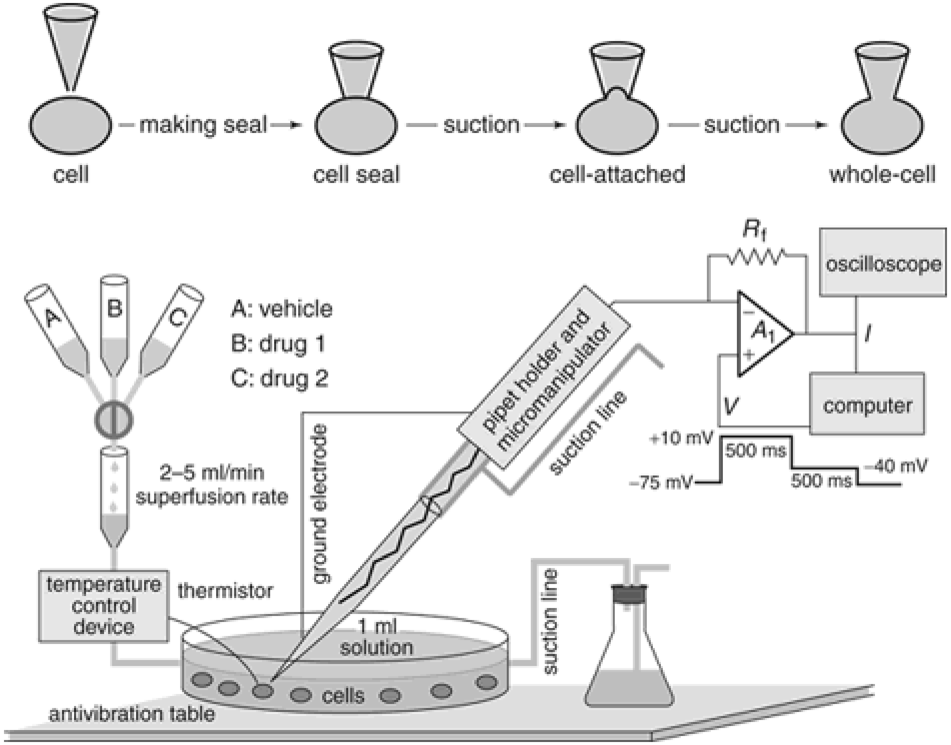
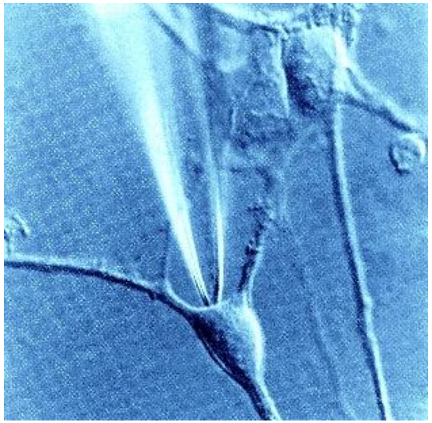
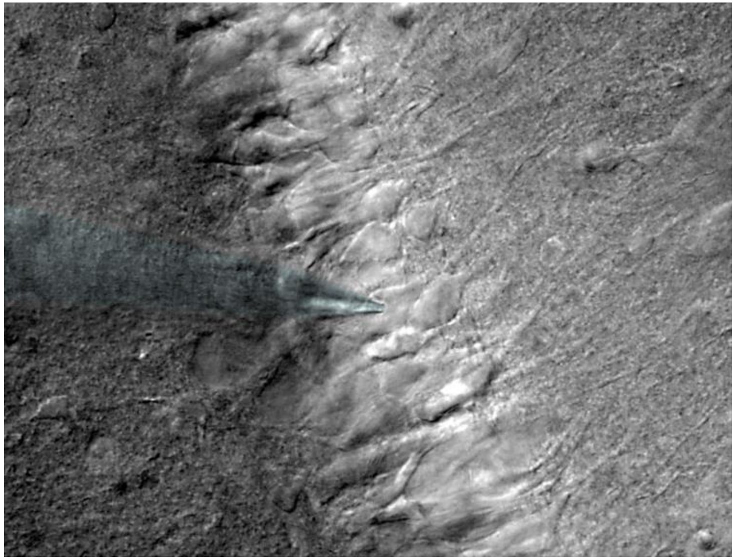
Técnicas para o estudo da neurotransmissão


cell
making seal
cell seal
suction
cell-attached
suction
whole-cell
A
B
C
A: vehicle
B: drug 1
C: drug 2
2–5 ml/min
superfusion rate
temperature control device
thermistor
ground electrode
pipet holder and micromanipulator
suction line
1 ml solution
cells
suction line
antivibration table
R_f
A_1
oscilloscope
I
V
computer
+10 mV
500 ms
−75 mV
500 ms
−40 mV





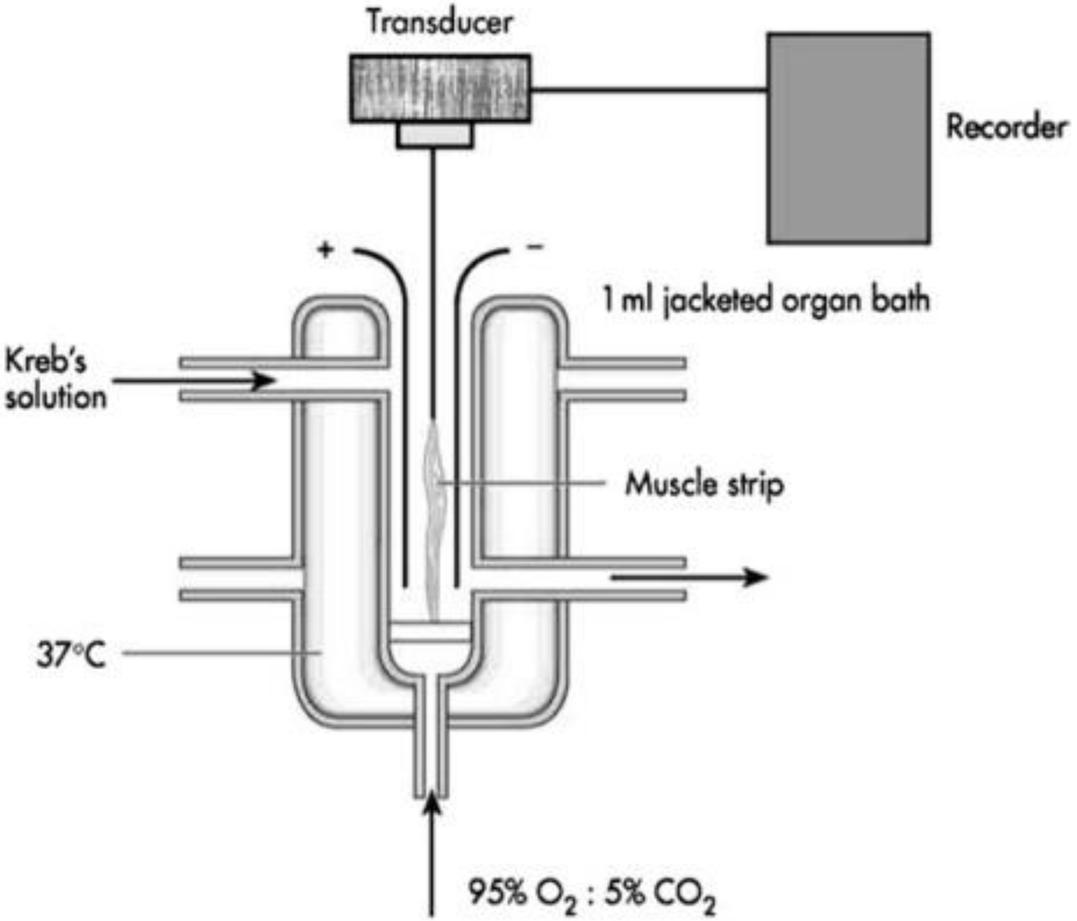
Técnicas para o estudo da neurotransmissão

- Estratégias:
  - **Detecção direta do neurotransmissor**
    - Neuroquímica
    - Eletroquímica
  - **Detecção indireta do neurotransmissor**
    - Efeito biológico pós-sináptico (o receptor como detector)
      - Efeito sobre um órgão alvo
      - Correntes e potenciais pós-sinápticos
  - **Detecção direta da exocitose**
      - Corantes de membrana
      - Medidas elétricas de capacitância

Medida indireta: contração muscular

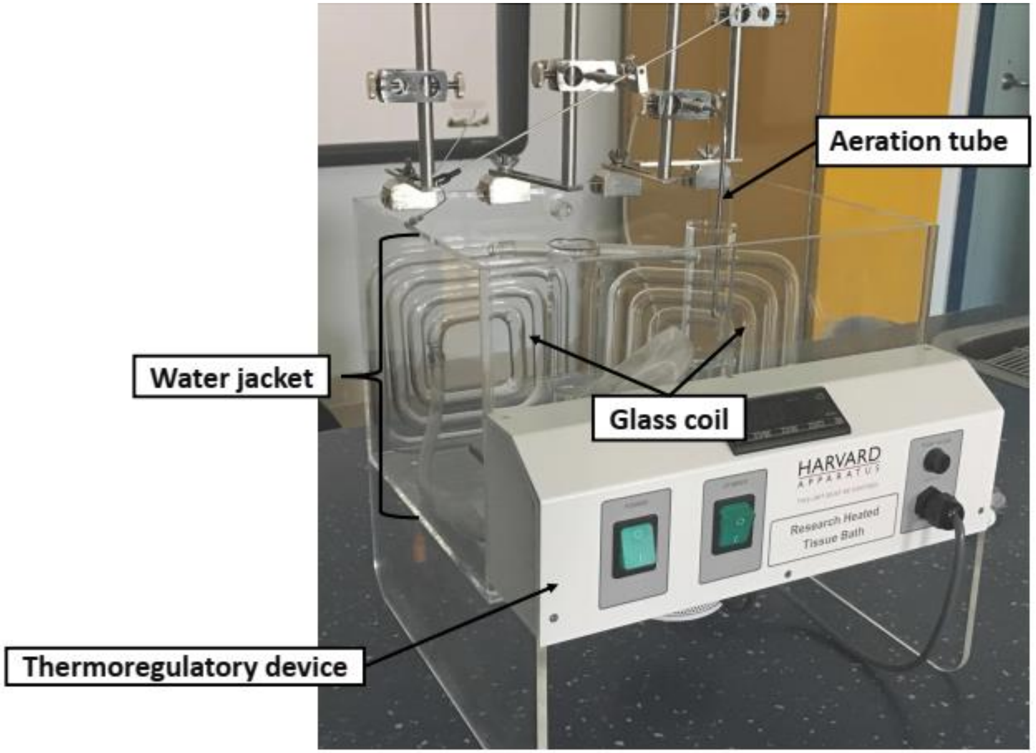
- Músculo liso ou esquelético
  - Preparação comum: Íleo de cobaia
    - Liberação pelo plexo mientérico
  - Músculo esquelético
    - Estimulação do nervo motor
  - Contração muscular ~ liberação de transmissor; calibração com padrões conhecidos de transmissor
  - Uso de antagonistas de receptores pós-sinápticos é essencial para caracterizar a contração como produto da liberação de neurotransmissores
- Alterações do batimento cardíaco e outras funções autonômicas
  - Experimentos pioneiros de Otto Loewi

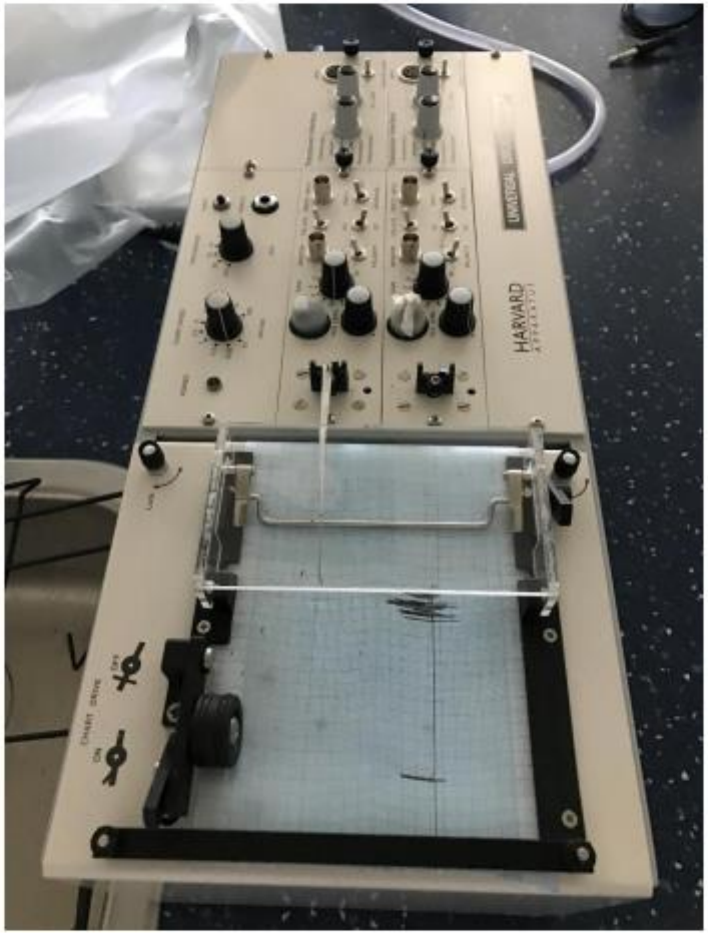
Aparato para estudar a contração do musculo liso (íleo de cobaia)


Transducer
Recorder
+
-
1 ml jacketed organ bath
Kreb's solution
Muscle strip
37°C
95% O₂ : 5% CO₂

Patra J.K., Das S.K., Das G., Thatoi H. (2019) Isolated Tissues and Organs. In: A Practical Guide to Pharmacological Biotechnology. Learning Materials in Biosciences. Springer, Singapore. https://doi.org/10.1007/978-981-13-6355-9_2

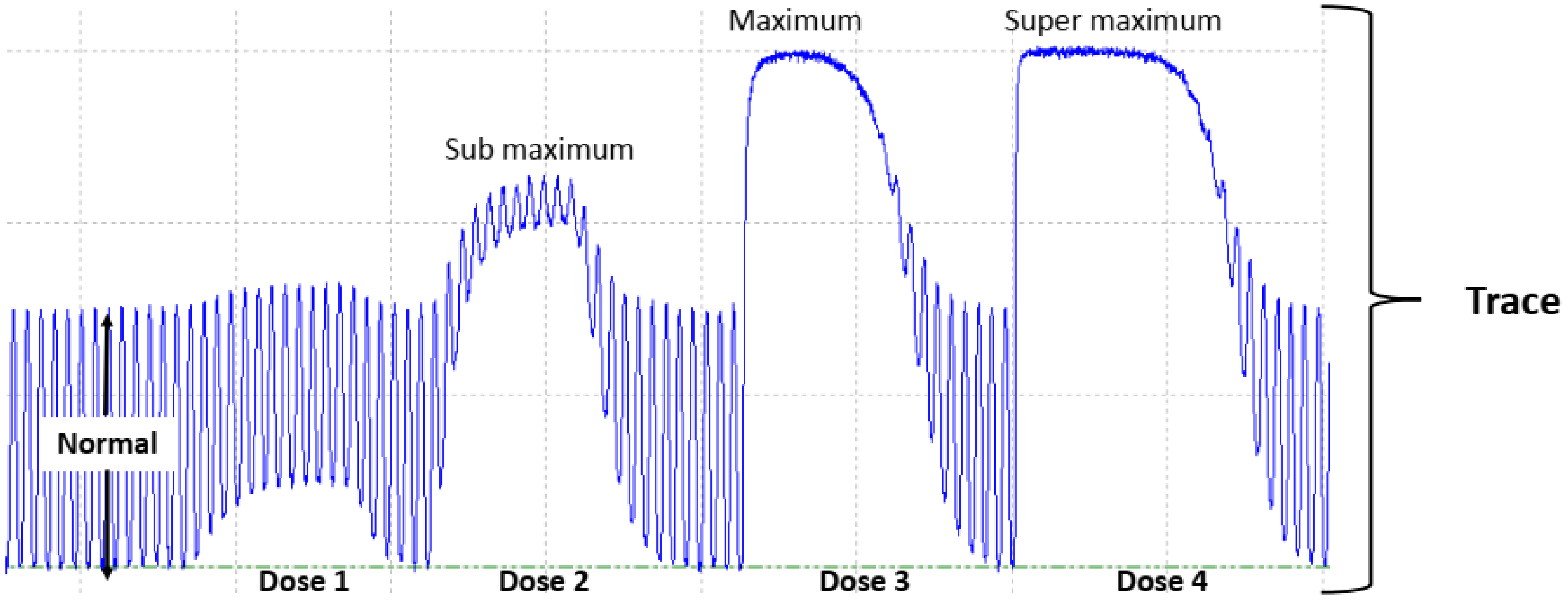
Aparato para estudar a contração do musculo liso (íleo de cobaia)


Aeration tube
Water jacket
Glass coil
Thermoregulatory device
HARVARD APPARATUS
Research Heated Tissue Bath


HARVARD APPARATUS
CHART DRIVE
ON
OFF

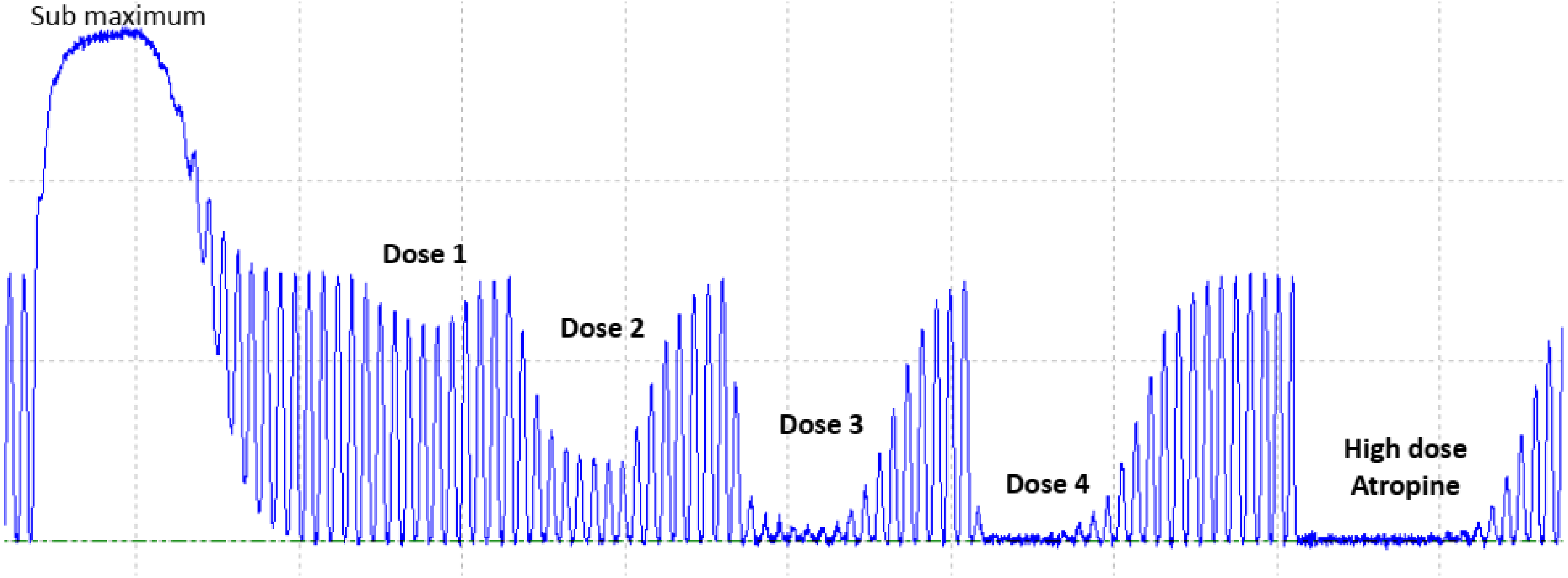
https://www.slideshare.net/OsamaAlZahrani5/pharmacology-practical-effect-of-cholinergic-drugs-on-isolated-rabbit-jejunum-drug-amp-drug-response-relationship?from_action=save

Agonist drug (Acetylcholine)



https://www.slideshare.net/OsamaAlZahrani5/pharmacology-practical-effect-of-cholinergic-drugs-on-isolated-rabbit-jejunum-drug-amp-drug-response-relationship?from_action=save

Antagonist drug (Atropine)

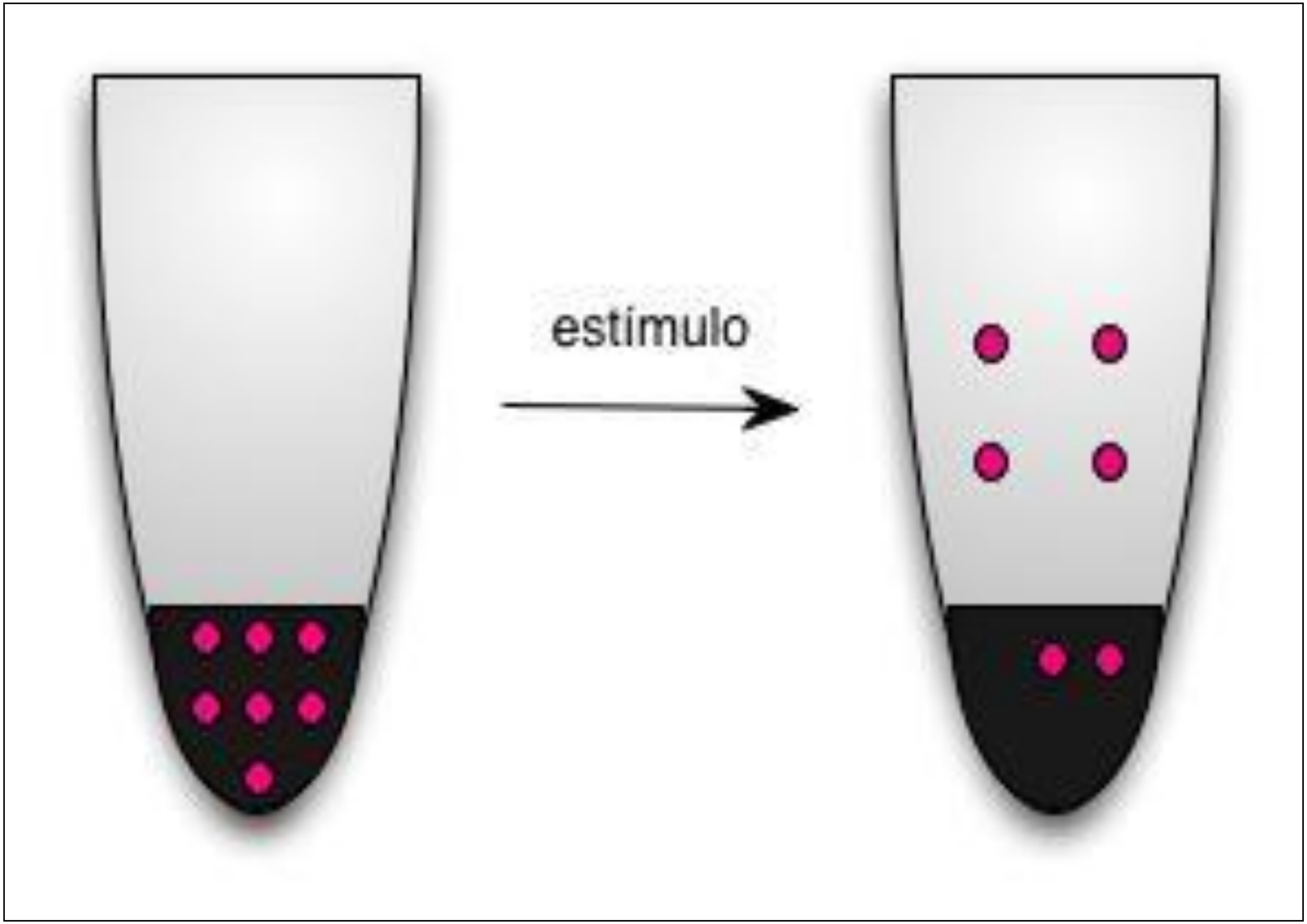


https://www.slideshare.net/OsamaAlZahrani5/pharmacology-practical-effect-of-cholinergic-drugs-on-isolated-rabbit-jejunum-drug-amp-drug-response-relationship?from_action=save

Neuroquímica

- Preparações *in vitro*:
    - Fatias ou prismas de tecido cerebral
        - *Prática e fácil porém grosseira*
    - Células em cultura
        - *Cara, precisa de um ensaio sensível*
    - Sinaptossomas
        - *Muito usada, isola o elemento pré-sináptico por isso ideal para estudar mecanismos pré-sinápticos. Não leva em consideração o lado pós. Não leva em consideração o resto do neurônio*
- Preparações *in vivo*:
    - Perfusão de gânglios periféricos
        - Gânglio cervical superior
    - Microdiálise
        - *Boas por se tratarem de preps in vivo, mas muito trabalhosas*
- *Em todas essas preparações o neurotransmissor é medido no sobrenadante do meio de incubação*

Neuroquimica – detecção por radioisótopos

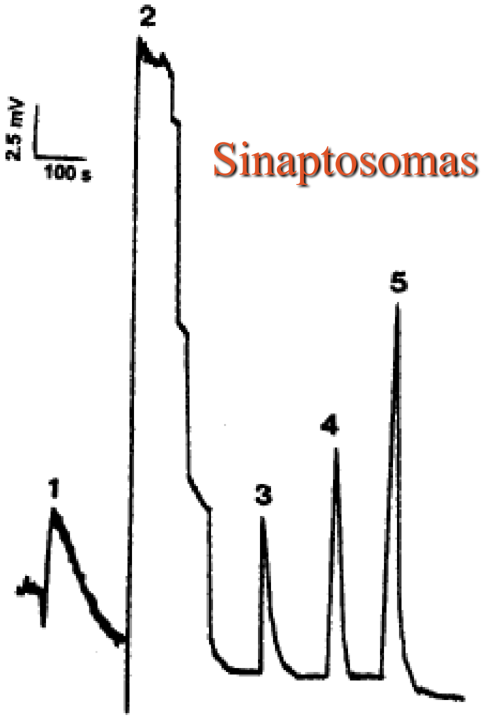


Neuroquímica

- HPLC
  - Para aminoácidos
    - Pouco usado. Mais usado para microdiálise *in vivo*.
- Radioimunoensaio
  - Para peptídeos
- Ensaio luminométrico para acetilcolina e ATP.
  - Reações de oxiredução que culminam na transferência de elétrons, gerando luz. Compara-se a resposta gerada pela amostra com padrões
    - ATP: reação da Luciferina/luciferase
      - Faz-se medidas em tempo real em sinaptosomas ou tecido nervoso
    - Acetilcolina: reação do luminol
      - Faz-se em sobrenadantes
  - *Método de alta sensibilidade, mas caro e muito sensível a impurezas*

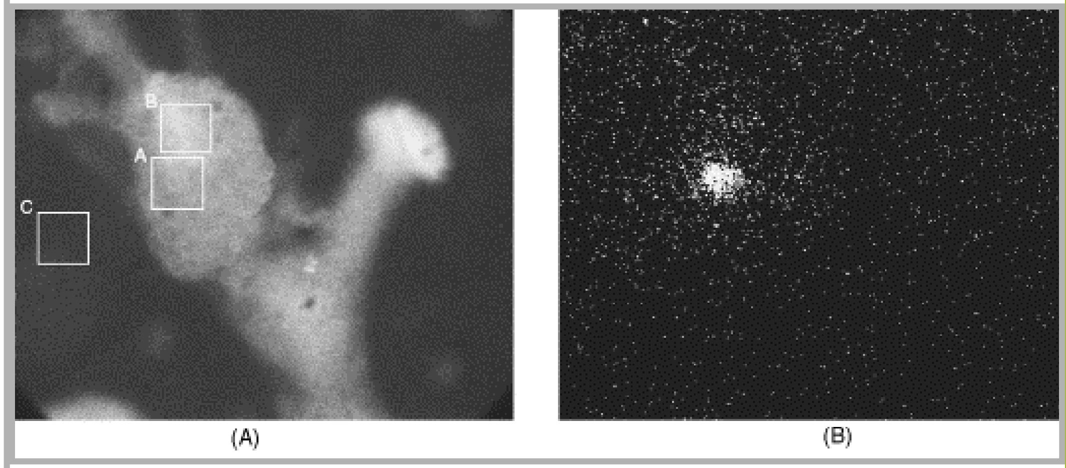
Ensaio luminométrico

- Ensaio luminométrico para ATP.



Sinaptosomas

Fig. 1. Trace recording of the continuous release of ATP from rat brain cortical synaptosomes depolarized with 33 mM KCl. Results of a typical experiment are shown. (1) 33 mM KCl depolarization in the absence of calcium (EGTA 1 mM); (2) 33 mM KCl depolarization in the presence of calcium (2.5 mM); (3) ATP standard (5 pmol); (4) ATP standard (10 pmol); (5) ATP standard (20 pmol).



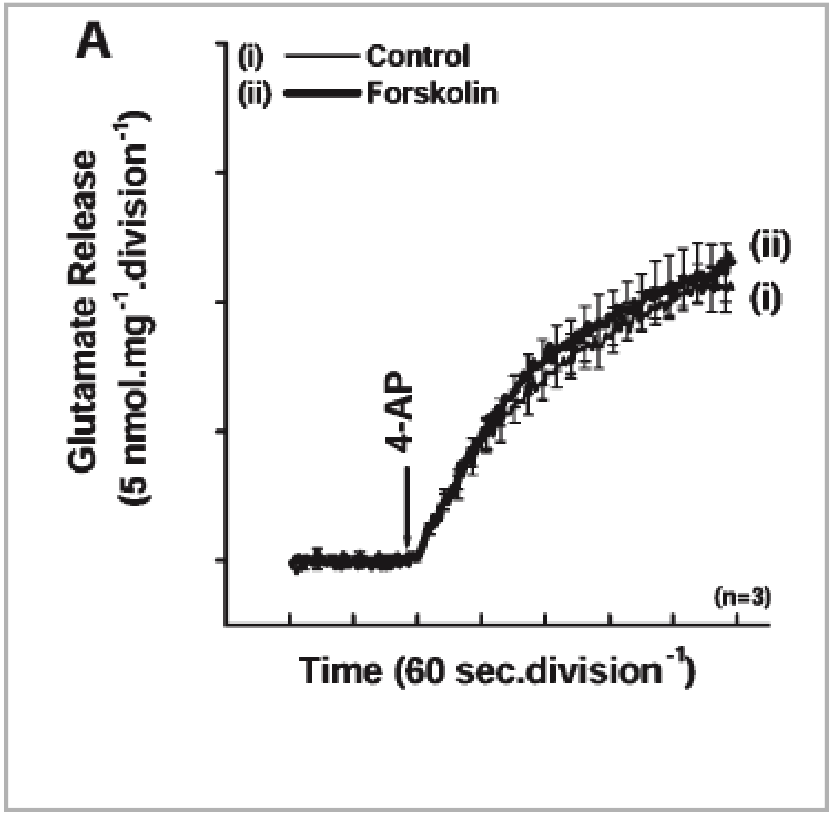
(A)

(B)

Visualização em tempo real de ATP sendo liberado em um gânglio nervoso de molusco

Ensaio luminométrico

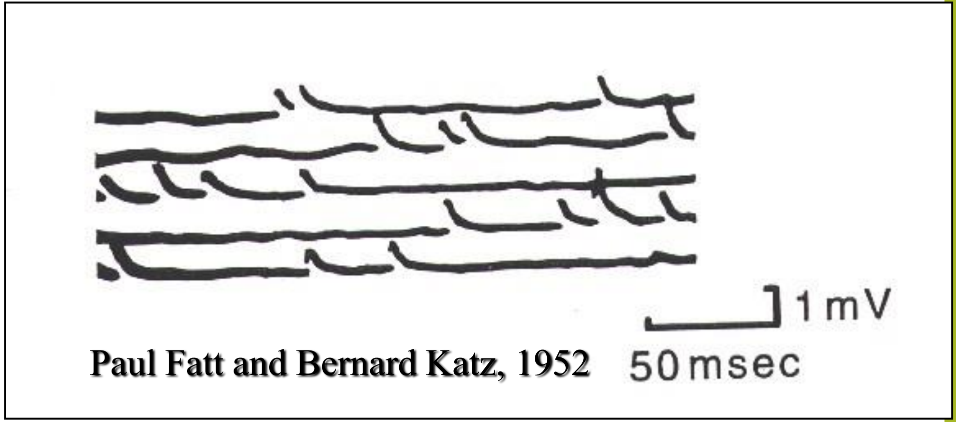
- Fluorimetria para glutamato
  - Extremamente sensível, mede em tempo real a liberação de glutamato.
    - Baseia-se medição da fluorescência do NAPH produzido pela oxidação descarboxilativa do glutamato pela glutamato desidrogenase.
    - Após o expto. faz-se uma curva de calibração adicionando L-glutamato



Eletrofisiologia

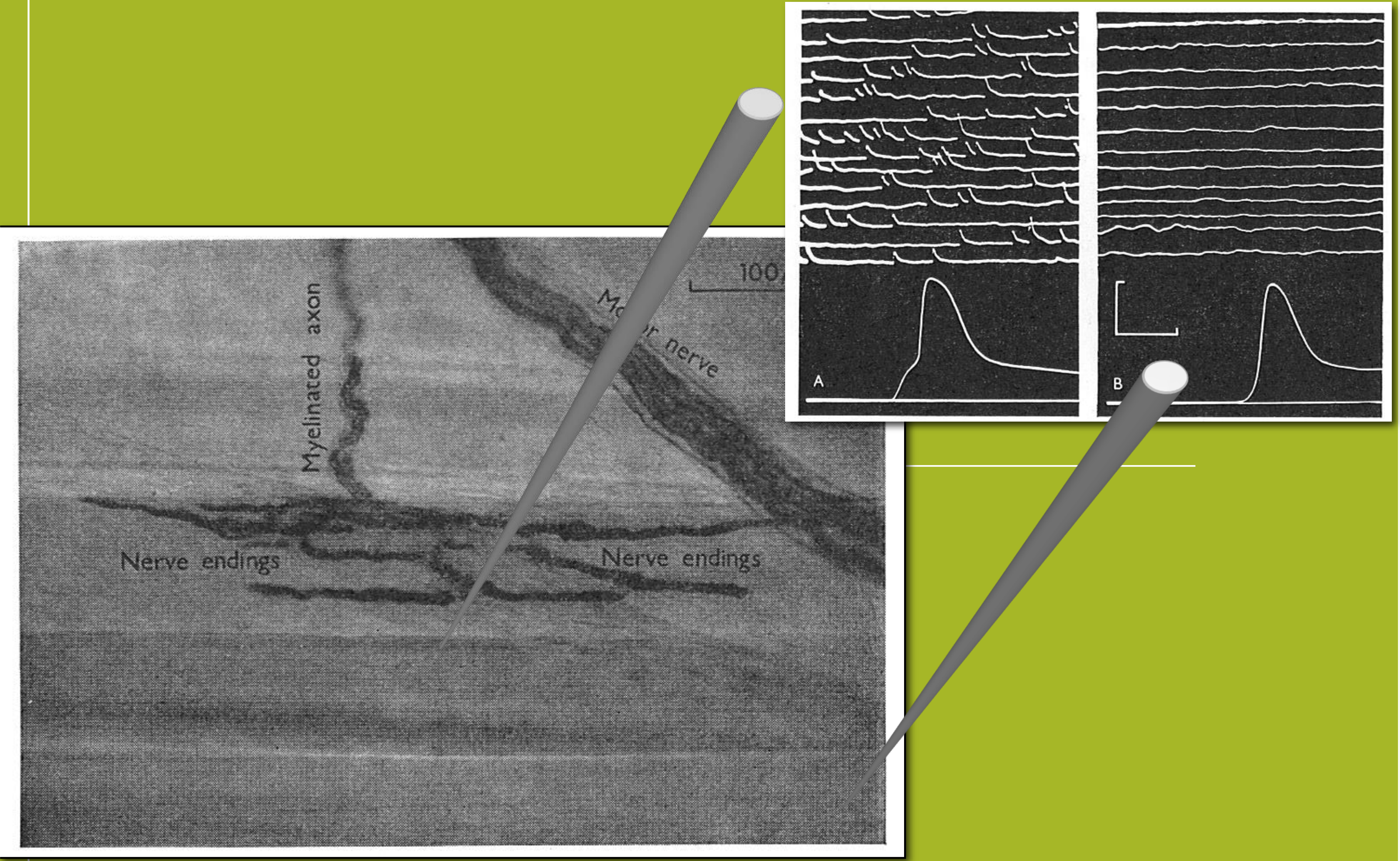
- Eletrofisiológicas
  - Neurotransmissão na junção neuromuscular
    - Medidas de potenciais miniaturas e potenciais de placa motora

Neuromuscular Junction (400X)
← Branching Motor Axon



Paul Fatt and Bernard Katz, 1952

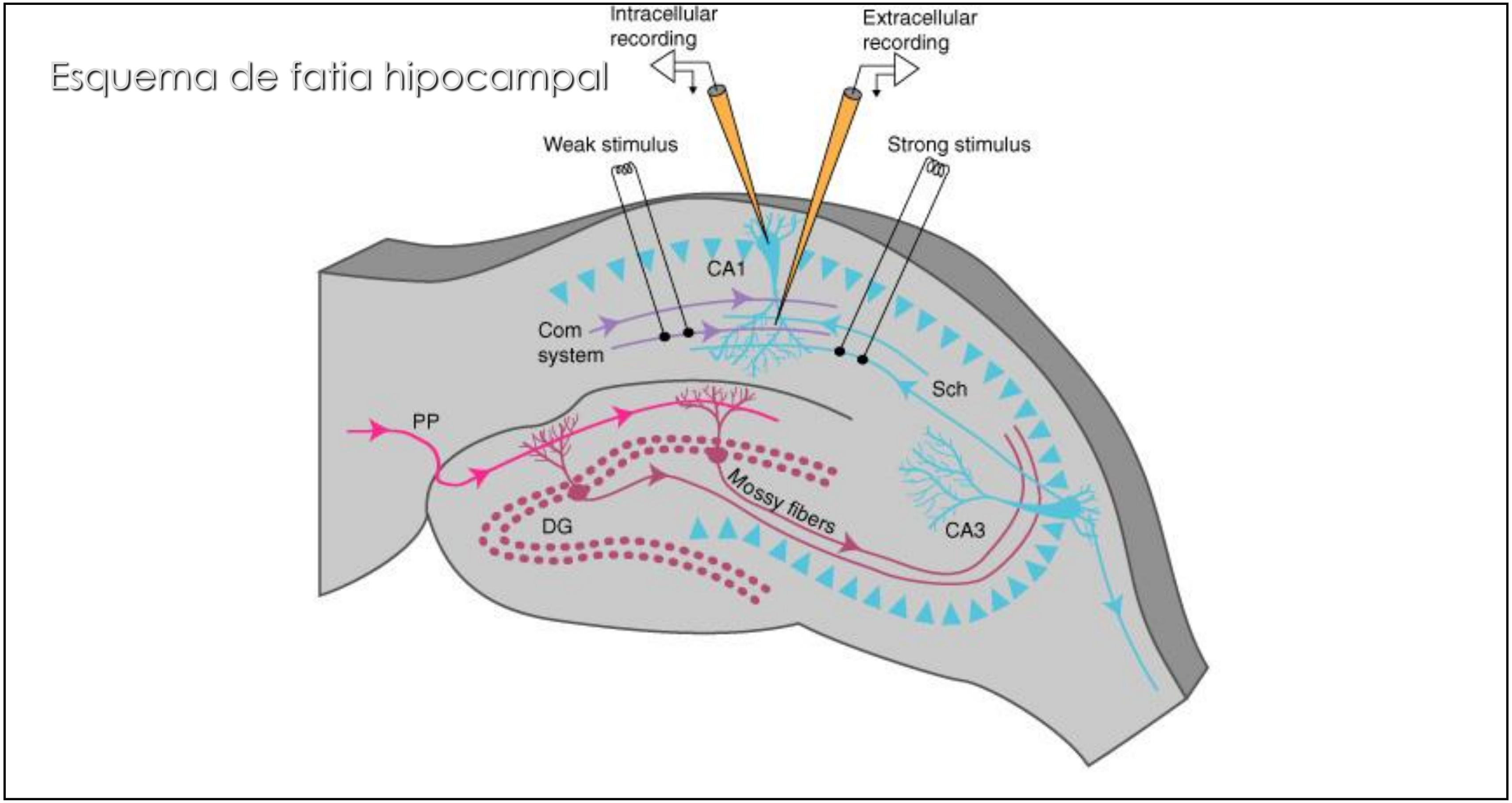
BERNARD KATZ E A NEUROTRANSMISSÃO QUANTAL NA JUNÇÃO NEUROMUSCULAR DA RÃ


100
Myelinated axon
Mo r nerve
Nerve endings
Nerve endings
A
B

Técnicas para o estudo da neurotransmissão

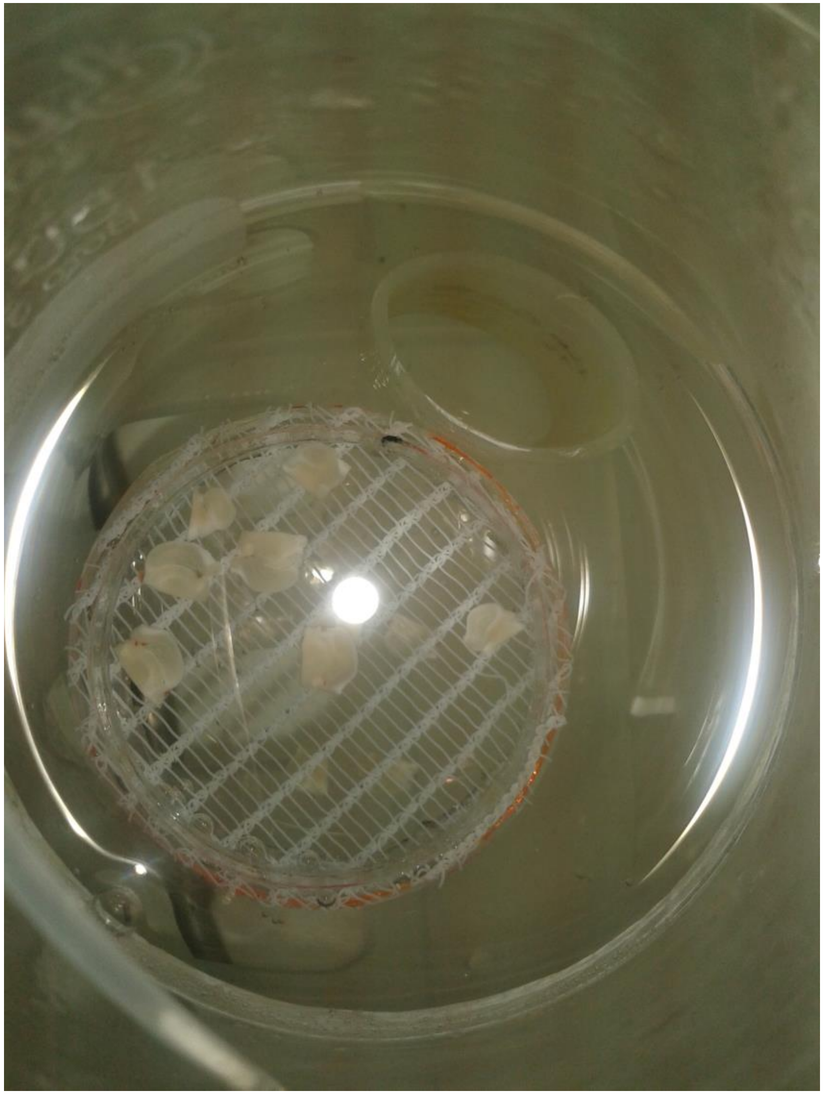
- Eletrofisiológicas
  - Neurotransmissão em fatias de sistema nervoso central

Esquema de fatia hipocampal

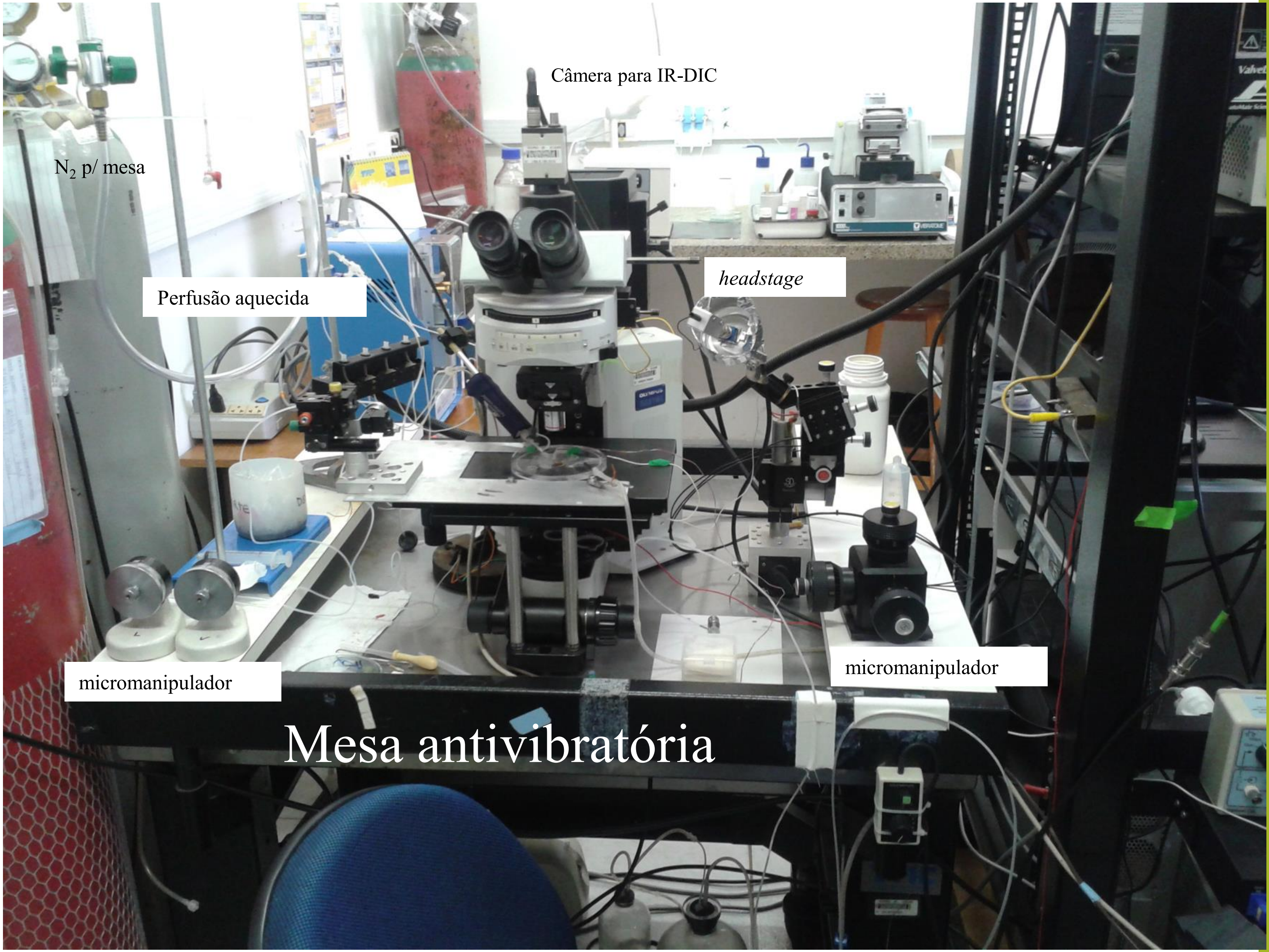

Intracellular recording
Extracellular recording
Weak stimulus
Strong stimulus
CA1
Com system
Sch
PP
DG
Mossy fibers
CA3

Fatias

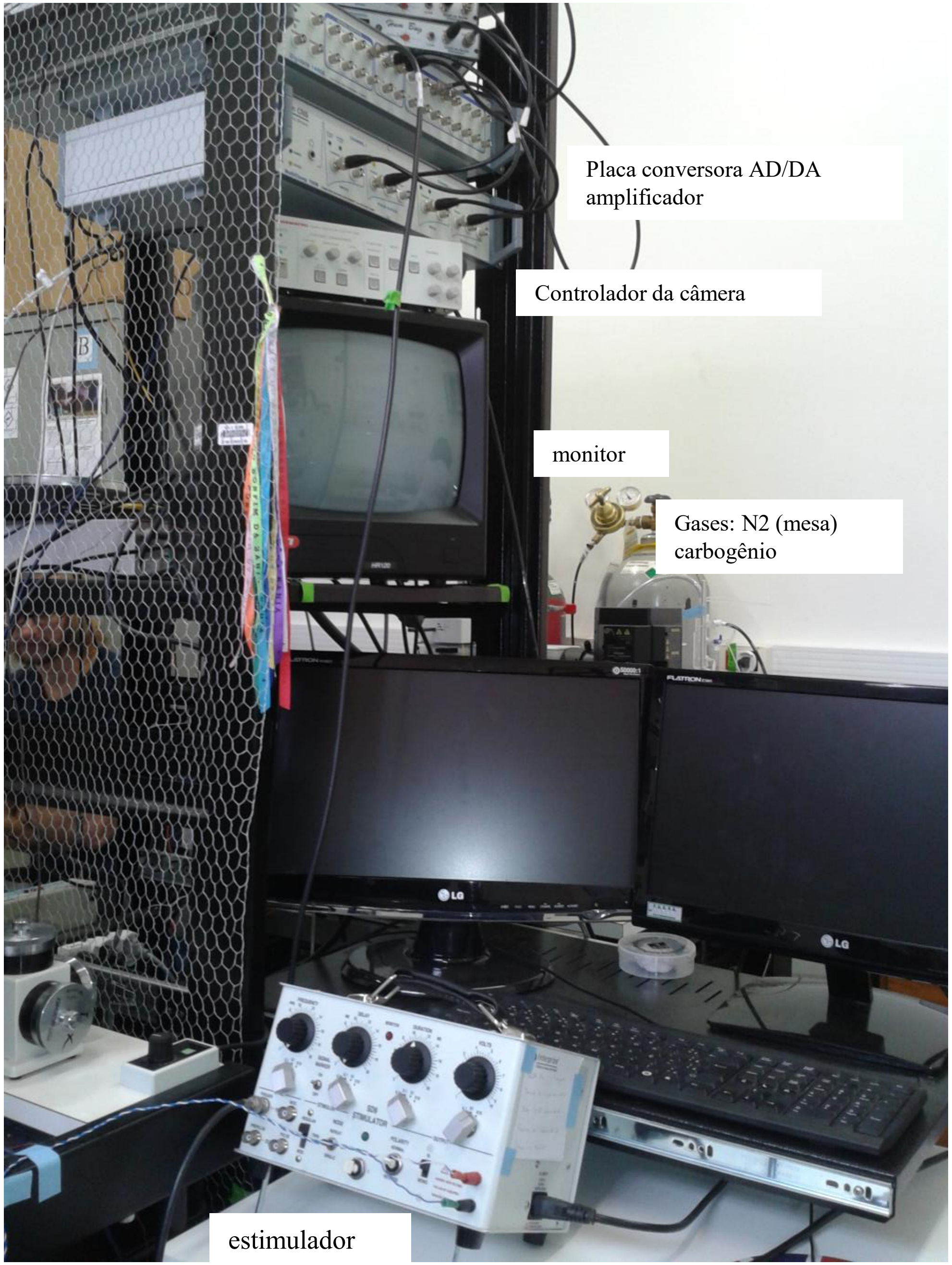
VIBRATOME
3000
VALET



O *rig* ou *set-up*


Câmera para IR-DIC
N_2 p/ mesa
Perfusão aquecida
headstage
micromanipulador
micromanipulador
Mesa antivibratória

O *rig* ou *set-up*



Placa conversora AD/DA
amplificador

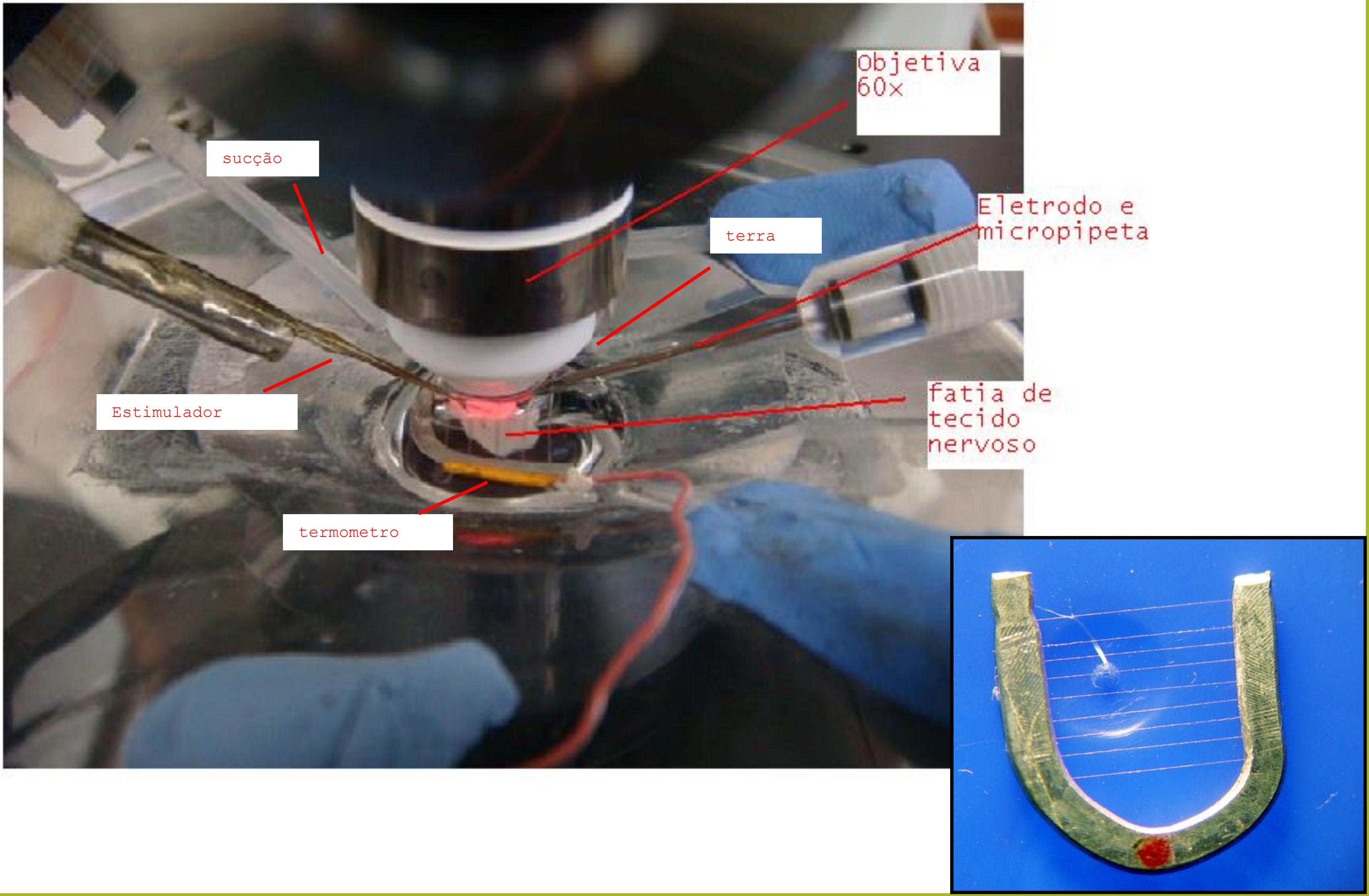
Controlador da câmera

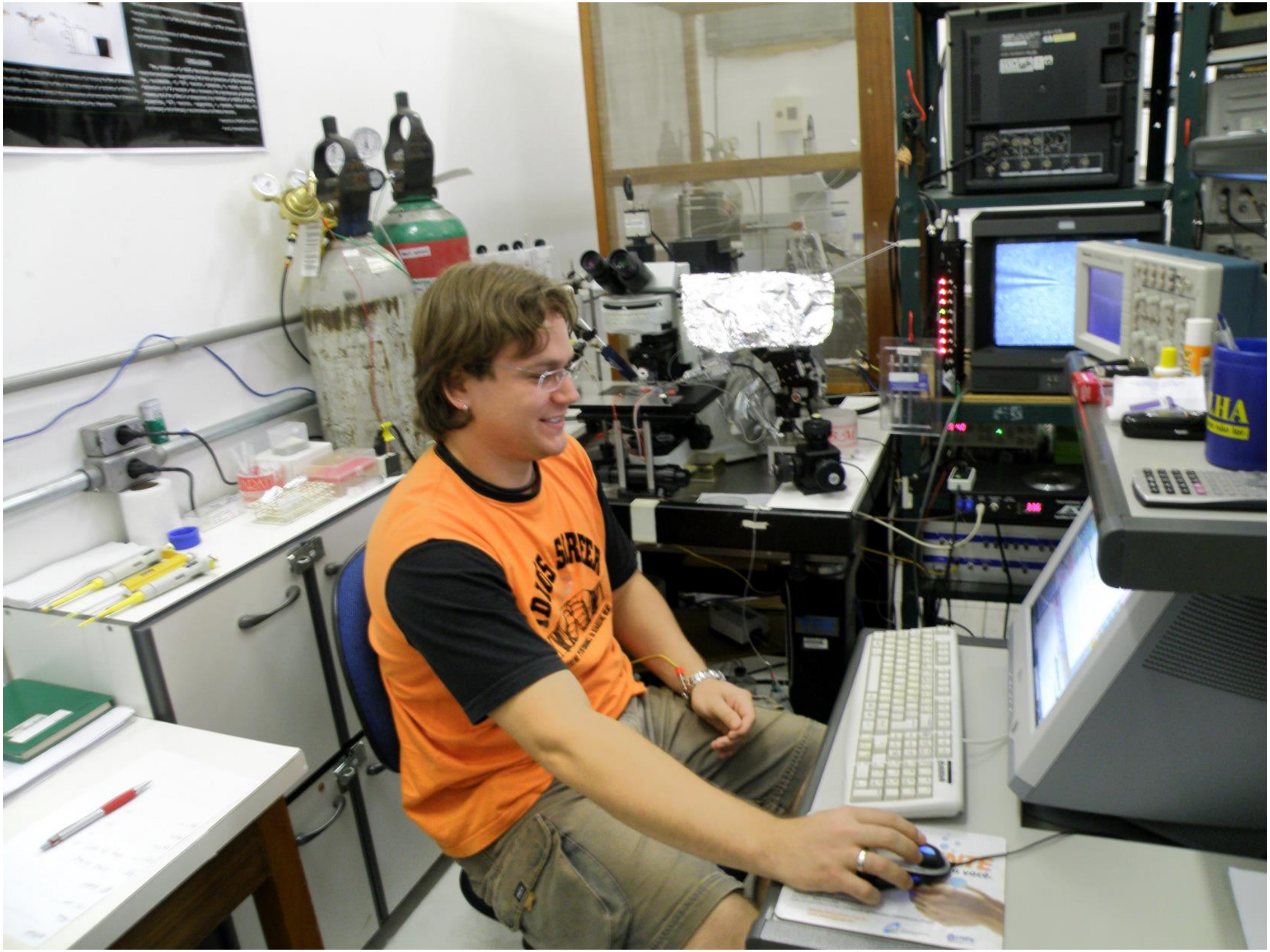
monitor

Gases: N2 (mesa)
carbogênio

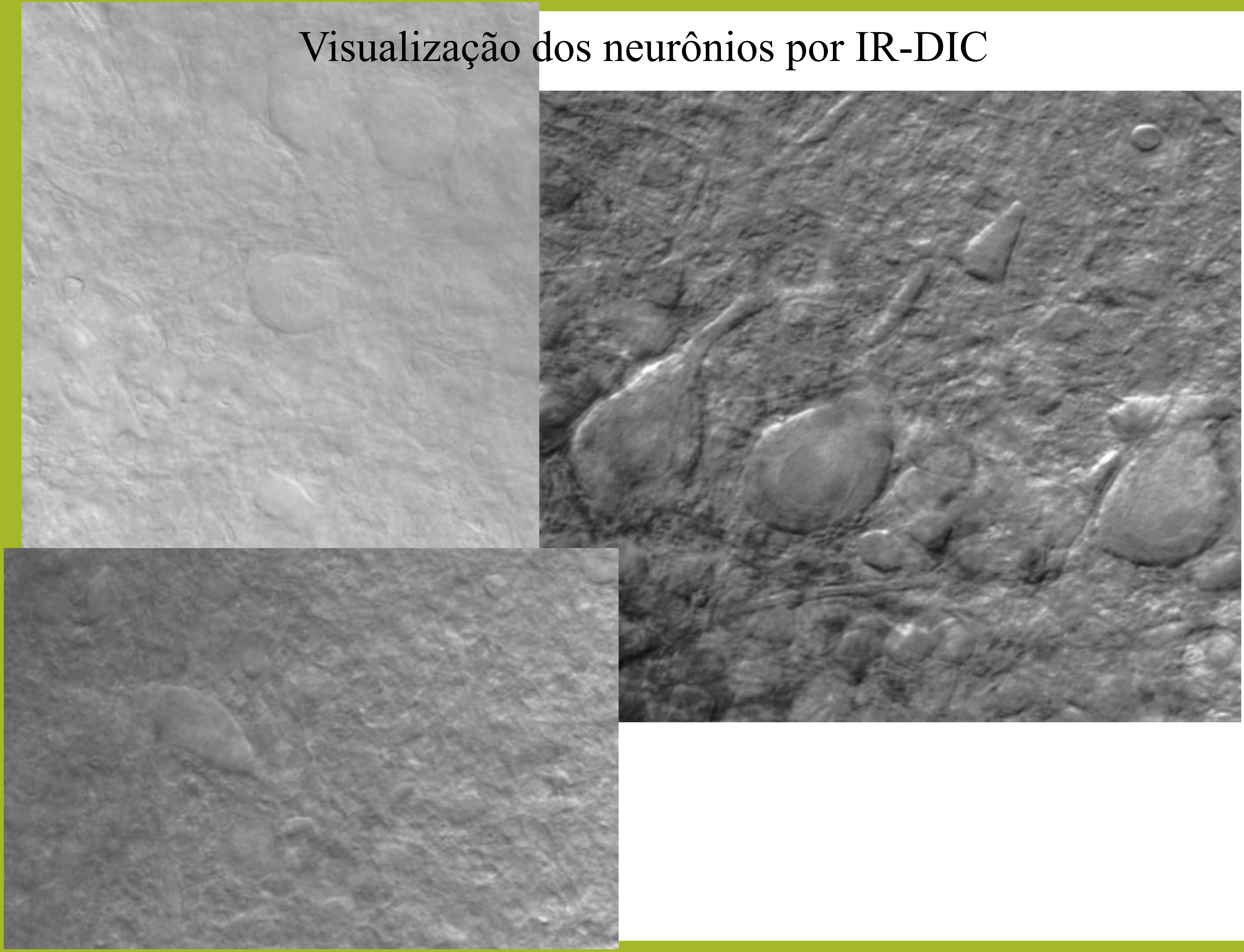
estimulador

Registro eletrofisiológico


Objetiva 60x
sucção
terra
Eletrodo e micropipeta
fatia de tecido nervoso
Estimulador
termometro



Visualização dos neurônios por IR-DIC

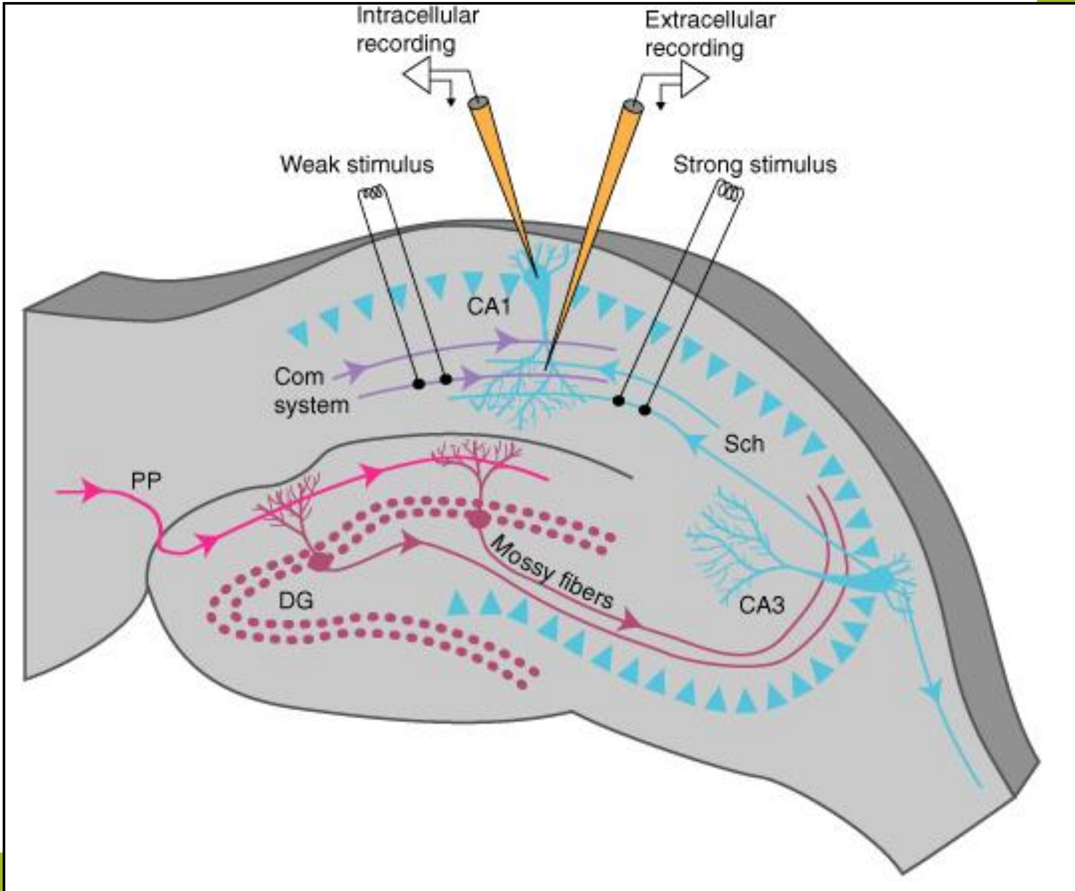


Eletrofisiologia

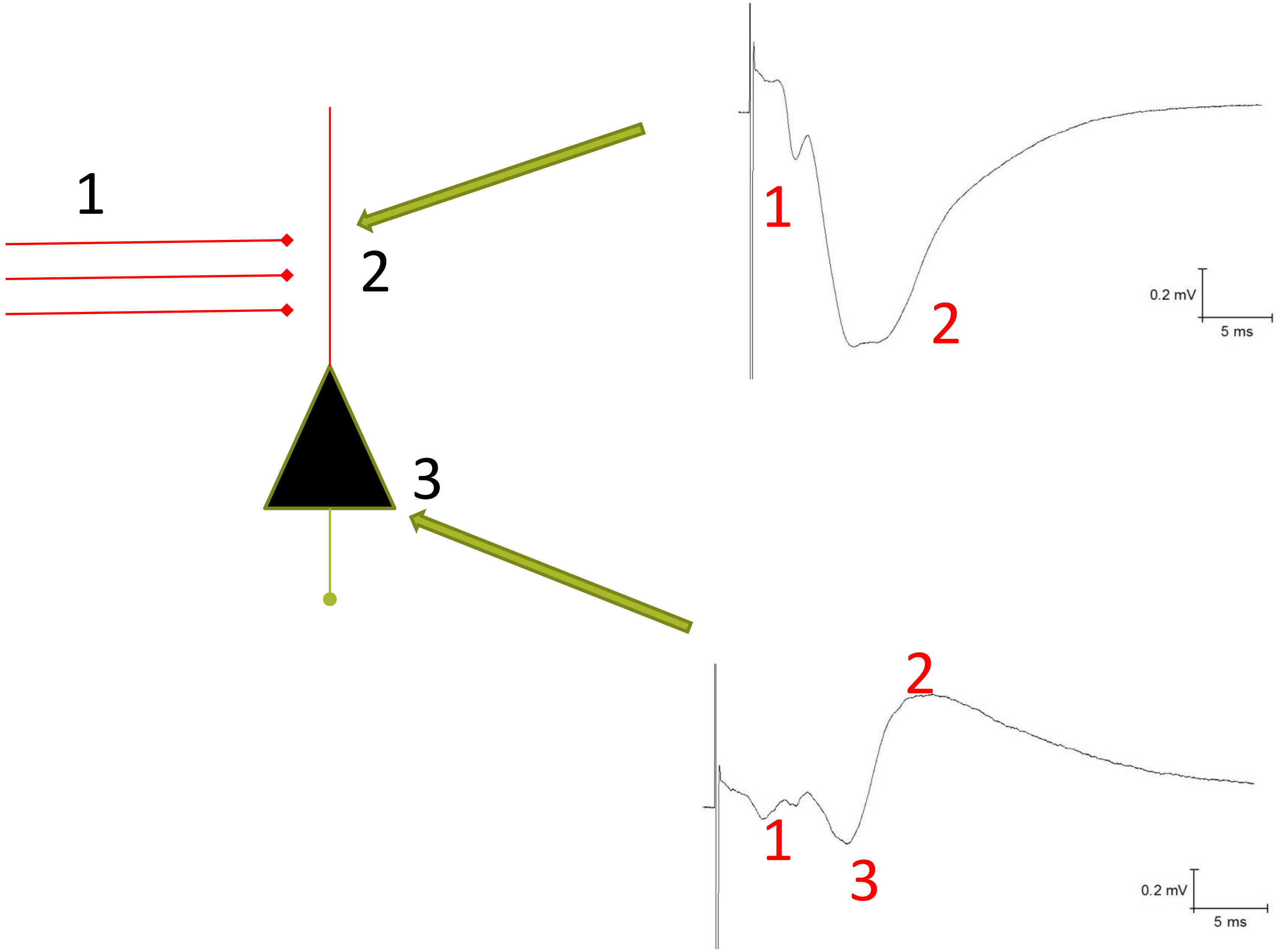
- Registros extracelulares (potenciais de campo)
    - Atividade de diversos *inputs*.
- Técnicas eletrofisiológicas de "single-cell"
    - Registros em neurônios em fatias de sistema nervoso central
        - *Voltage-clamp* (correntes a potencial fixo)double-electrode
        - *Current clamp* (potenciais) - "sharp-electrode" - empalamento
        - *Patch-clamp* (voltage e current-clamp)
            - Registro de eventos evocados (estimulação dos axônios pré-sinápticos) e espontâneos
    - Registros em neurônios em cultura primária
        - *Patch-clamp* (*voltage e current-clamp*).
            - Registro de eventos sinápticos espontâneos
            - Registro de eventos sinápticos evocados

Registros em fatias do hipocampo

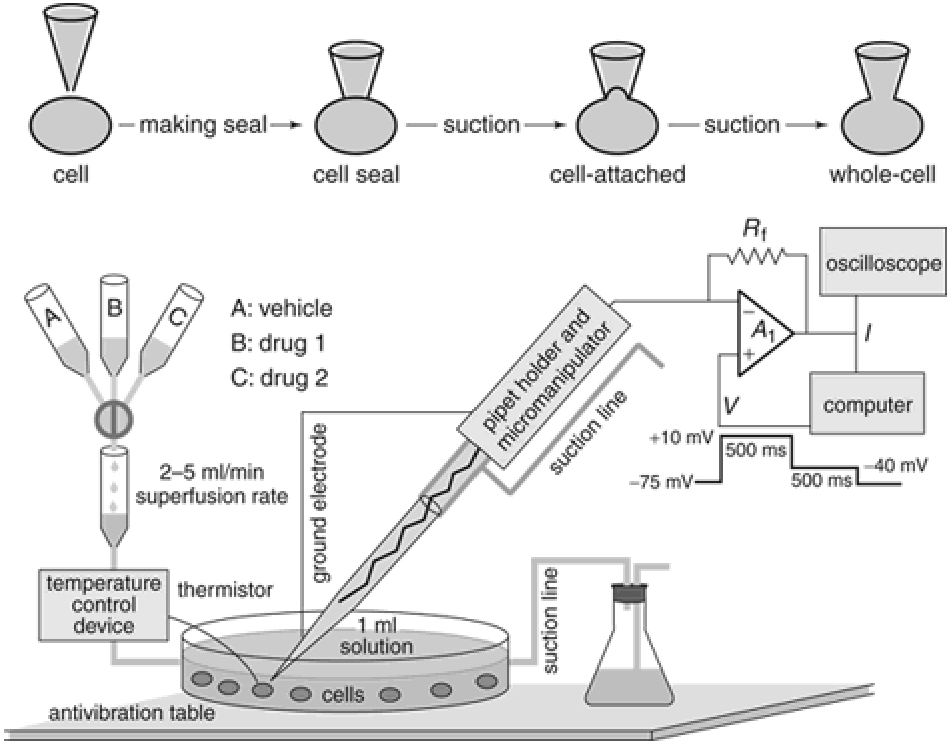
BRIGH DETA ENHANC GAMMA IMPRO S

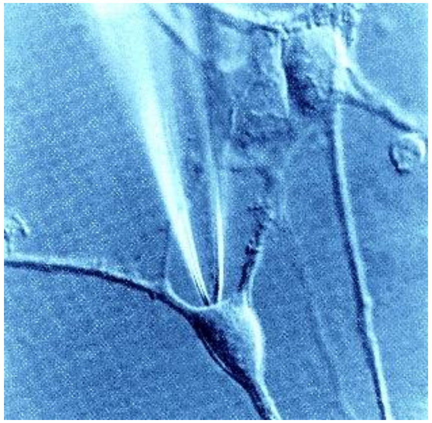

Intracellular recording
Extracellular recording
Weak stimulus
Strong stimulus
CA1
Com system
Sch
PP
DG
Mossy fibers
CA3

Registros extracelulares

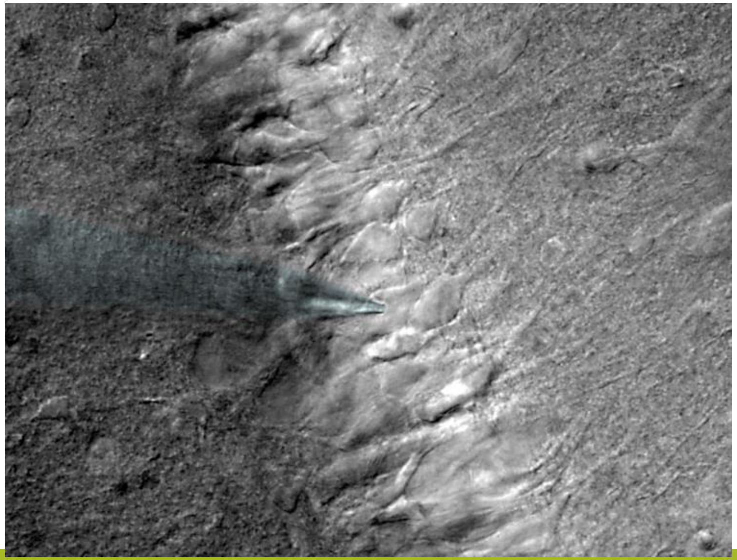


A técnica de *patch clamp*


cell
making seal
cell seal
suction
cell-attached
suction
whole-cell
A
B
C
A: vehicle
B: drug 1
C: drug 2
2–5 ml/min
superfusion rate
temperature control device
thermistor
ground electrode
pipet holder and micromanipulator
suction line
1 ml solution
cells
suction line
antivibration table
R_f
oscilloscope
A_1
I
V
computer
+10 mV
500 ms
–75 mV
500 ms
–40 mV

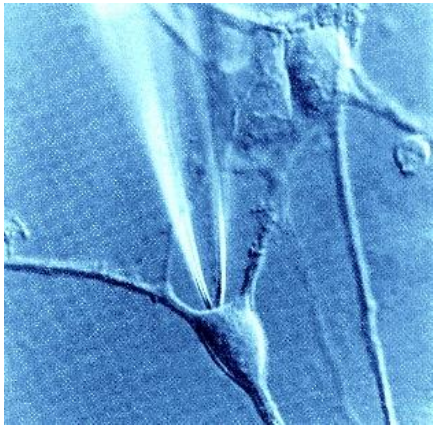


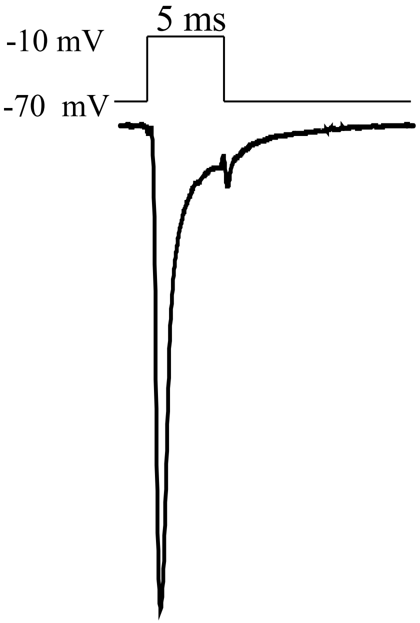
Neurônios em cultura



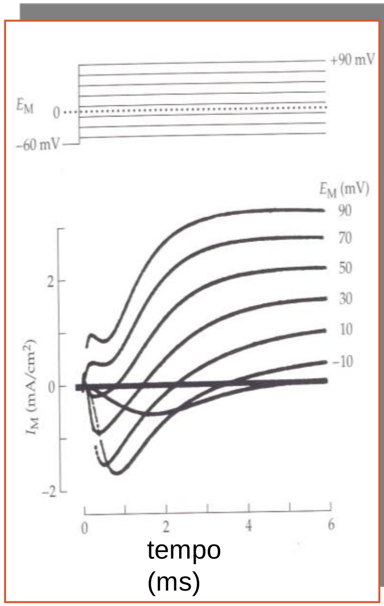
Neurônios em “fatias”

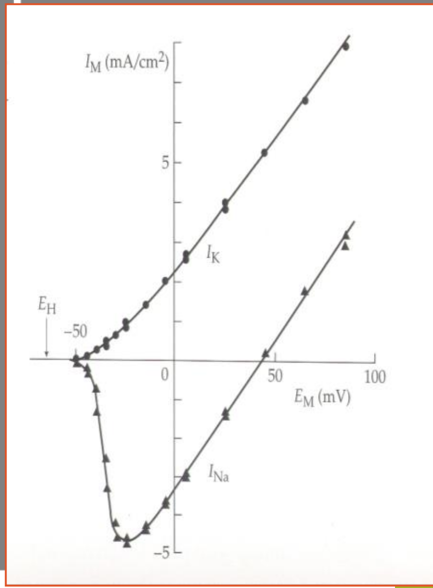
Voltage clamp = Mantém o potencial de membrana constante.





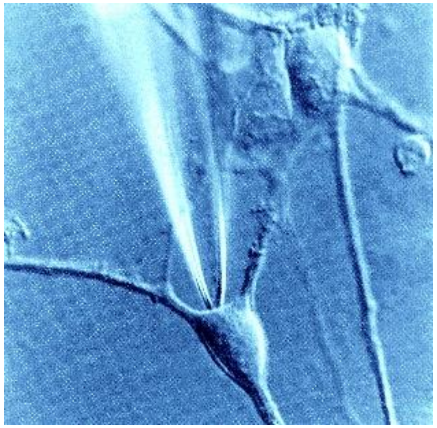
Corrente de sódio

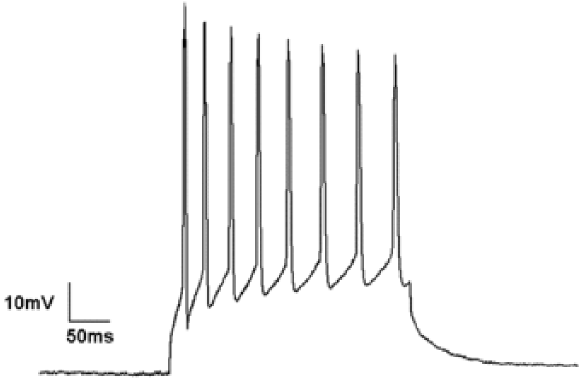




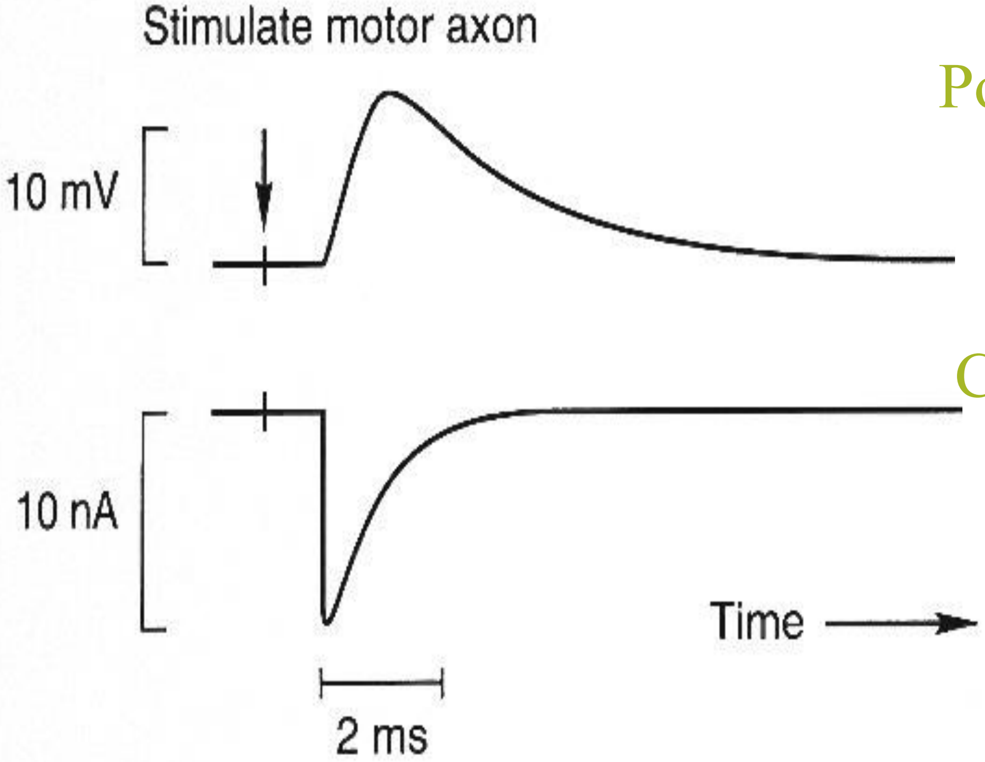
Correntes **positivas** = corrente de **saída** = saída de cátions ou entrada de ânions = **hiperpolarização** da membrana
Correntes **negativas** = corrente de **entrada**= entrada de cátions ou saída de ânions = **despolarização** da membrana

Current clamp = o potencial da membrana varia livremente com a passagem de corrente.





Registros intracelulares



Potencial pós-sináptico excitatório

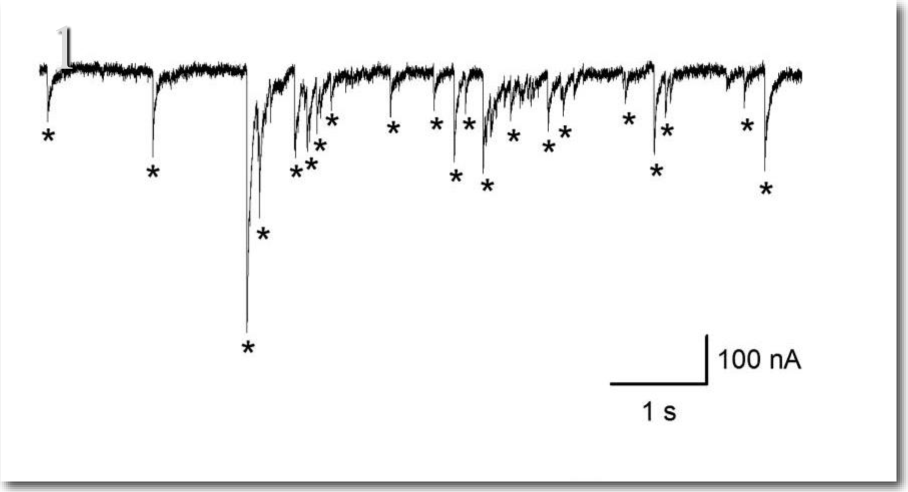
(medido em current-clamp)

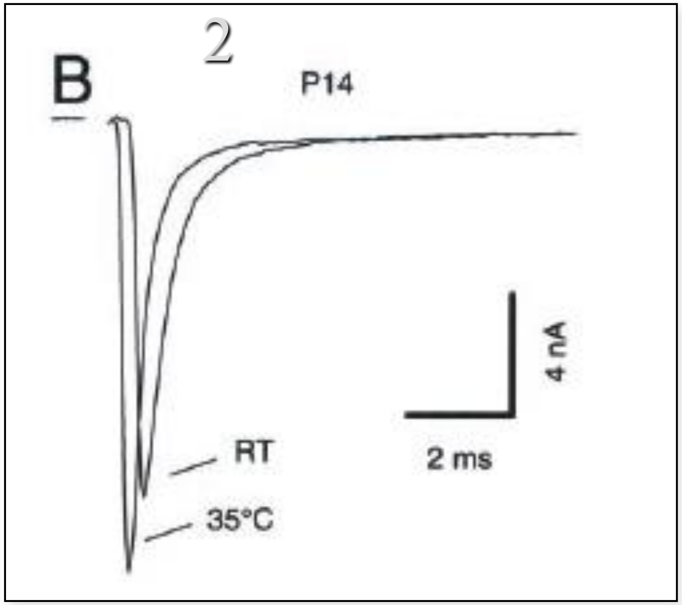
Corrente pós-sináptica excitatória

(medido em voltage-clamp)

Técnicas para o estudo da neurotransmissão

- Eletrofisiológicas
  - Técnicas eletrofisiológicas de "single-cell" -**whole-cell patch-clamp**
    - Registros em neurônios centrais
      - Whole-cell Patch-clamp (voltage-clamp)
      - Registro de eventos espontâneos (1) e evocados (2)



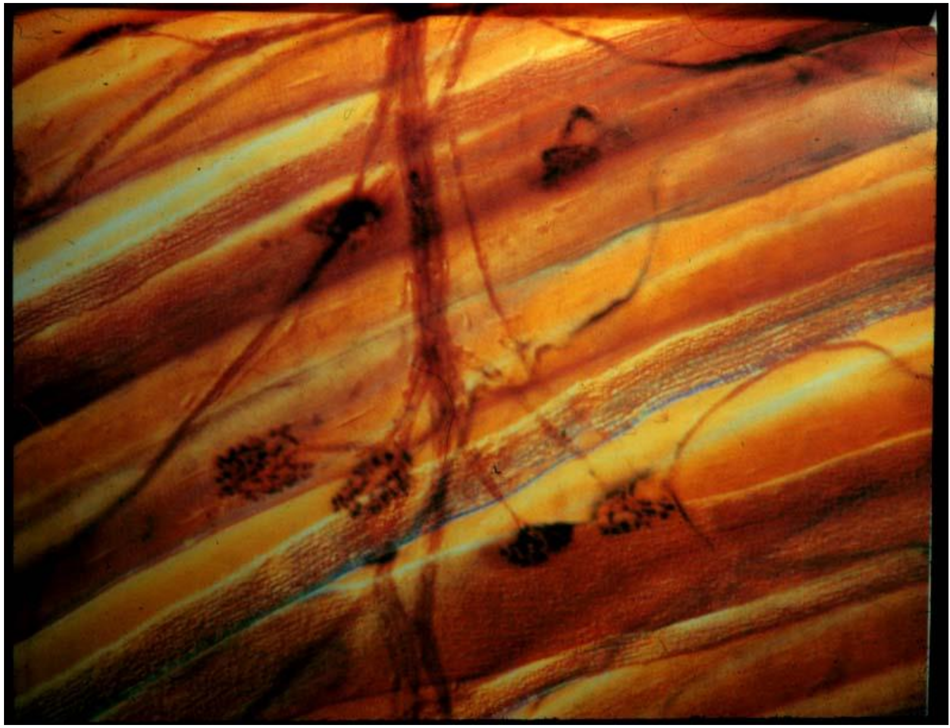


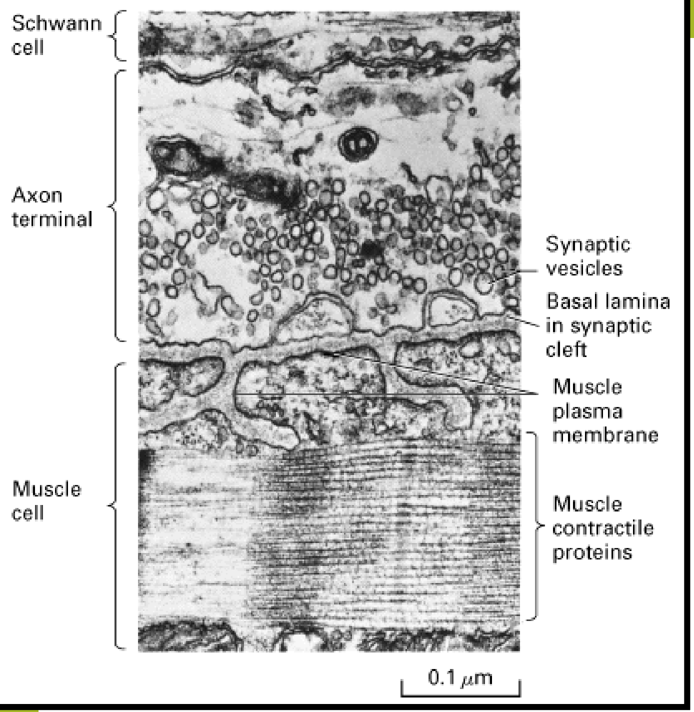
Sinapses pouco convencionais

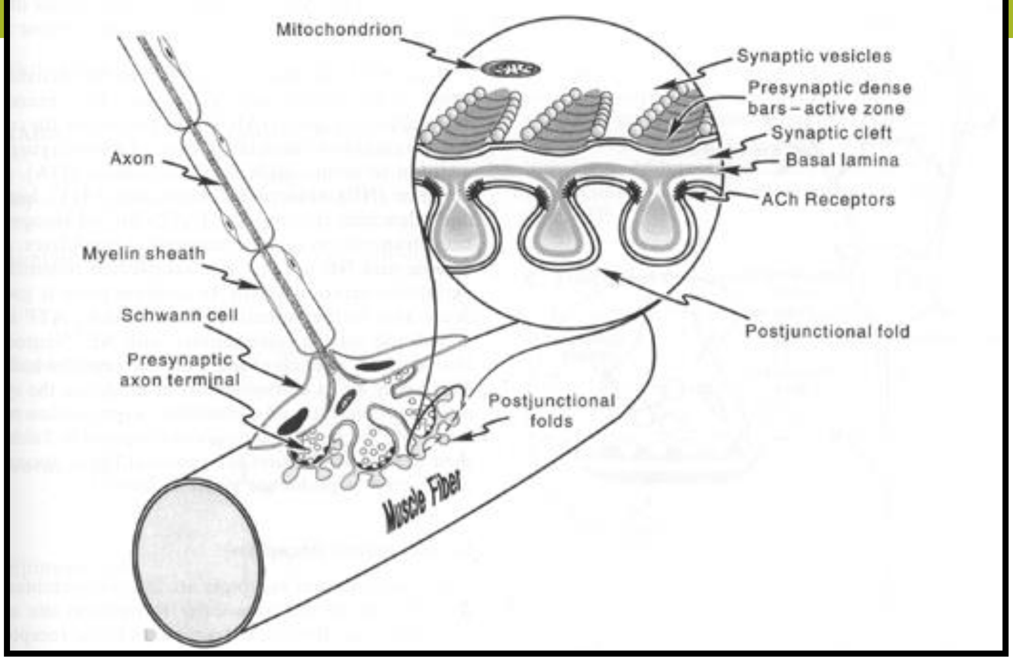
- sinapses muito maiores do que as convencionais são modelos importantes da investigação da fisiologia pré-sináptica.

A junção neuromuscular foi uma das primeiras sinases a serem estudadas

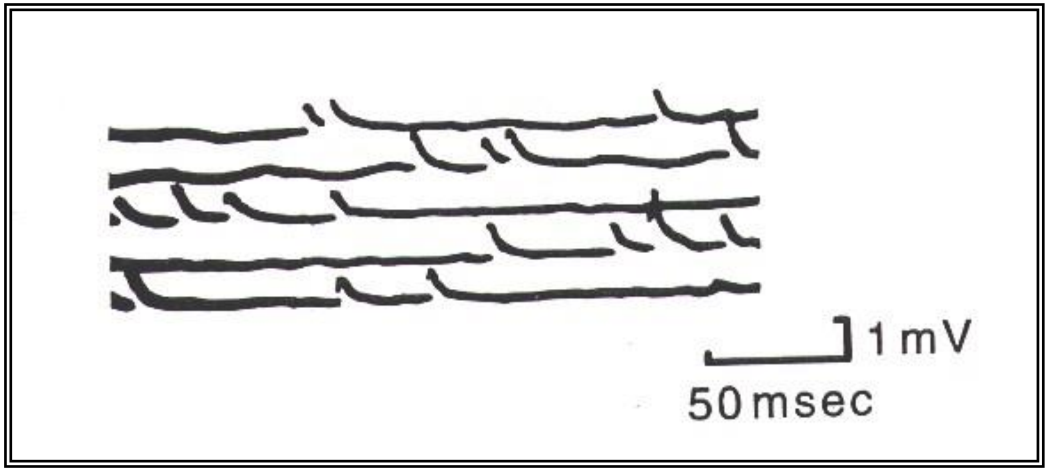
- Cada fibra recebe um terminal. Cada terminal possui diversos sítios de liberação vesicular
- O registro da neurotransmissão é feito na fibra muscular, por impalamento, um método fácil levando-se em comta a robustez da fibra muscular



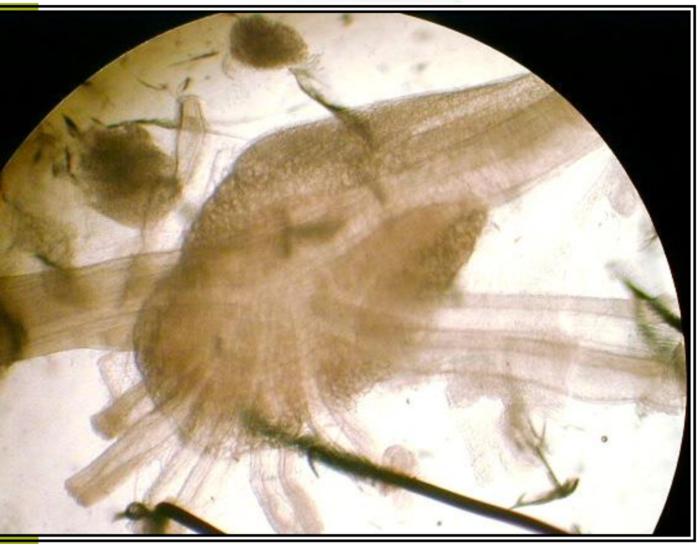

Schwann cell
Axon terminal
Muscle cell
Synaptic vesicles
Basal lamina in synaptic cleft
Muscle plasma membrane
Muscle contractile proteins
0.1 μm

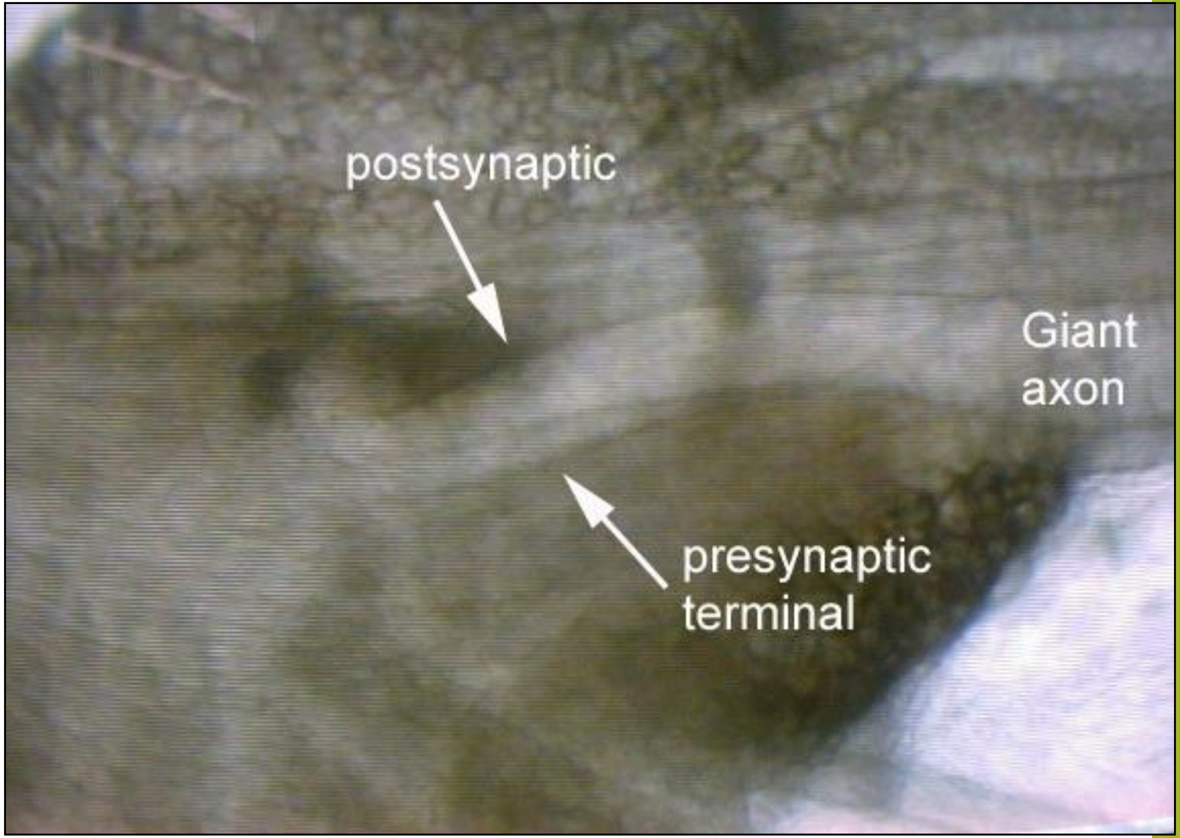

Mitochondrion
Synaptic vesicles
Presynaptic dense bars – active zone
Synaptic cleft
Basal lamina
ACh Receptors
Axon
Myelin sheath
Schwann cell
Presynaptic axon terminal
Postjunctional fold
Postjunctional folds
Muscle Fiber

Potenciais miniatura da placa motora ajudaram a entender os mecanismos de liberação de neurotransmissores



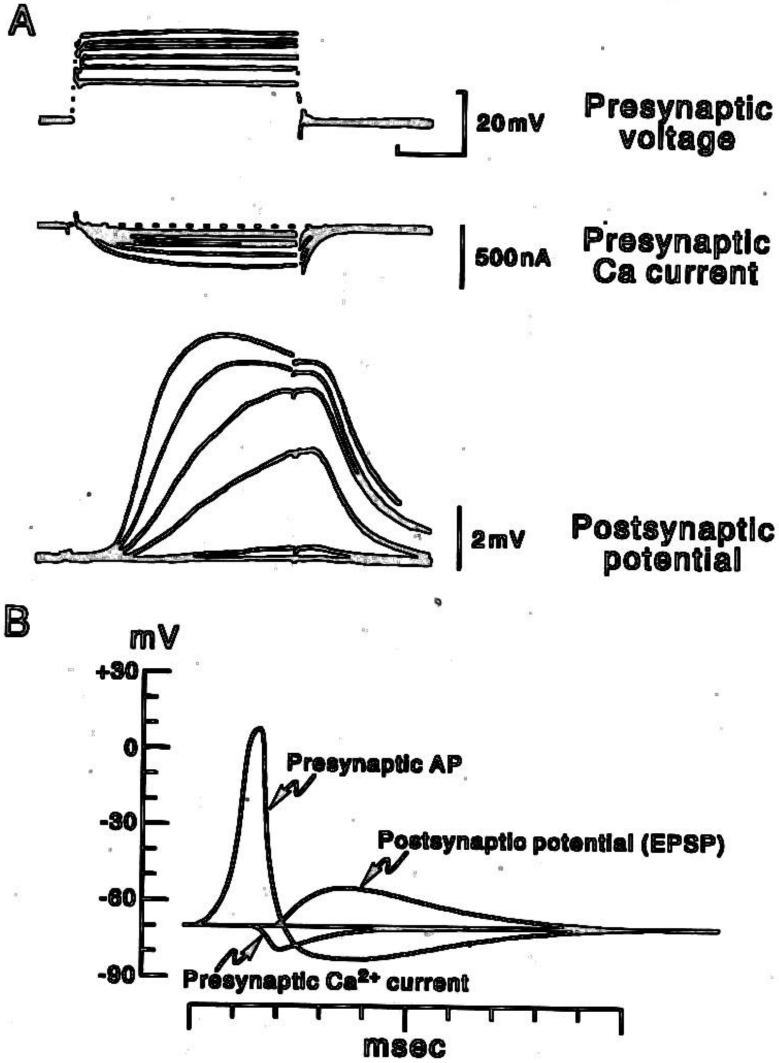
Sinapse gigante da lula (gânglio estelado)



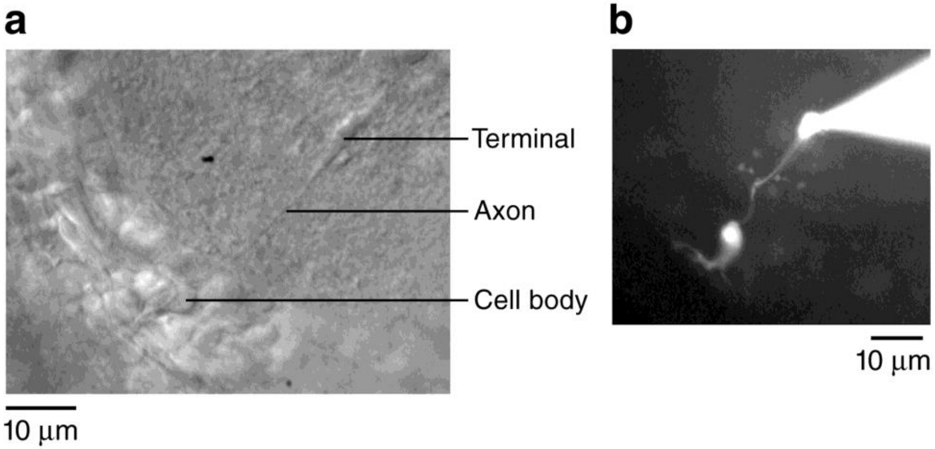

postsynaptic
Giant axon
presynaptic terminal

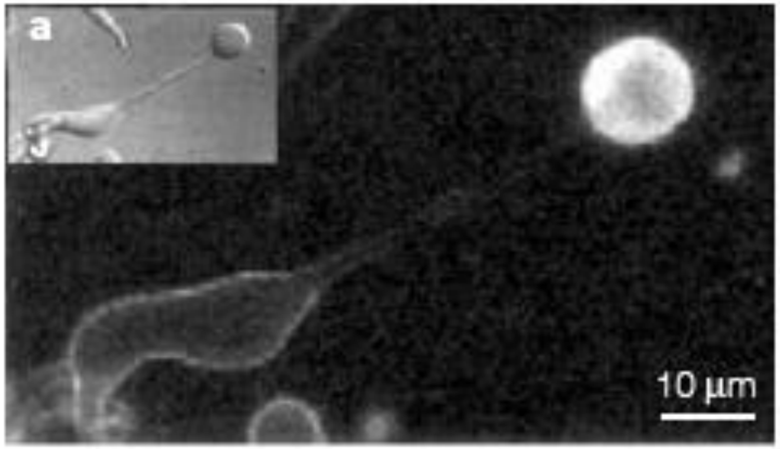
Sinapse gigante da lula (gânglio estelado)

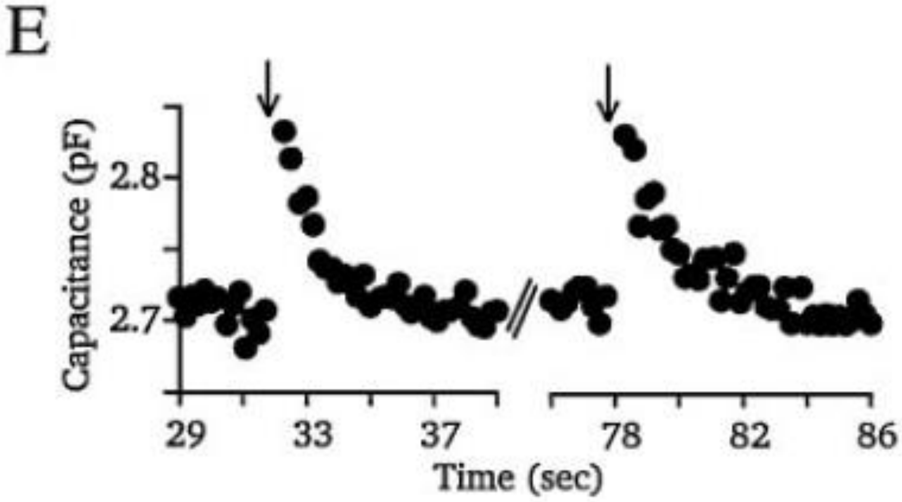
Registros simultâneos pré e pós sinápticos revelaram a dependência de cálcio na neurotransmissão



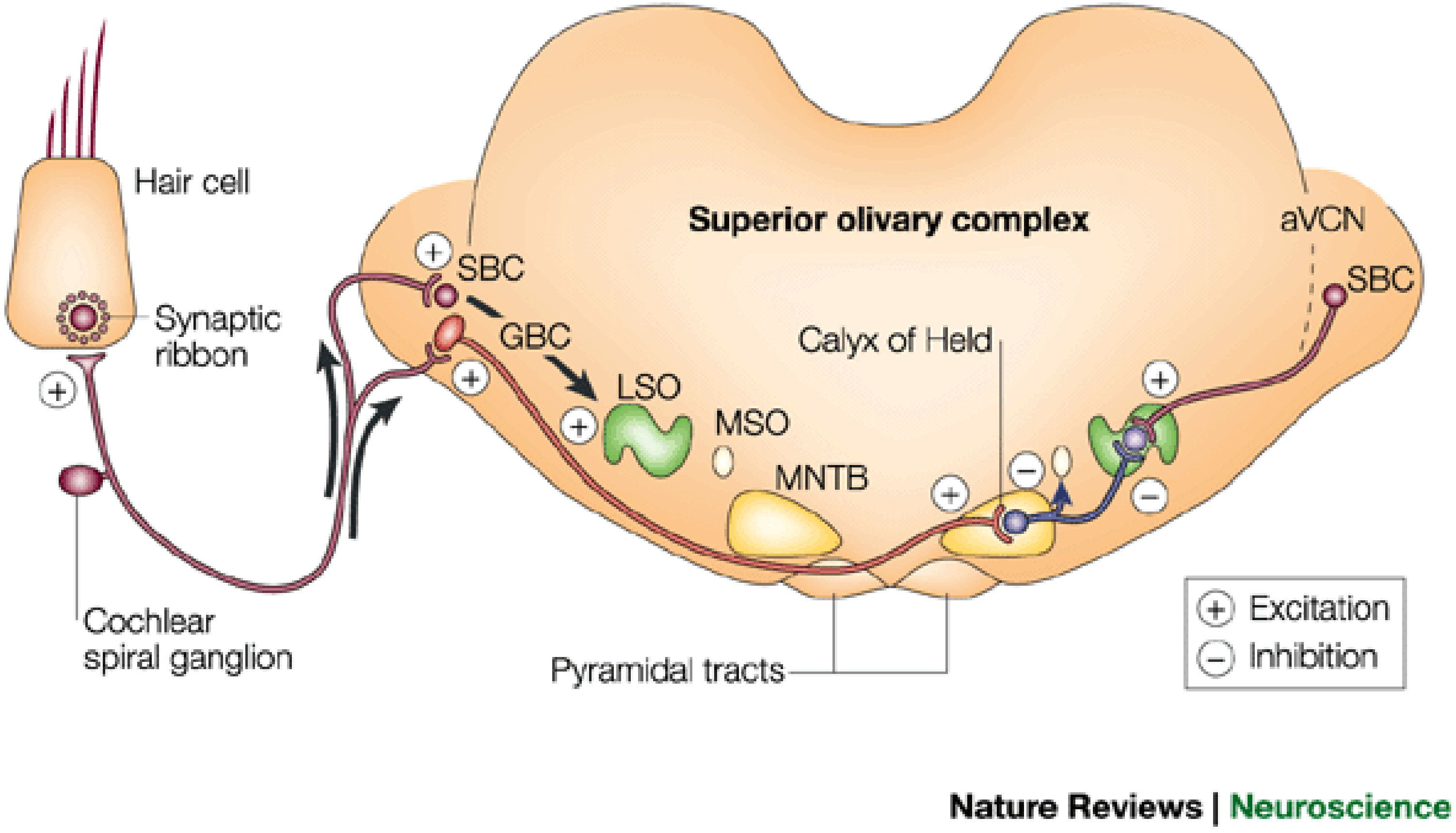
O Terminal gigante dos neurônios bipolares da retina do peixe permitiram os primeiros registros diretos de exo e endocitose por medidas de capacitância


a
Terminal
Axon
Cell body
10 µm
b
10 µm



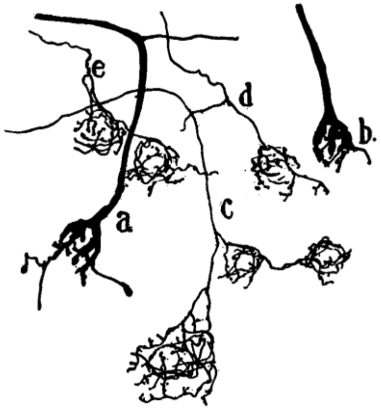


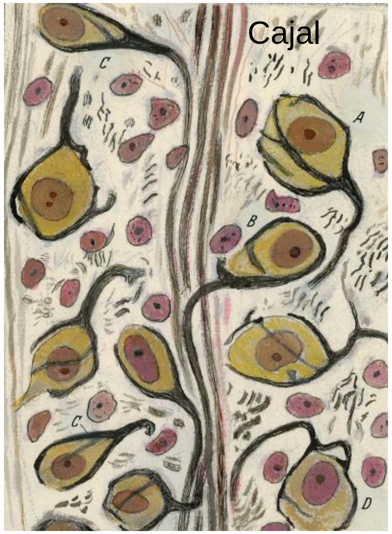
O Cálice de Held é uam sinapse gigante central que tem ajudado a entender os mecanismos de exocitose central

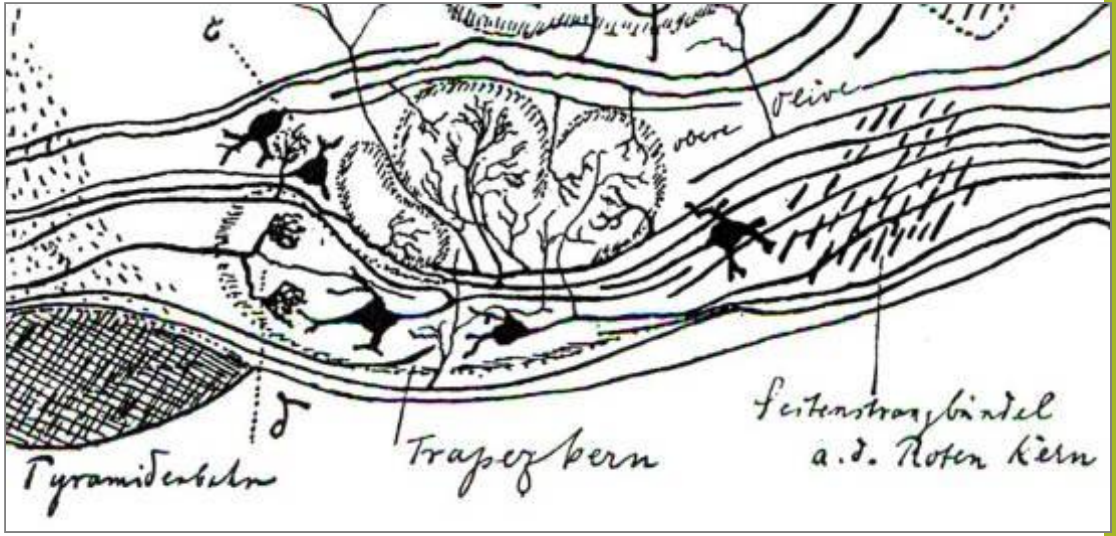

Hair cell
Synaptic ribbon
Cochlear spiral ganglion
Superior olivary complex
SBC
GBC
LSO
MSO
MNTB
Calyx of Held
aVCN
SBC
Pyramidal tracts
+
−
⊕ Excitation
⊖ Inhibition
Nature Reviews | Neuroscience

vonGersdorff & Borst, 2002

The calyx of Held

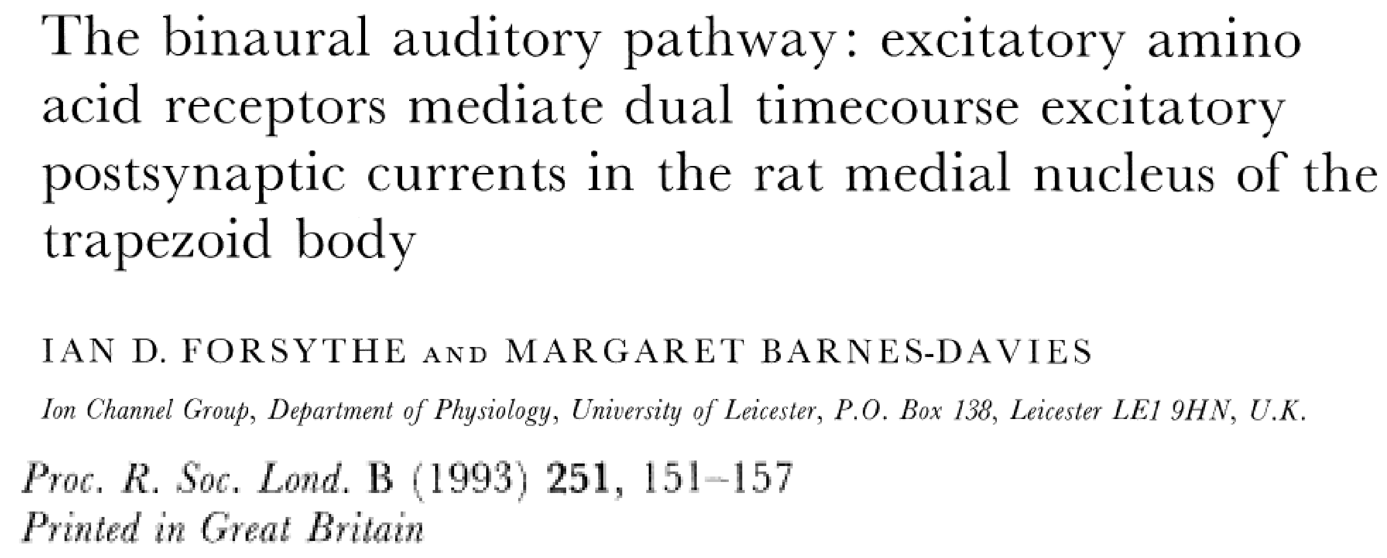



Cajal


ε
olive
olive
Pyramidenbahn
δ
Trapezkern
Seitenstrangbündel
a.d. Roten Kern

Held, H. (1893). Die zentrale Gehörleitung. Arch. Anat. u. Physiol. 17, 201-248.

A “redescoberta” do cálice de Held

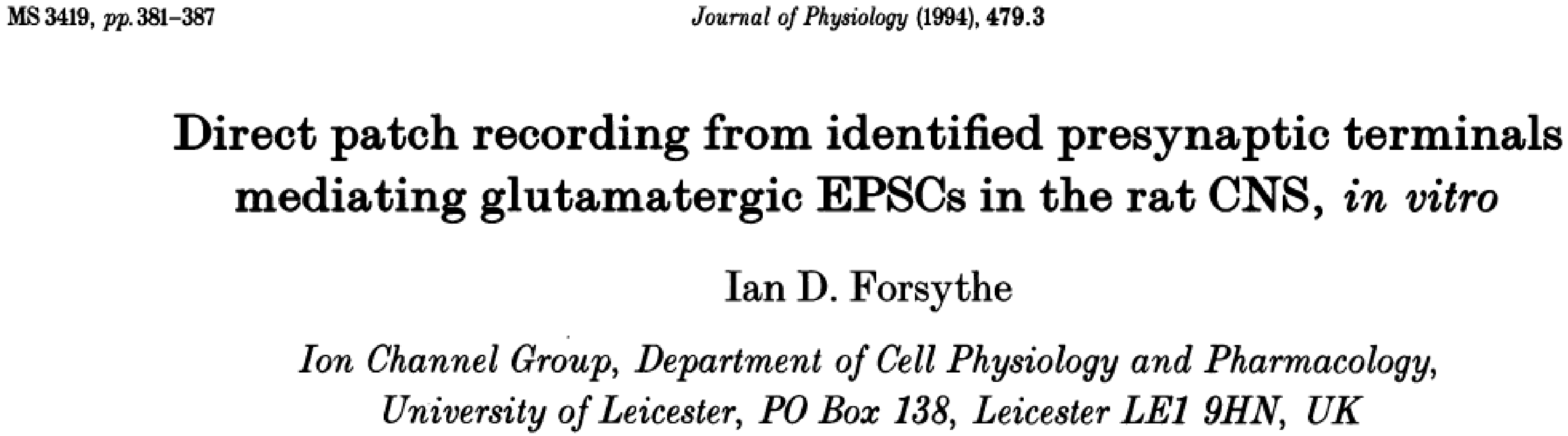


The binaural auditory pathway: excitatory amino acid receptors mediate dual timecourse excitatory postsynaptic currents in the rat medial nucleus of the trapezoid body

IAN D. FORSYTHE AND MARGARET BARNES-DAVIES

Ion Channel Group, Department of Physiology, University of Leicester, P.O. Box 138, Leicester LE1 9HN, U.K.

Proc. R. Soc. Lond. B (1993) **251**, 151–157
Printed in Great Britain



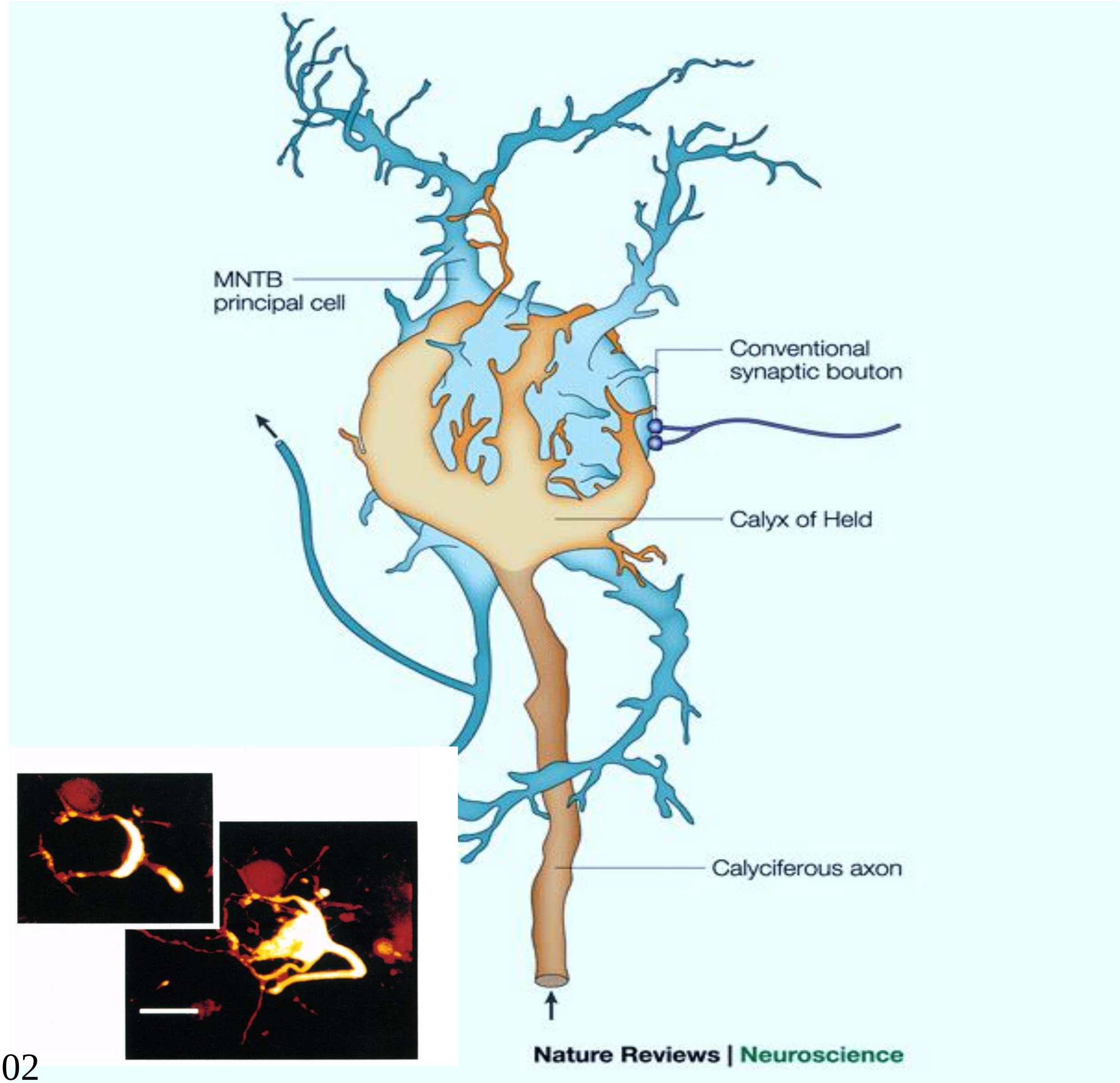
MS 3419, *pp.* 381–387 *Journal of Physiology* **(1994), 479.3**

Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, *in vitro*

Ian D. Forsythe

Ion Channel Group, Department of Cell Physiology and Pharmacology,
University of Leicester, PO Box 138, Leicester LE1 9HN, UK

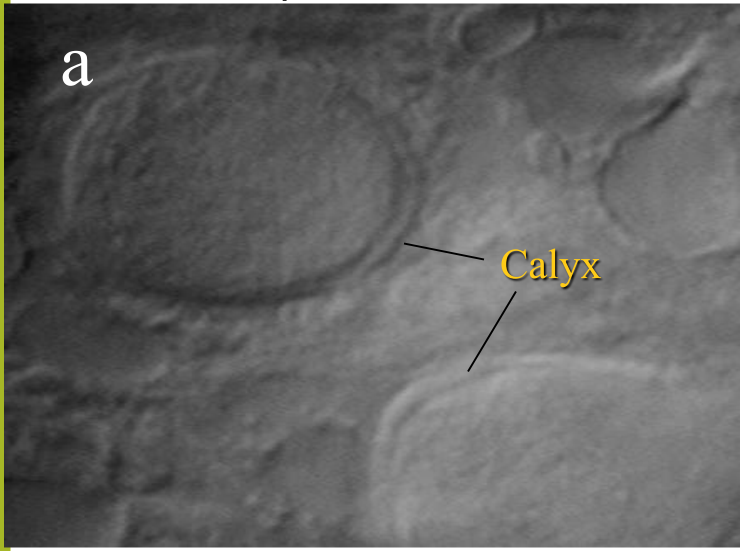
The calyx of Held

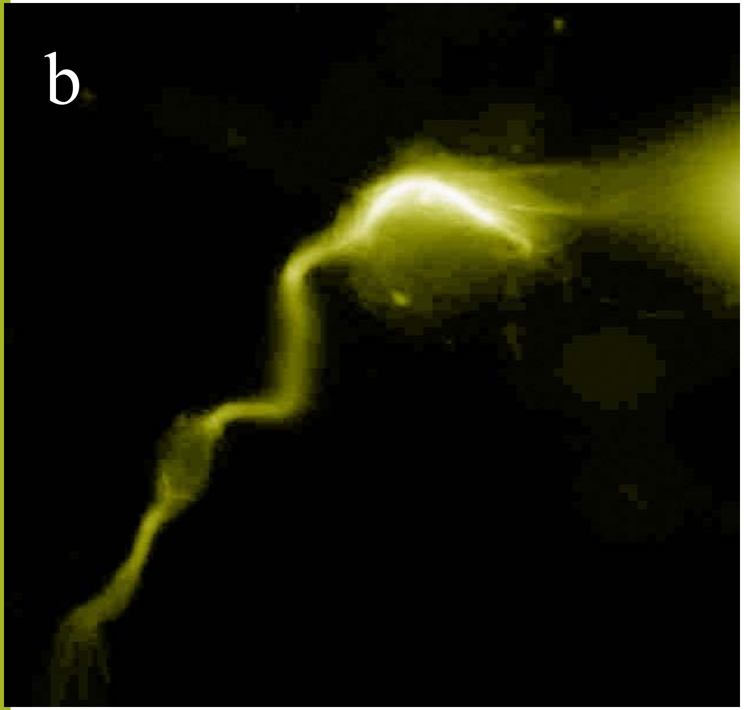

MNTB
principal cell
Conventional
synaptic bouton
Calyx of Held
Calyciferous axon

Nature Reviews | Neuroscience

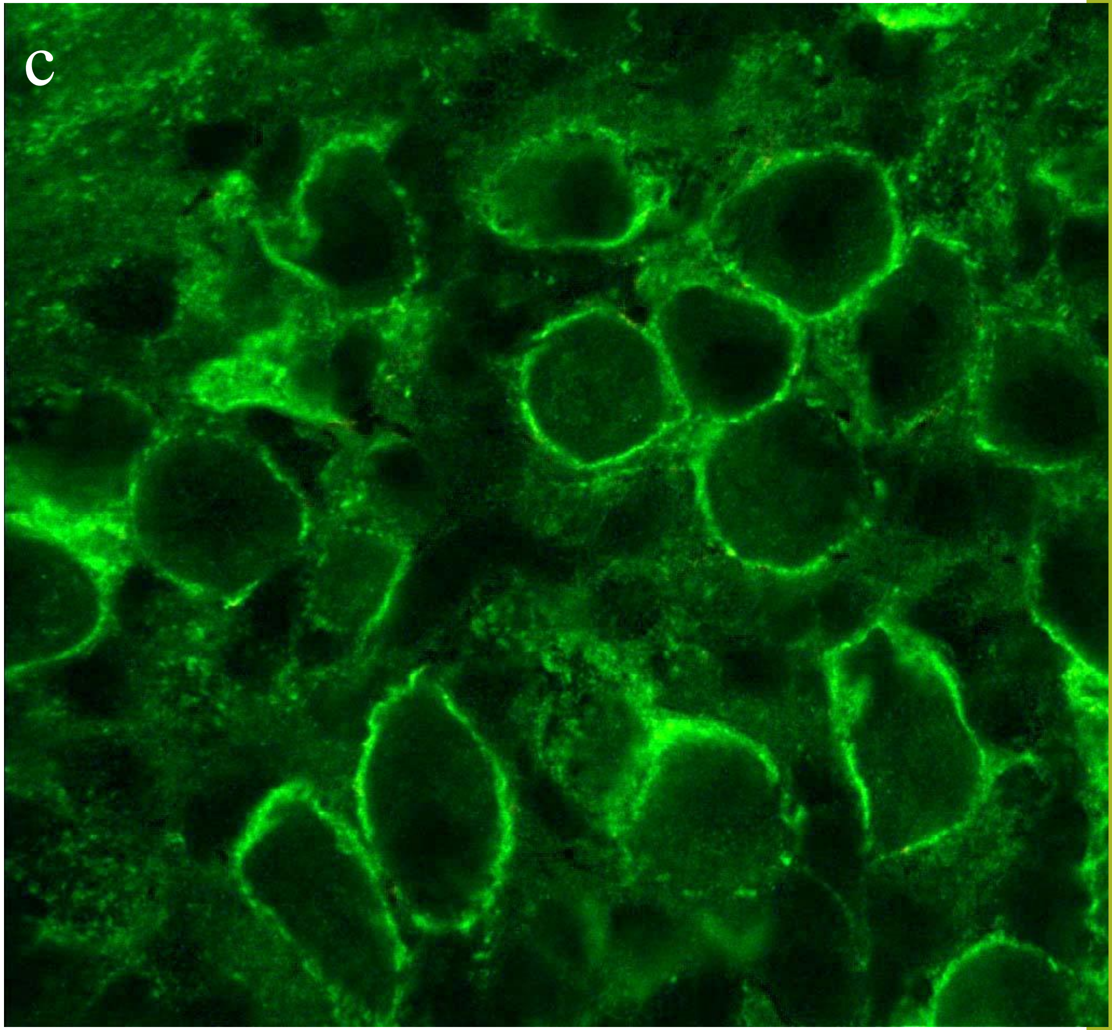
vonGersdorff & Borst, 2002

The Calyx of Held

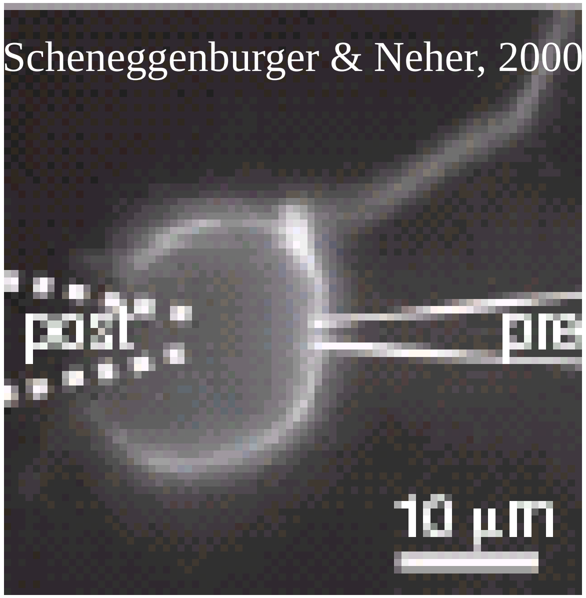


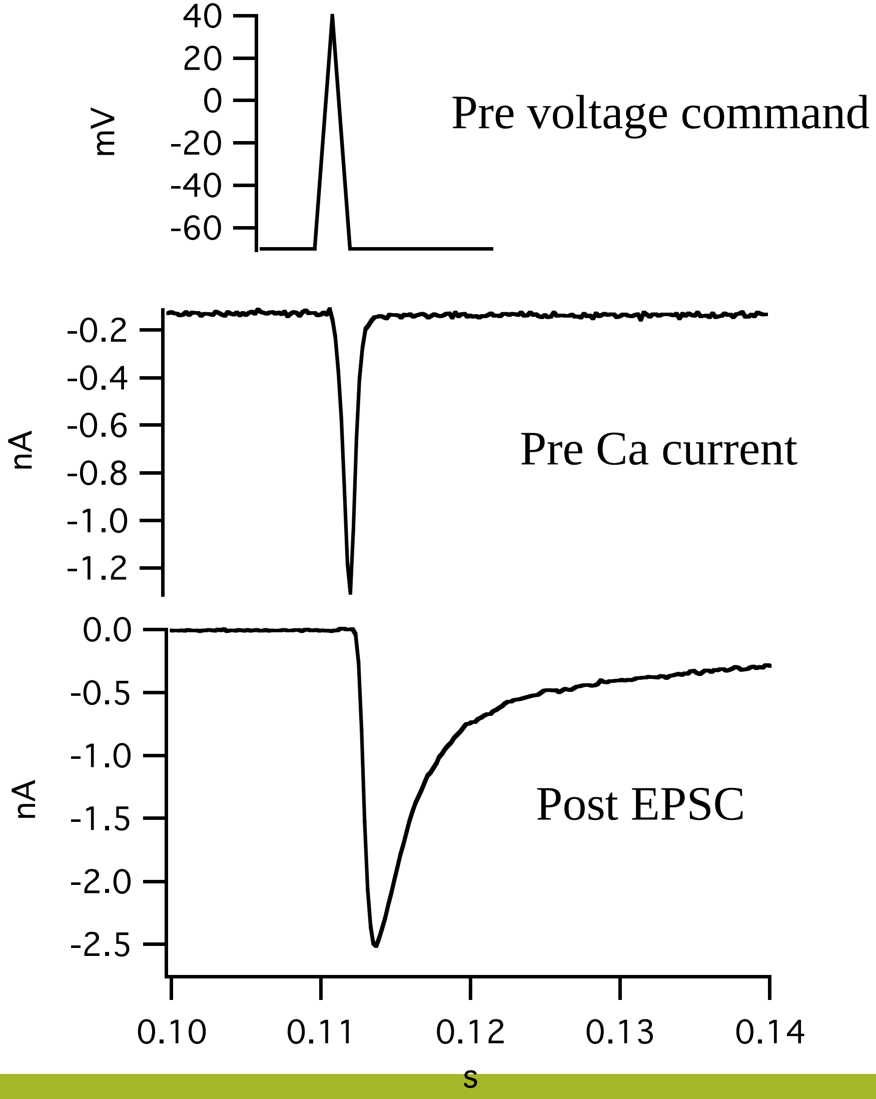


a) IR-DIC image
b) Luciffer yellow filling
c) Anti-SV2 stainning

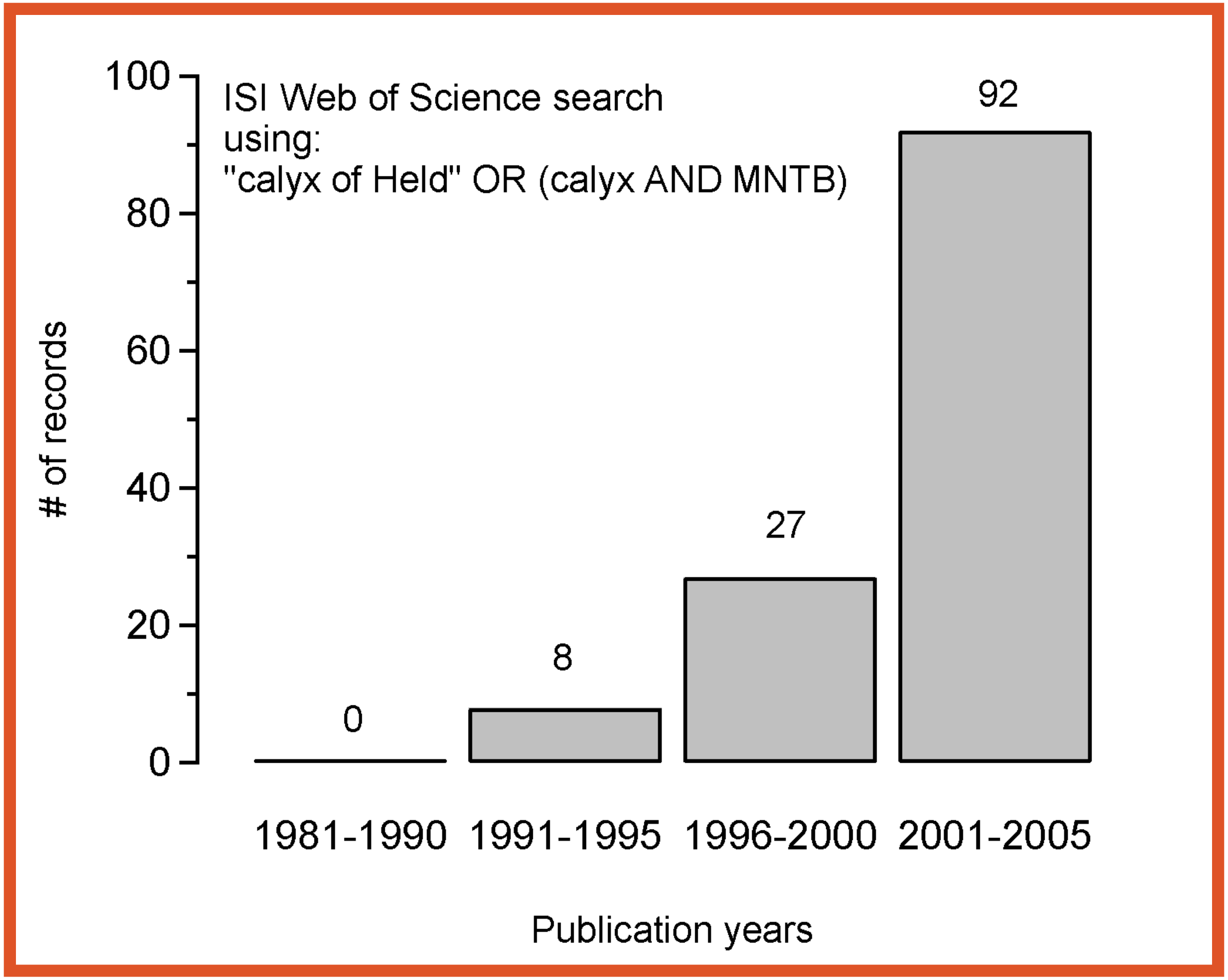


The calycel terminal is big enough to allow electrophysiological recording


Scheneggenburger & Neher, 2000
post
pre
10 µm



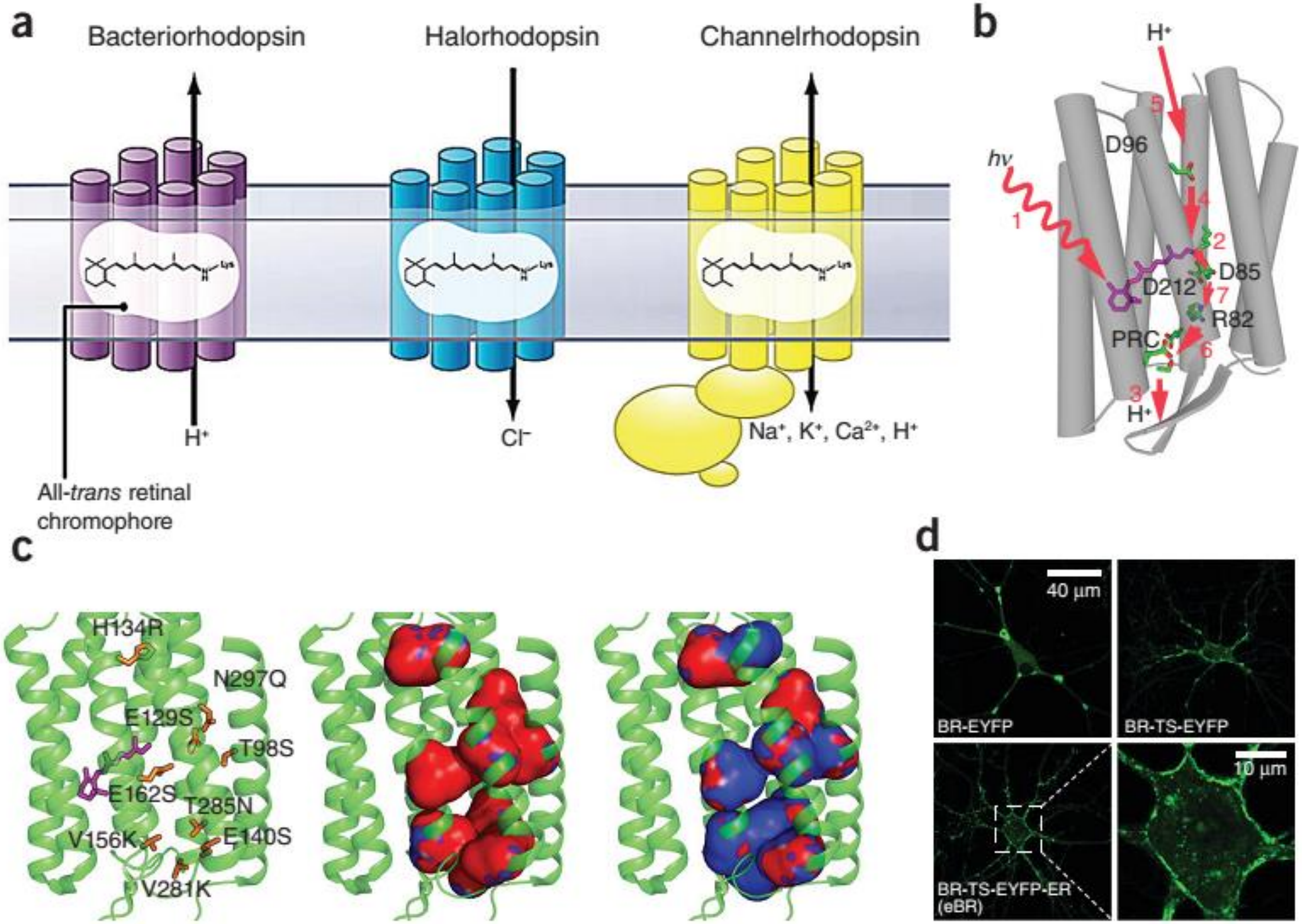
O Número de publicações sobre o cálice de Held tem aumentado vertiginosamente desde sua “redescoberta”



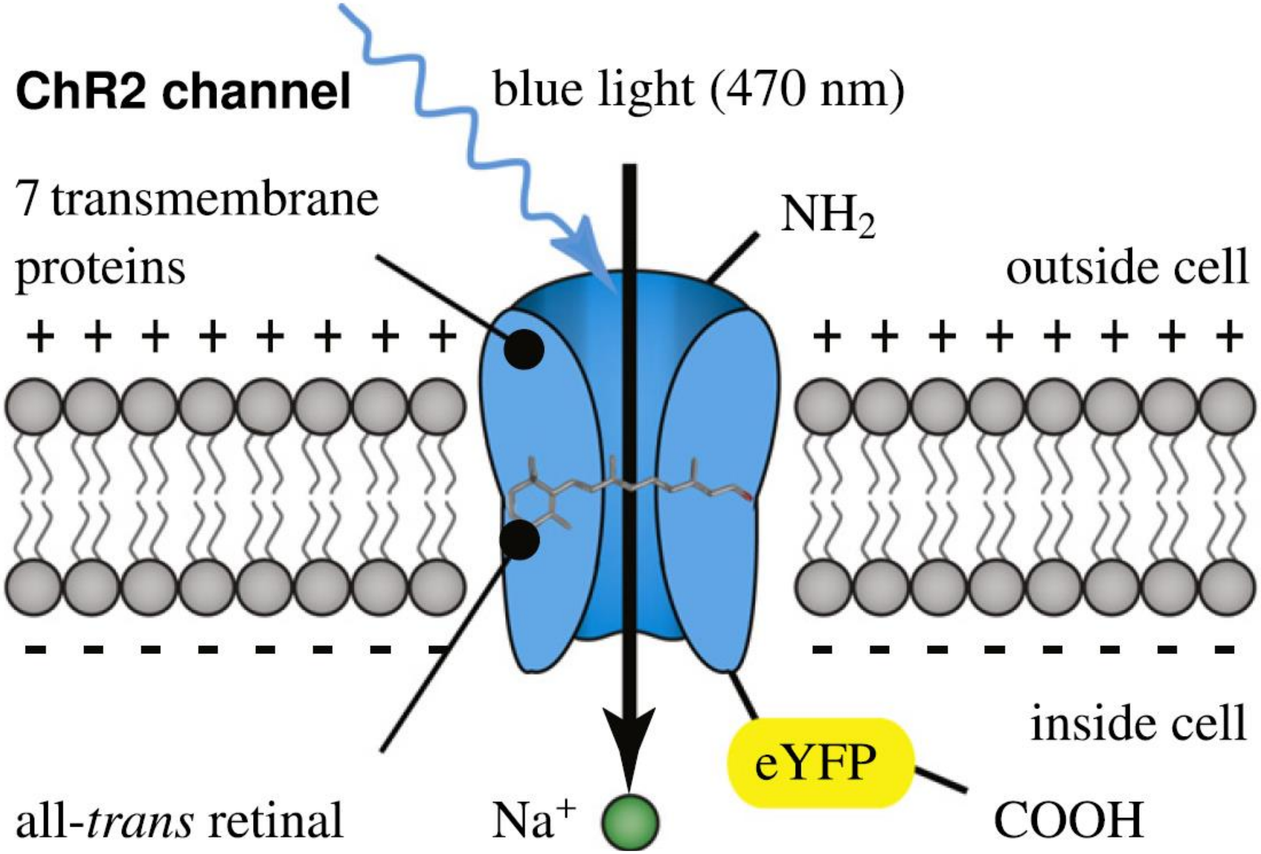
OPTOGENÉTICA

Karl Deisseroth

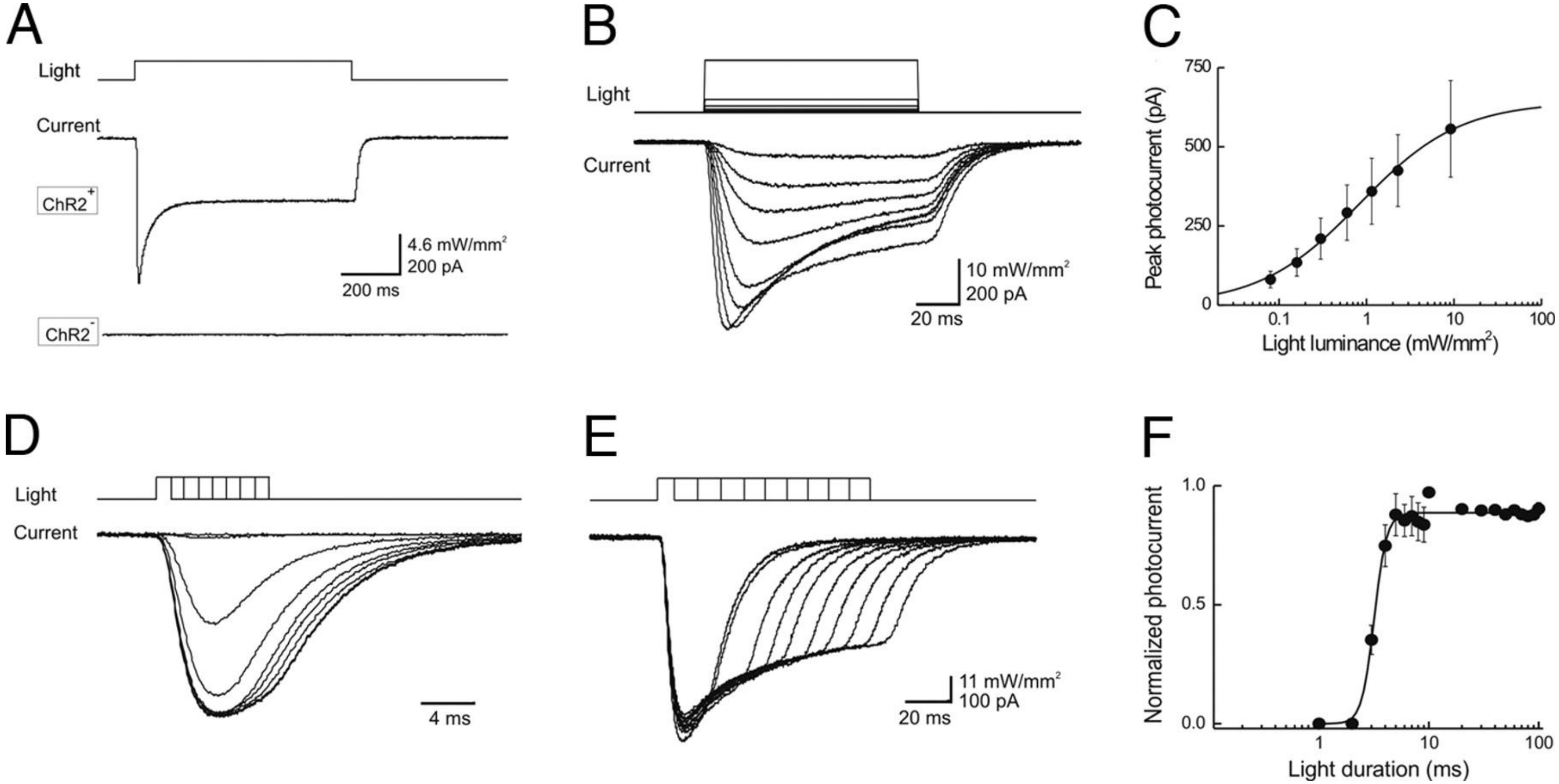
Opsinas provenientes de algas ou bactérias controladas por luz


a
Bacteriorhodopsin
Halorhodopsin
Channelrhodopsin
H⁺
Cl⁻
Na⁺, K⁺, Ca²⁺, H⁺
All-*trans* retinal chromophore
b
H⁺
hν
D96
D85
D212
R82
PRC
H⁺
1
2
3
4
5
6
7
c
H134R
N297Q
E129S
T98S
E162S
T285N
V156K
E140S
V281K
d
40 µm
BR-EYFP
BR-TS-EYFP
BR-TS-EYFP-ER (eBR)
10 µm

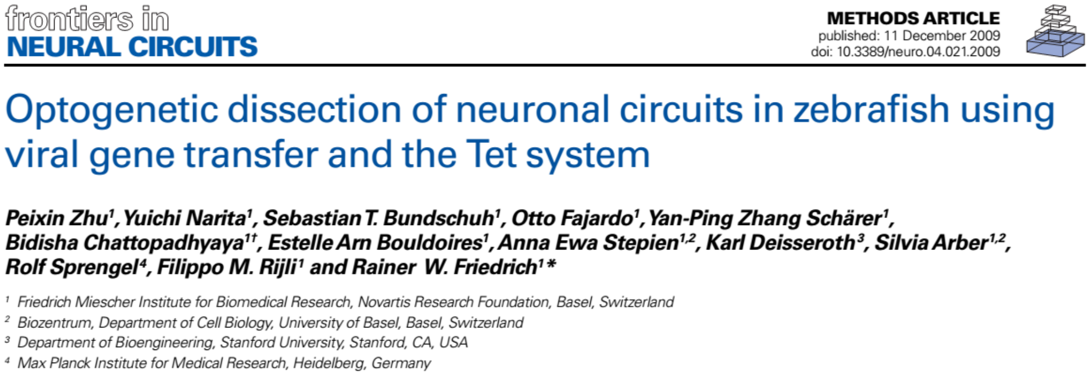
A canalorodopsina é um canal catiônico ativado pela luz azul (470 nm)


ChR2 channel
blue light (470 nm)
7 transmembrane proteins
NH_2
outside cell
+ + + + + + + +
+ + + + + + + +
- - - - - - - -
- - - - - - - -
inside cell
eYFP
all-*trans* retinal
Na^+
COOH

Illumination evokes photocurrents in ChR2-positive cortical neurons.



H. Wang et al. PNAS 2007;104:8143-8148



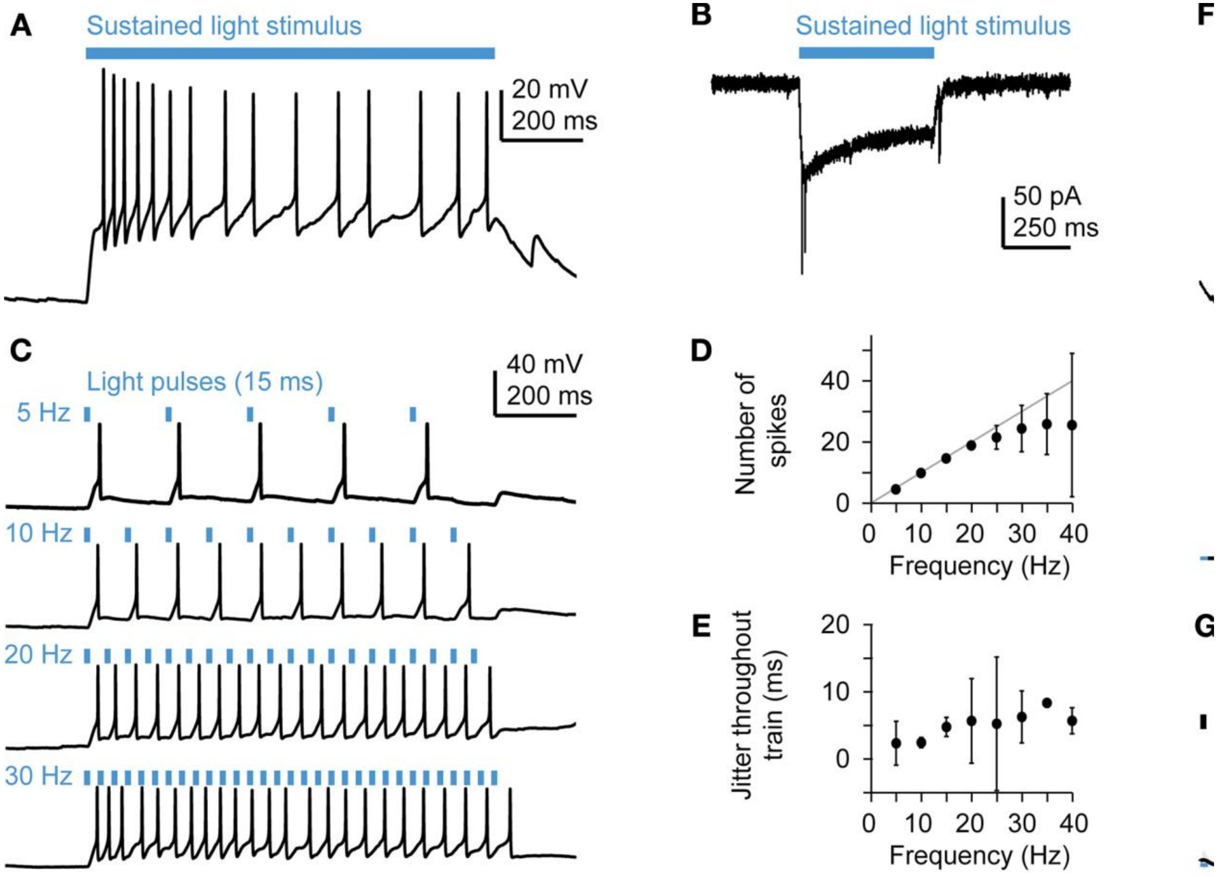
frontiers in
NEURAL CIRCUITS

METHODS ARTICLE
published: 11 December 2009
doi: 10.3389/neuro.04.021.2009

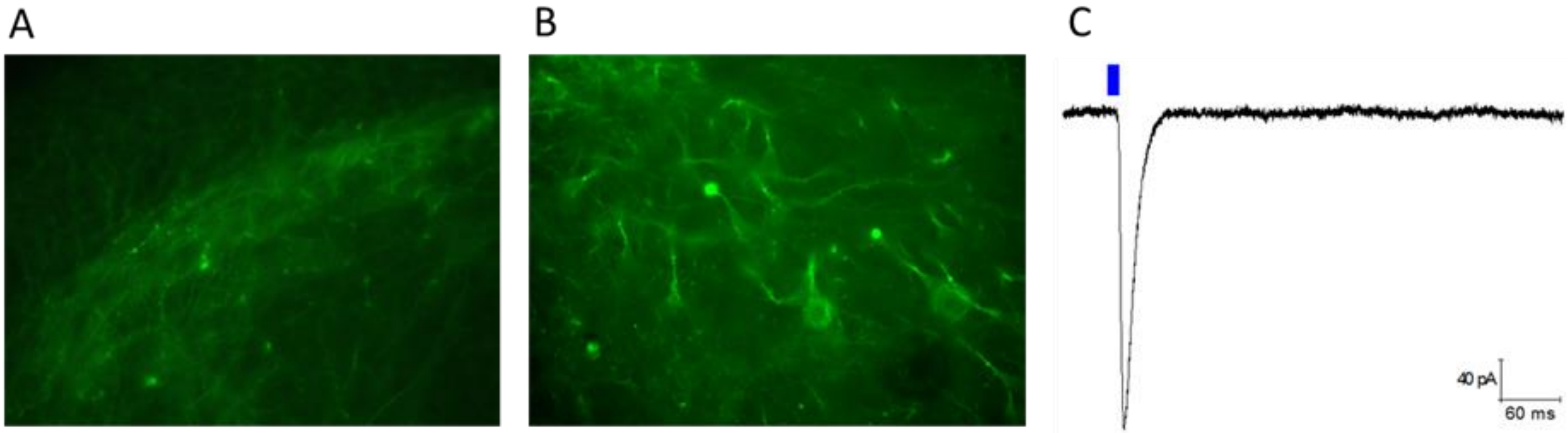
Optogenetic dissection of neuronal circuits in zebrafish using viral gene transfer and the Tet system

Peixin Zhu[1], Yuichi Narita[1], Sebastian T. Bundschuh[1], Otto Fajardo[1], Yan-Ping Zhang Schärer[1], Bidisha Chattopadhyaya[1†], Estelle Arn Bouldoires[1], Anna Ewa Stepien[1,2], Karl Deisseroth[3], Silvia Arber[1,2], Rolf Sprengel[4], Filippo M. Rijli[1] and Rainer W. Friedrich[1]*

[1] Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Basel, Switzerland
[2] Biozentrum, Department of Cell Biology, University of Basel, Basel, Switzerland
[3] Department of Bioengineering, Stanford University, Stanford, CA, USA
[4] Max Planck Institute for Medical Research, Heidelberg, Germany

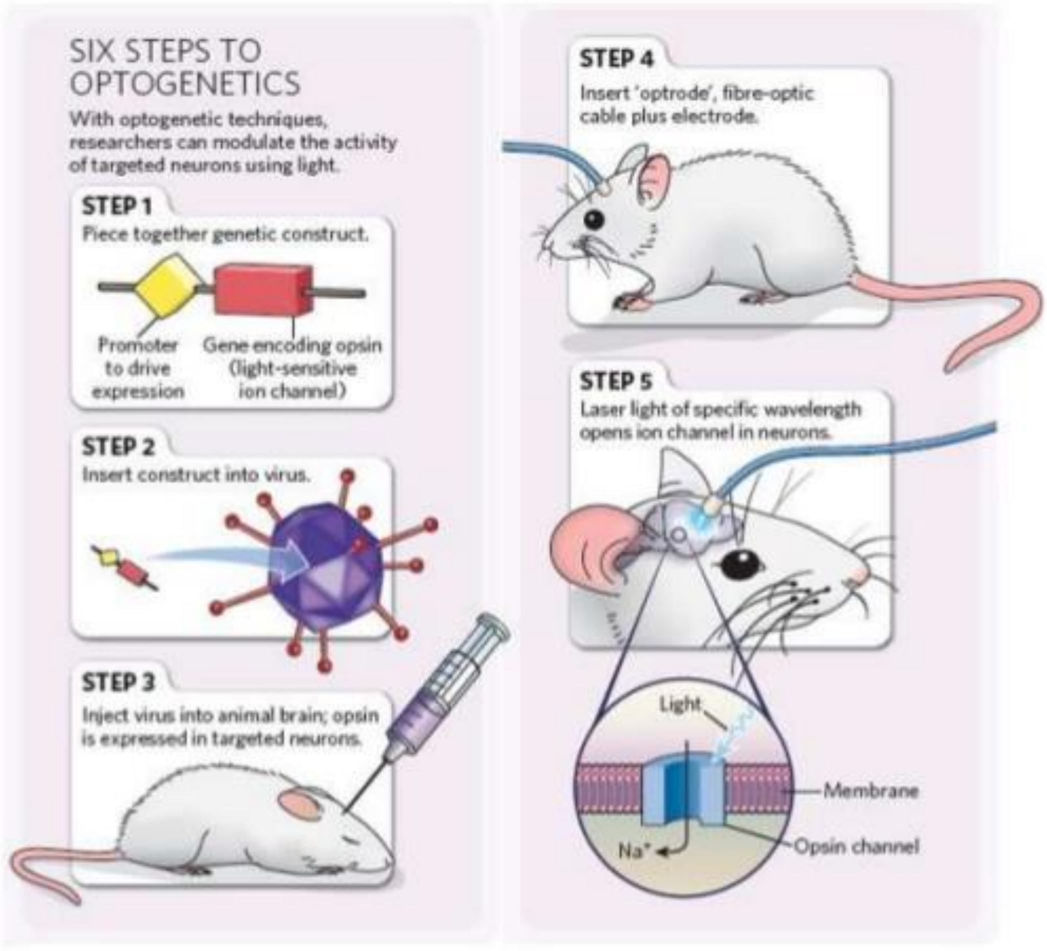


Estimulação com luz azul resulta em correntes sinápticas evocadas em fatias de hipocampo de animais transfectados com Chr2



Projeções de células do *hilus* do giro dentado transfectadas com o hSyn-ChR2-GFP. A e B Nossos resultados preliminares mostram a expressão da GFP em corpos celulares e dendritos. C. Corrente sináptica excitatória evocada por um pulso de luz azul com duração de 10 ms, registrada em neurônio piramidal da região da CA1, em fatias hipocampais, usando-se *whole-cell patch-clamp*. Neste experimento foi utilizada uma fonte de LED, 488nm, com 20-30% da potência total.

A micoinjeção em áreas específicas de virus (AAV ou lentvirus) contendo plasmideos que expressam Chr2 ligadas a promotores especificos, permite a estimulação de vias específicas neurais



SIX STEPS TO OPTOGENETICS

With optogenetic techniques, researchers can modulate the activity of targeted neurons using light.

STEP 1
Piece together genetic construct.

STEP 2
Insert construct into virus.

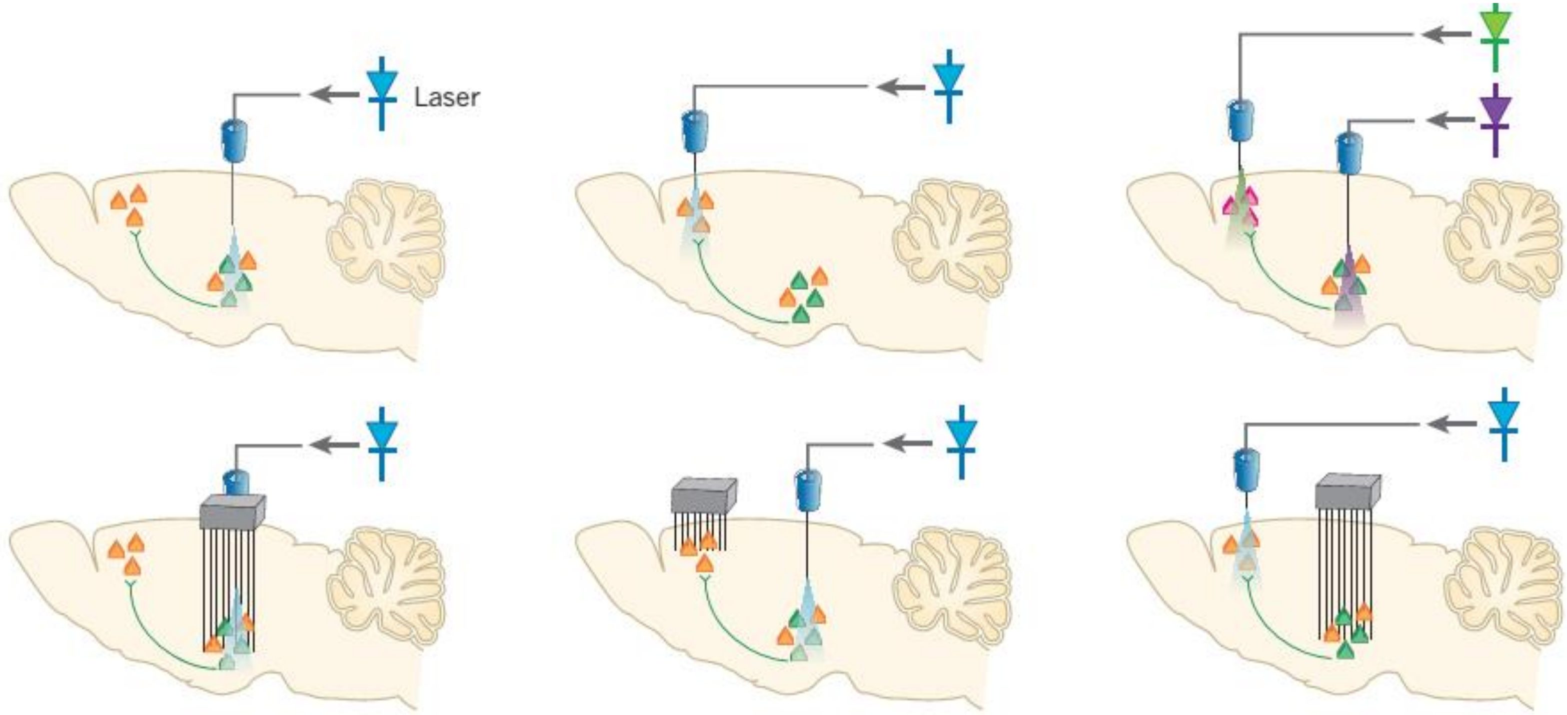
STEP 3
Inject virus into animal brain; opsin is expressed in targeted neurons.

STEP 4
Insert 'optrode', fibre-optic cable plus electrode.

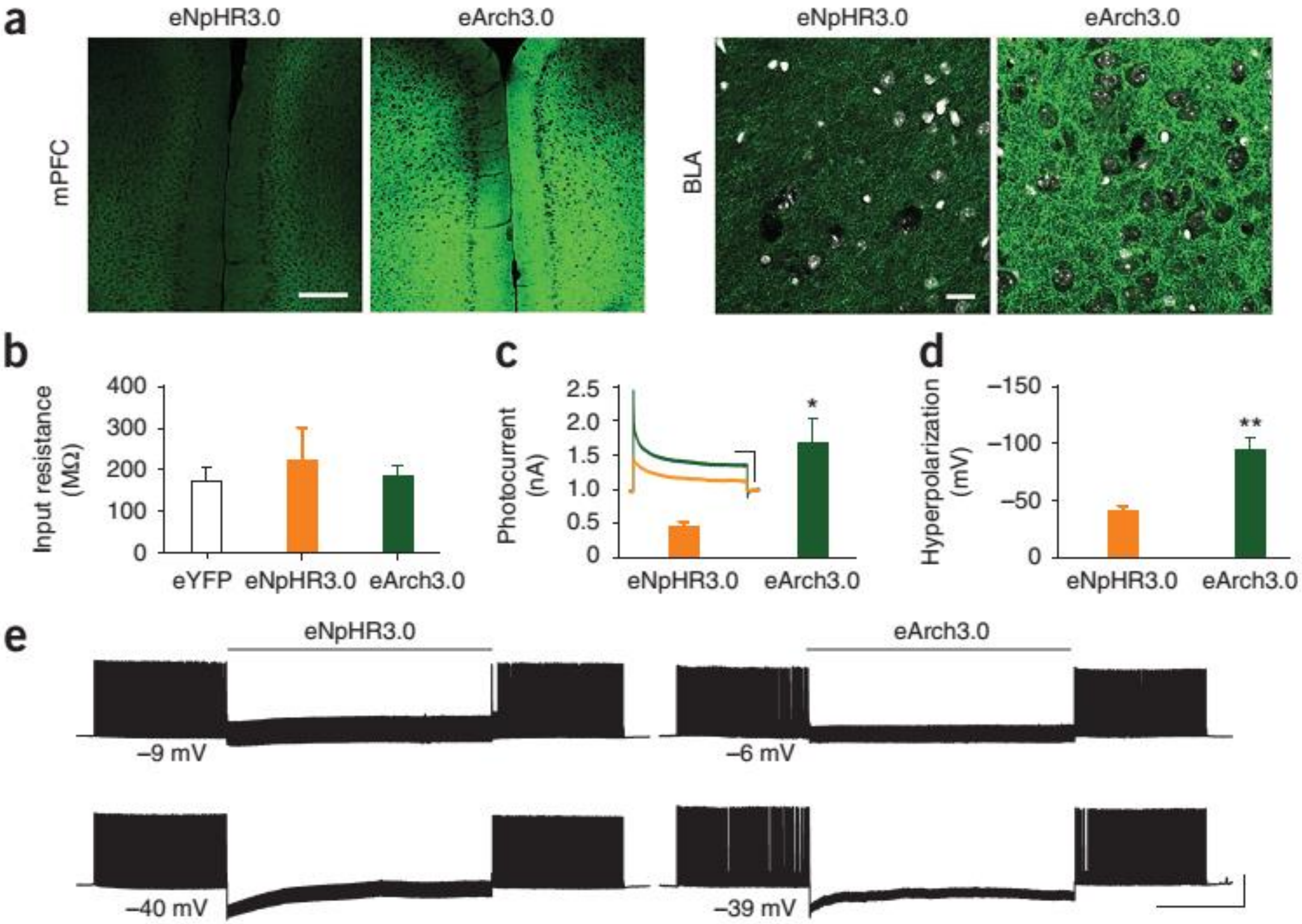
STEP 5
Laser light of specific wavelength opens ion channel in neurons.

Promoter to drive expression
Gene encoding opsin (light-sensitive ion channel)
Light
Membrane
Na^+
Opsin channel

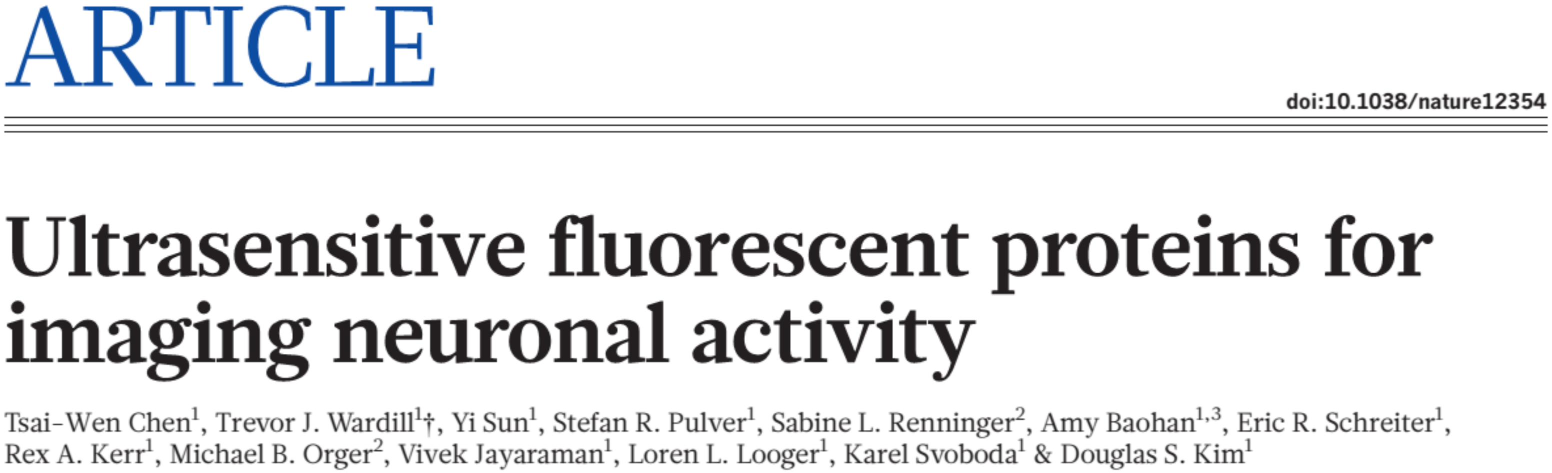
Experimentos de optogenética *in vivo*



Halorodopsina (canal de Cl^-) e archrodopsina (bomba extrudora de H^+) produzem hiperpolarização da membrana e consequente inibição neuronal



GCamp6 é uma proteína engenheirada para ser sensora de cálcio

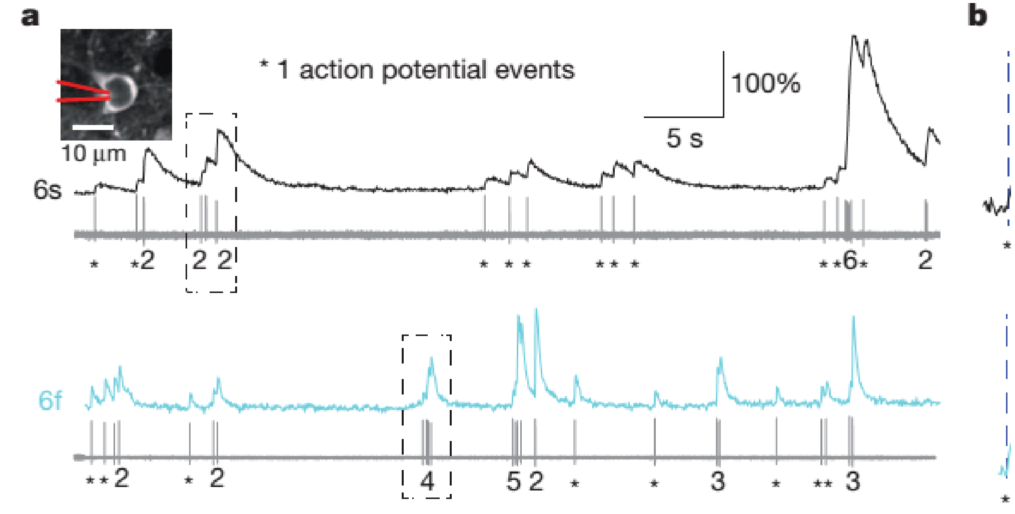


ARTICLE

doi:10.1038/nature12354

Ultrasensitive fluorescent proteins for imaging neuronal activity

Tsai-Wen Chen[1], Trevor J. Wardill[1]†, Yi Sun[1], Stefan R. Pulver[1], Sabine L. Renninger[2], Amy Baohan[1,3], Eric R. Schreiter[1], Rex A. Kerr[1], Michael B. Orger[2], Vivek Jayaraman[1], Loren L. Looger[1], Karel Svoboda[1] & Douglas S. Kim[1]

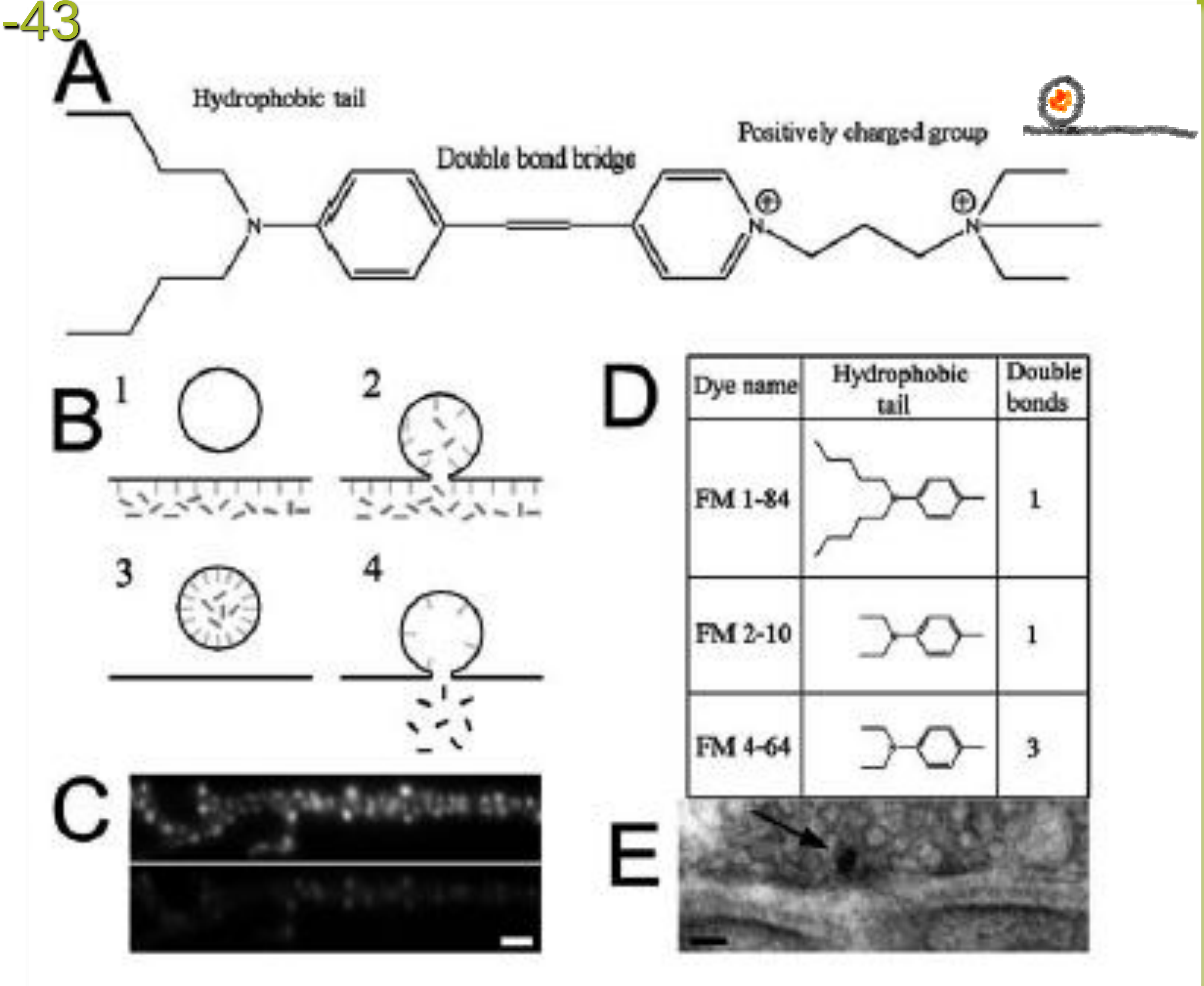


Detecção da exocitose por corantes FM1

- FM1-43. We have developed and characterized fluorescent dyes that stain the membranes of recycled synaptic vesicles. The best dye is **FM1-43**, which was synthesized by Dr. Fei Mao of Molecular Probes, Inc. Three properties make FM dyes useful for studying exocytosis, endocytosis, and endosomal trafficking:
- The dyes partition reversibly in membranes
- The dyes do not 'flip flop' and so are never free in the cytoplasm
- The dyes are far more fluorescent in membranes than in water (From Bill Betz Web page))

Bill Betz

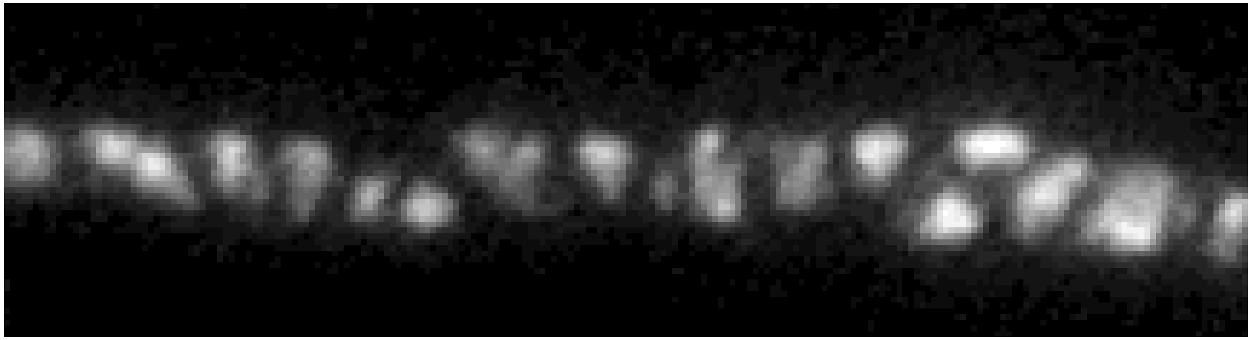
FM1-43

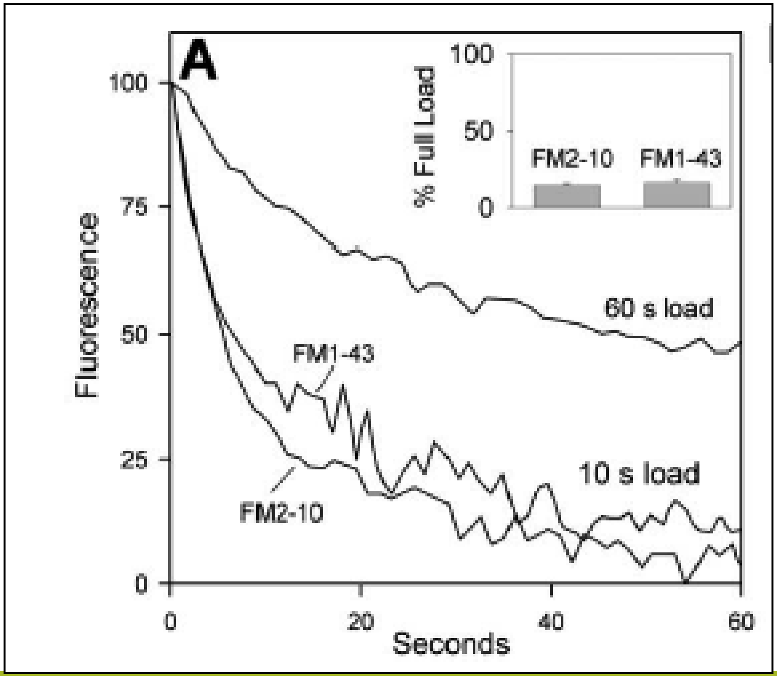

A
Hydrophobic tail
Double bond bridge
Positively charged group
N
N
N
B
1
2
3
4
C
D
E

Dye name	Hydrophobic tail	Double bonds
FM 1-84		1
FM 2-10		1
FM 4-64		3

FM1-43

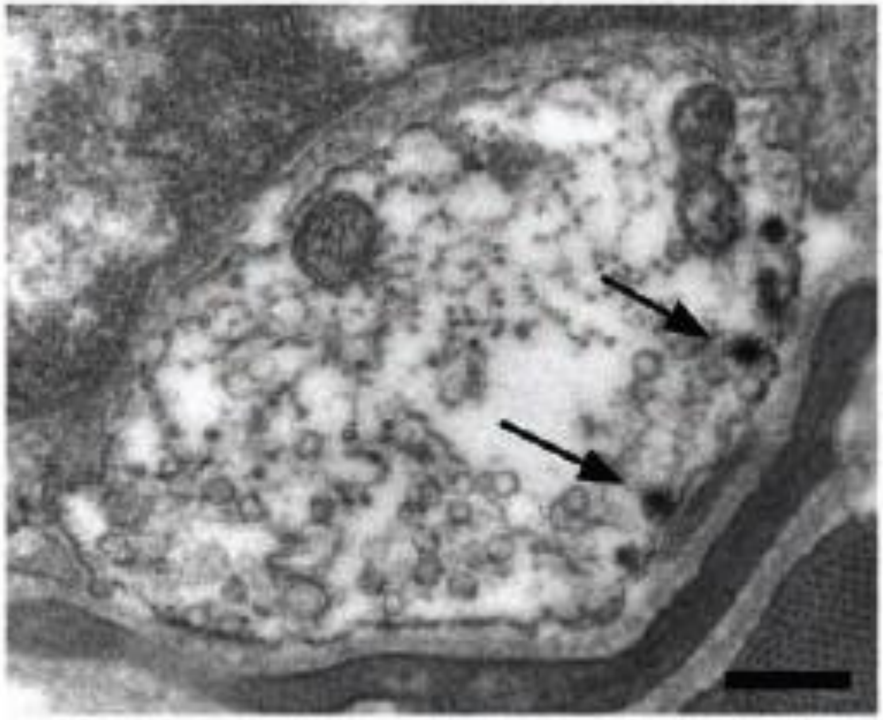
Junção neuromuscular





FM1-43

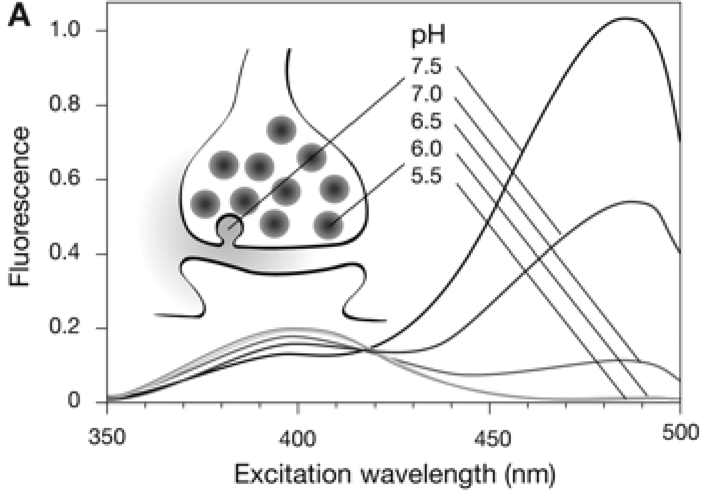
A fotoconversão do FM1-43 promove a sua fixação

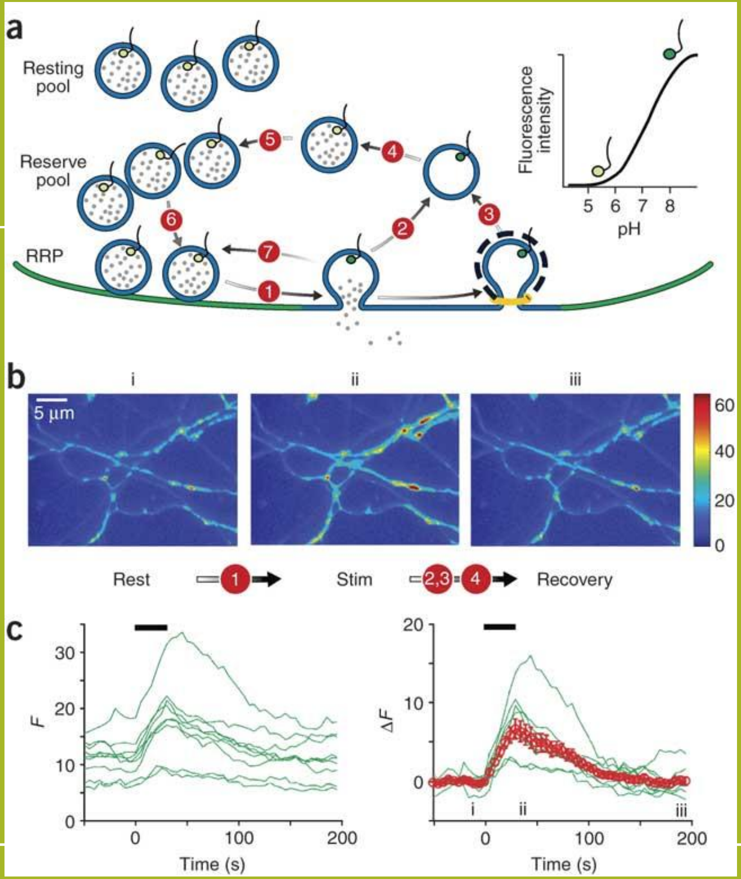


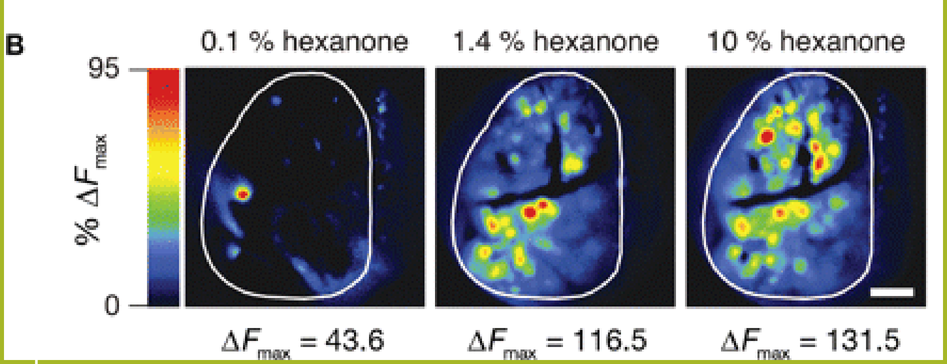
SYNAPTO-PHLUORIN

Proteína derivada da GFP que fluoresce em meio alcalino.

Mede exocitose já que só fluoresce quando o interior da vesícula entra em contato com o meio extracelular, já que o interior da vesícula é ácido

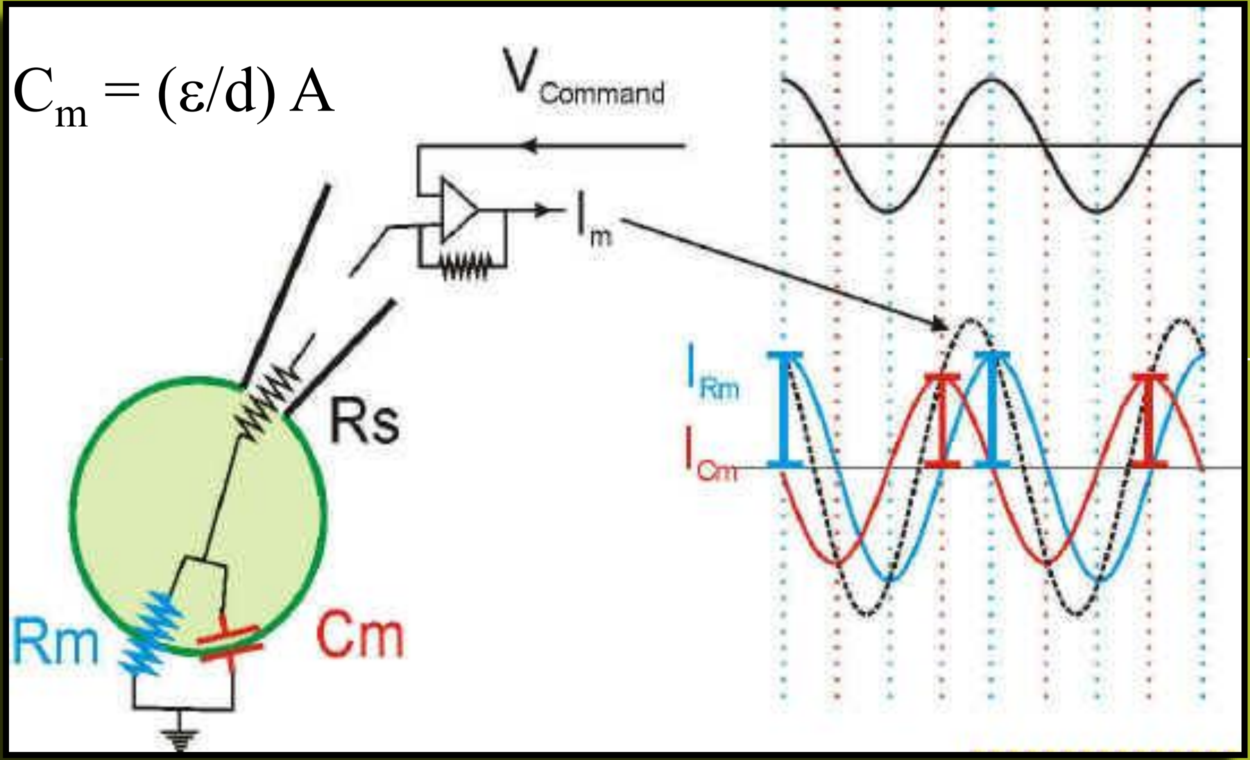



a
Resting pool
Reserve pool
RRP
1
2
3
4
5
6
7
Fluorescence intensity
5
6
7
8
pH
b
i
ii
iii
5 µm
60
40
20
0
Rest
1
Stim
2,3
4
Recovery
c
30
20
10
0
F
0
100
200
Time (s)
20
10
0
ΔF
i
ii
iii
0
100
200
Time (s)


B
0.1 % hexanone
1.4 % hexanone
10 % hexanone
95
0
% ΔF_max
ΔF_max = 43.6
ΔF_max = 116.5
ΔF_max = 131.5

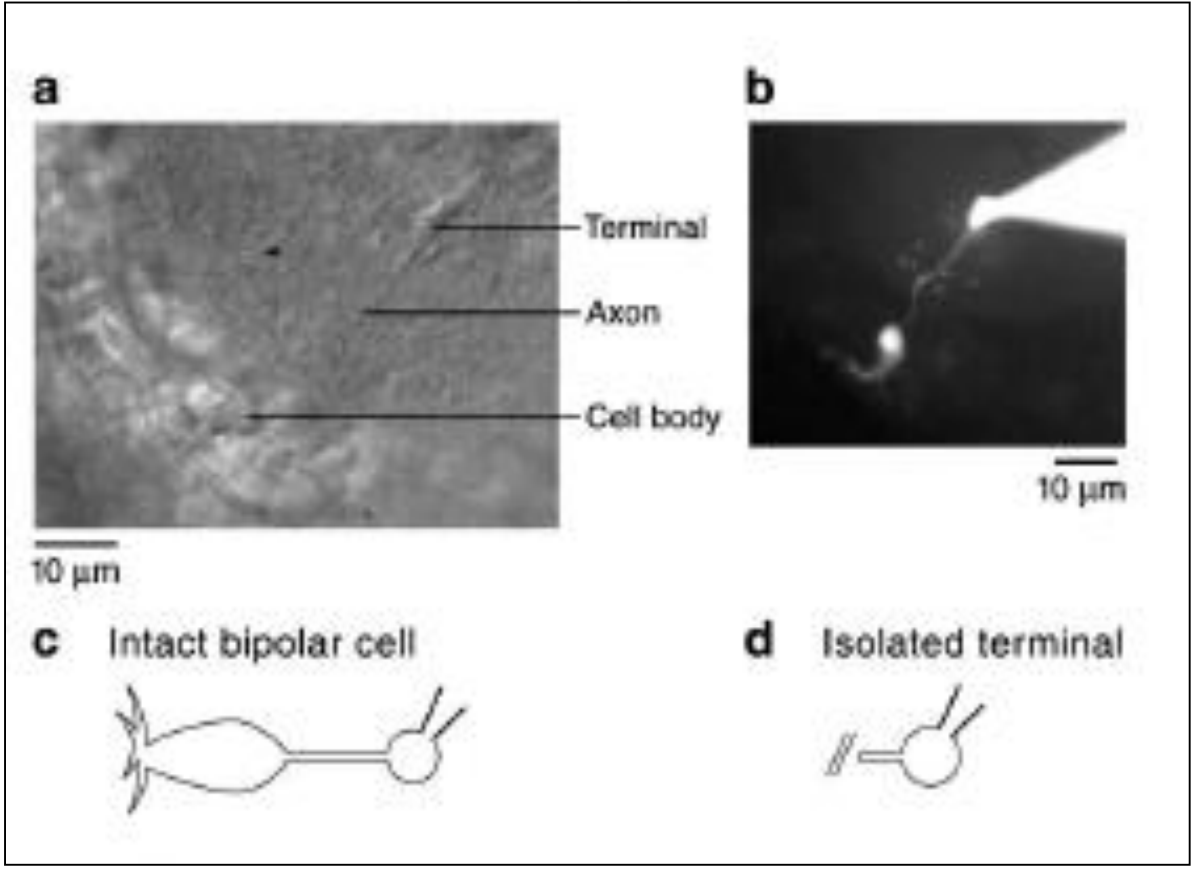
synaptopHluorina expressa no bulbo olfatório de camundongo

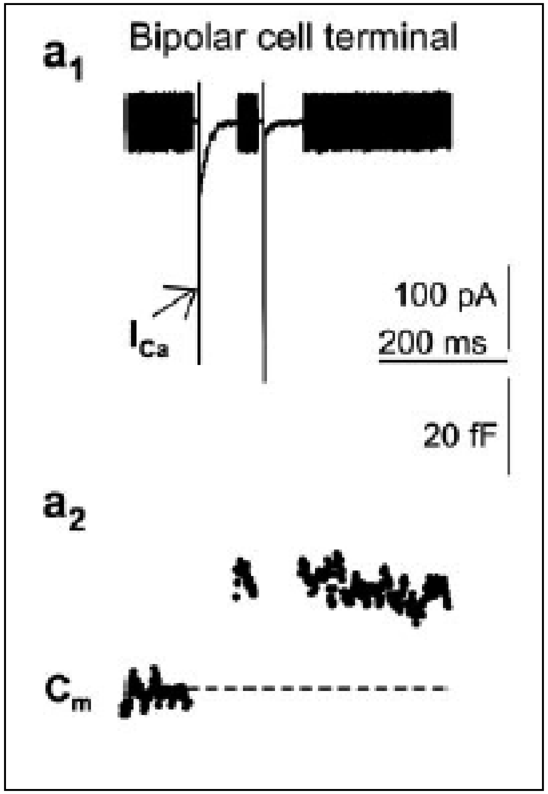
DETECÇÃOD A EXOCITOSE POR MEDIDAS DE CAPACITÂNCIA DA MEMBRANA- REFLETE UM AUMENTO DA ÁREA (EXOCITOSE)


$C_m = (\varepsilon/d)\ A$
$V_{Command}$
I_m
Rs
I_{Rm}
I_{Cm}
Rm
Cm

Medidas de capacitância da membrana refletem um aumento da área (exocitose)
Usa-se terminais gigantes ou células neuroendócrinas

Terminais gigantes do neurônio bipolar da retina de peixe


a
Terminal
Axon
Cell body
10 µm
b
10 µm
c Intact bipolar cell
d Isolated terminal

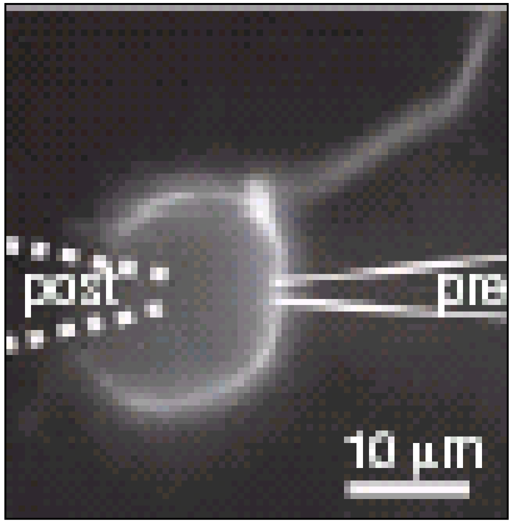


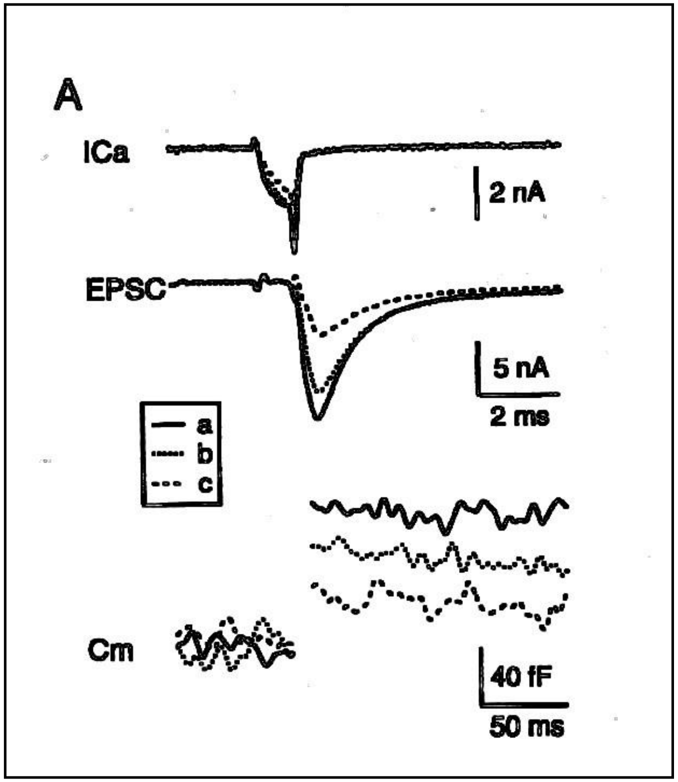
Palmer et al J Neuroscience 23 (2003)

Medidas de exocitose no terminal gigante cálice de Held

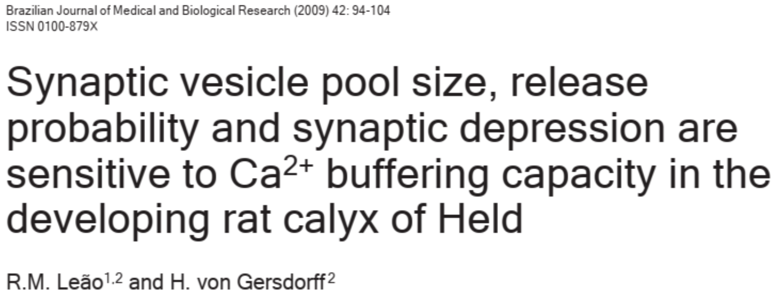
Medidas pré e pós-sinápticas simultâneas
Correlação direta da exocitose com correntes pós-sinápticas

Sun JY, Wu LG. Fast kinetics of exocytosis revealed by simultaneous measurements of presynaptic capacitance and postsynaptic currents at a central synapse. *Neuron* 2001; 30: 171-182.





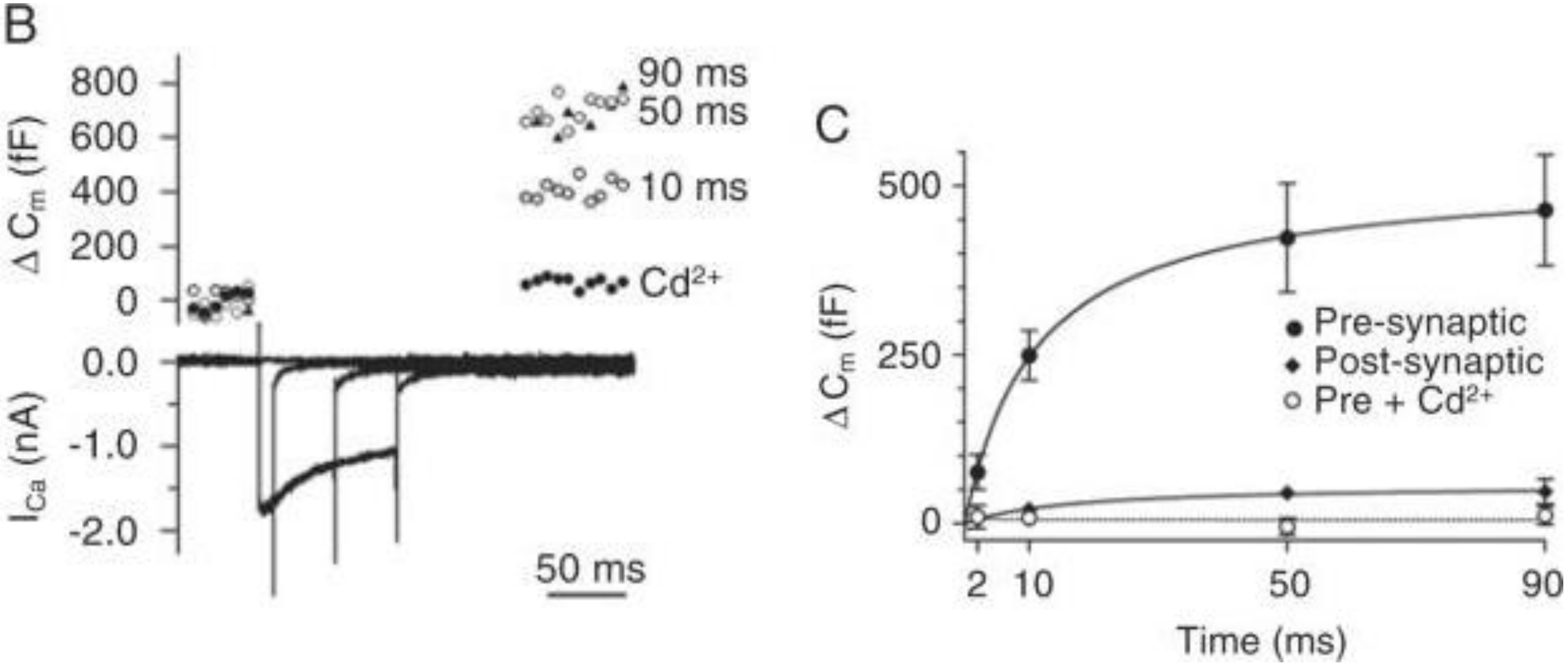
Medidas de exocitose no terminal gigante cálice de Held



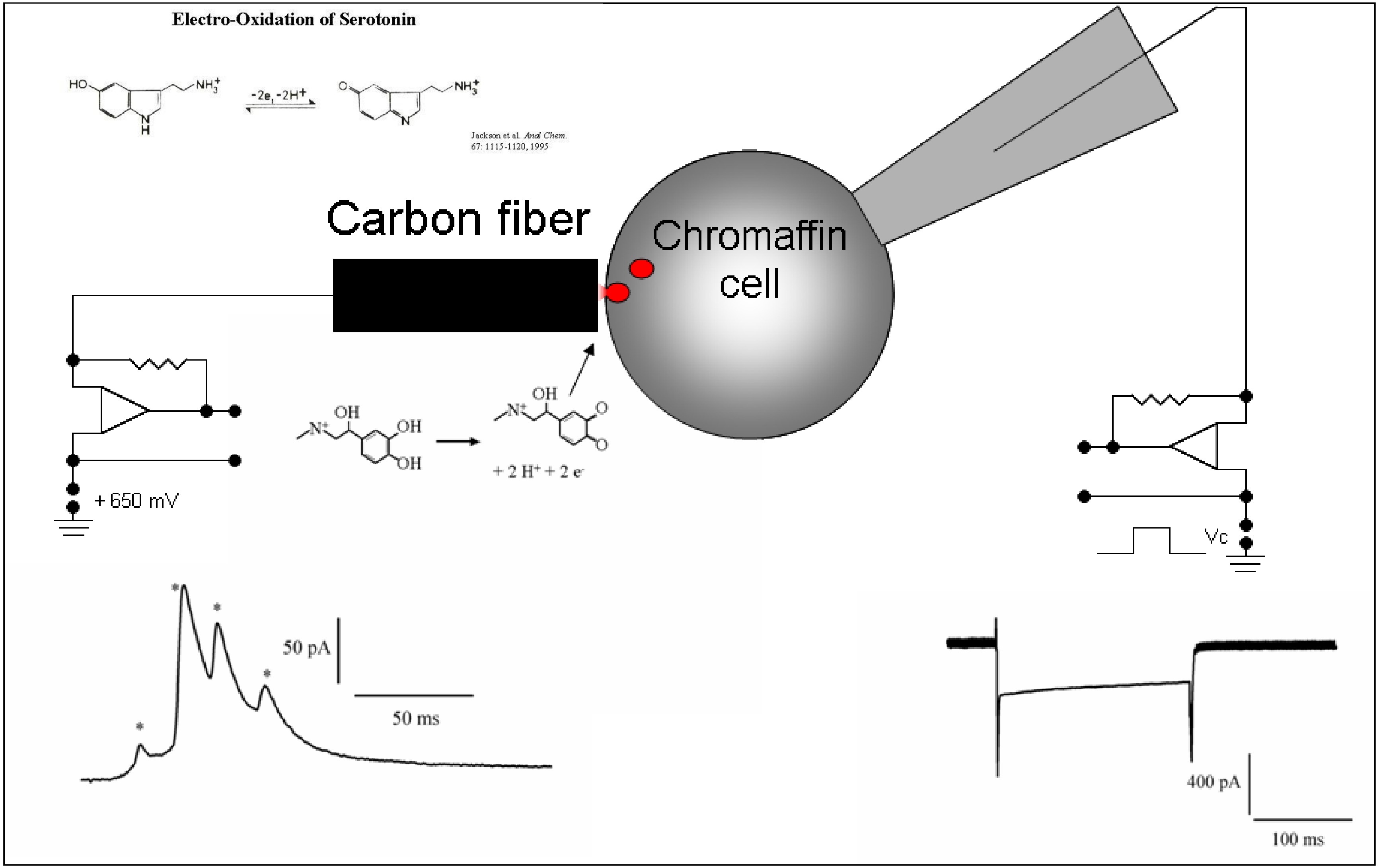
Brazilian Journal of Medical and Biological Research (2009) 42: 94-104
ISSN 0100-879X

Synaptic vesicle pool size, release probability and synaptic depression are sensitive to Ca^{2+} buffering capacity in the developing rat calyx of Held

R.M. Leão[1,2] and H. von Gersdorff[2]



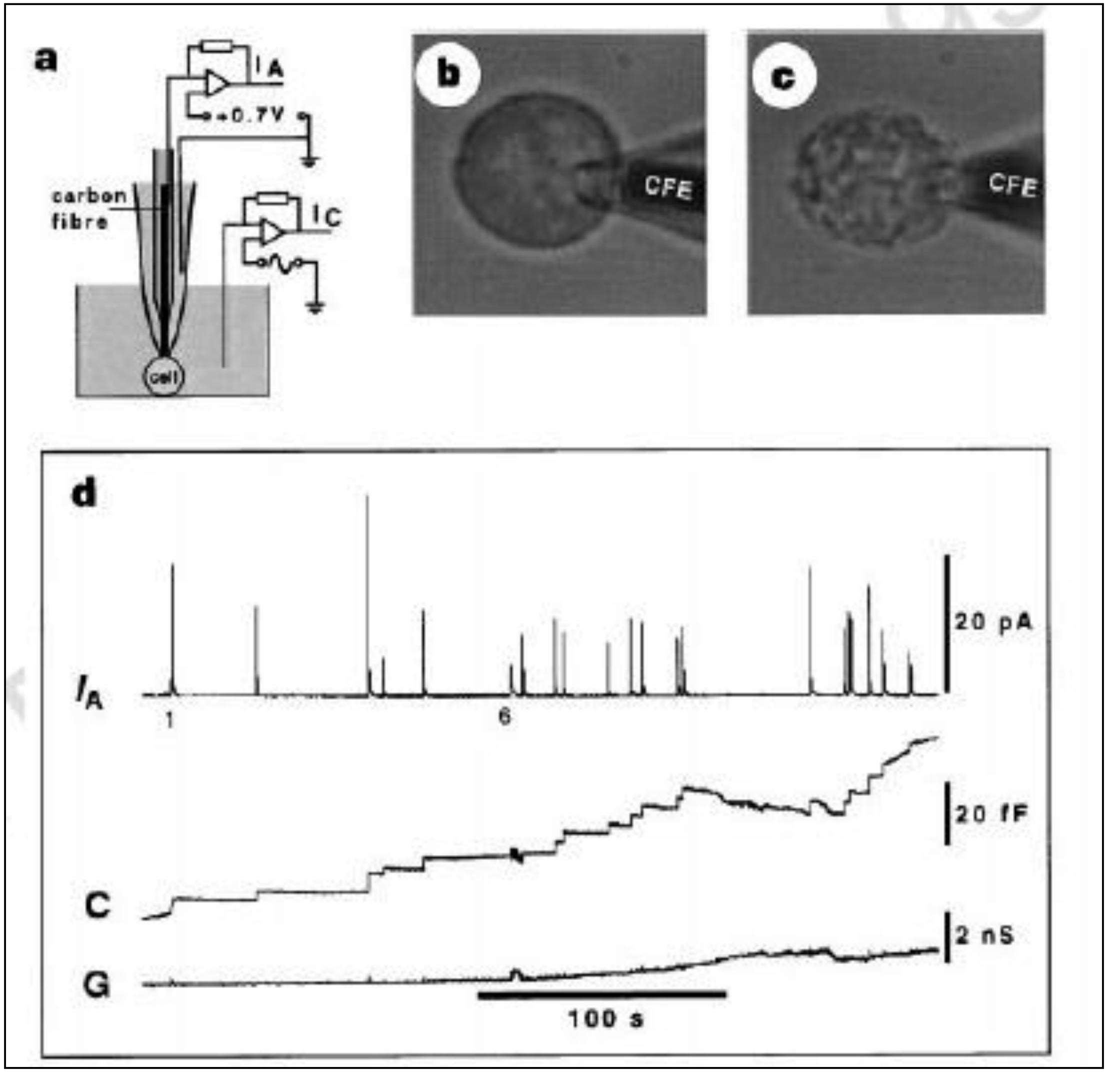
Detecção de catecolaminas por amperometria



Electro-Oxidation of Serotonin
HO
NH3+
N
H
-2e, -2H+
O
NH3+
N
Carbon fiber
Chromaffin cell
+650 mV
OH
N+
OH
OH
OH
N+
O
O
+ 2 H+ + 2 e-
Vc
50 pA
50 ms
400 pA
100 ms

Jackson et al. *Anal Chem.* 67: 1115-1120, 1995

Detecção de catecolaminas por amperometria e capacitância simultaneamente



Albillos et al., Nature 389 (1997)