The Spread of RAS

The SPREAD OF RAS Activity Triggered by Activation Of a Single Dendritic Spine

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Watson School at Cold Spring Harbor PhD in 2008
"Dynamics of Plasticity and Signaling at Individual Synapses"

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Svoboda received a BA in physics from Cornell University and a PhD in Biophysics from Harvard University. For his thesis work, with Steven Block and Howard Berg, he measured the molecular movements and forces of individual kinesin molecules, a molecular motor common to all eukaryotic cells. His postdoctoral work, with Winfried Denk and David Tank at Bell Labs, focused on synaptic and dendritic function in the cortex. From 1997 until 2006 he was a Professor at Cold Spring Harbor Laboratory and an HHMI Investigator.

What is the mechanism of learning and memory?
Neuron with dendritic ‘spines’

A Golgi-stained pyramidal cell in the parietal cortex of a rat.
The high power images at the right show dendritic spines on apical (A) and basilar (B) dendritic branches.

Photo by Grazyna Gorny c/o Terry Robinson, Univ. Michigan Dept. Psychology

Proc Natl Acad Sci U S A. 2003 September 2; 100(18): 10523–10528.

Amphetamine or cocaine limits the ability of later experience to promote structural plasticity in the neocortex and nucleus accumbens

Bryan Kolb,*† Grazyna Gorny,* Yilin Li,‡ Anne-Noël Samaha,‡ and Terry E. Robinson‡

PNAS Received January 22, 2003

Synaptic plasticity in the Hippocampus

Stimulation at any one of the three excitatory connections onto CA1 neurons shows plasticity (Bliss and Lømo, 1973)

Brief, high frequency burst causes increased EPSP. Can last for hours.

Long Term Potentiation (LTP).

Hippocampus
LTP Characteristics

1. Cooperativity
   - Weak stimulation does not generate LTP.
   - Strong stimulation does generate LTP.

2. Specificity
   - Strong stimulation at one site produces LTP there, but there is no LTP at a different site.
   - The effect is plasticity at the postsynaptic site.

3. Associativity
   - If a weak input arrives at the same time as a strong input, there will be association between the two. LTP will be produced at the weak input.

NMDA Receptor

Hebbian Learning

"Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability. When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."

- Donald O. Hebb (1949)

Spike Timing Dependent Plasticity

- Post-synaptic spike before pre-synaptic spike
- Pre-synaptic spike before post-synaptic spike

Hebbian Synapse

cells that fire together, wire together
• Cooperativity and Associativity are effects of STDP

• All Pre-Post spiking

• But one characteristic left unexplained: SPECIFICITY

Why is this paper important?

1) It is generally assumed that Hebbian learning must be “synapse specific”.
2) Thus, A, C, and D all connect to B, but only A is strengthened.
3) Spines are important for compartmentalizing calcium influx leading to local effect only.
4) Yet, modeling studies suggest that cooperative plasticity between nearby spines should lead to correlated inputs mapping onto neighboring synapses. This could lead to branch-specific non-linear integration. The single neuron might then be able to make many different computations depending on the dendritic branch”

Kenji Morita – from “perspective notes”

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Fluorescence Microscopy

Two-photon laser scanning fluorescence microscopy.

Denk W, Strickler JH, Webb WW.

School of Applied and Engineering Physics, Department of Physics, Cornell University, Ithaca, NY 14853.

Molecular excitation by the simultaneous absorption of two photons provides intrinsic three-dimensional resolution in laser scanning fluorescence microscopy. The excitation of fluorophores having single-photon absorption in the ultraviolet with a stream of strongly focused subpicosecond pulses of red laser light has made possible fluorescence images of living cells and other microscopic objects. The fluorescence emission increased quadratically with the excitation intensity so that fluorescence and photo-bleaching were confined to the vicinity of the focal plane as expected for cooperative two-photon excitation. This technique also provides unprecedented capabilities for three-dimensional, spatially resolved photochemistry, particularly photolytic release of caged effector molecules.


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Confocal Microscopy

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Two- (or multi-) photon microscopy
Two, or multi-photon microscopy

**Conventional fluorescence microscopy**

Multiphoton microscopy

**Watt W. Webb**
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(4-methoxy-5,7-dinitroindolinyl-glutamate, or MDNI-glu, 1)


- For glutamate: two-photon microscope used to uncage glutamate onto single spine
- For post-synaptic spike: patch-clamp electrode

**Synthesis of a caged glutamate for efficient one- and two-photon photorelease on living cells**

**COMMUNICATION**

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(4-methoxy-5,7-dinitroindolinyl-glutamate, or MDNI-glu, 1)
Inducing LTP

- Classically:
  - Pre-Post spiking
  - High frequency stimulation

- Non-classical:
  - Uncaging Neurotransmitter
    - Persistent stimulation, .5Hz for 30 seconds, WITHOUT MG BLOCK

Structural LTP

- Simple: spines grow bigger
- Contours around synapse
- More AMPA receptors
- Effect: stronger EPSP

Dendritic Spines

Viewing alexa dye in dendritic spine using 2-photon microscope.

LTP Pathway

- Complicated… won’t go over
- But basically NMDA -> recruitment of Actin/Myosin + AMPA receptors -> more sensitive synapse

Spine formation and growth; shrinkage and elimination

Addressed question: SPECIFICITY

- LTP is input-specific
  - 1 axon to 1 dendritic spine
  - CALCIUM is compartmentalized.

- But what about the specificity of LTP’s output?
  - One spine to itself?
  - OR Affect others near it?
Candidate Molecules
- If output nonspecific, molecules diffuse out
- Likely molecules? NMDA-related
- RAS! Known to be a plastic molecule

RAS
- BUT we know, when active, Ras
  1. Promotes synaptic connections
  2. Increases synaptic strength
  3. Prevents neuronal apoptosis

What is RAS
- Small GTP-ase
- Need NMDA Ca+2

What to Test
- Need to know where ACTIVE RAS goes is
- Don't care about inactive RAS... does 'nothing'
  - Problem: Label "RAS" and "RAS+RBD"
  - Solution: FRET

FRET
- Forster Resonance Energy Transfer
- Like Tuning forks
- Energy NOT transferred by photons, by resonance

Lower to Higher wavelength
Biological Application

Quantification

• Ratio of Red:Green
  EQUALS
  Ratio of Active: Inactive
  • See if red dots migrate out of spine head

Procedure

1. Uncage glutamate persistently to induce LTP by 2-photon excitation

2. See RAS migratory pattern by FRET

Initial study: Synaptic Plasticity

• two photon imaging
• uncaging pulses: 30 pulses; ½ second intervals directed at single spine
• Low extracellular Mg

Observed transient inward calcium current of 7.3 pA. ~5 NMDA-Receptors.
Calcium was restricted to single spine.

Single Spine Calcium Currents

Ras Spread


Figure S1
Spine Volume increases after uncaging G

RAS Activation
- Calcium influx followed by increase in RAS
- RAS = small guanosine triphosphate binding protein.
- Increase peaks at 1 m.; returns to baseline in 15 m.
  Time constant = 5.6 m.
- RAS activation correlated with sustained spine enlargement.
- A competitive blocker FRAS-F
- Inhibition of downstream RAS pathway (RAS-ERK – RAS extracellular signal activated kinase), blocks spine enlargement.
- Other requirements for spine enlargement: CAM Kinase II, PKC signaling, actin polymerization.

Role of RAS
- Correlated with for spine head growth in LTP
  \[
  \text{SRN}_{\text{A}} \quad \text{(Binding fraction)}
  \]
  \[
  \text{SRN}_{\text{B}} \quad \text{(Binding fraction)}
  \]

Length constant ≈ 11 microns at 4 min

Tomoko Tada and Morgan Sheng (2006)
Current Opinion in Neurobiology 2006, 16:95–101

Spine formation and growth; shrinkage and elimination

Tomoko Tada and Morgan Sheng (2006)
Current Opinion in Neurobiology 2006, 16:95–101
**RAS Activation**

- Low Calcium –
  - inhibitors of CaM K2 (KN62)
  - inhibitors of phosphoinositide 3-kinase (PI3K; 20 mM LY294002)
  - inhibitors of protein kinase C (1 mM Gö6976)

**RAS**

- Multiple signaling pathways, particularly those involving Rho and Ras family small GTPases, converge on the actin cytoskeleton to regulate spine morphology and dynamics bidirectionally. Numerous cell surface receptors, scaffold proteins and actin binding proteins are concentrated in spines and engaged in spine morphogenesis.
- Tomoko Tada and Morgan Sheng – Current Opinion in Neurobiol 2006 16:95
  - GTPase protein.
  - cell growth, cell survival, cell differentiation
  - signal → RAS → nucleus
  - HRAS subfamily; cell proliferation

**Controls for Results**

- Used modified RAS and RBD
- Affect Diffusion Coefficient? Activation Decay?

**CONTROLS:** Does the RAS sensor used in this study distort spatial profile of RAS diffusion and expression?

\[ L \sim \sqrt{D\tau_{\text{inactivation}}} \]

**diffusion equation prediction:**

- Space constant proportional to \( D^{1/2} \) and \( \tau_{\text{inactivation}}^{1/2} \)

**General Test for diffusion**

- Does it increase spread?
- No!
- Concentrations of Ras-GFP and RBD-RFP do not affect Ras spread

**It is reasonable to assume that the tagged sensor modifies spatial spread**

- It has a RAS binding domain
- It (FRAS-F) could saturate RAS scaffolds and RAS inactivators
- RBD might compete with GTP-activating proteins, thereby increasing the estimate of the inactivation time constant.
- To test: modulate FRAS-F levels and measure.
- The spread was independent of FRAS-F concentration.
How fast does RAS diffuse?

- Fused RAS with ON/OFF-GFP
- Shine brief flash of light, GFP fluoresce
- When the spine head is not green, no RAS left
- Repeated with RAS+RBD complex

Direct Test of RAS spatial spread

- H-Ras tagged with photoactivatable GFP (paGFP-Ras)
- Photo activate, measure time and space constant.

- RAS diffused freely
- RAS+RBD also diffused freely
- No immobile portion of active RAS in spine head

Test for Activation Decay

- More RBD = more competition with RAS inactivators (GTPases)
- Eliminated loss of fluorescence due to diffusion by stimulating everything (bAPs)
- Compared time decay constants (tau) over varying RAS-GFP RBD-RFP expression levels
Ras Spread is affected

- D is unaffected
- Tau is increased by a factor of 2
- L increased by ~1.5

\[ L \sim D_{\text{inactivation}} \]

Conclusions

- RAS diffuses out in seconds
- RAS inactivates in minutes
- Thus, can affect neighboring synapses before inactivated
- After recalibration, invades 10 um, ~15 synapses

What is the function of spread of RAS?

SYNAPTIC CROSSTALK EXPERIMENT:

- Can SPREAD of RAS lower the threshold of plasticity in a neighboring spine?
- Uncage glutamate near spine 1
give sub-threshold uncaging near spine 2
- Spine 2 increases in volume.

Function of the SPREAD of RAS

Fig. 4. Spread of Ras signaling and synaptic crosstalk. (A) Time-lapse images of a GFP-expressing pyramidal neuron in an acute hippocampal brain slice. At time = 0, 30 uncaging pulses (0.5 Hz, 4-ms pulse duration, LTP protocol) were applied to the spine marked by a white arrowhead (LTP spine) in a low concentration (nominally 0mM) of extracellular Mg2+. At time = 3 min, vehicle (0.1% dimethyl sulfoxide) was pressure-applied locally from a glass micropipette until time = 17 min. At time = 5.5 min, the subthreshold protocol (30 uncaging pulses, 0.5 Hz, 1-ms pulse duration) was applied to a nearby spine (sub spine, red arrowhead). (B) Spine-volume changes in the vehicle condition (11 spines, mean ± SEM).

Conclusion from sub-threshold stimulation experiment.

- “Together, these data indicate that the spread of Ras-dependent signaling is necessary for the local regulation of the LTP induction threshold.”

Overall Conclusion

- Stimulation of LTP in one synaptic spine is likely to influence neighboring spines within a distance of 10 microns along the same dendrite.
- Activation of neighboring spines is much delayed – minutes rather than seconds.
- Spines sharing a common stretch of dendrite may be co-regulated.
- Possible functional units within a single neuron.
Points to take home

- RAS is important to synaptic plasticity
- RAS diffusion = proof of non-compartmentalized synapses

- A functional unit = not a synapse, but a cluster of synapses in a general vicinity?

- But too early to tell: don’t know enough 😊

- Just one signaling molecule…