# Study of the Antisecretory Factor effects on $GABA_A$ receptor by using RuBi-GABA uncaging with non-linear photoactivation in rat cerebellar granule cells *in vitro*

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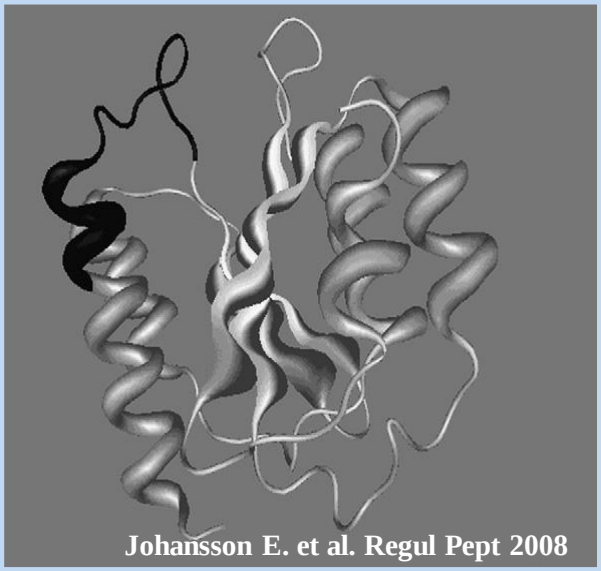
SIF – 106° Congresso Nazionale
14-18 Settembre 2020

# Antisecretory Factor (1)

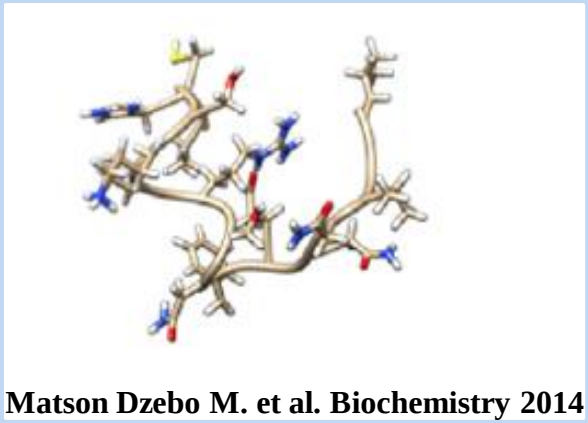
- Identified in the 80s by Stefan Lange e Ivar Lönnroth
- Protein: 41 kDa
- Expressed in various mammalian tissues and in plasma
- **Inhibitory action** against intestinal hypersecretion (i.e., dysentery)
- **Anti-inflammatory action** (i.e., tinnitus)

# Antisecretory Factor (2)




*Antisecretory Factor*

- N – terminal: antisecretory site
- C – terminal: part of the regulatory 19S subunit of the proteasome
- von Willebrand Factor type A domain between AA 5 and 188
- AF–16: peptide of 16 AA containing the antisecretory site



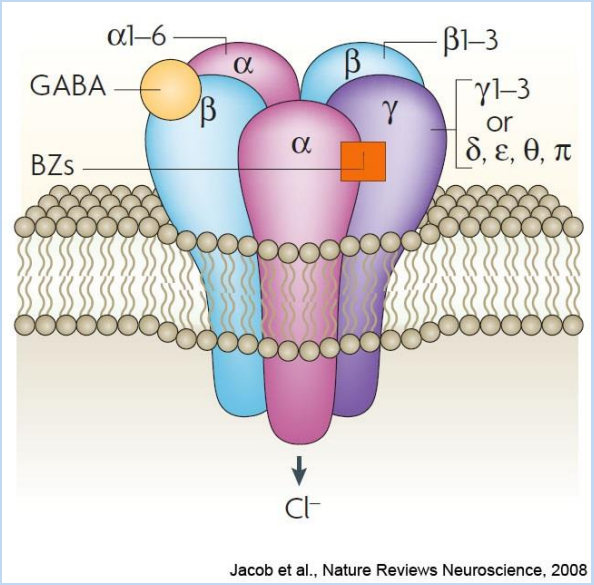

*AF-16 Peptide*

| V | C | H | S | K | T | R | S | N | P | E | N | N | V | G | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

**Active site**

*Amino acid sequence of AF-16*

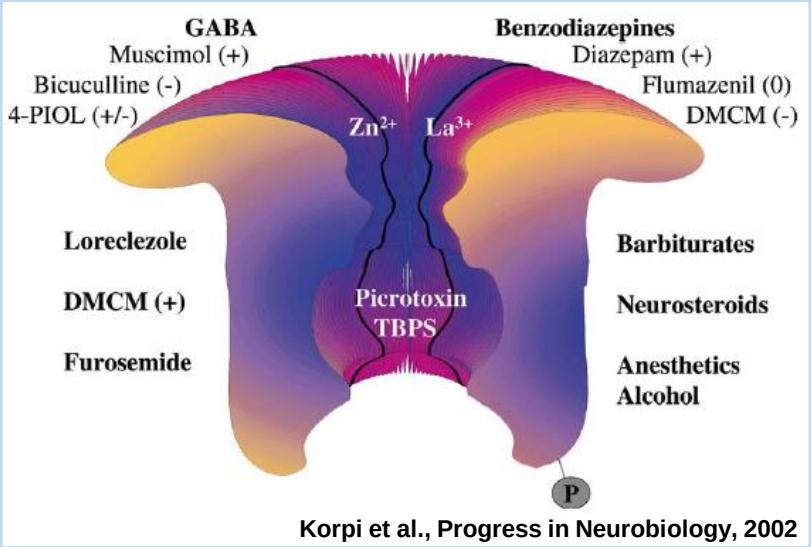
# $GABA_A$ receptor



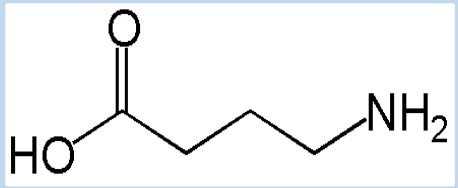
Jacob et al., Nature Reviews Neuroscience, 2008

$GABA_A$ receptor subtypes are composed of **different** protein **subunits**

- 6 α subunits
- 3 β ″
- 3 γ ″
- 1 δ ″
- 1 ε ″
- 1 θ ″
- 1 π ″

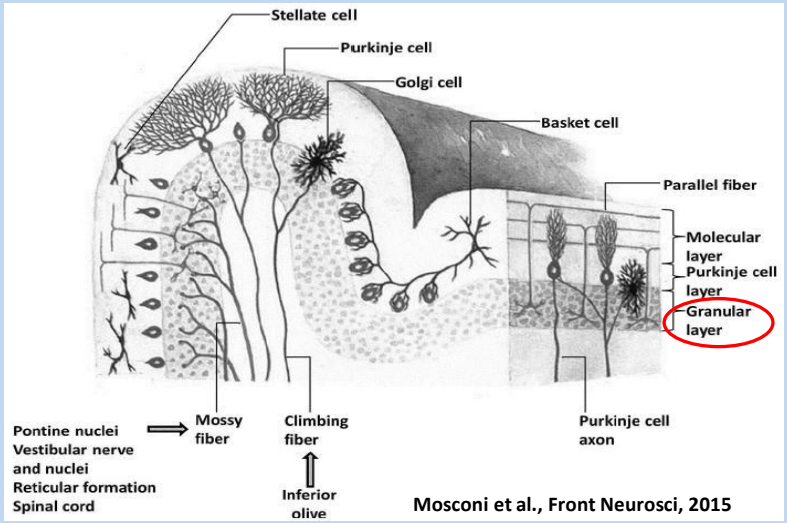


Korpi et al., Progress in Neurobiology, 2002

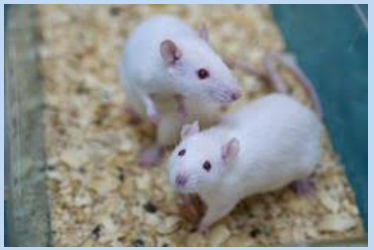



**GABA**
Main **inhibitory neurotransmitter** in the Central Neuron System

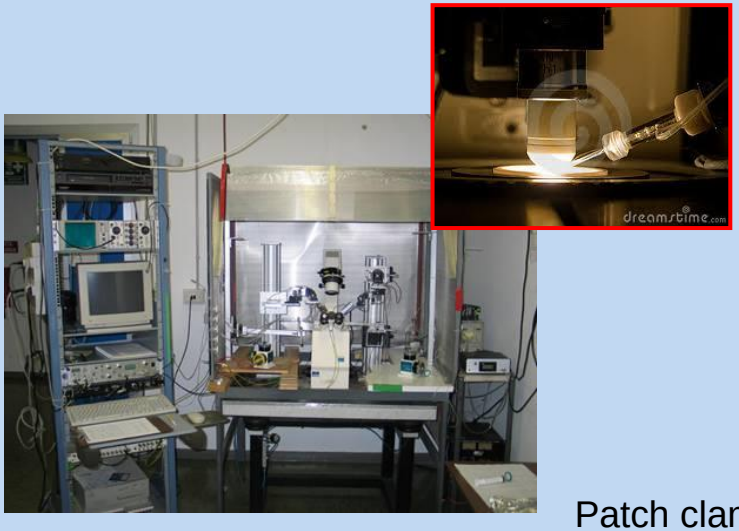
# Experimental approach (1)

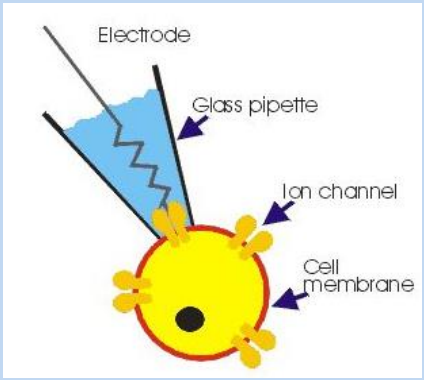



Cerebellar cortex

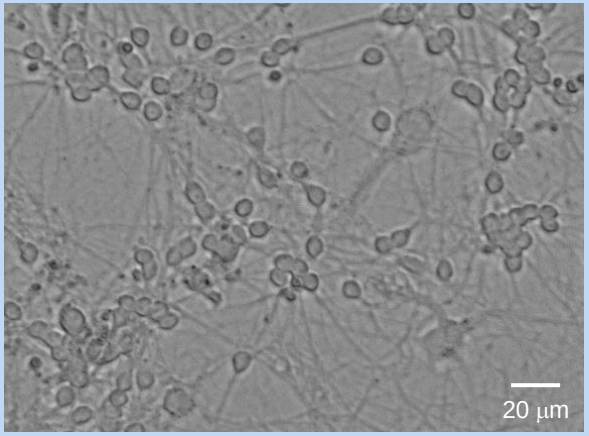


7d postnatal rats



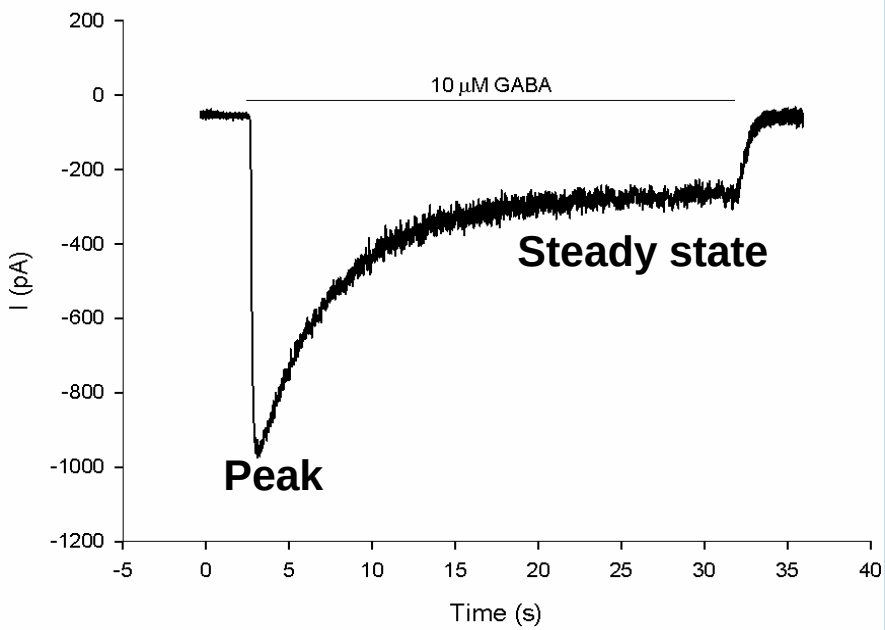



Patch clamp technique

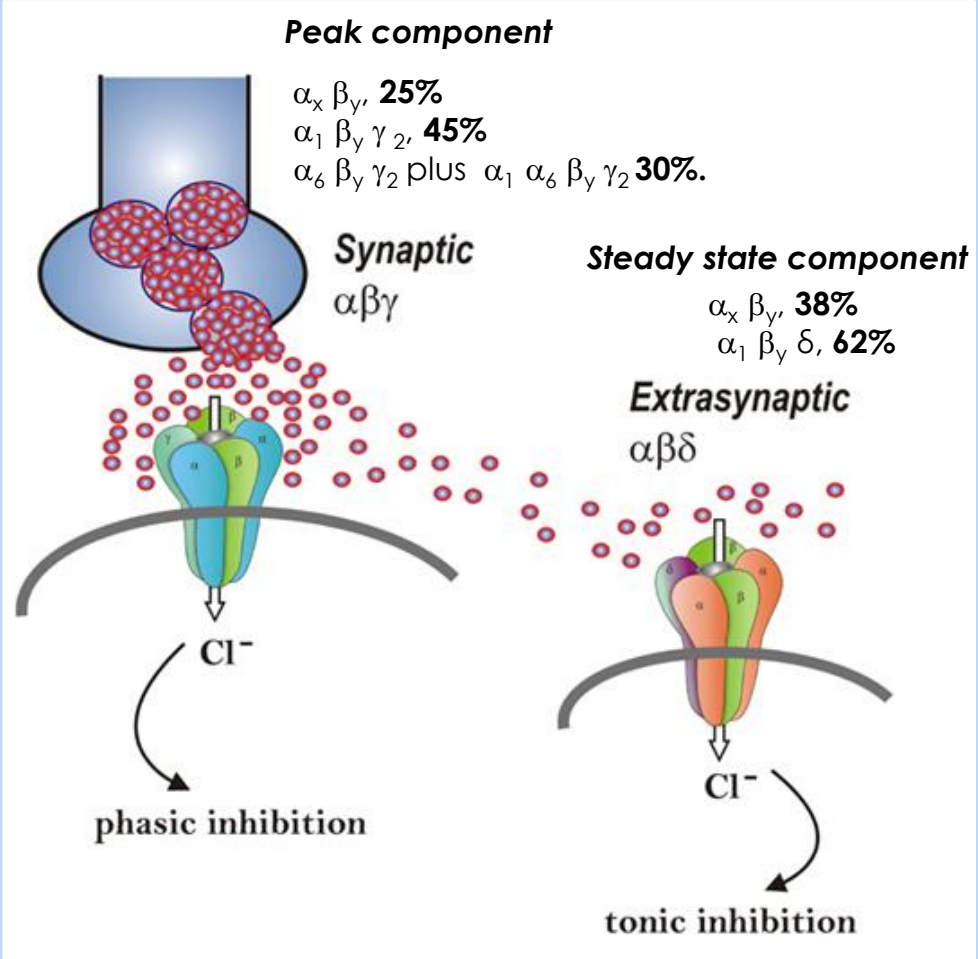



Cerebellar granule cells in culture

# Distribution of $GABA_A$ receptor subtypes

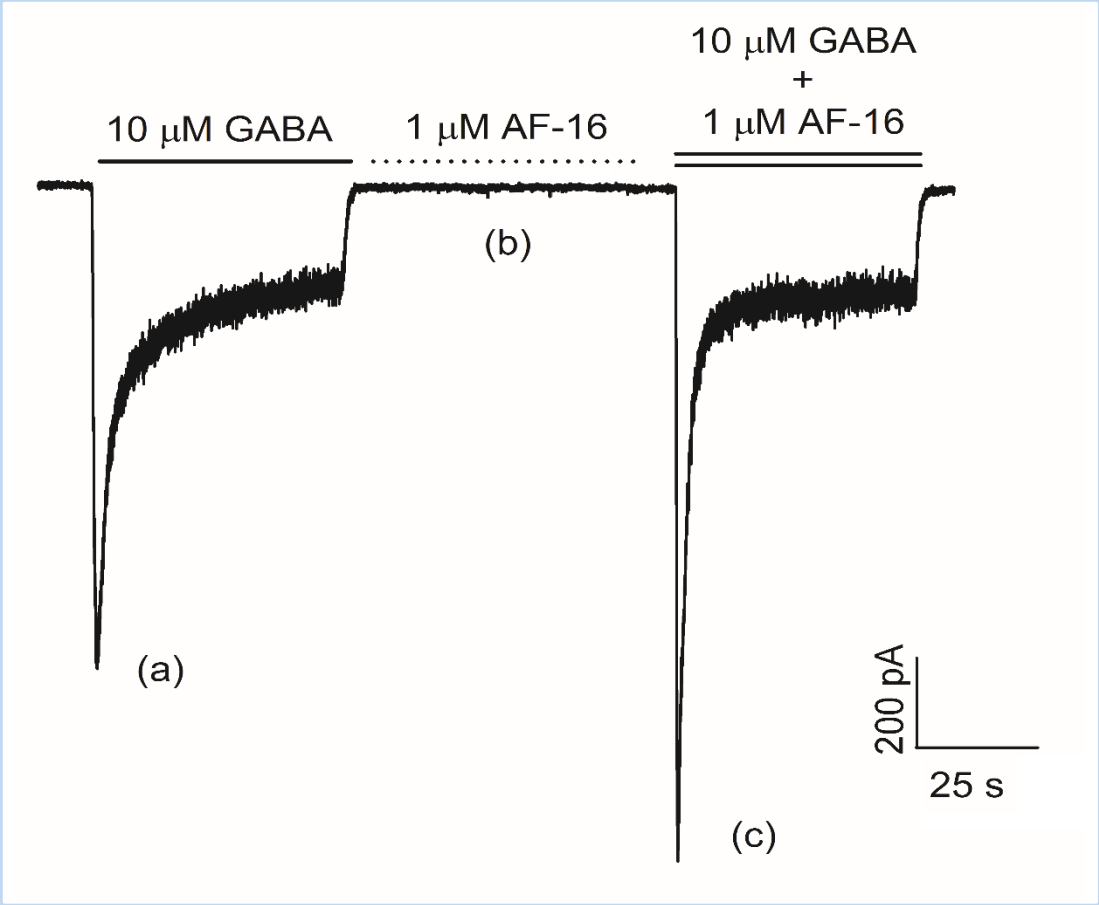



A typical profile of chloride current activated by **10 μM GABA**




**Reddy D.S. (2011).** Front Endocrinol; 2: 38
**Gatta E. et al. (2009).** Neuroscience;162: 1187-1191

# Effect of AF-16 treatment on $GABA_A$ receptors




(a) Current activated by **10 μM GABA**

(b) 3 min perfusion with **1 μM AF-16**

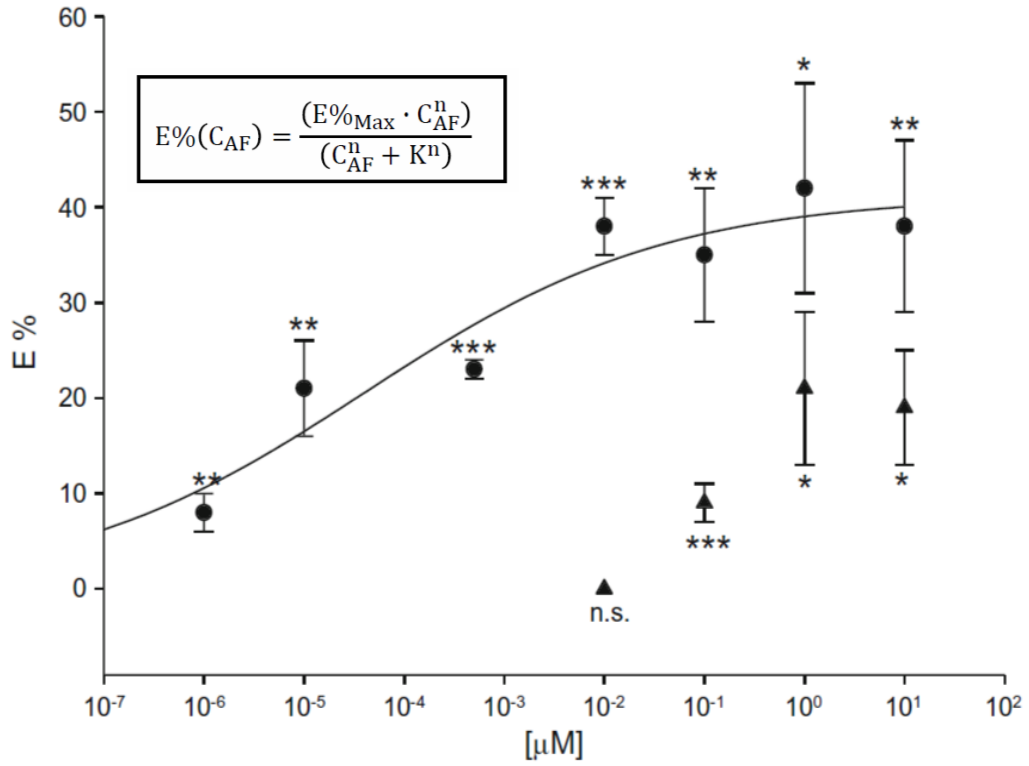
(c) Response evoked by **10 μM GABA + 1 μM AF-16**

**Bazzurro V. et al. (2018).** J Mol Neurosci; 64(2):312-320.

# AF-16 Dose-response curves

E%= Percentage Effect

$$E\% = \frac{(I_{AF,GABA} - I_{GABA})}{I_{GABA}} \cdot 100$$

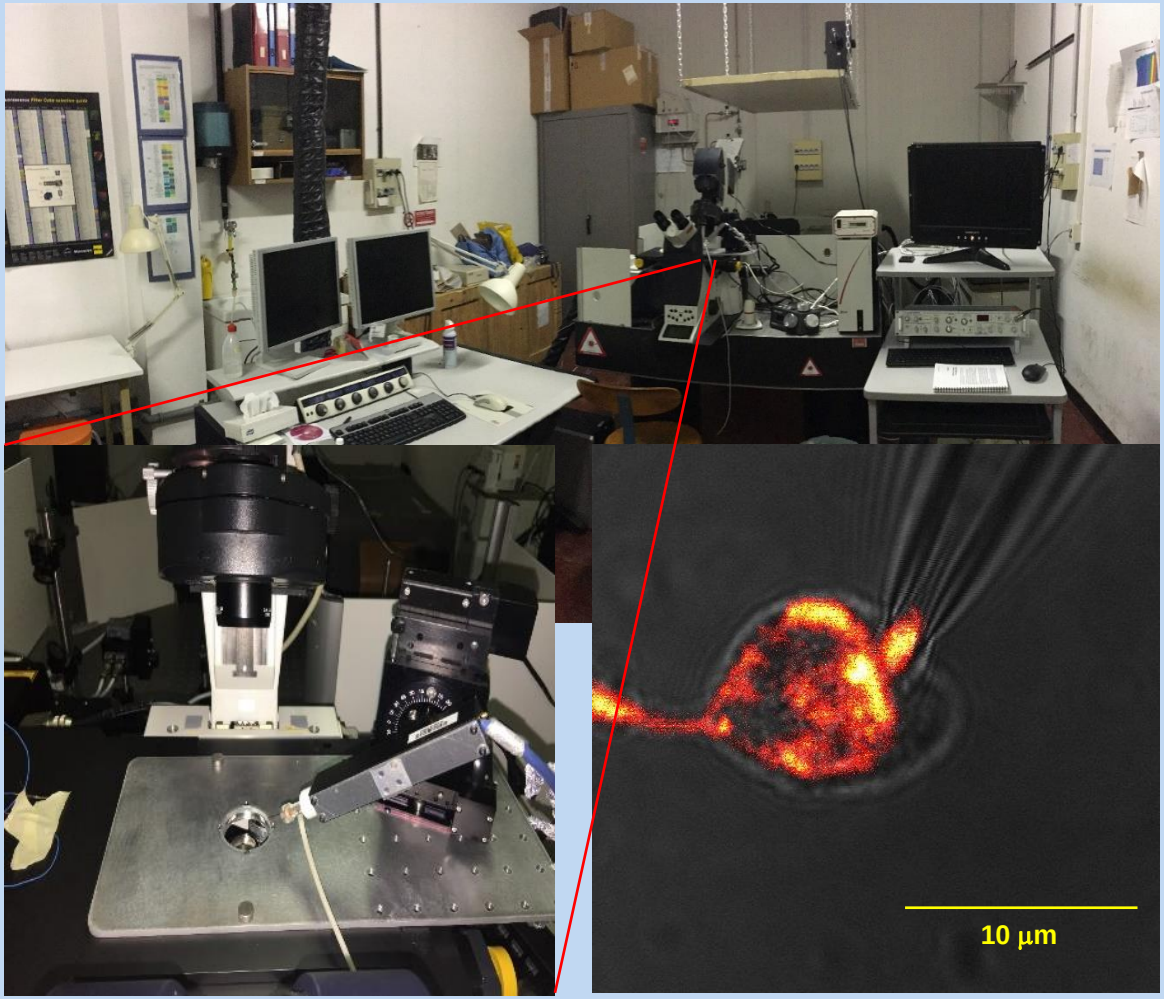



●: AF-16 3 minutes perfusion

▲: AF-16 30 seconds perfusion

**Bazzurro V. et al. (2018).** J Mol Neurosci; 64(2):312-320.

# Experimental approach (2)




- Vibration isolation table
- Confocal and two-photon microscope for imaging and uncaging (Leica TCS SP5)
- Micromanipulators for moving and positioning the electrode
- Low-noise amplifier (Axon Axopatch 200B)
- Computer (for controlling and generating the stimulus waveform and data acquisition)

# Caged compounds

Caged compounds = molecules biologically or functionally inert until they are activated by light

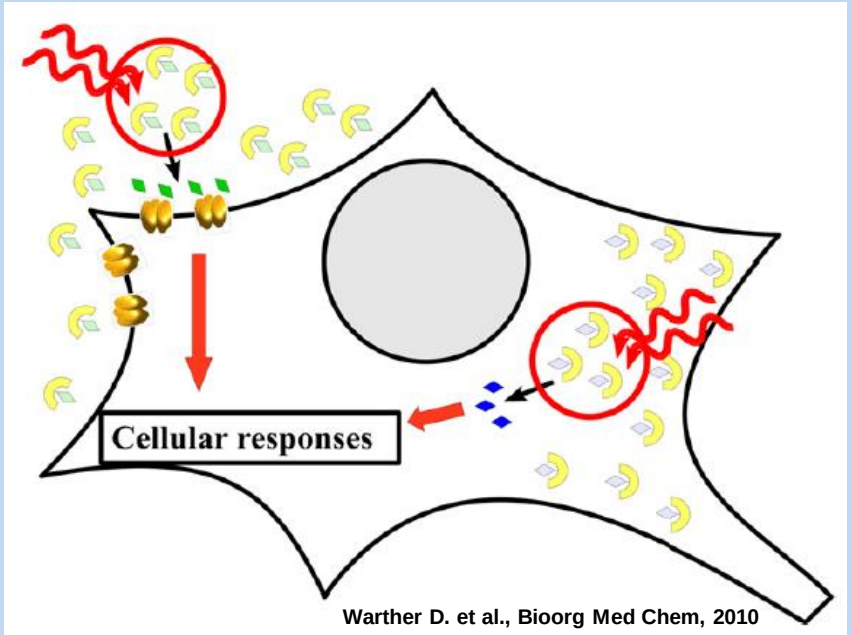
## Advantages of caged compounds

- **Rapid** responses
- **Spatial** and **temporal control** of cell activation
- Channel kinetics **quantification**
- Inert until photoactivated
- Quantification of uncaged molecules
- In the whole cell configuration they can be introduced into the cytoplasm through the pipette

Warther D. et al., Bioorg Med Chem, 2010

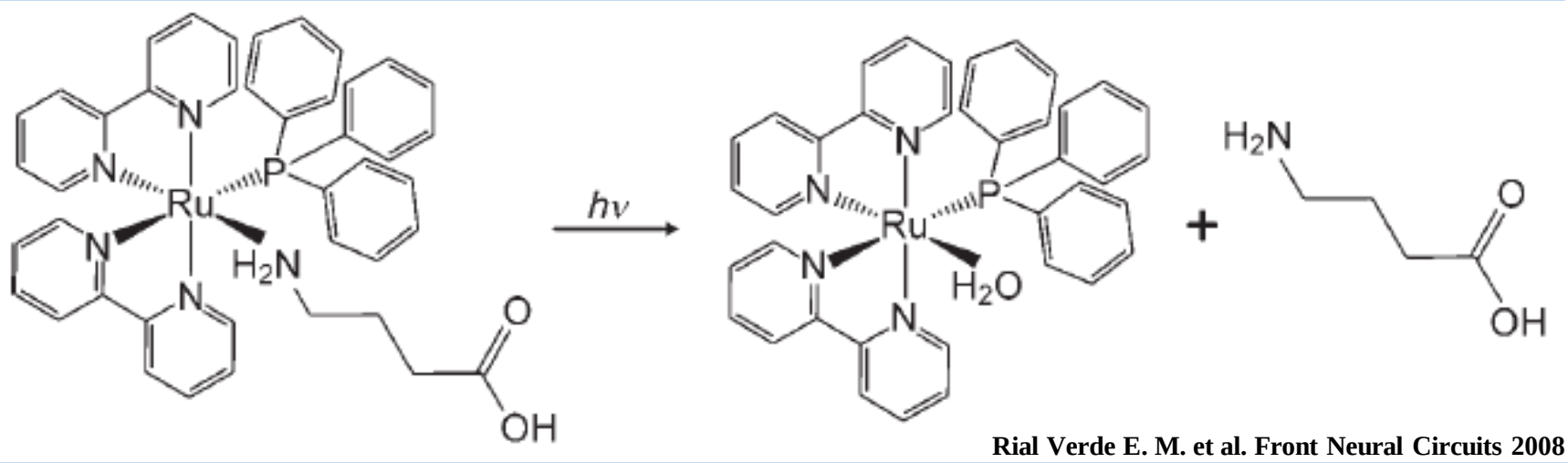
## Characteristics of caged compounds

- They must not produce a response before illumination
- They must be hydrolytically and enzymatically stable during the experiment



Warther D. et al., Bioorg Med Chem, 2010

# RuBi-GABA




Rial Verde E. M. et al. Front Neural Circuits 2008

- **Visible light** can be used to photorelease GABA
- It is less damaging for DNA or proteins with no activation of stress-response
- It can penetrate farther into tissues than UV light reducing the relative energy required to uncage as a function of depth
- Possible use **Two-Photon excitation**

# RuBi-GABA Uncaging

## Precise 3D modulation of electro-optical parameters during neurotransmitter uncaging experiments with neurons in vitro

Marco Cozzolino[1,2,3], Virginia Bazzurro[1,3], Elena Gatta[1], Paolo Bianchini[2], Elena Angeli[1], Mauro Robello[1] & Alberto Diaspro[1,2]✉

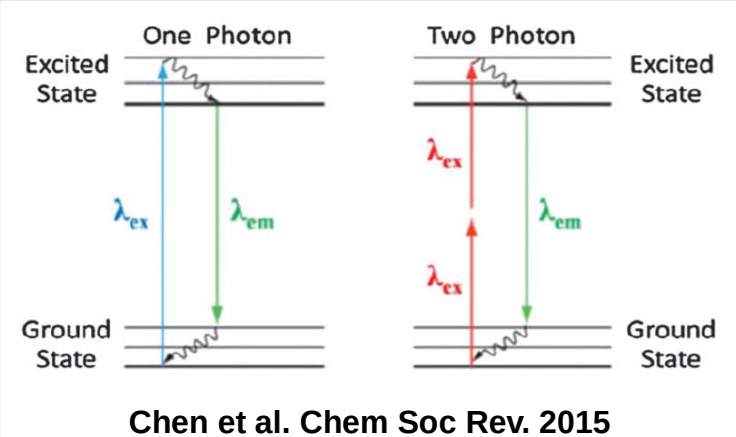
Ruthenium–bipyridinetriphenylphosphine–GABA (RuBi–GABA) is a caged compound that allows studying the neuronal transmission in a specific region of a neuron. The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is bound to a caged group that blocks the interaction of the neurotransmitter with its receptor site. Following linear—one-photon (1P)—and non-linear—multi-photon—absorption of light, the covalent bond of the caged molecule is broken, and GABA is released. Such a controlled release in time and space allows investigating the interaction with its receptor in four dimensions (X,Y,Z,t). Taking advantage of this strategy, we succeeded in addressing the modulation of $GABA_A$ in rat cerebellar neurons by coupling the photoactivation process, by confocal or two-photon excitation microscopy, with the electrophysiological technique of the patch-clamp in the whole-cell configuration. Key parameters have been comprehensively investigated and correlated in a temporally and spatially confined way, namely: photoactivation laser power, time of exposure, and distance of the uncaging point from the cell of interest along the X, Y, Z spatial coordinates. The goal of studying specific biological events as a function of controlled physical parameters has been achieved.

**Cozzolino M., Bazzurro V. et al. (2020).** Sci Rep; 10(1):13380.

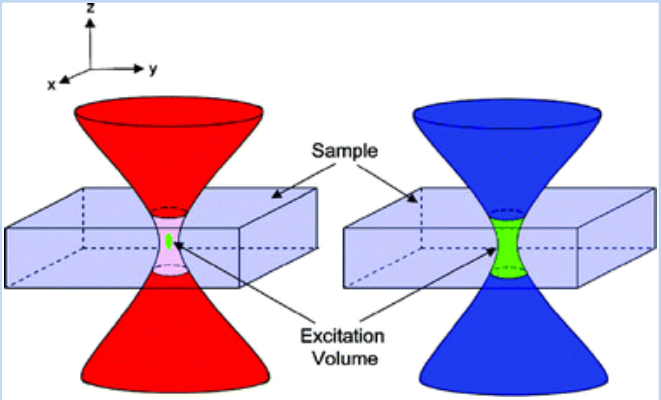
**See also** the communication of Dr. Elena Angeli

# Why Two-Photon Microscopy ?

- **Tiny focal volume** in thick sample
- **Deep penetration**, minimizing phototoxicity, preserving sample and less photobleaching
- The **excitation** of the fluorophore is **near the focal plane** where the laser light is more concentrated
- High spatial resolution imaging *in vivo*



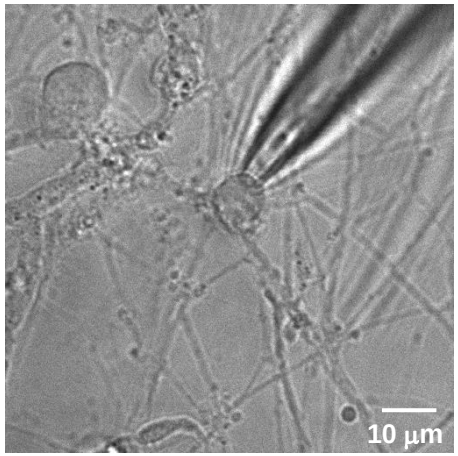

Chen et al. Chem Soc Rev. 2015

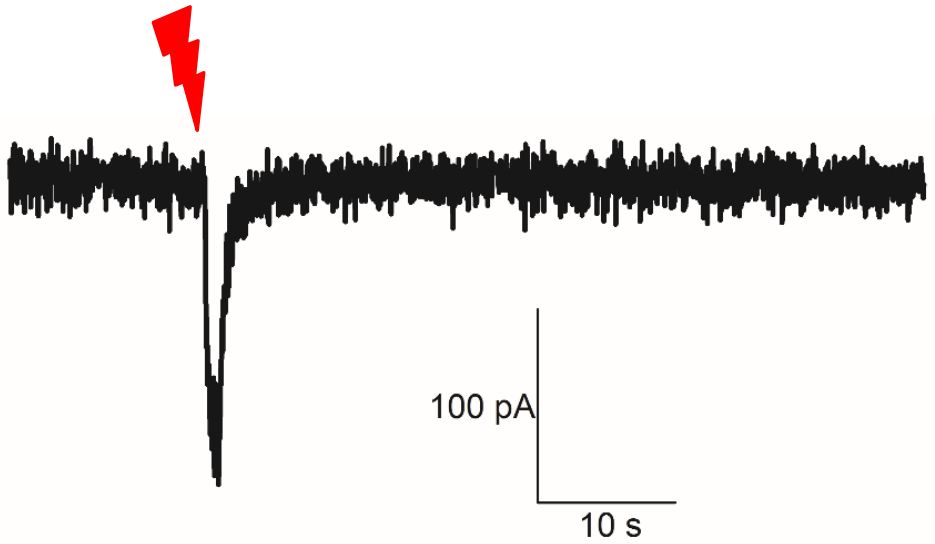



Pedersen et al. Free Radic Res. 2010

**Diaspro A. and Robello M. (2000).** J Photochem Photobiol B; 55:1-8

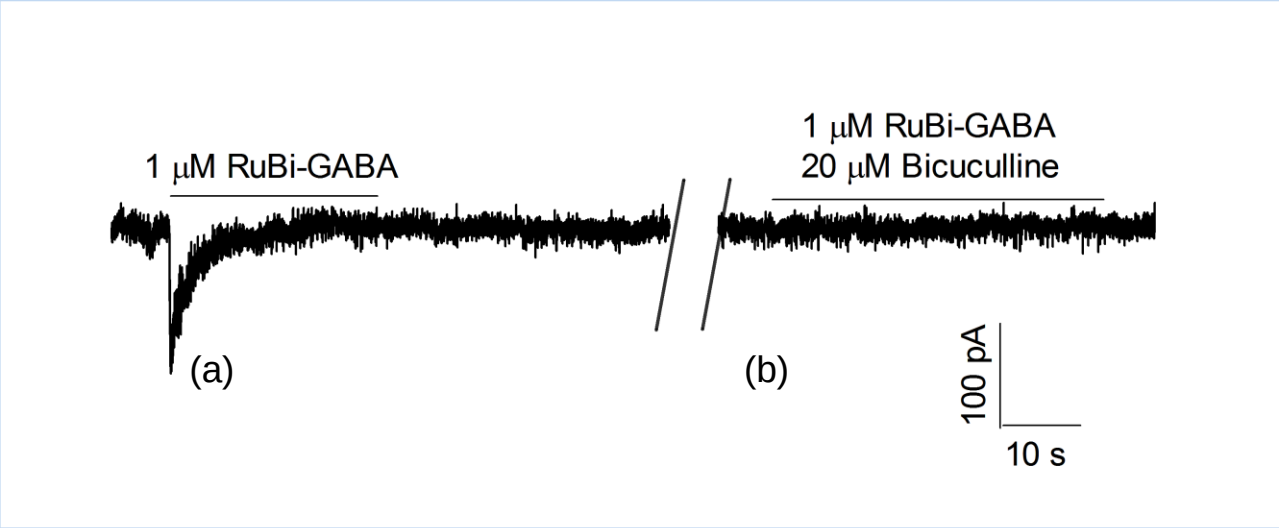
# 10 μM RuBi-GABA response




Localized Uncaging
**Wavelength** 750 nm
**Laser Power** 30 mW
**Exposure Time** 100 ms

# GABA antagonist: Bicuculline

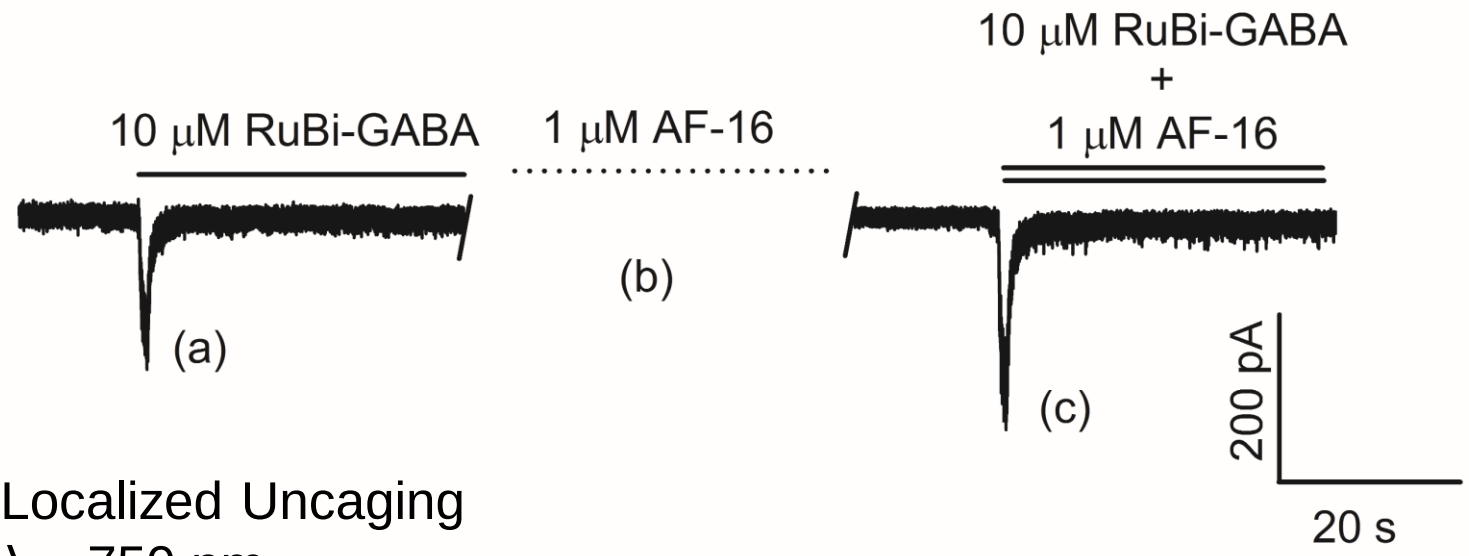



(a) Response evoked by **1 μM RuBi-GABA**

(b) Response evoked by **1 μM RuBi-GABA + 20 μM Bicuculline**

**Cozzolino M., Bazzurro V. et al. (2020).** Sci Rep; 10(1):13380.

# 1 μM AF-16 and RuBi-GABA



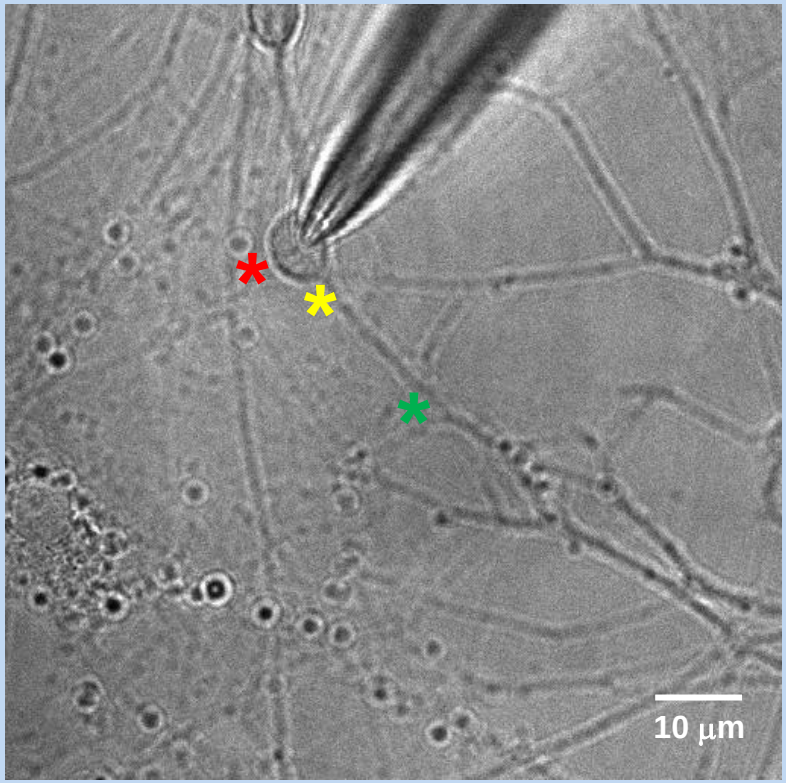

Localized Uncaging
λ = 750 nm
Laser Power = 30 mW
Exposure Time = 100 ms

(a) Current activated after the uncaging of **10 μM RuBi-GABA**

(b) 3 min perfusion with **1 μM AF-16**

(c) Response evoked by **10 μM RuBi-GABA + 1 μM AF-16**

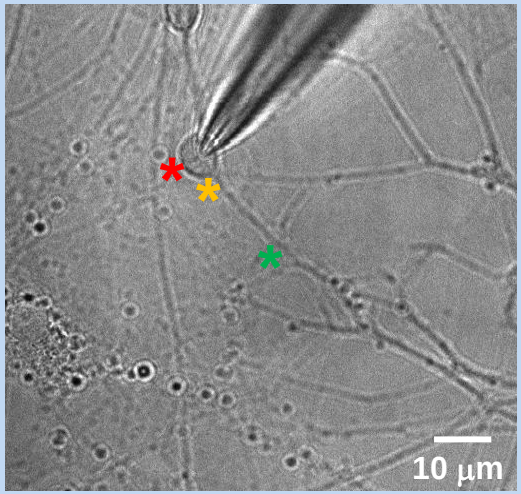
# Uncaging on soma, cone and neurite (1)

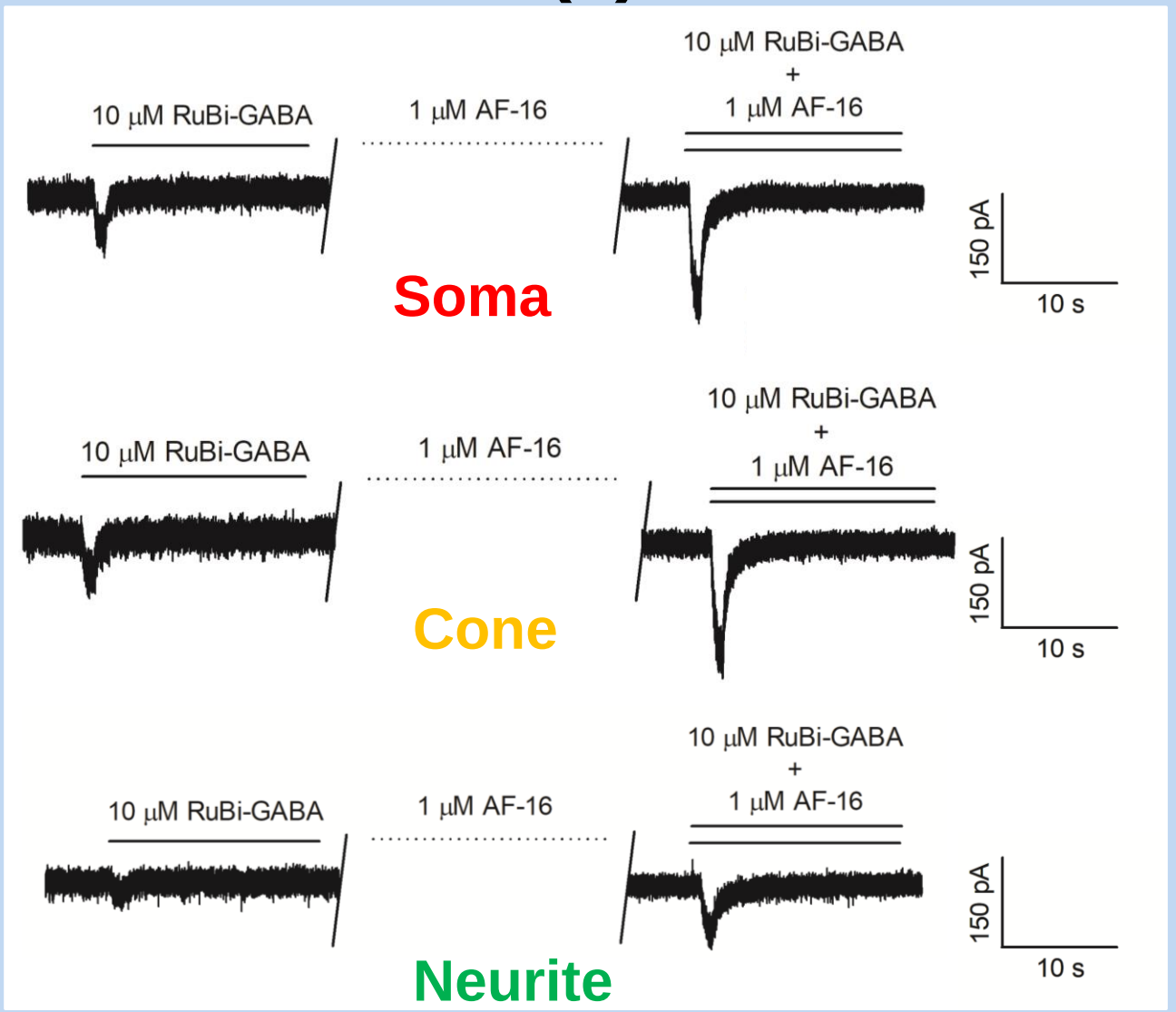



* **Soma**
* **Cone**
* **Neurite**

Localized Uncaging:
**λ** = 750 nm
**Laser Power** = 30 mW
**Exposure Time** = 100 ms

# Uncaging on soma, cone and neurite (2)



# RuBi-GABA, AF-16 and Furosemide

**Furosemide**:

**$\alpha_6$ selective blocker**

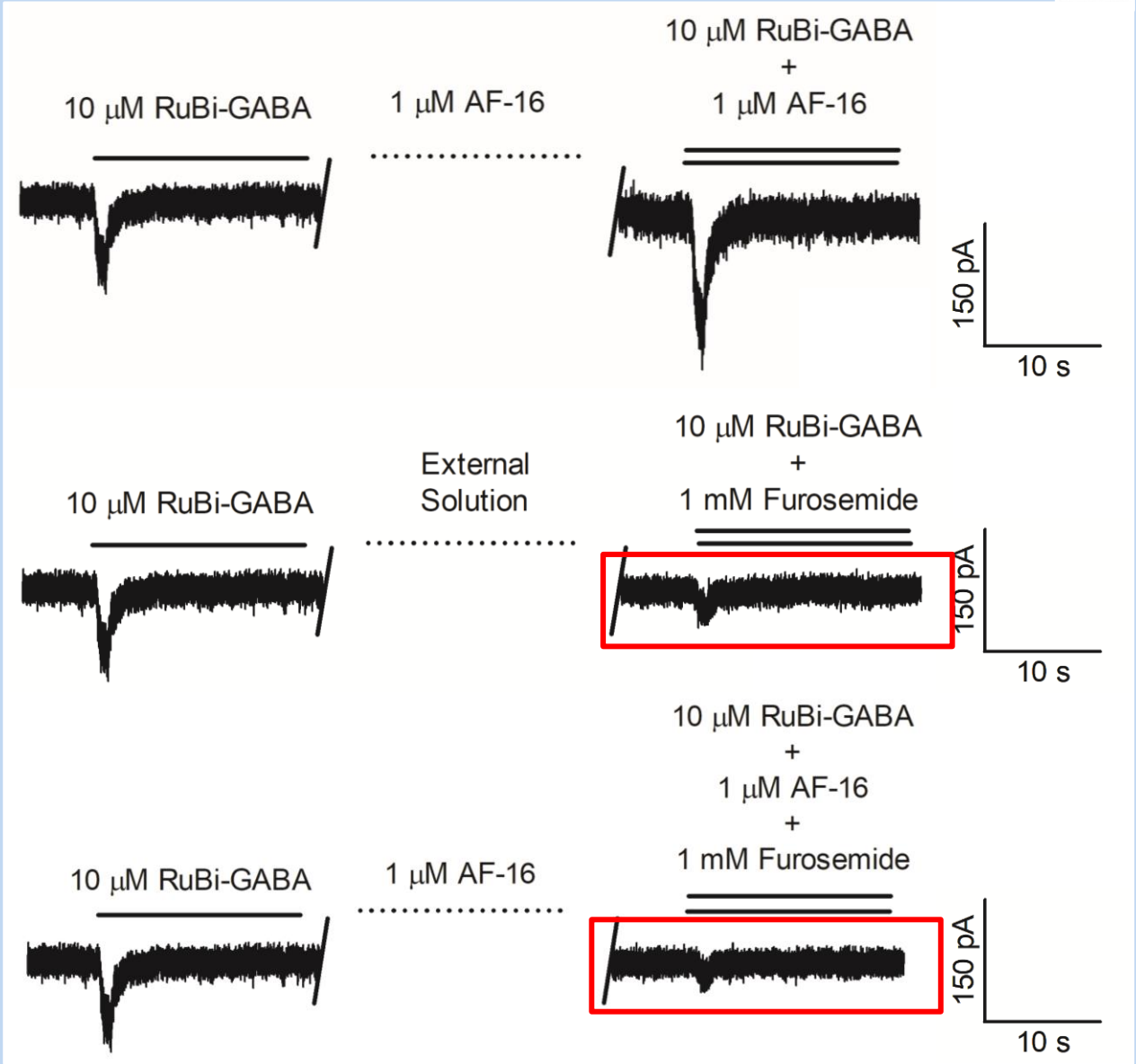
of $GABA_A$ receptors

Localized Uncaging:

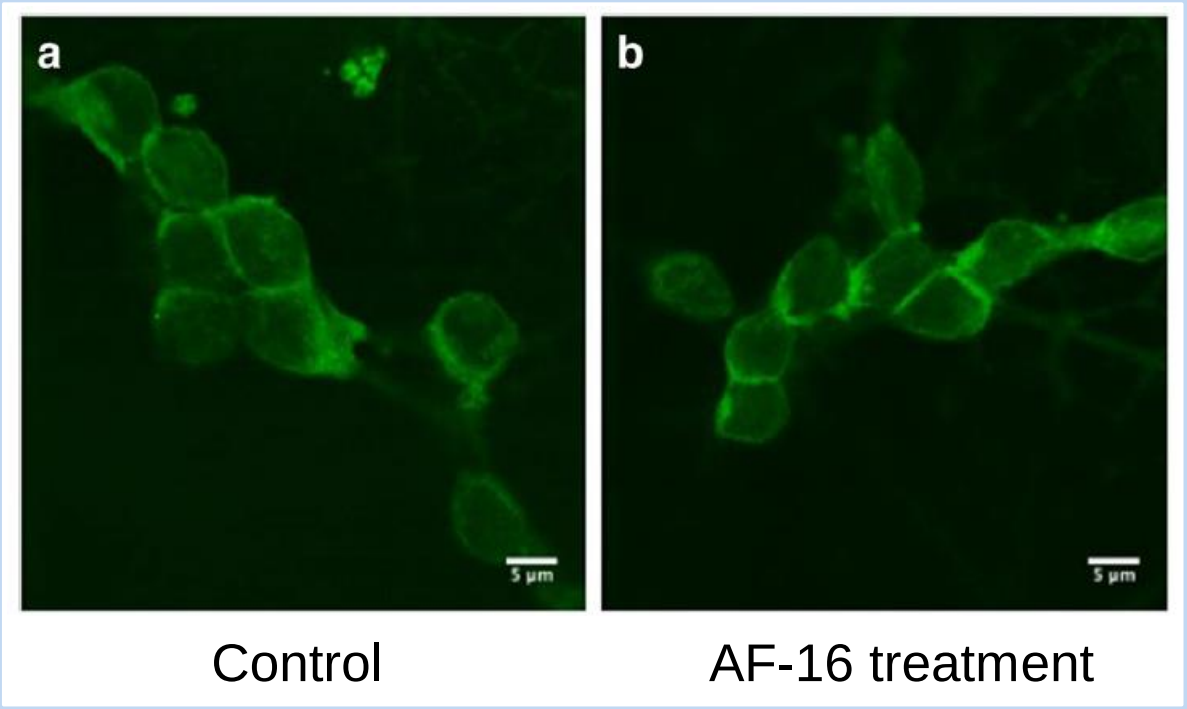
**λ** = 750 nm

**Laser Power** = 30 mW

**Exposure Time** = 100 ms



# $\gamma_2$ subunit distribution increases after 1 μM AF-16 treatment




**Fluorescence Increase = (17.4 ± 0.4)%**
**n = 32 cells**

**Bazzurro V. et al. (2018).** J Mol Neurosci; 64(2):312-320.

# Conclusions

- The **uncaging** combined with non-linear microscopy and the patch-clamp is a **useful tool** for studying the receptor localized response
- The Antisecretory Factor increases the response of $GABA_A$ receptors
- The Antisecretory Factor effect is different on soma, cone and neurite

# Thanks to….

Dipartimento di Fisica
Università degli Studi di Genova

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