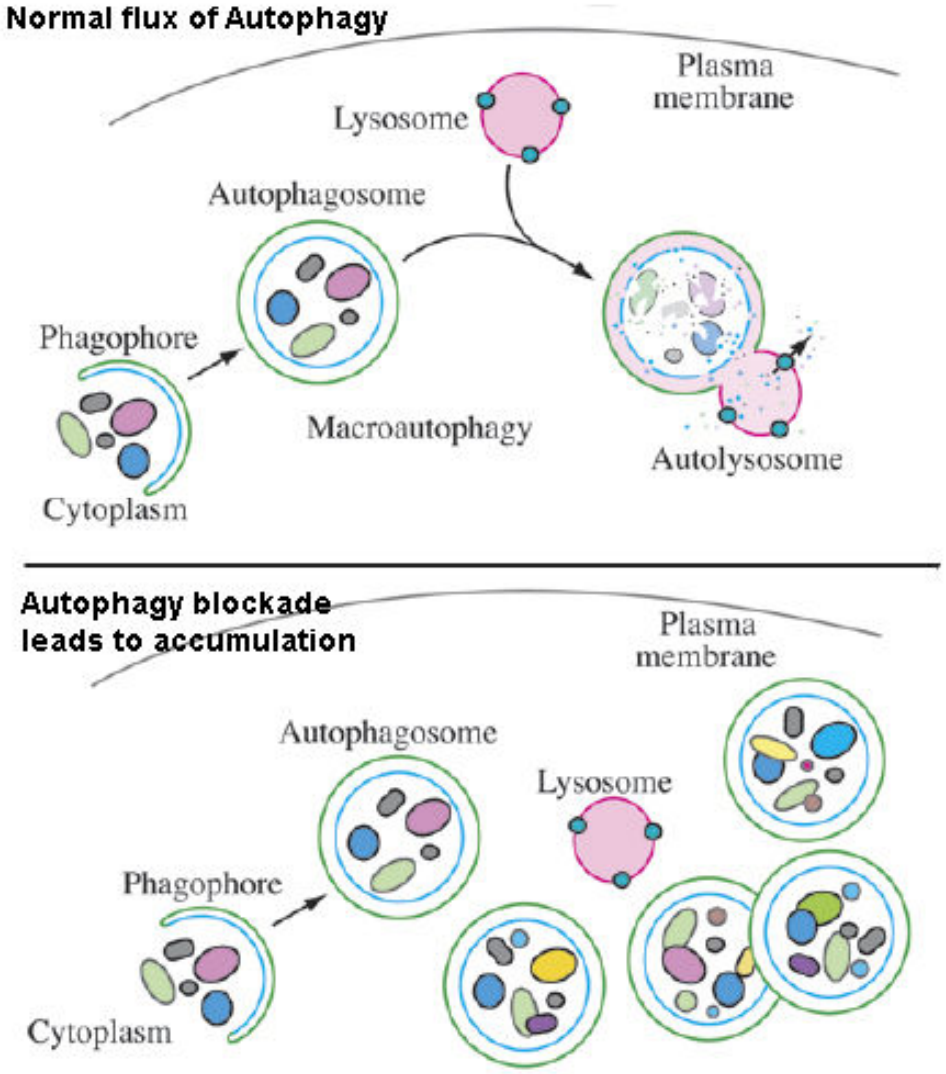


CCT inhibition blocks the progress of autophagy





Flux of Autophagy: productive or blockade



[Autophagy 4:2, 151-175; 16 February 2008]; ©2008 Landes Bioscience

Review

Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes

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Naqvi,[135] Thomas P. Neufeld,[136] Ichizo Nishino,[137] Ralph A. Nixon,[138] Takeshi Noda,[139] Bernd Nürnberg,[140] Michinaga Ogawa,[141] Nancy L. Oleinick,[142] Laura J. Olsen,[143] Bulent Ozpolat,[113] Shoshana Paglin,[144] Glen E. Palmer,[145] Issidora Papassideri,[146] Miles Parkes,[147] David H. Perlmutter,[148] George Perry,[5] Mauro Piacentini,[149] Ronit Pinkas-Kramarski,[150] Mark Prescott,[151] Tassula Proikas-Cezanne,[152] Nina Raben,[153] Abdelhaq Rami,[154] Fulvio Reggiori,[128] Bärbel Rohrer,[155] David C. Rubinsztein,[156] Kevin M. Ryan,[157] Junichi Sadoshima,[158] Hiroshi Sakagami,[159] Yasuyoshi Sakai,[160] Marco Sandri,[161] Chihiro Sasakawa,[162] Miklós Sass,[98] Claudio Schneider,[163] Per O. Seglen,[164] Oleksandr Seleverstov,[165] Jeffrey Settleman,[166] John J. Shacka,[167] Irving M. Shapiro,[168] Andrei Sibirny,[169] Elaine C.M. Silva-Zacarin,[170] Hans-Uwe Simon,[171] Cristiano Simone,[172] Anne Simonsen,[173] Mark A. 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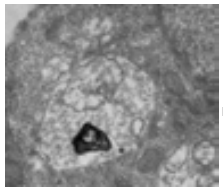
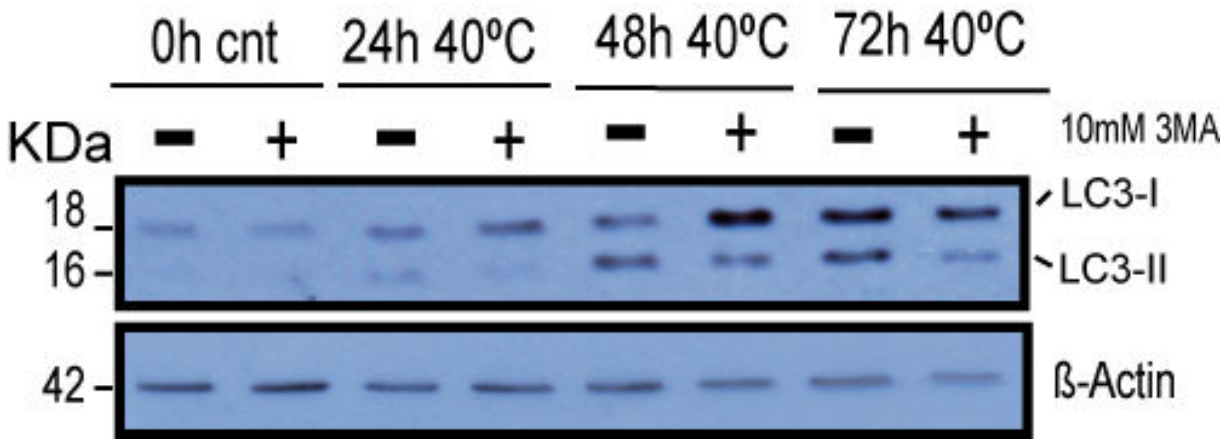
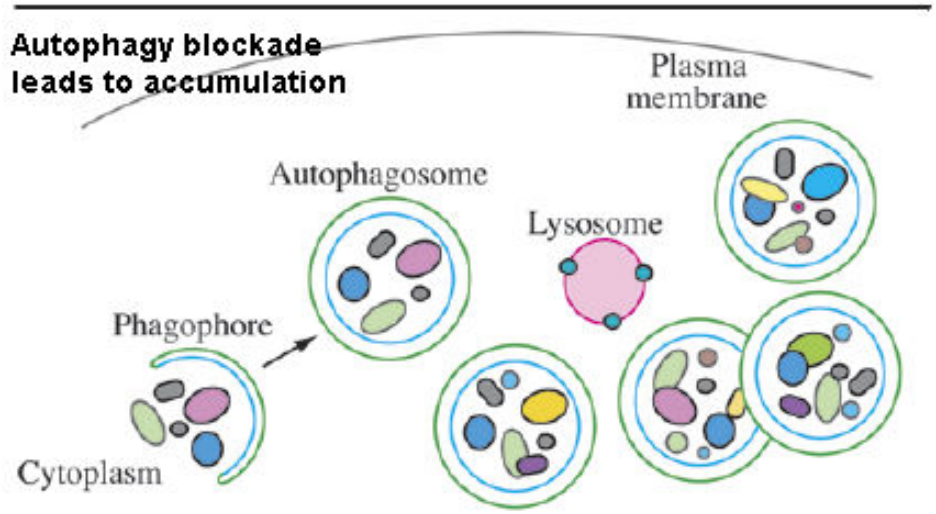
Wiley,[203] Zhijun Xi,[204] Gutian Xiao,[205] Joachim Yahalom,[206] Jin-Ming Yang,[207] George Yap,[208] Xiao-Ming Yin,[209] Tamotsu Yoshimori,[139] Li Yu,[107] Zhenyu Yue,[210] Michisuke Yuzaki,[211] Olga Zabirnyk,[212] Xiaoxiang Zheng,[213] Xiongwei Zhu[174] and Russell L. Deter[214]

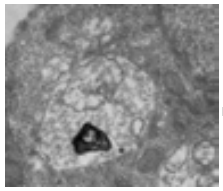
Klionsky et al., (2008). *Autophagy*



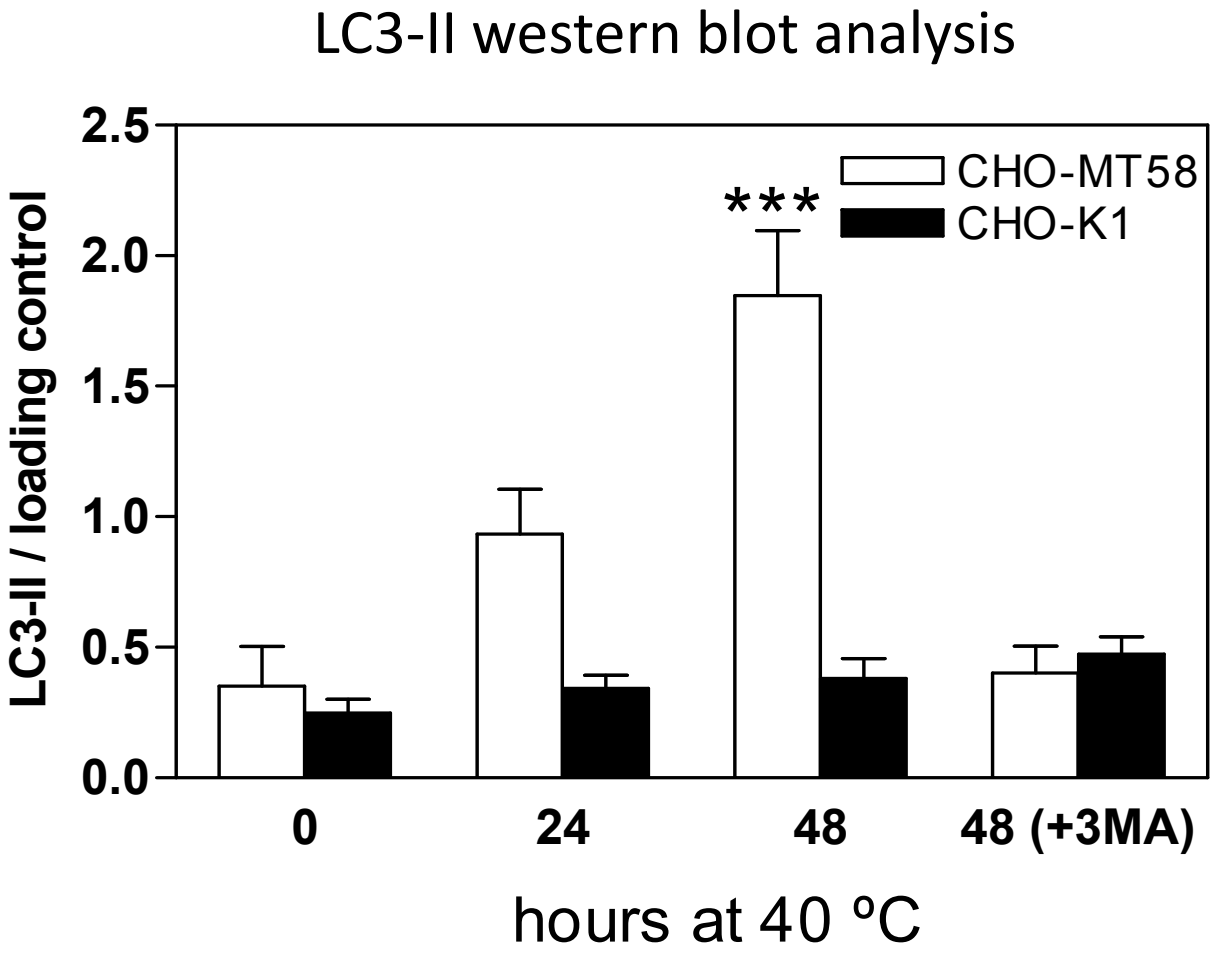
CCT inhibition blocks the progress of autophagy

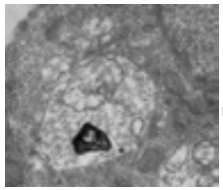






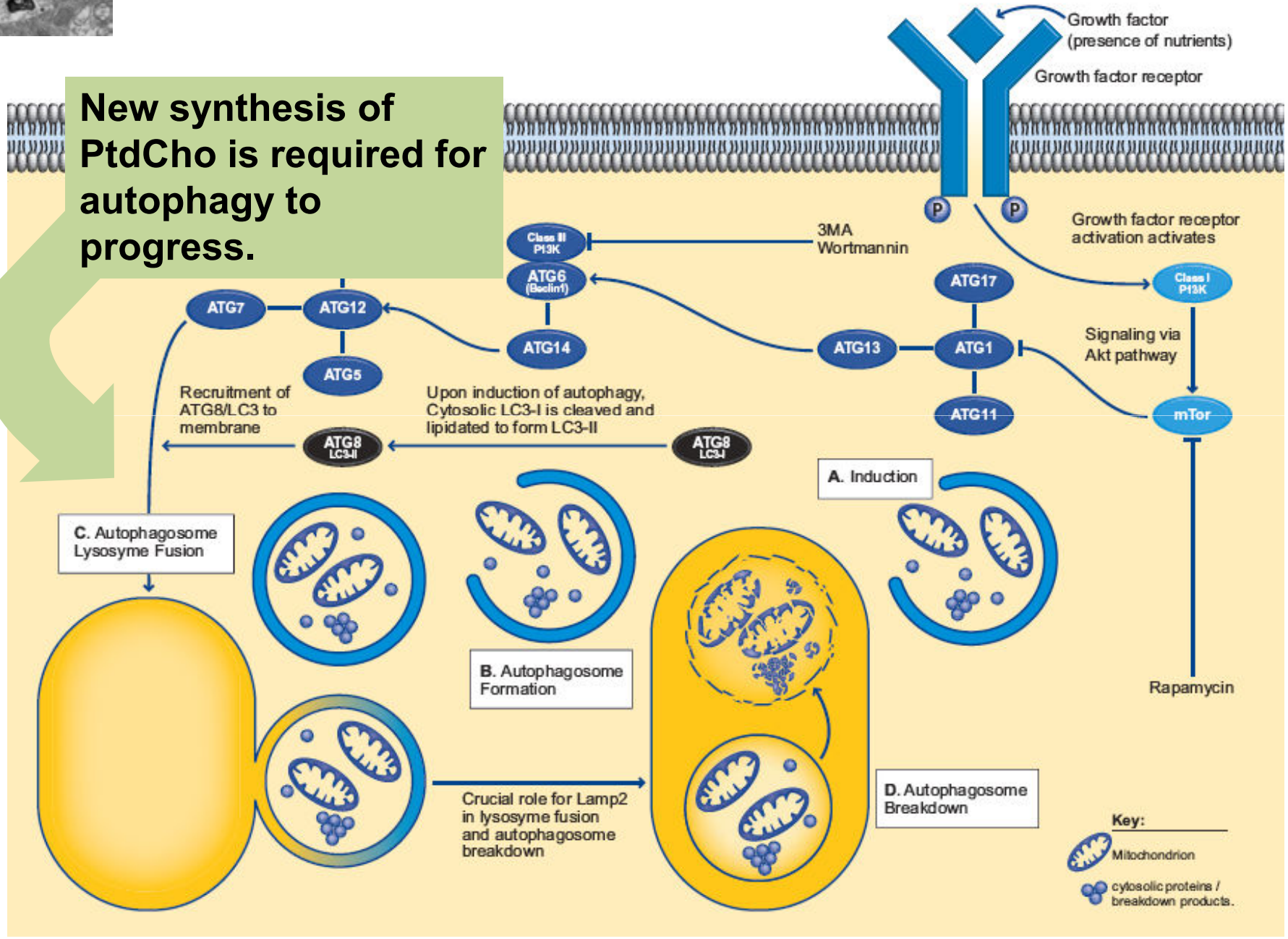
Temperature alone does not recapitulate the MT58 phenotype

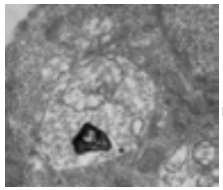




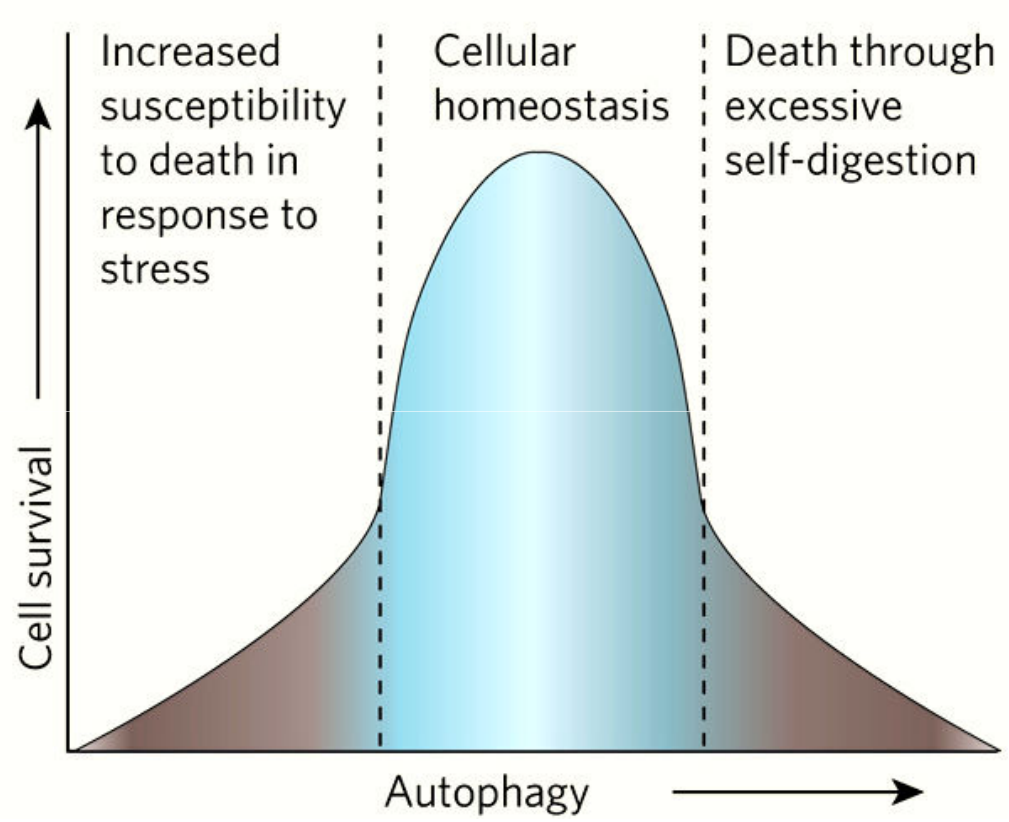
Our working model

New synthesis of PtdCho is required for autophagy to progress.

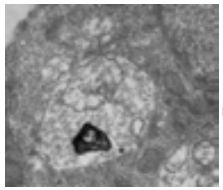




Why do cells die?



Autophagy is a constitutive survival mechanism

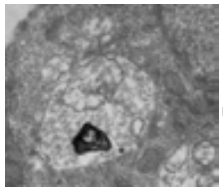
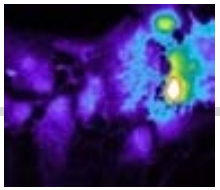


Does it all have any interest for clinical science?

Autophagy defends cells against invading Group A Streptococcus (2004) Science 306, 1037-1040.

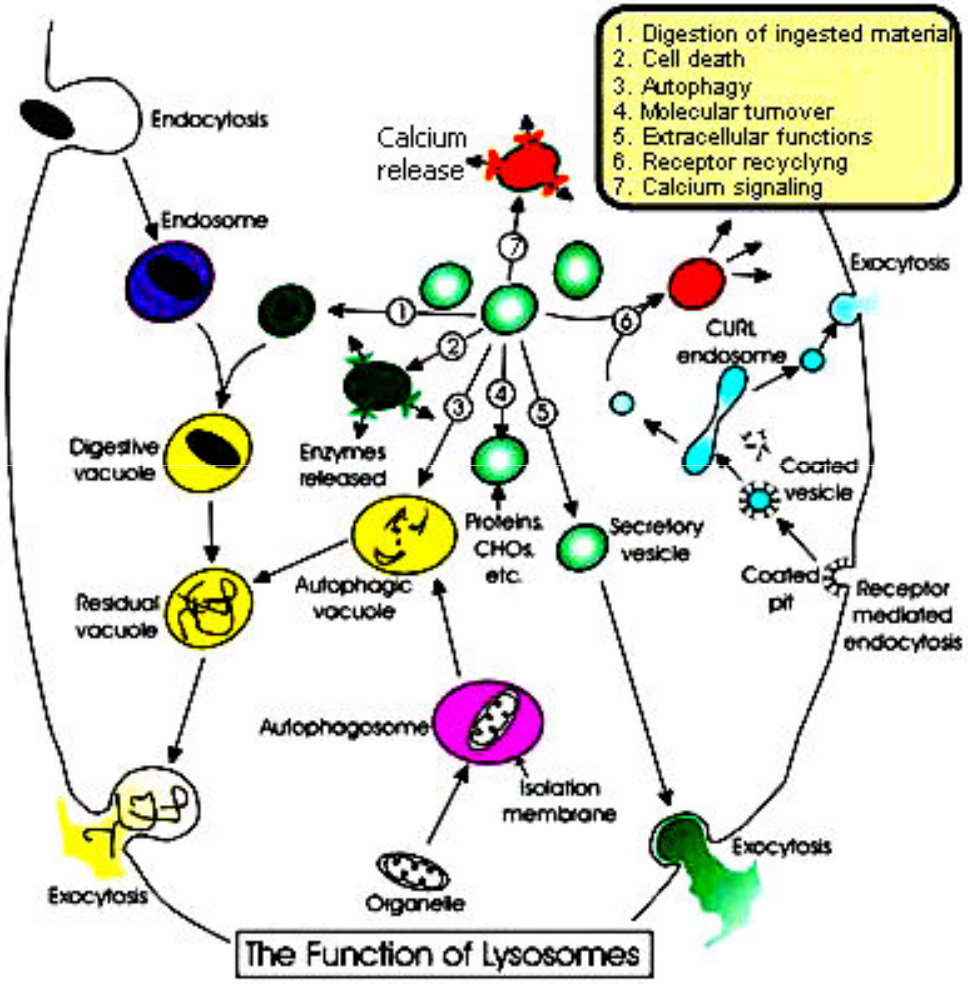
Bacterial inhibition of phosphatidylcholine synthesis triggers apoptosis in the brain (2004) J. Exp. Med. 200, 99-106.

Our results show that inhibition of PtdCho synthesis may well underlie bacterial escaping from the autophagic machinery

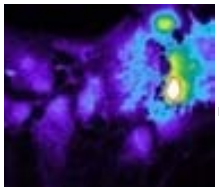



Functions of Lysosomes

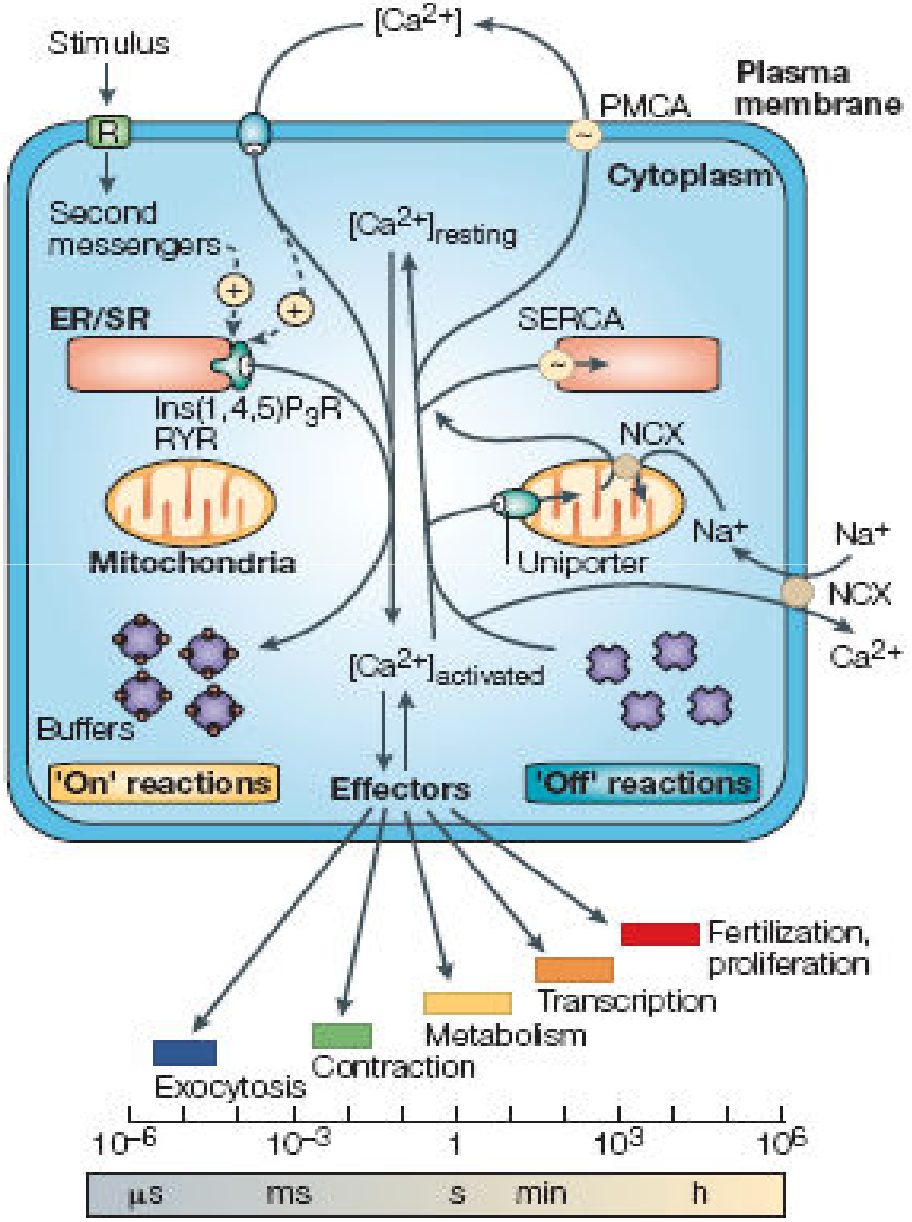
- Degradation of cell components
  - Cell death — 1st
  - Autophagy
- Exocytosis / Secretory pathway
  - Calcium signalling — 2nd

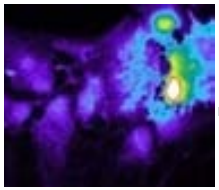


O'Bay DH., (2009)

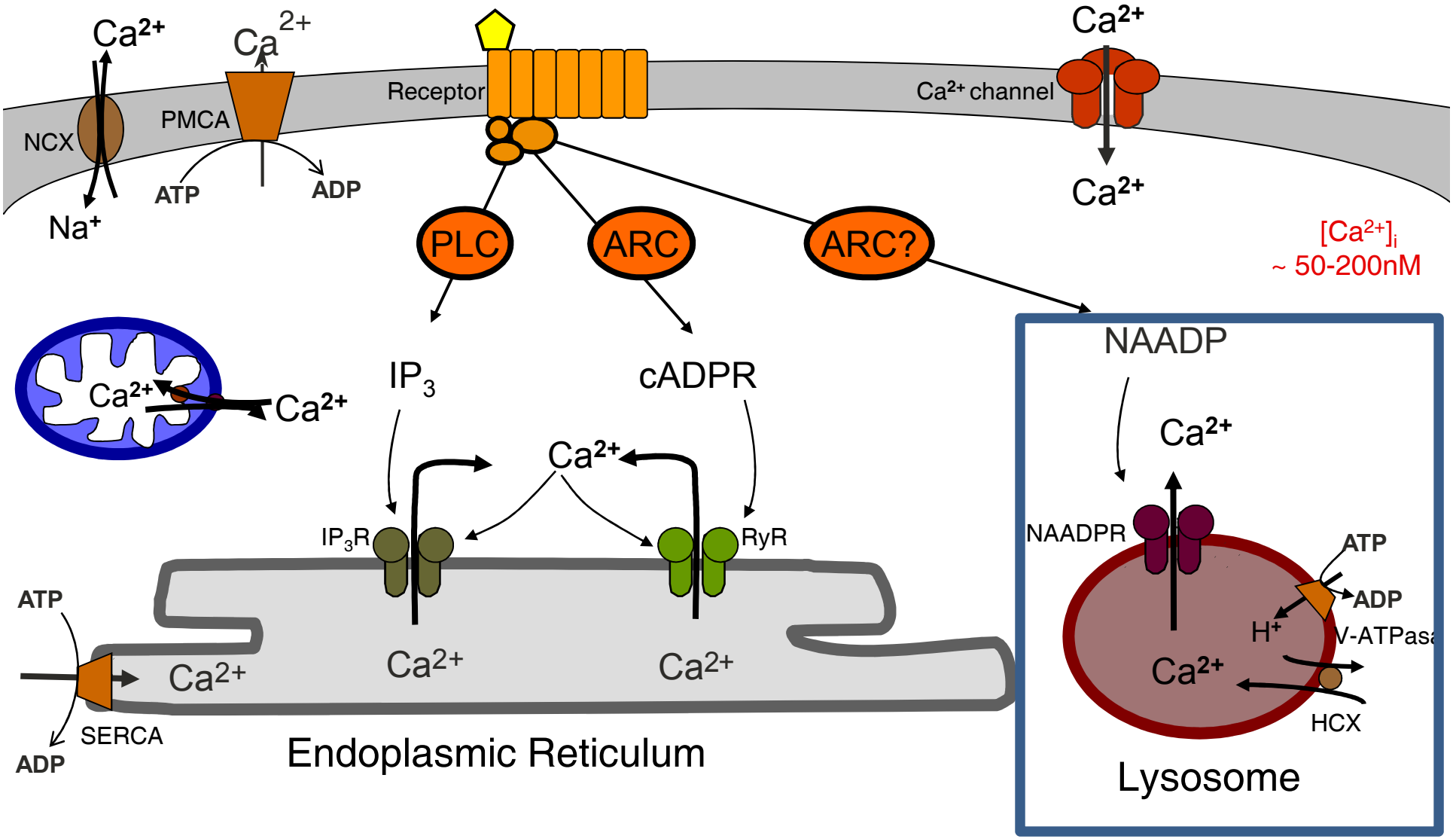


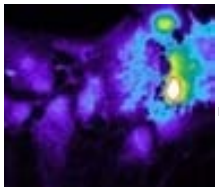
Ca^{2+} is a universal messenger



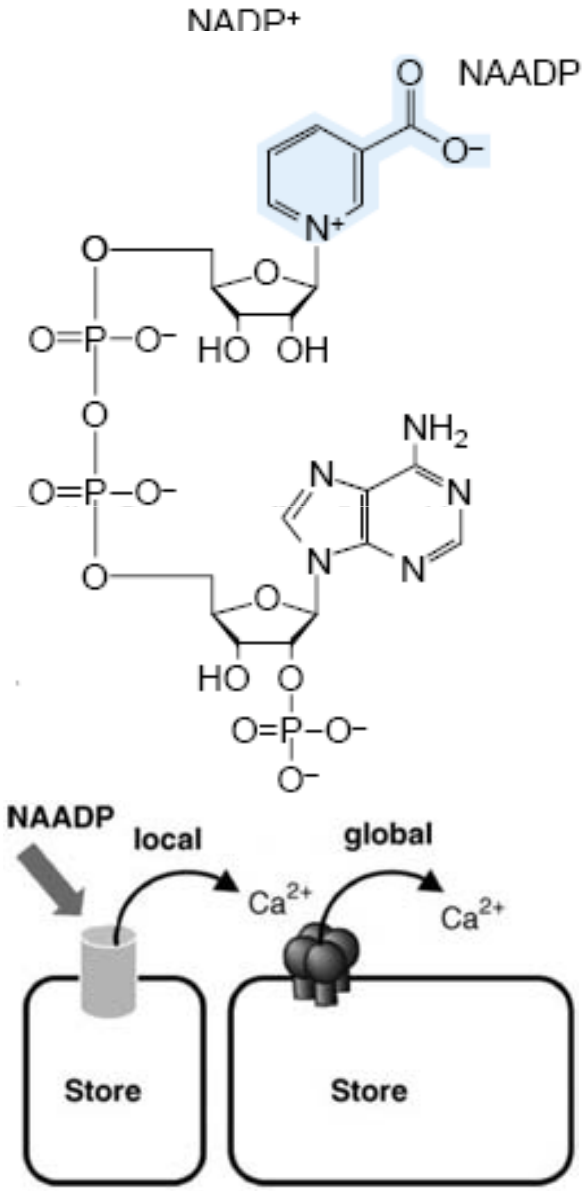


Basic mechanisms of Ca^{2+} regulation

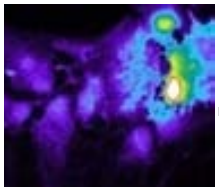




What is NAADP?



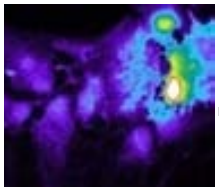
- NAADP is a new potent Ca^{2+} second messenger derived from NADP.
- NAADP receptor is localised in lysosomes.
- The NAADP receptor is not known yet. Recently, up to four putative receptors have been proposed: TRP-ML1; TPC1; TPC2 and RyR.
- NAADP-R is inactive at high agonist concentrations, generating a bell-shaped concentration-response curve.
- NAADP signalling is thought to be a trigger Ca^{2+} signal that is subsequently amplified by CICR and Ca^{2+} influx.



Some physiological functions of NAADP

- Insulin secretion
- Egg fertilization
- Neuron differentiation
- Neurite outgrowth

Has NAADP a role in astrocytes?

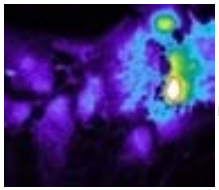


Astrocytes

- Traditional functions of Astrocytes:
  - Structural and Metabolic support of neurons
  - Neurotransmitter reuptake
  - Regulation of ion concentration in the extracellular space
- New evidences:
  - Astrocytes contain many neurotransmitter receptors
  - Astrocytes respond to agonist by increasing Ca^{2+} concentrations, which can expand in neighbouring astroyctes to produce Ca^{2+} waves

… Astrocyte excitability is based on changes in Ca^{2+} concentrations

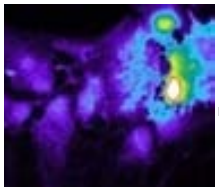
- New functions (tripartite synapse):
  - Release of neurotransmitters (gliotransmission)
  - Modulation of synaptic transmission



To determine whether NAADP mobilises Ca^{2+} from lysosome-related vesicles in cortical astrocytes.

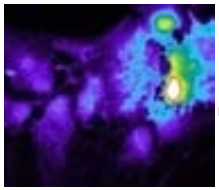
To find out whether NAADP and lysosome-related vesicles mediate neurotransmitter-induced Ca^{2+} responses in cortical astrocytes.

To elucidate the participation of NAADP and lysosome-related vesicles in mechanically induced Ca^{2+} waves between cortical astrocytes.



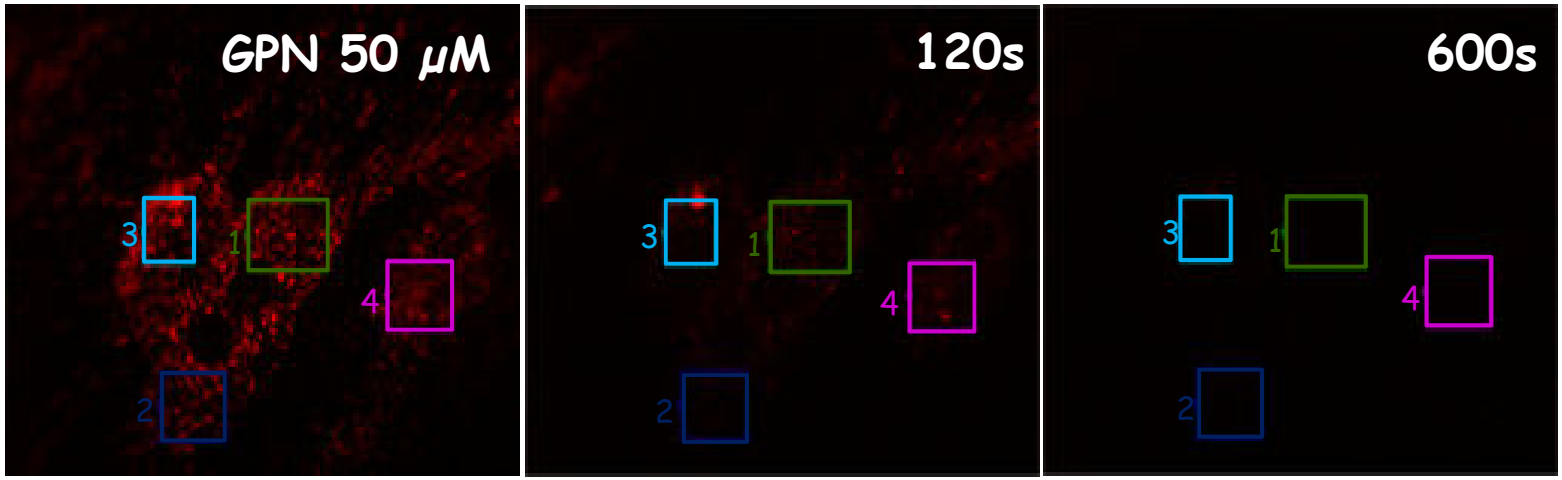
Measurement of Ca^{2+} concentration

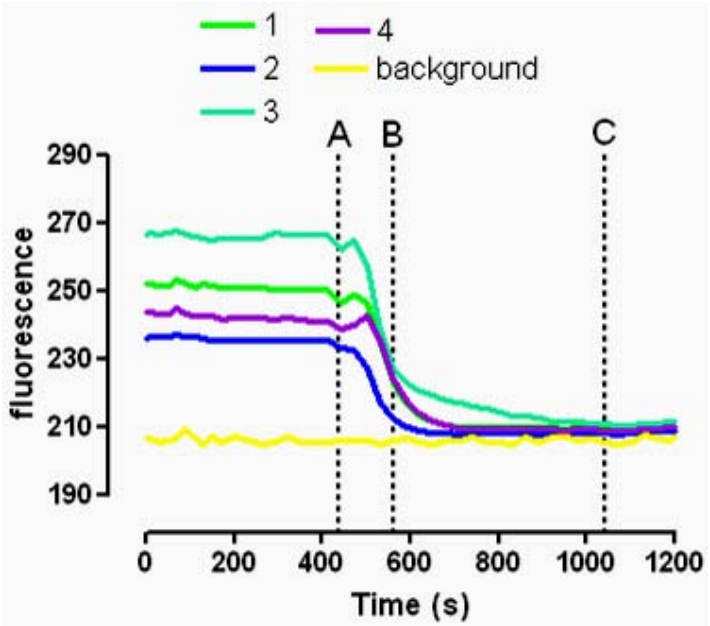
- Ca^{2+} dyes:
  - Fura-2-AM
  - Fluo-4-AM
- Ca^{2+} imaging system

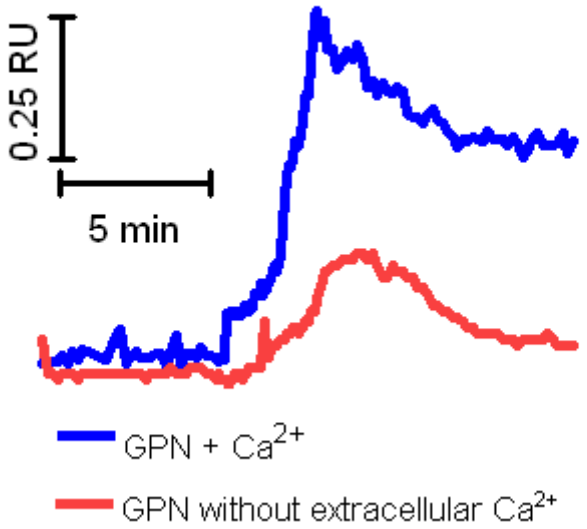


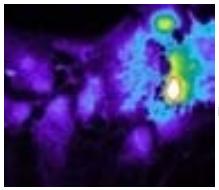
Astrocyte lysosomes contain Ca^{2+}

Lysotracker Red staining





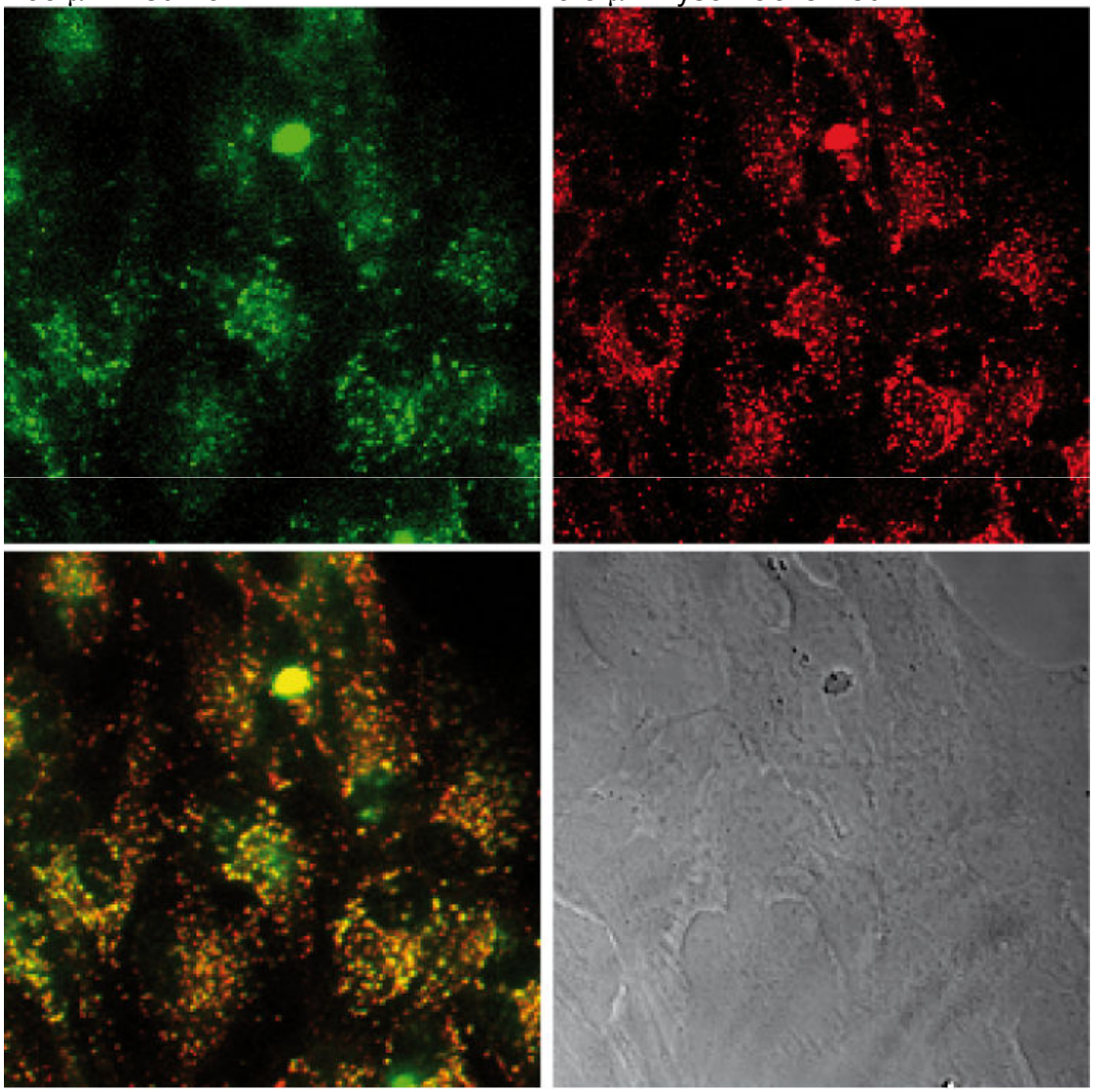




Astrocyte lysosomes express NAADP-R

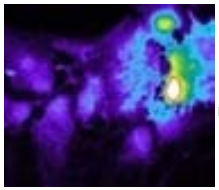
100 μM Ned-19

0.5 μM LysoTracker red

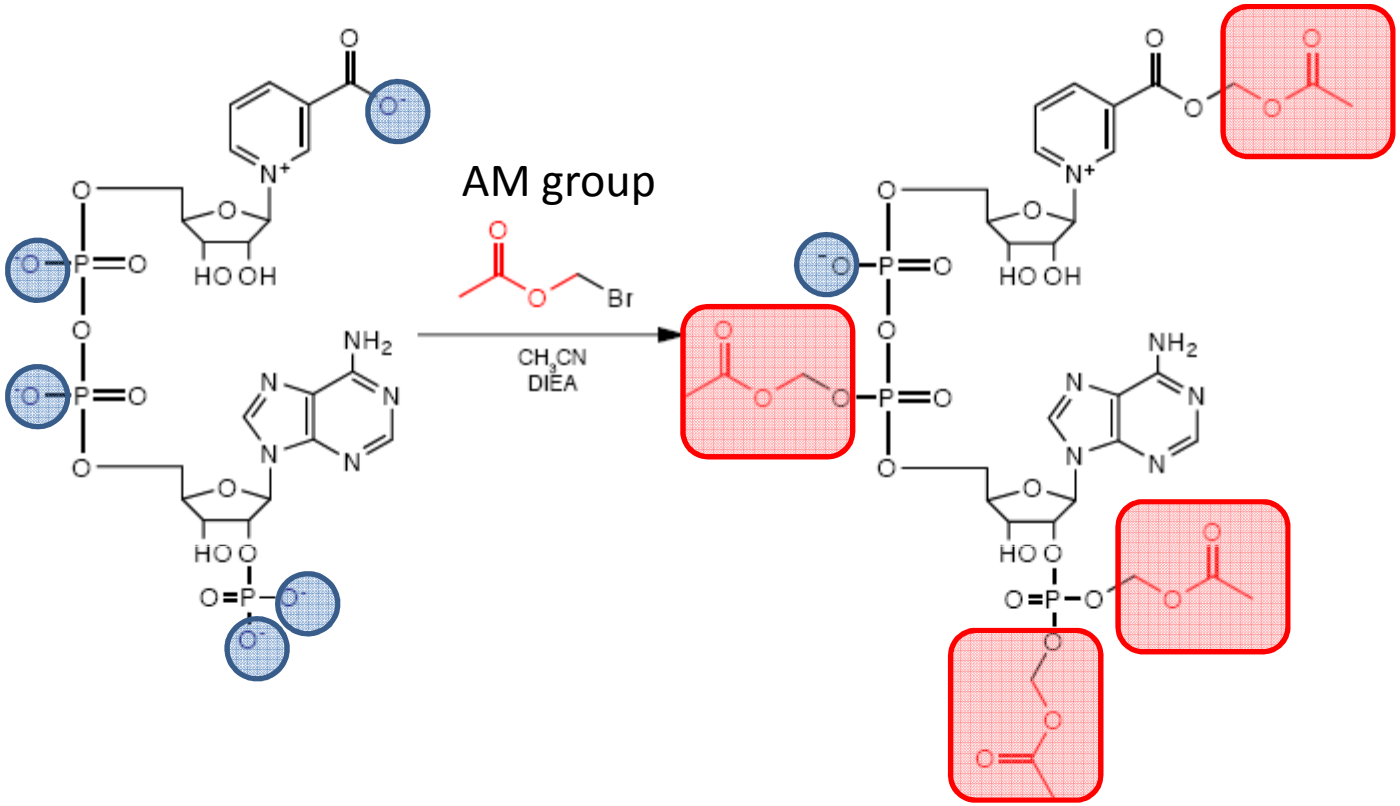


overlay

bright field



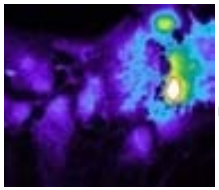
Making NAADP permeable



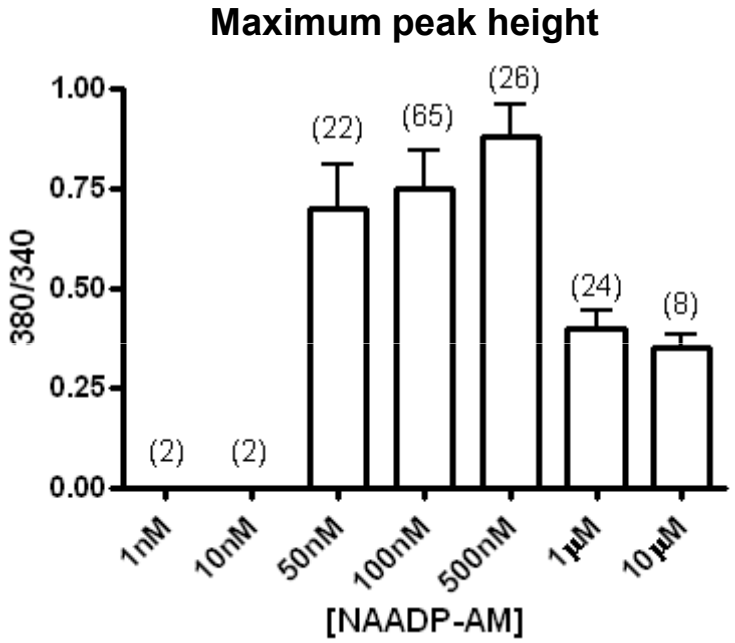
NAADP
Charge: -5

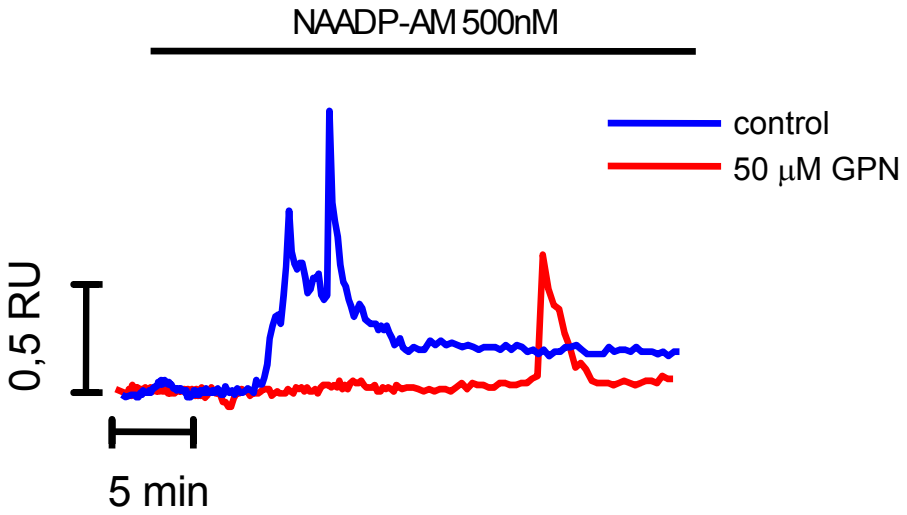
NAADP-AM
(permeable)

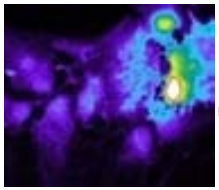
Parkesh *et al.*, 2008



NAADP releases Ca^{2+} from astrocyte lysosomes

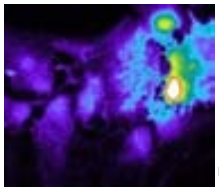




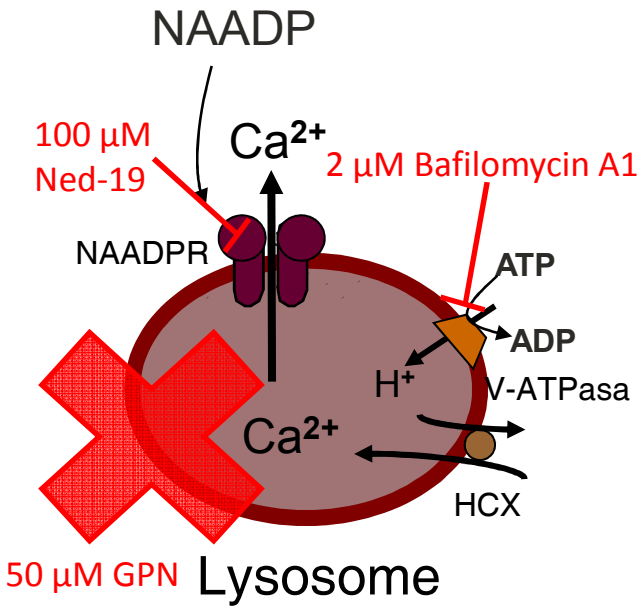


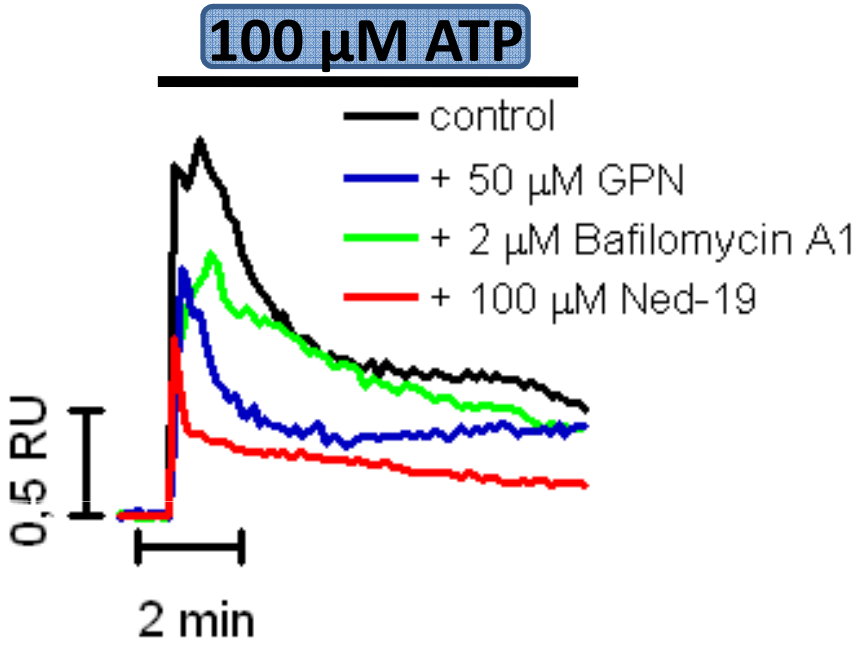
Neurotransmitters induce Ca^{2+} rise in Astrocytes

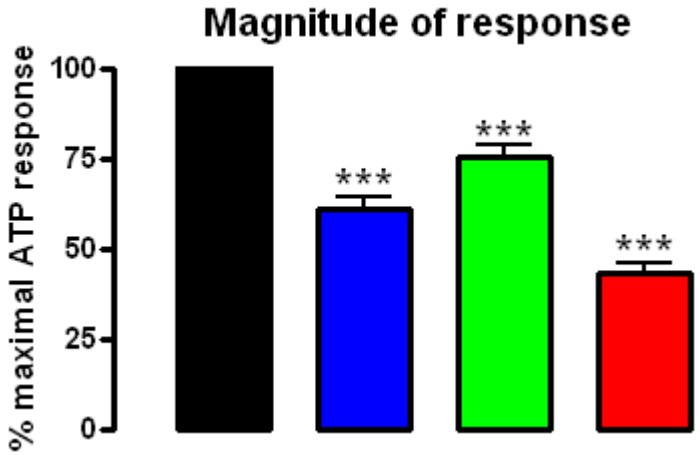
Can neurotransmitters release Ca^{2+} from lysosomes through activation of NAADP receptors?

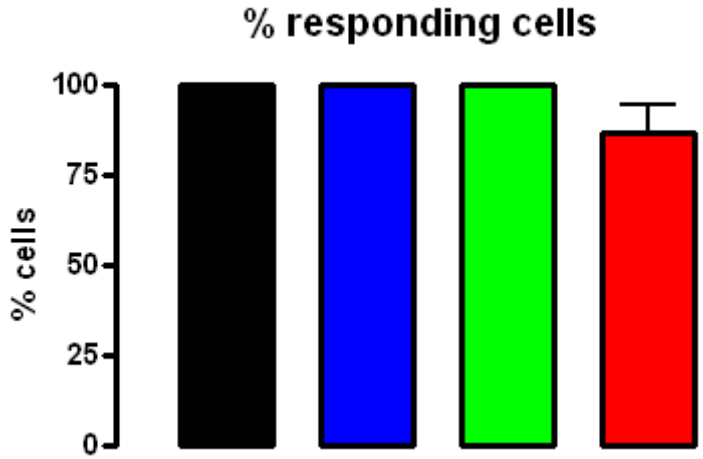


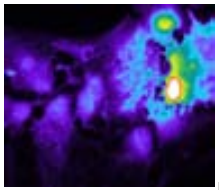
ATP-induced Ca^{2+} responses are mediated by NAADP and lysosomes



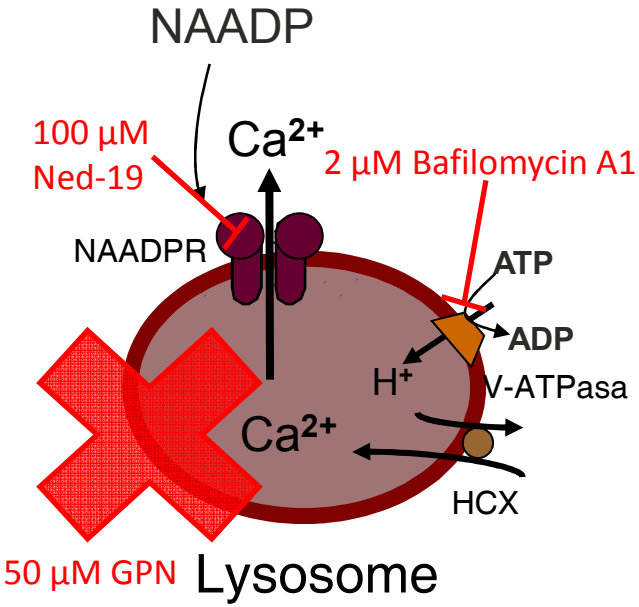


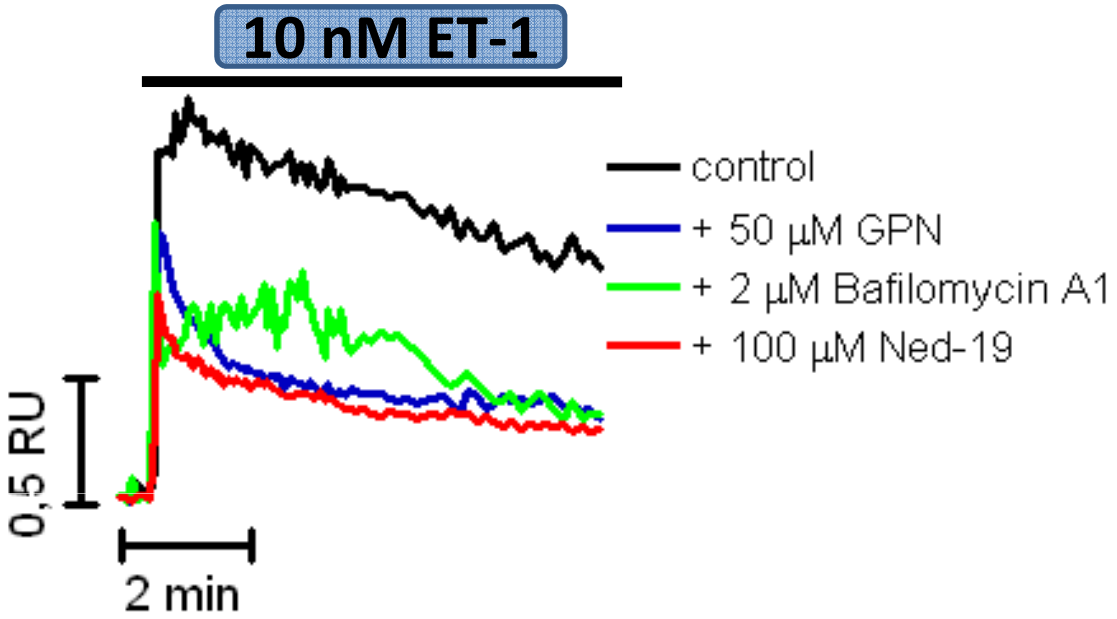


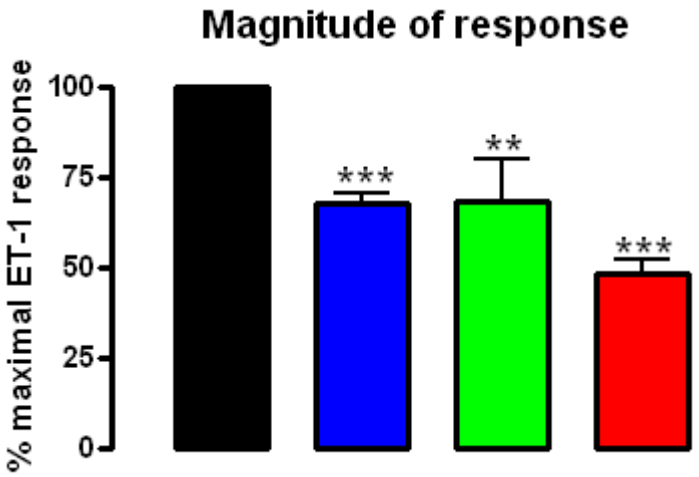


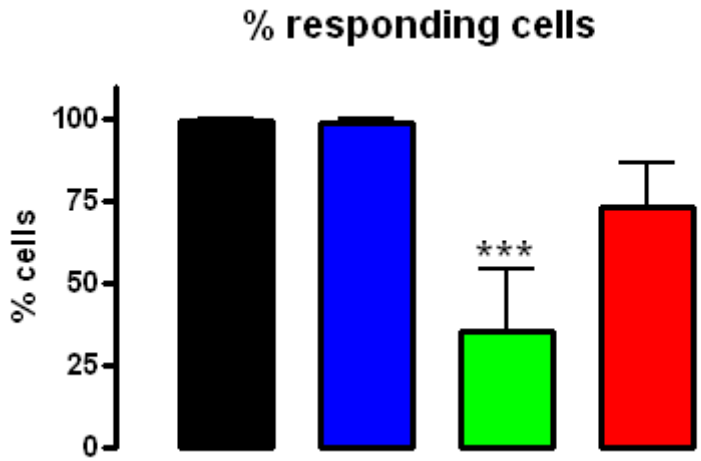


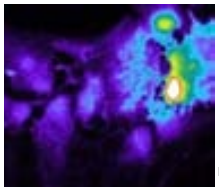
Endothelin-1-induced Ca^{2+} responses are mediated by NAADP and lysosomes



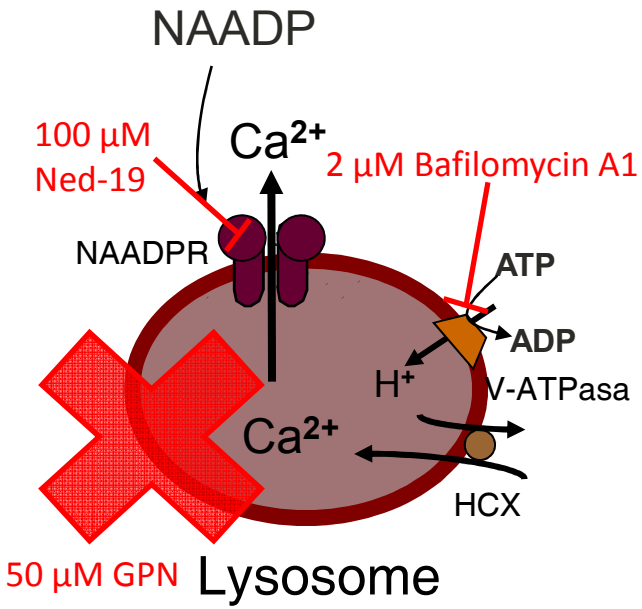


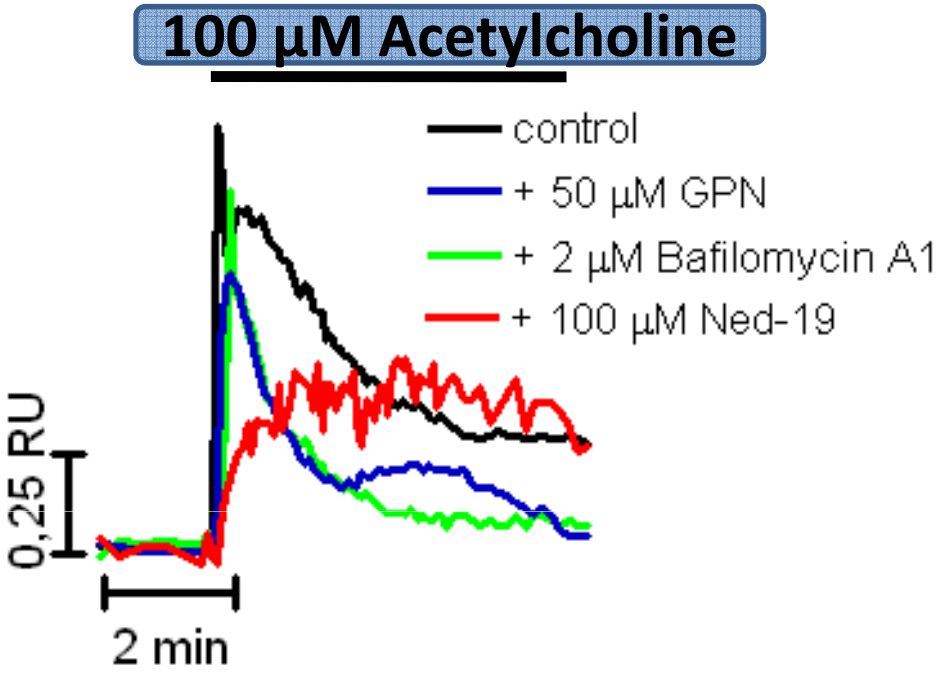


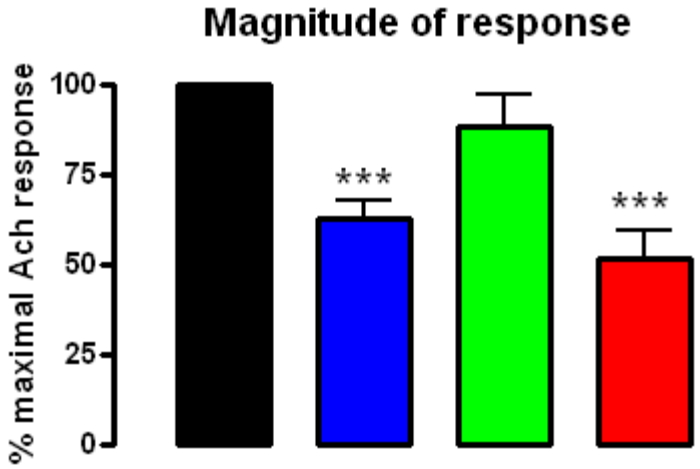


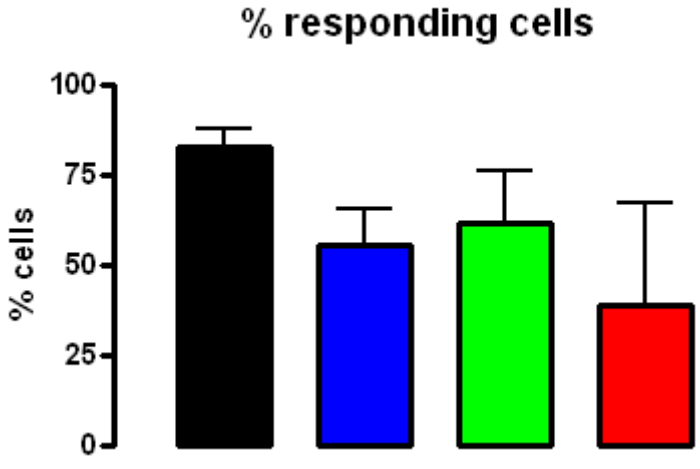


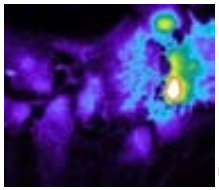
Acetylcholine-induced Ca^{2+} responses are mediated by NAADP and lysosomes



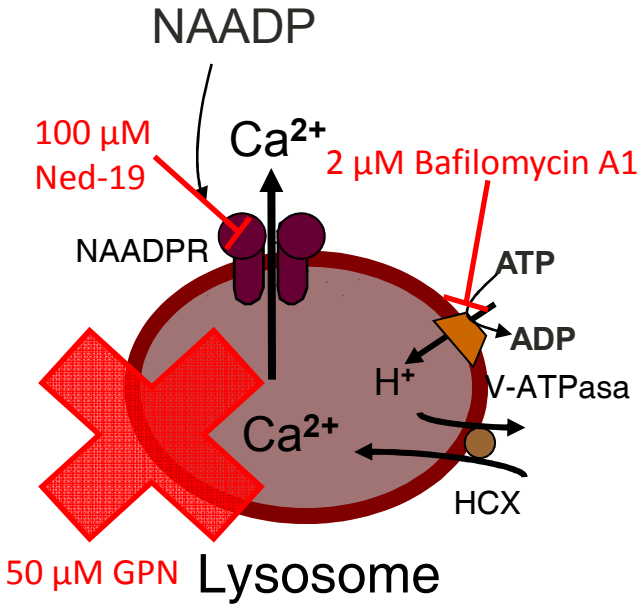


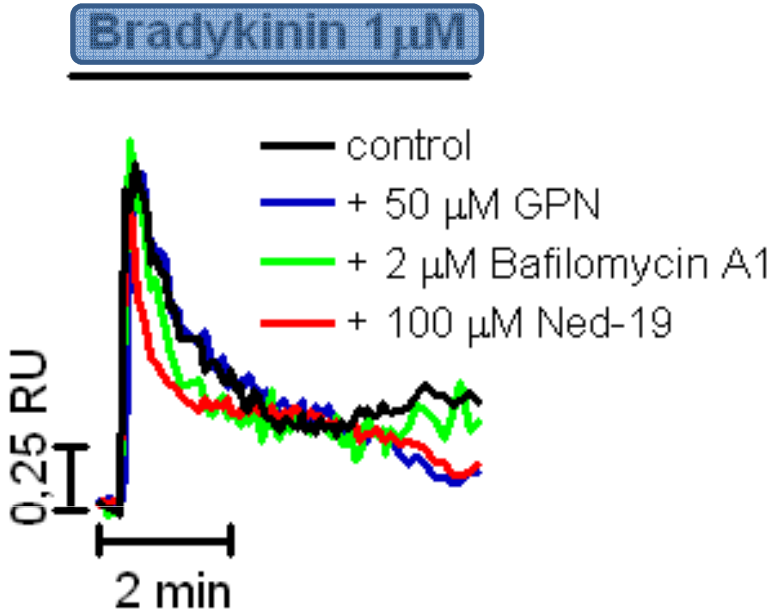


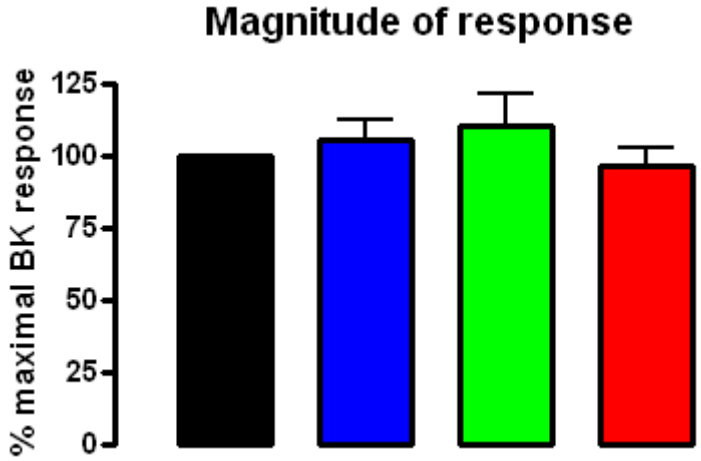


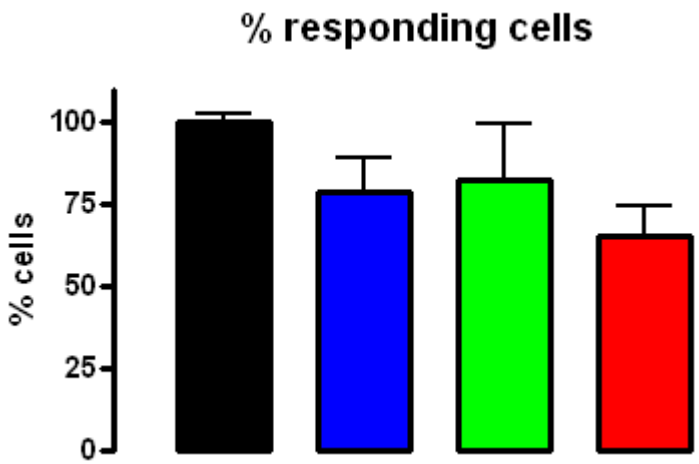


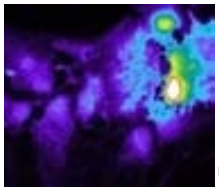
Bradykinin-induced Ca^{2+} responses ARE NOT mediated by NAADP and lysosomes











Some Neurotransmitters use NAADP signalling to induce Ca^{2+} responses in astrocytes

External Stimuli

ATP
ET-1
Ach

Internal Signal

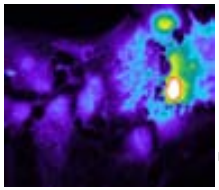
NAADP

Lysosome

NAADPR

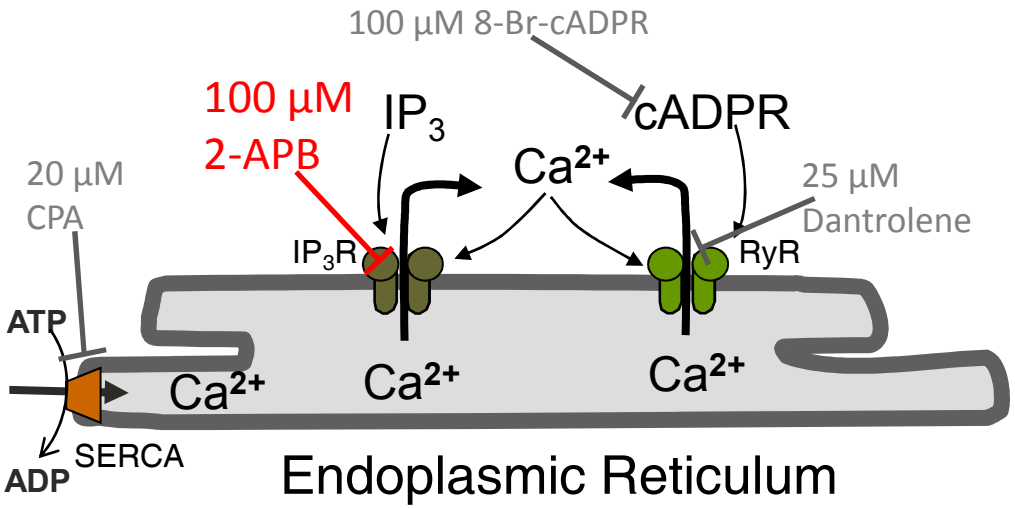
Ca^{2+} Ca^{2+} Ca^{2+}

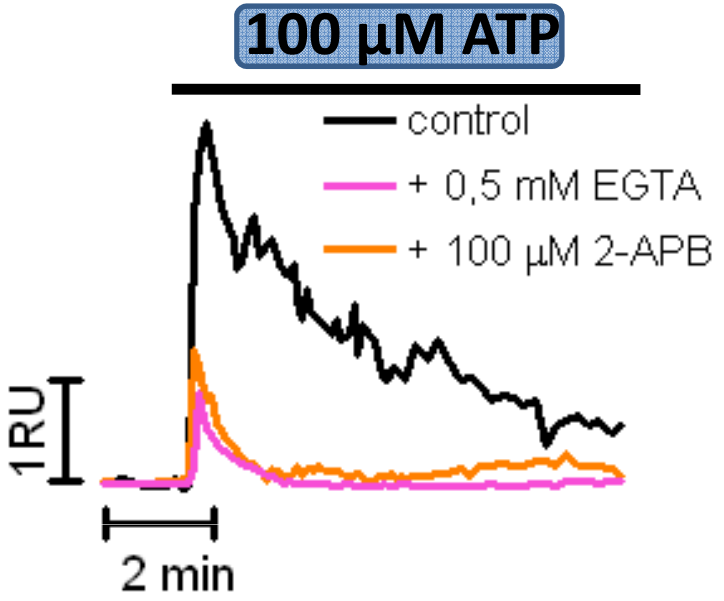
Ca^{2+}

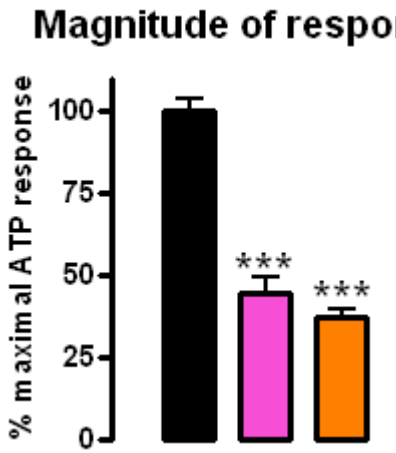


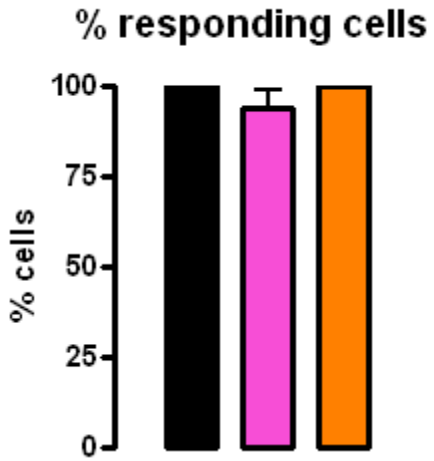
ATP-induced Ca^{2+} responses are also mediated by IP_3 signalling and extracellular Ca^{2+}

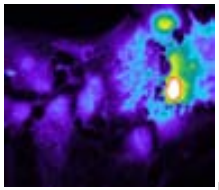
EGTA: extracellular Ca^{2+} chelator



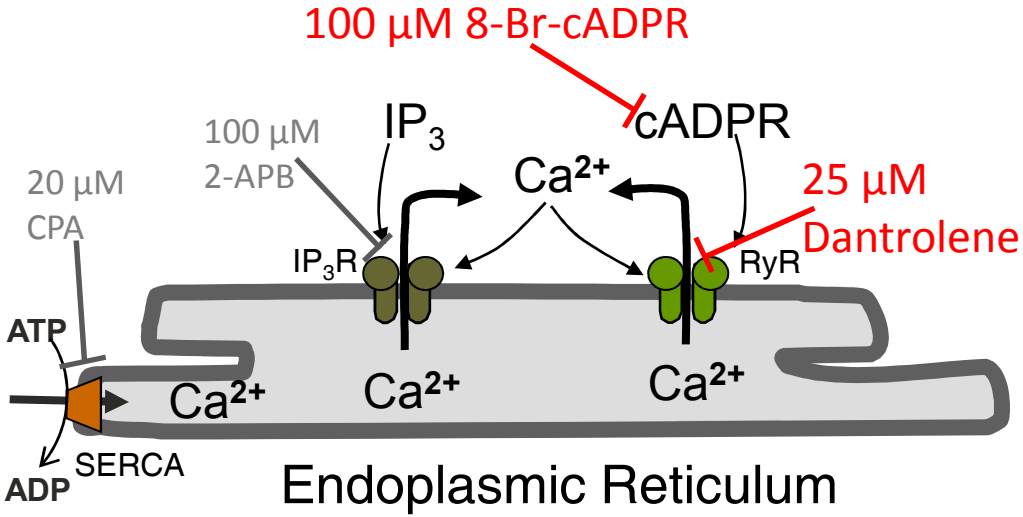


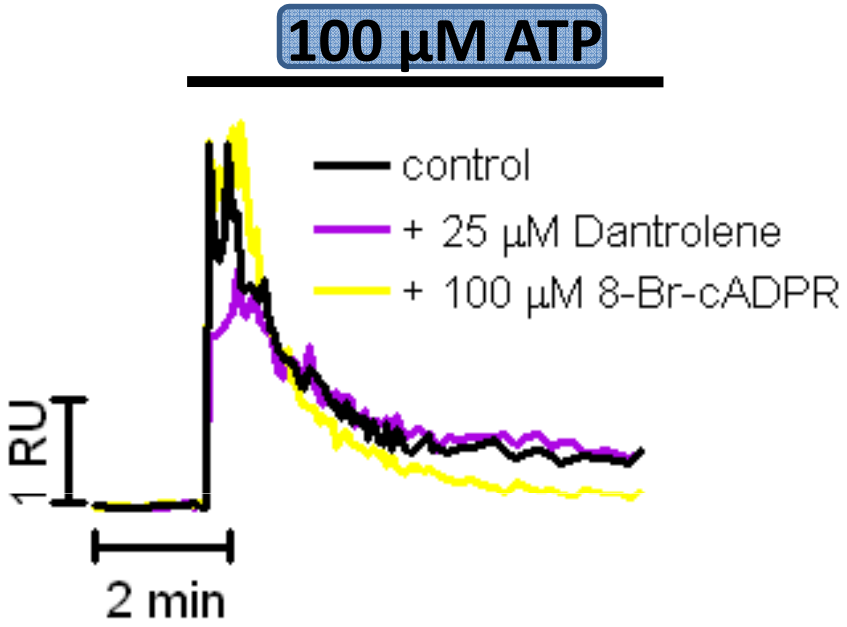


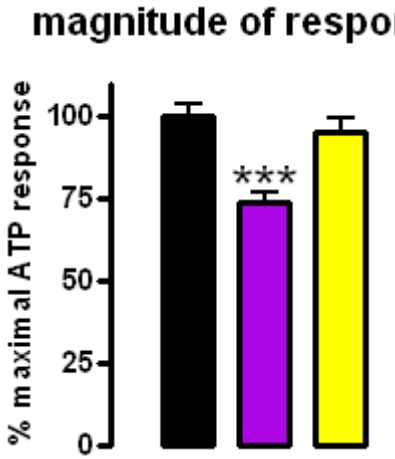


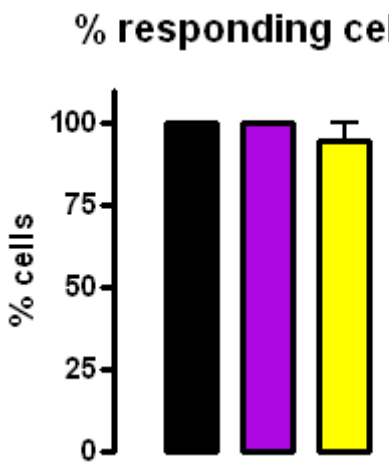


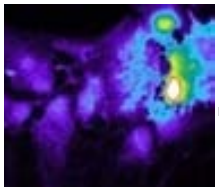
ATP-induced Ca^{2+} responses are slightly influenced by cADPR signalling and Ryanodine receptors











Not all neurotransmitters signal Ca^{2+} through NAADP

External Stimuli

BK

ATP
ET-1
Ach

Internal Signal

IP_3/ cADPR

NAADP

Endoplasmic Reticulum

IP_3R

RyR

Ca^{2+} Ca^{2+} Ca^{2+}

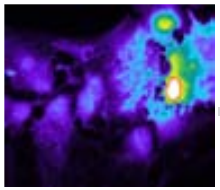
Ca^{2+}

Lysosome

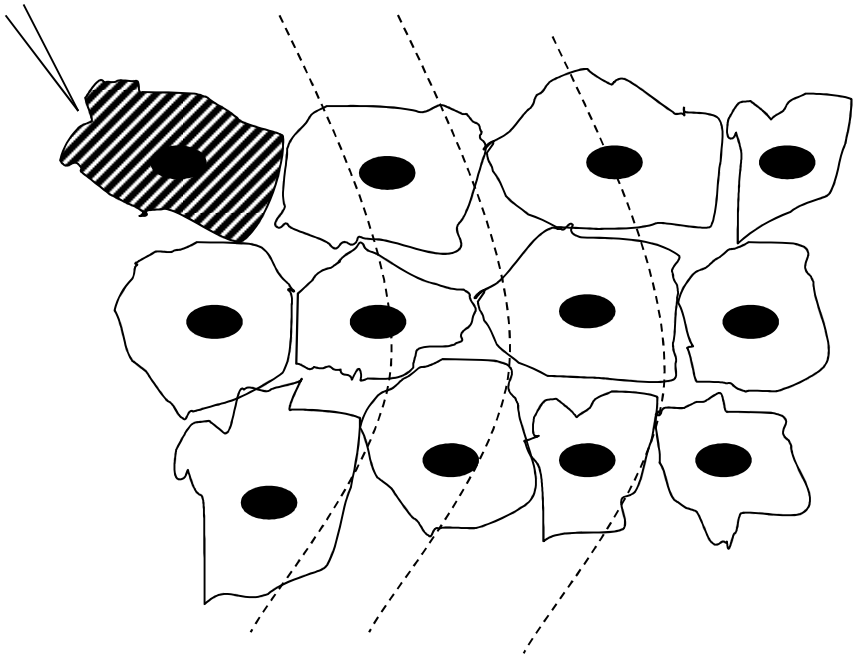
NAADPR

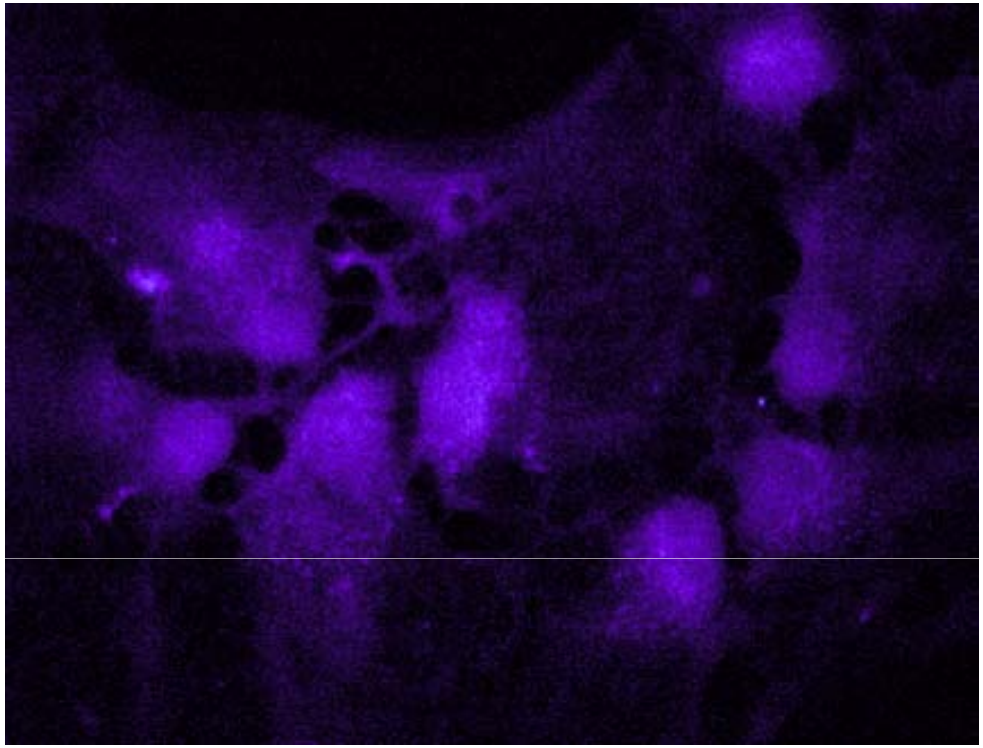
Ca^{2+} Ca^{2+}

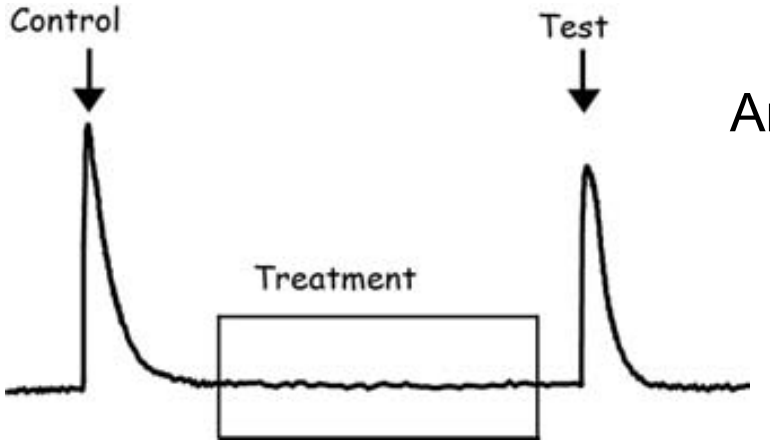
Ca^{2+}



Mechanically-induced Ca^{2+} waves

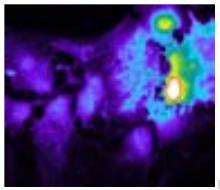




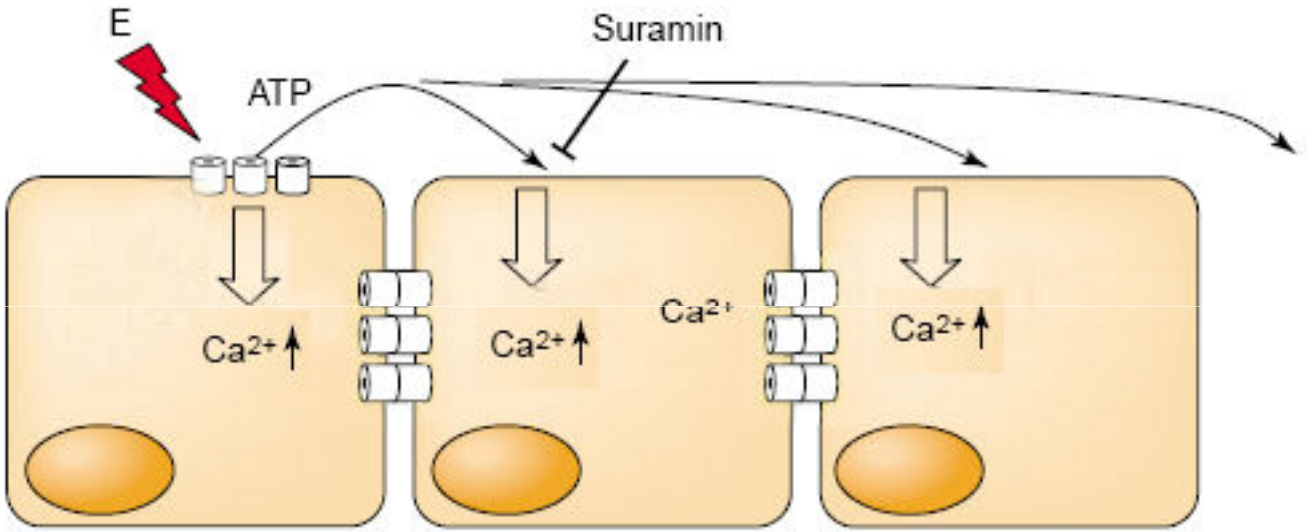


Analysed Parameters:

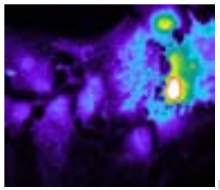
- magnitude of response of each cell
- number of responding cells
- velocity of propagation (Δ_{time} from estimulated cell)



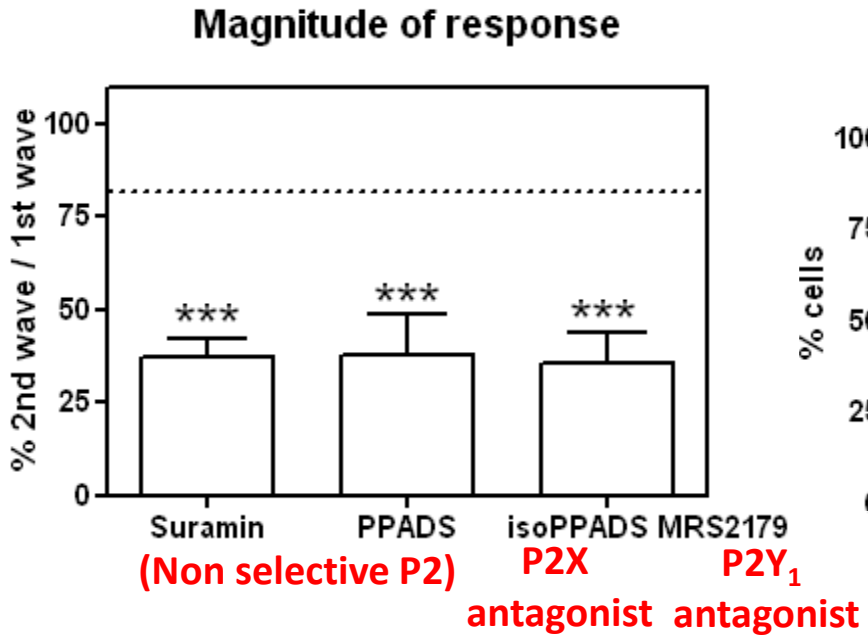
ATP release from astrocytes mediates the propagation of Ca^{2+} waves

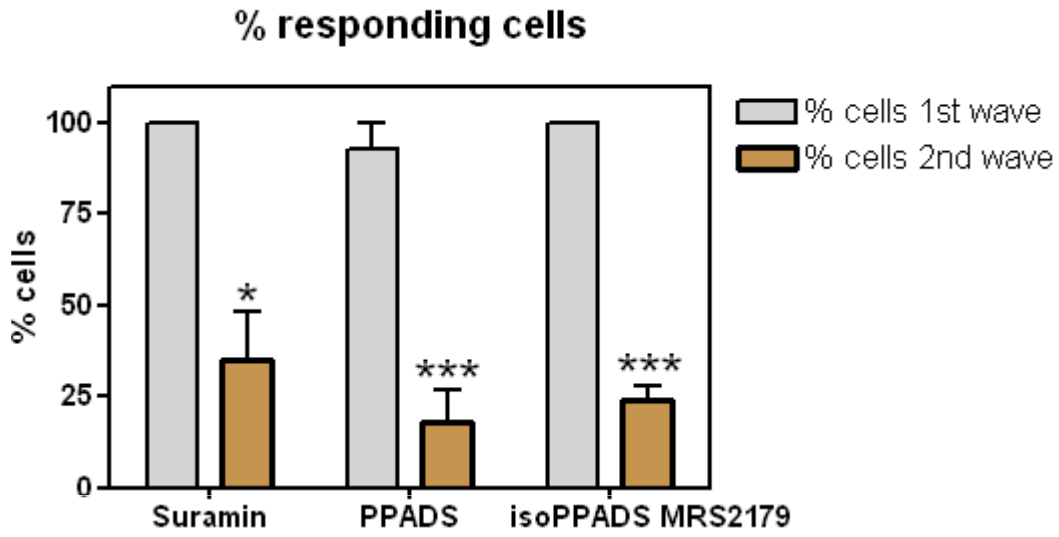


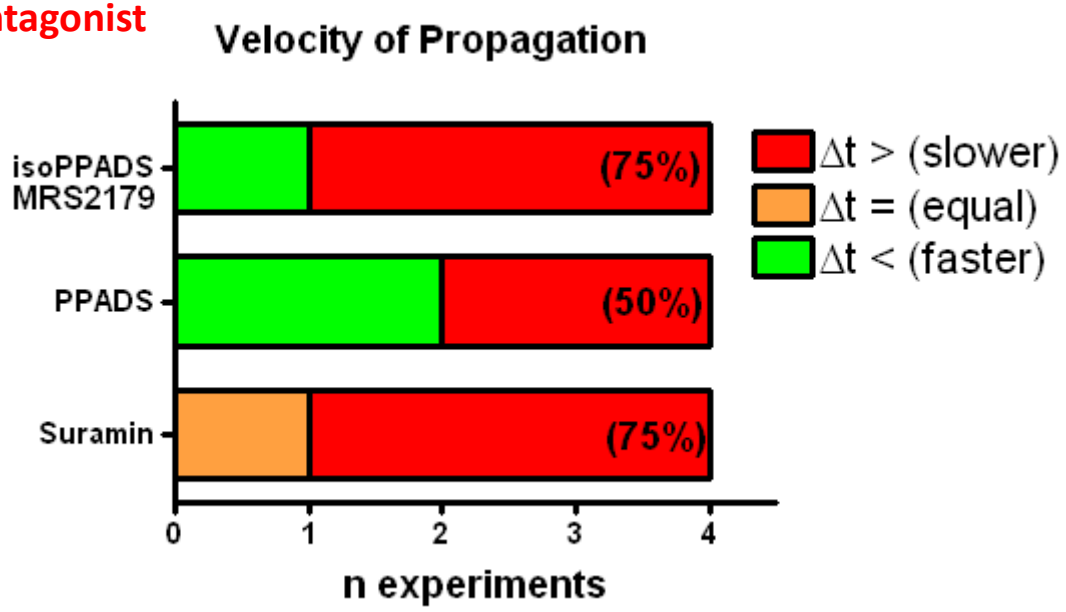
Bennett et al., (2003)

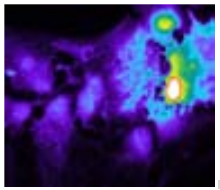


Purinergic receptor activation is needed for Ca^{2+} wave propagation

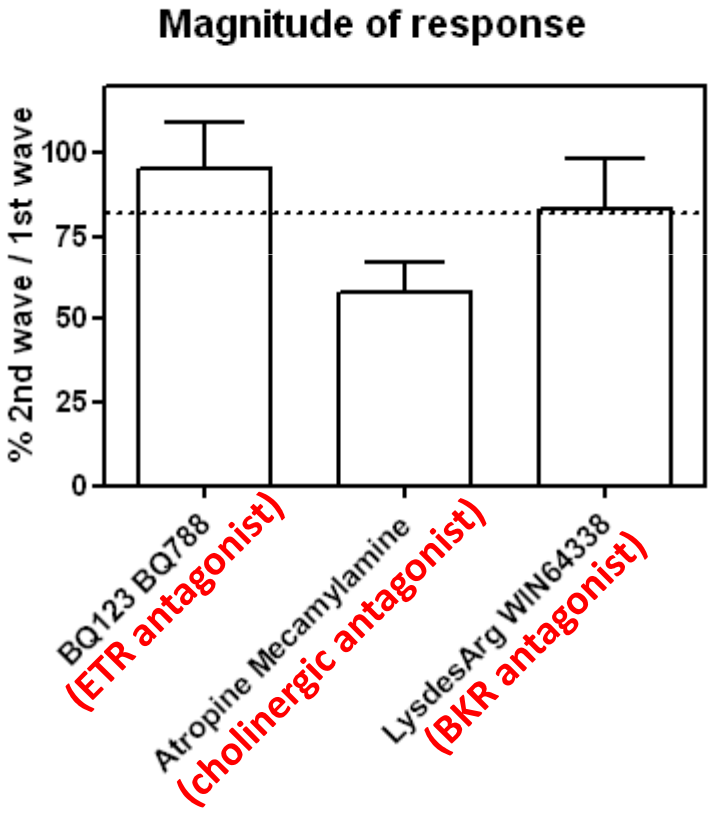


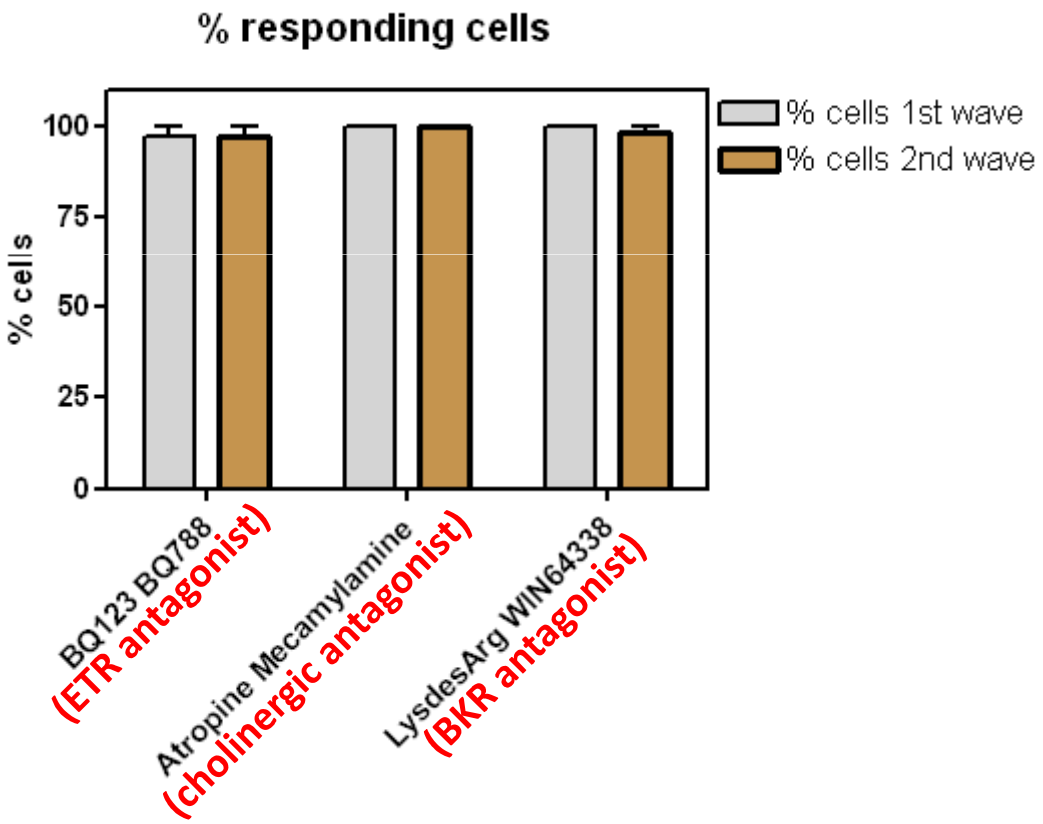


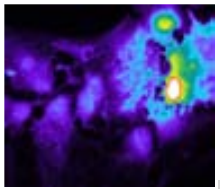




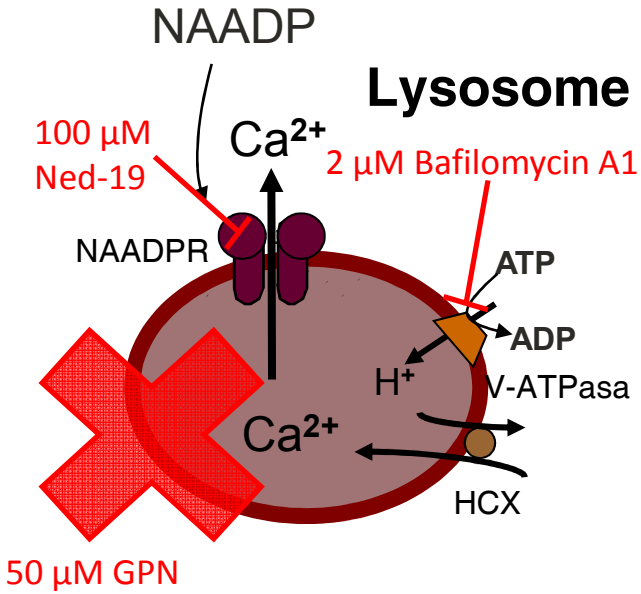
No need for ET-1, Ach or BK receptor activation in Ca^{2+} wave propagation

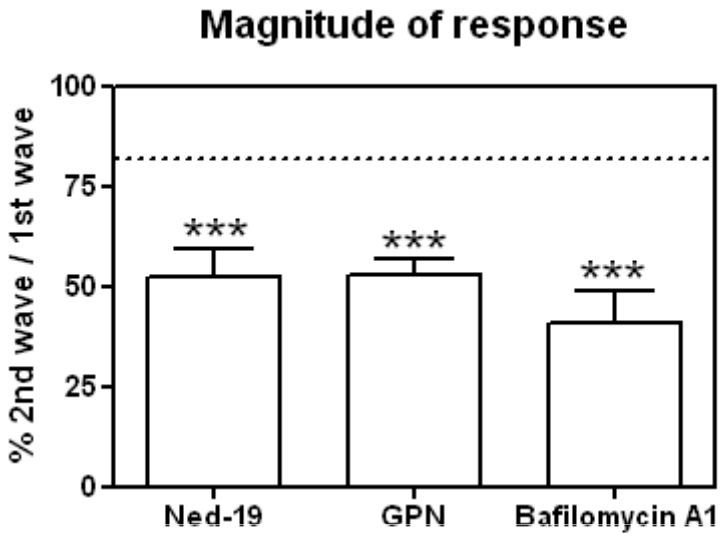


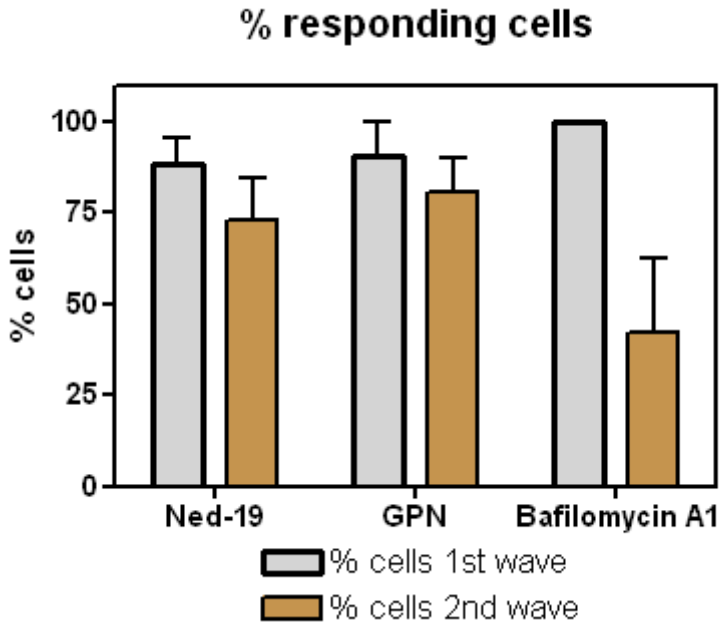


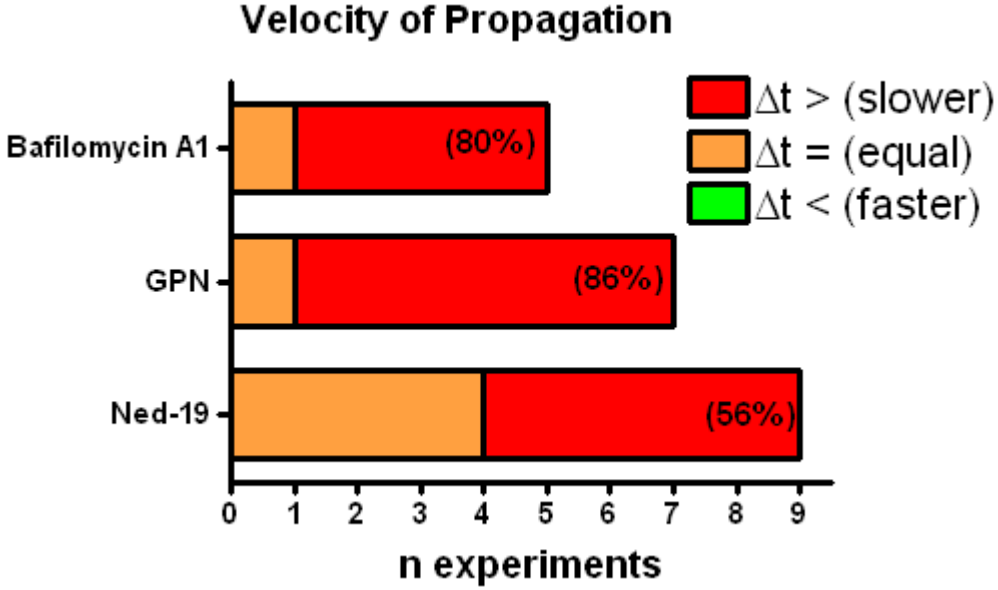


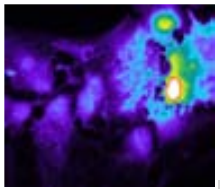
NAADP signalling and lysosomal Ca^{2+} participate in Ca^{2+} wave propagation



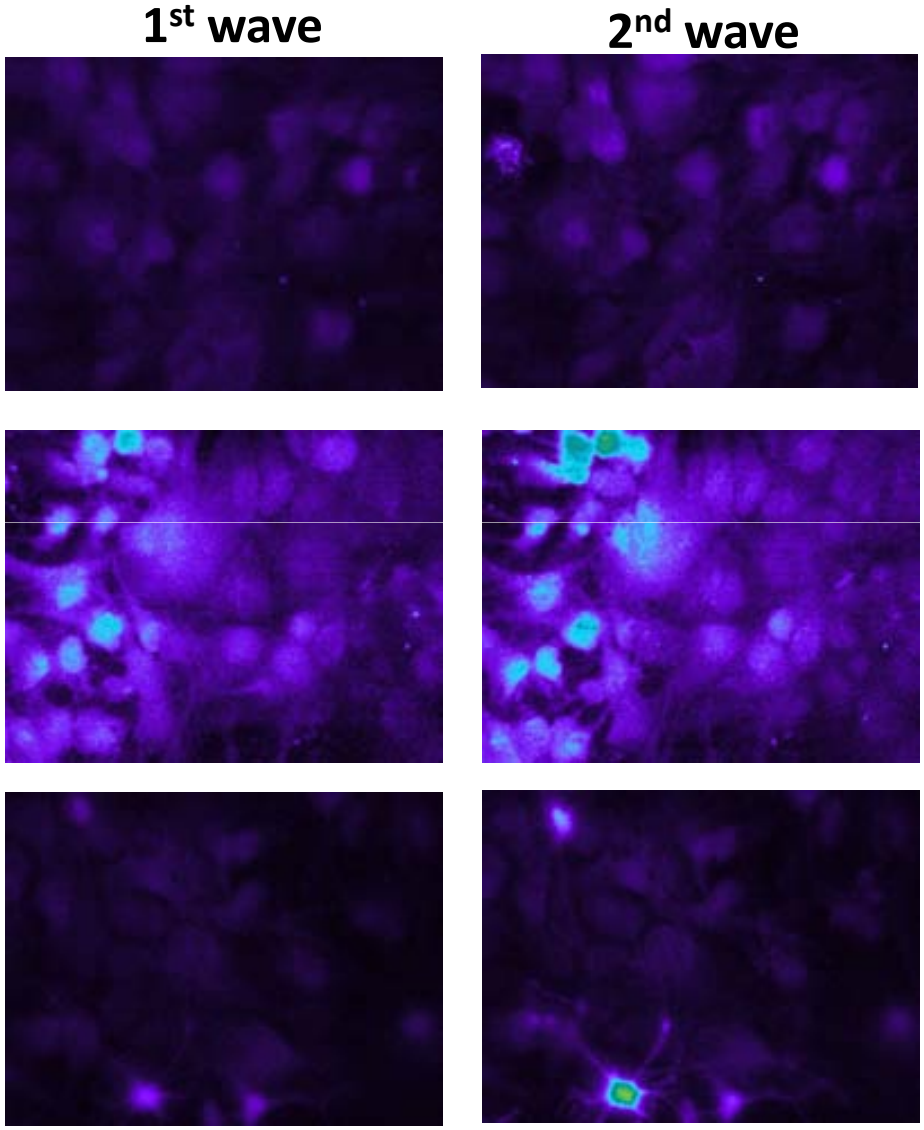


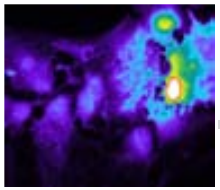




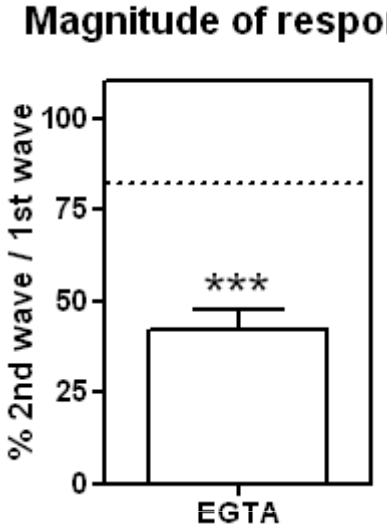


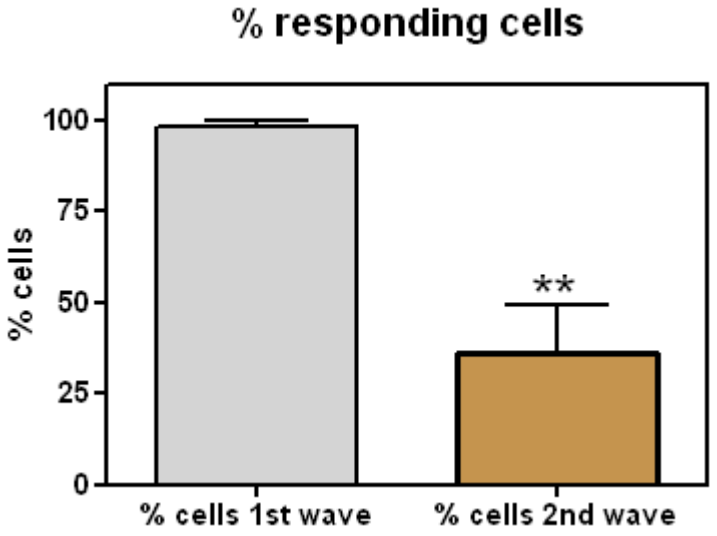
NAADP signalling and lysosomal Ca^{2+} participate in Ca^{2+} wave propagation

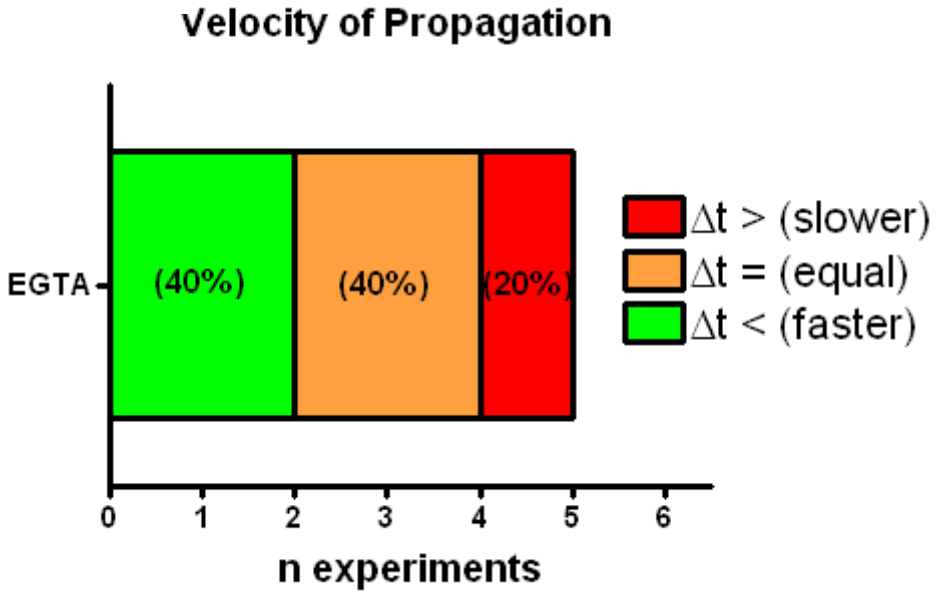


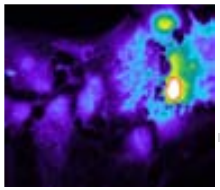


Extracellular Ca^{2+} is needed for Ca^{2+} wave propagation

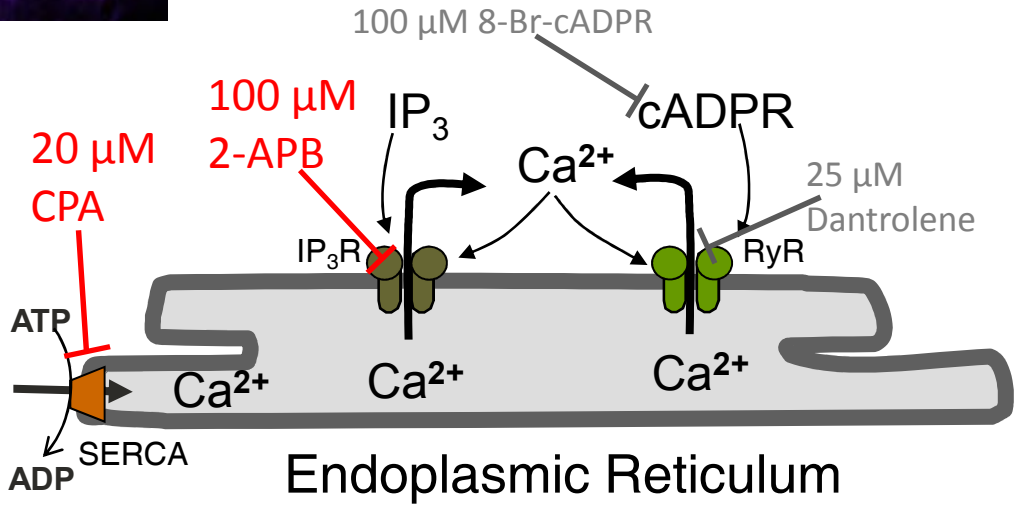


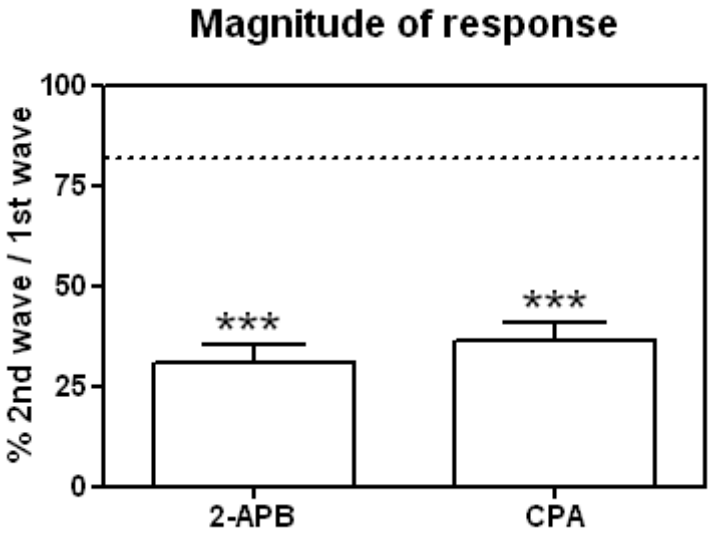


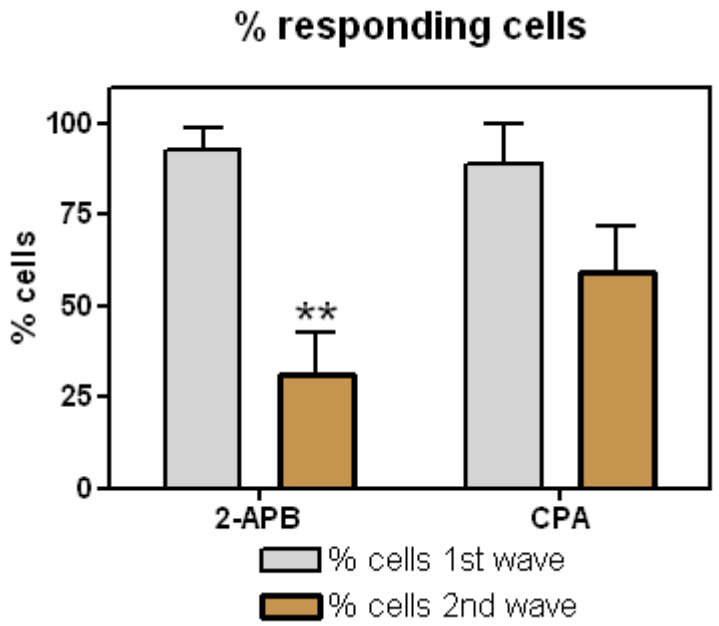


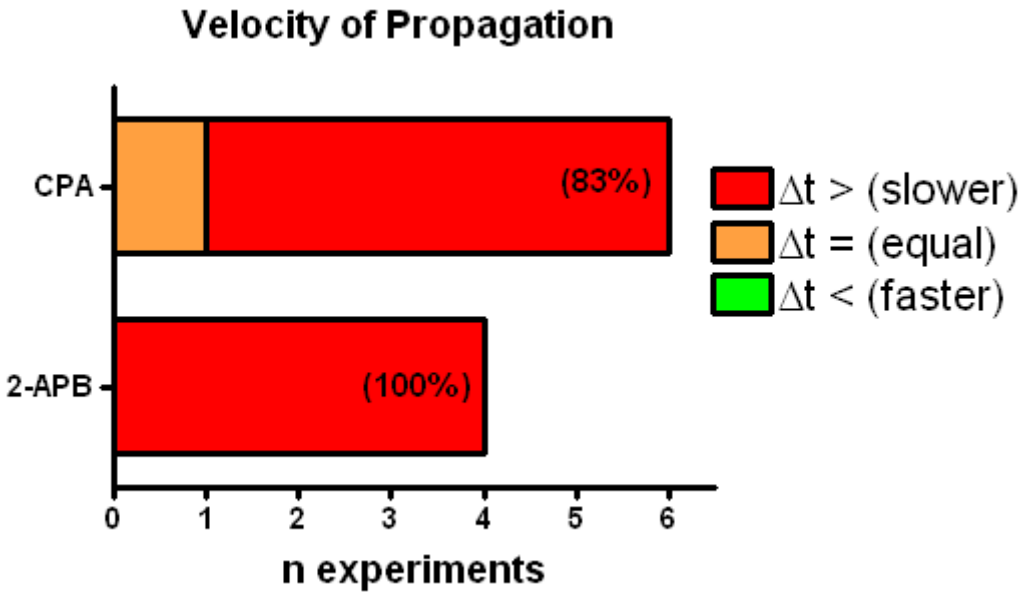


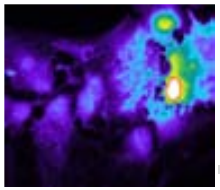
IP_3 signalling mediates Ca^{2+} wave propagation



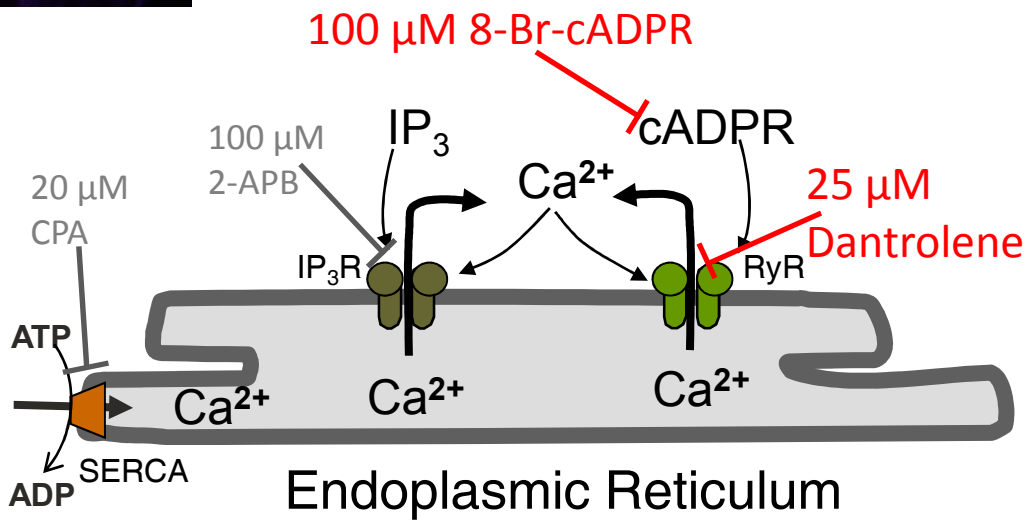


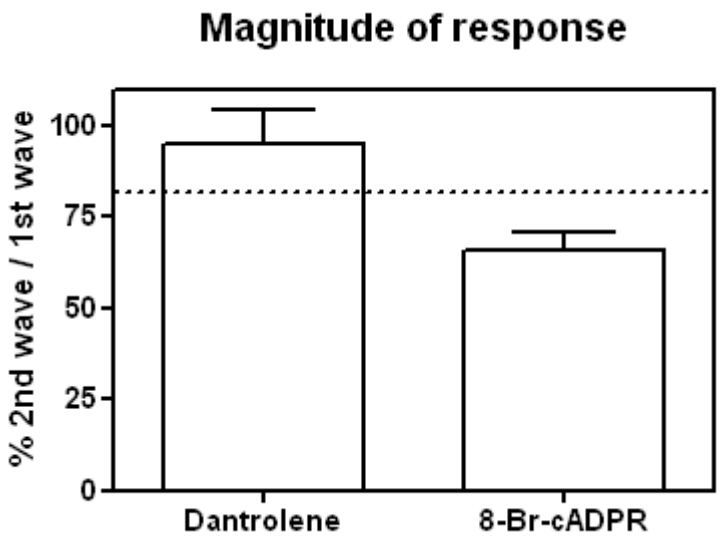


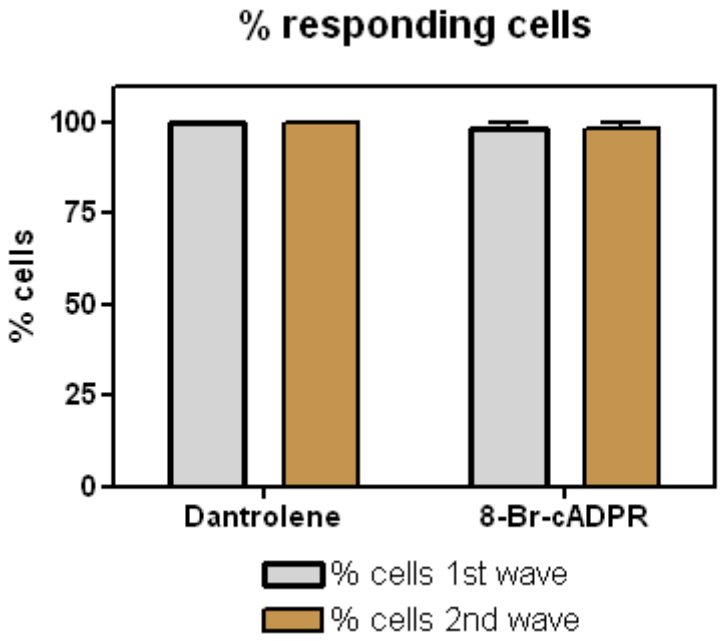


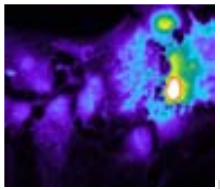


Ryr/cADPR signalling does not seem to take part in Ca^{2+} wave propagation

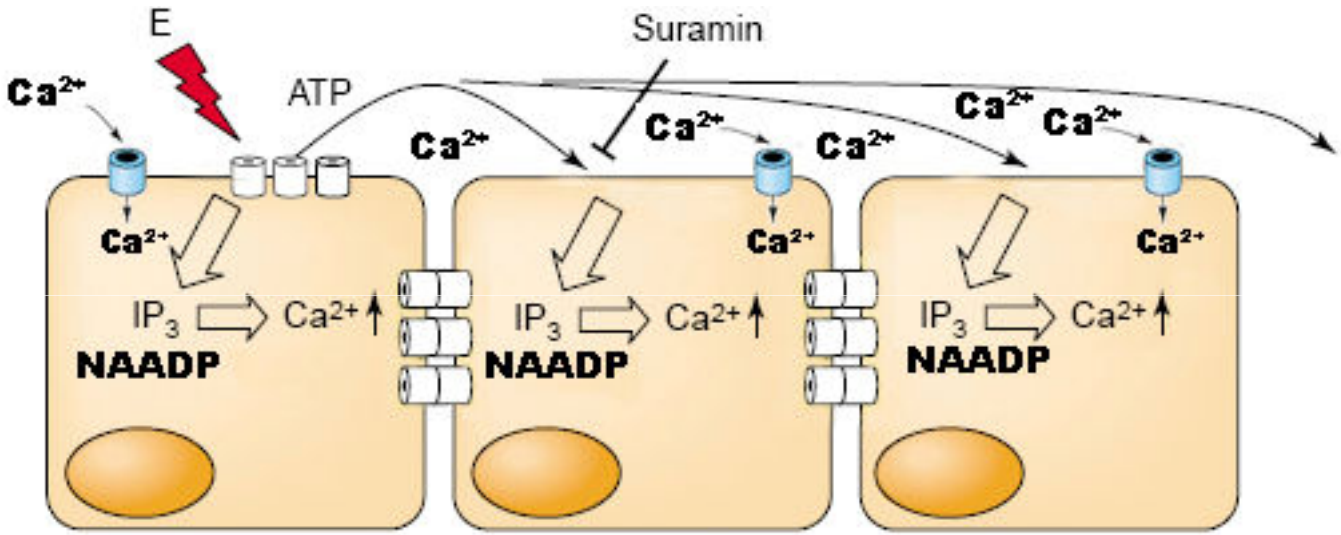


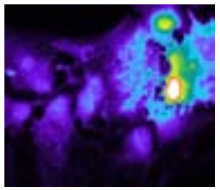






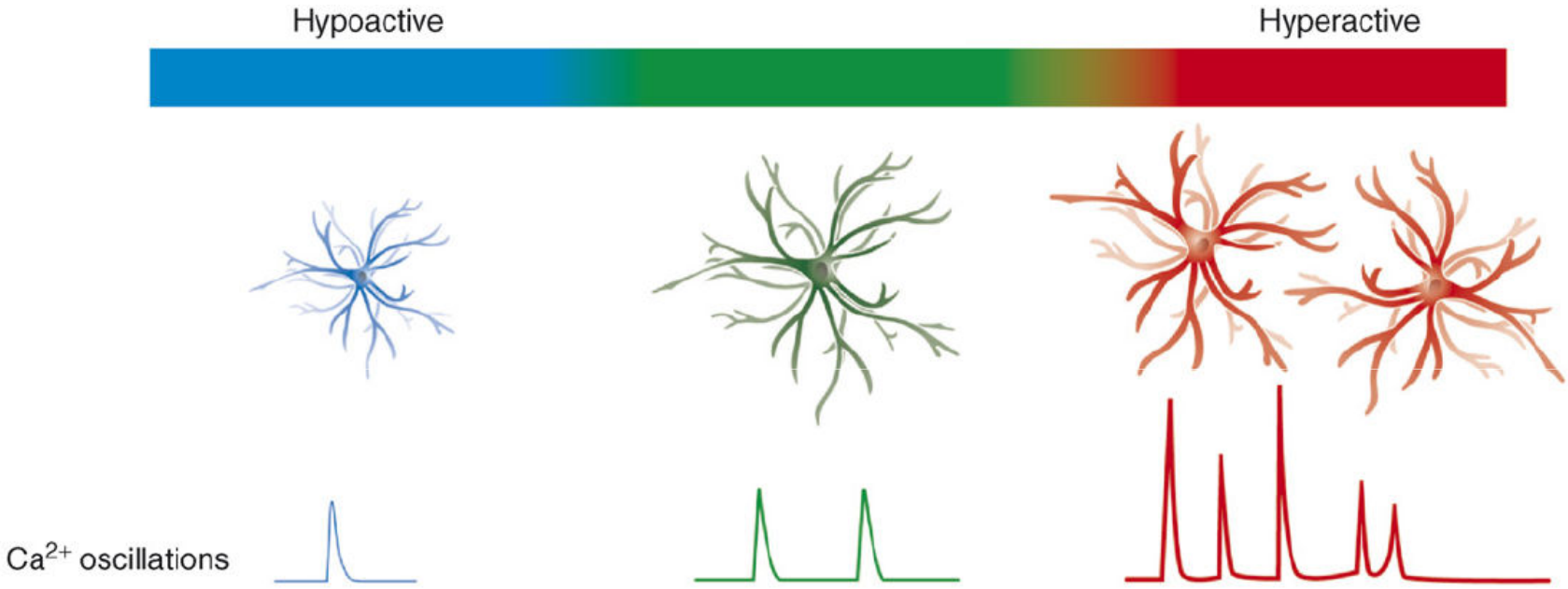
NAADP signalling participates in Ca^{2+} wave propagation through purinergic receptors





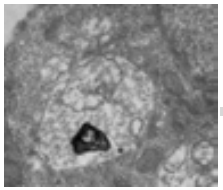
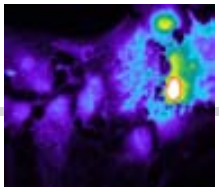
Does it all have any interest for clinical science?

Astrocytic Ca^{2+} activation spectrum: a biological model



NAADP modulates astrocyte Ca^{2+} excitability

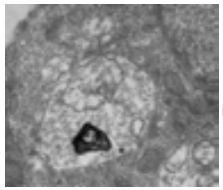
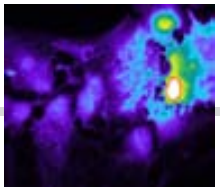
NAADP and lysosomal Ca^{2+} may be effective pharmacological targets

1. CHO MT58 cells at 40 ^{o}C undergo a rapid inhibition of PtdCho synthesis, followed by a decrease in the mass of PtdCho and a loss of viability.

2. Cell death caused by inhibition of PtdCho synthesis takes place with very little chromatin condensation and DNA degradation, together with no activation of caspase-3. Therefore, cells die by a non apoptotic mechanism.

3. Inhibition of PtdCho synthesis involves overexpression of cathepsin D and the appearance of big autophagic vesicles with high levels of LC3-II. High LC3-II over time shows that inhibition of PtdCho synthesis blocks the progress of autophagy.

4. Astrocyte lysosomes contain Ca^{2+} and express NAADP receptors that can be activated by a cell permeable analogue of NAADP, NAADP-AM.

5. The neurotransmitters ATP, Endothelin-1 and Acetylcholine induce Ca^{2+} responses that in part are due to NAADP-mediated Ca^{2+} release from lysosomes.

6. The propagation of Ca^{2+} waves in rat cortical astrocytes requires the activation of purinergic receptors, but it does not depend on endothelin, cholinergic or bradykinin receptors.

7. NAADP-mediated Ca^{2+} release from lysosomes has a key role in the astrocytic excitability by modulating the velocity of propagation of Ca^{2+} waves, as well as the strength of response.