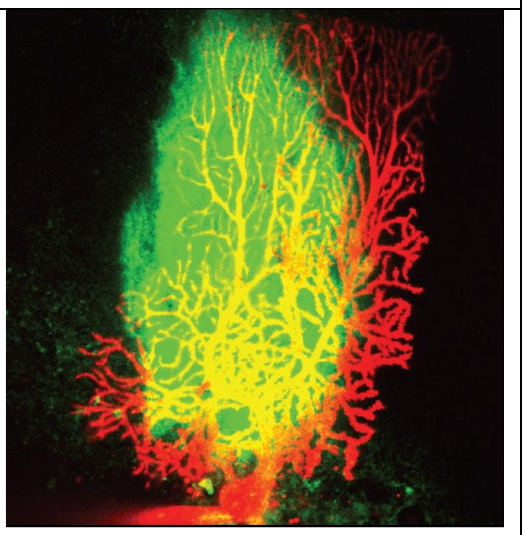
Site map for translation of

Hires SA, Zhu Y, Tsien R (2008). Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters
Proc Natl Acad Sci USA 105:4411–4416

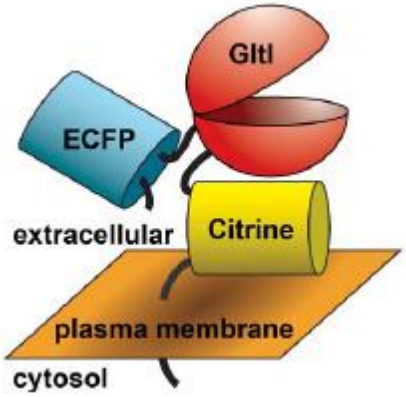
Front page

Text	Graphics
Significance Nerves typically communicate with muscles and other nerves through chemicals (neurotransmitters) that are released at the nerve tips and are sensed by cell surfaces that lie immediately adjacent to them. In the brain, however, neurotransmitters are sometimes released to a larger neighborhood of cells. This is often true of the neurotransmitter glutamate, the most abundant neurotransmitter in the central nervous system [1]. To understand the action of glutamate in neurotransmission and neural plasticity, it is essential to know how its concentration changes during the course of neural stimulation and afterwards. Hires et al (2008) devised a method of measuring glutamate in real time and found that at least with certain neurons, glutamate concentration rises and falls rapidly. **Contents** Abstract Introduction Experiments: Construction of glutamate sensors Initial testing of sensors in vitro Optimization of sensor characteristics Expression of sensor in living cells Glutamate measurement in dendrites49 Glutamate measurement in response to frequency of action potentials Implications: Glutamate function Sensor design	 Visualization of glutamate (green fluorescence) in cerebellum slice, using a different fluorophore than Hires et al. From Okubo Y & Iino M, cover of J Physiol (2011) 589 #3.

Abstract

Text	**Graphics**
[Translate abstract]	No graphic

Introduction

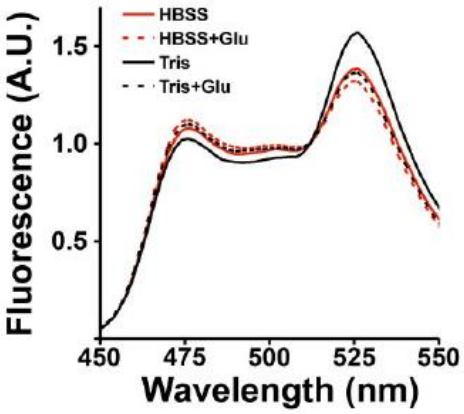
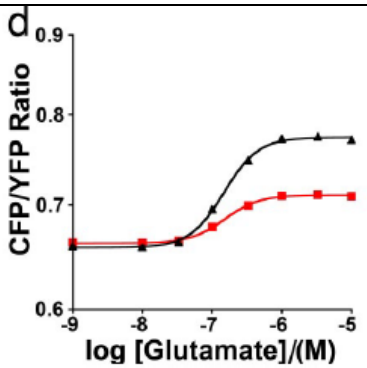
Text	Graphics
The classical junction between one nerve and another or one nerve and a muscle is the synapse. Neurotransmitter is released from the cell on one side of the synapse and detected by a receptor on the membrane on the opposite side. The neurotransmitter glutamate also excites other nearby cells, by spillover of glutamate from the synapse [1]. To understand how glutamate spillover works, it would be helpful to be know how much glutamate is present and at what times during and after nervous stimulation.	[Simple synapse with neurotransmitter spilling out of the synaptic cleft and interacting with neighboring cells]
[Principle behind measurement of glutamate through a glutamate-binding protein that fluoresces blue-green or yellow, depending on the binding of glutamate. Explanation of Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) and principle behind fluorescence resonance energy transfer [2]. Nature of glutamate periplasmic binding protein (GltI), how it changes its shape upon binding glutamate [need ref].]	 [need to improve on this picture to show +/- glutamate and energy transfer. Remove plasma membrane.]

1. Pál B (2018). Involvement of extrasynaptic glutamate in physiological and pathophysiological changes of neuronal excitability. Cell Molec Life Sci 75:2917-2949.
2. Deuschle K, Okumoto S, Fehr M, Looger LL, Kozhukh L, Frommer WB (2005). Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering. Prot Sci, 14:2304–2314.

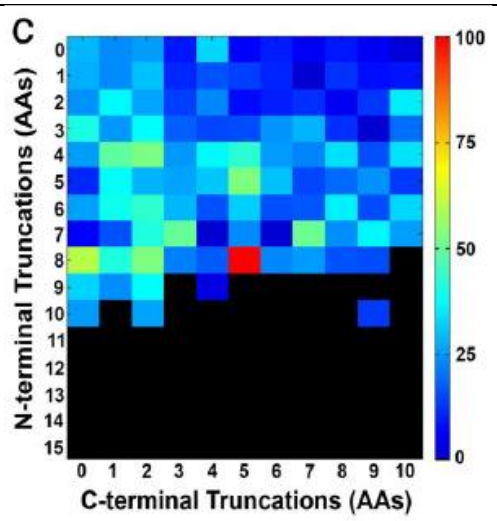
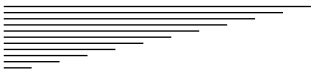
Experiment: Construction of glutamate sensors

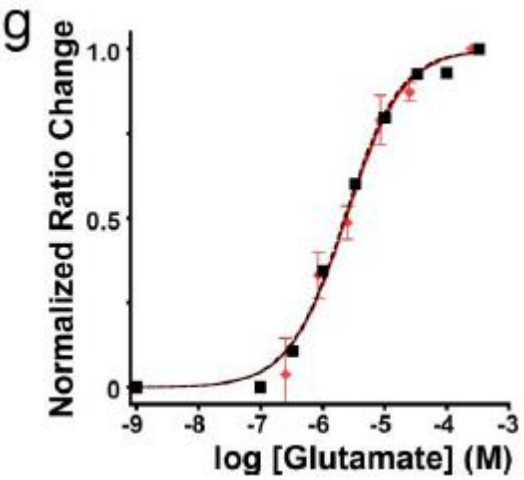
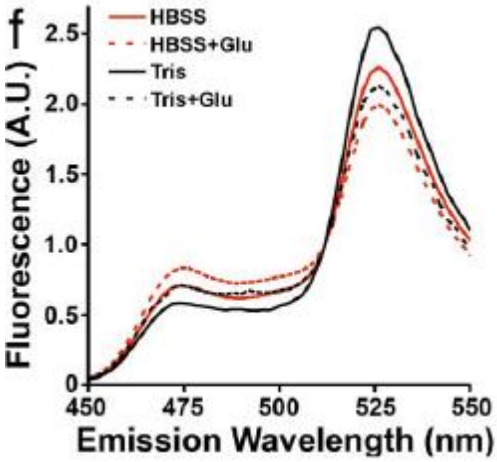
Text	Graphics
[Two sensors: one soluble, one attached to cell membrane. Soluble: Good for characterizing sensor in the test tube. Genes encoding CFP, GltI, and Citrine (a form of YFP) fused together. Preceded by six histidine residues strung one after the other (His-His-His-His-His-His = His6). Link to use of His tags in protein purification. This was accomplished by cloning into a special-purpose plasmid, pRSETB, used to express large amounts of protein in E. coli.]	Soluble: 
[Membrane bound: Good for placing sensor where it can detect extra-synaptic glutamate. Same three genes fused, preceded by signal peptide (from Immunoglobulin = Ig) to direct the protein to the membrane) and a protein to provide transmembrane regions so the sensor will get stuck in the membrane. The protein used for this purpose is Platelet-Derived Growth Factor Receptor (PDGFR), but the nature of the protein is not important. This was accomplished by cloning into a special purpose plasmid, pDISPLAY, used to express proteins on the surface of mammalian cells.]	Membrane-bound: 

Experiment: Initial testing of sensors in vitro

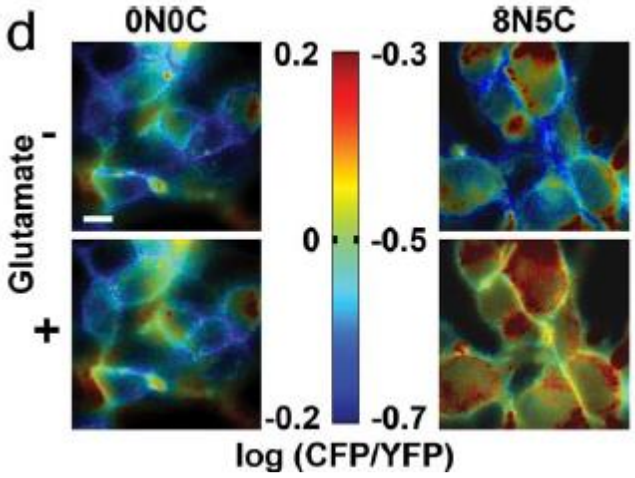
Text	Graphics
Significance [Fluorescence energy transfer emission from soluble glutamate sensor produced as described above measured +/- glutamate. Show how to calculate ratio of CFP/YFP.]	 [Get rid of Tris lines (may have to trace), label red lines + glutamate and – glutamate. Make horizontal color bar for wavelength, superimpose on graph. Point to downward difference in emission at 526 nm from YFP and upward difference at 476 nm from CFP in response to glutamate. Draw lines to Y axis]
[CFP/YFP ratio is sensitive to level of glutamate. Show that above graph represents extremes of graph to right. Problem: glutamate binding too good. No change in range of glutamate that is physiologically relevant. Need to improve. Also maximum response is about 0. Omit Fig. 1e.]	 [Erase "d". Erase black line. Expand Y axis. Change X axis to 10^{-9}, 10^{-8}, etc, no log. Arrow at ½ maximal increase with line to X axis (150 nM) = K_D. Label maximum response = +7%.]

Experiment: Optimization of sensor characteristics

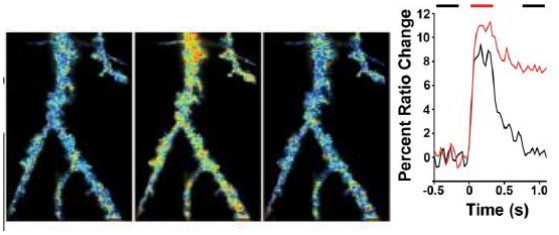
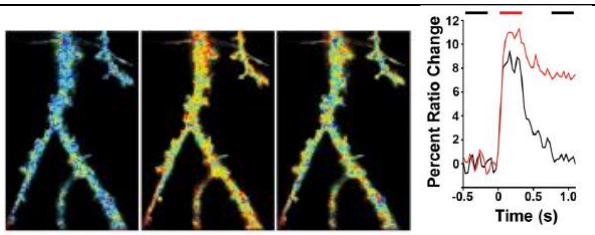
Text	Graphics
[Need to decrease affinity of glutamate binding protein for glutamate. Site-specific mutagenesis: S73T had K_D of 2.5 μM (17x decrease in affinity).	[Picture of GltI bound to glutamate from PDB. Highlight Ser-73.]
[Need to increase response magnitude. Truncate GltI(S73T) on N terminus and C terminus.]	[Need figure for truncation of sequence. Use graphical convention like that below.]
[Measure maximum response to glutamate as in Fig. 1d of all 176 possible truncation combinations. One big winner: 8 amino acids truncated on N terminus, 5 amino acids on C terminus. 44% maximum response to glutamate. Omit Fig. 2e	 Show truncations graphically, like:  Label color bar with absolute percentages: 7.1% = blue, 44% = red. Draw line from red box to X- and Y-axes

Performance of final optimized sensor with the following modifications (as described above): S73T, -8N, -5C. Optimized range of sensitivity to glutamate. Optimized range of fluorescent response +/- glutamate.	g  Change X-axis to 10^{-9} etc. Draw line from midpoint down to X axis, label K_D = 2.5µM. f  Color bar. Erase black. Lines to Y axis. Maximum response = 44%

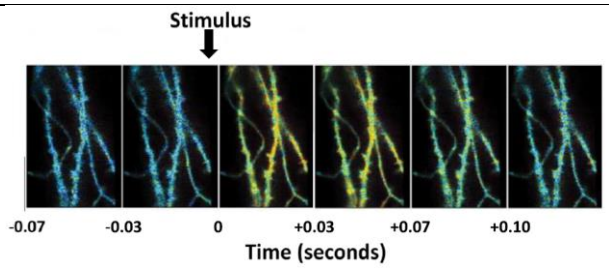
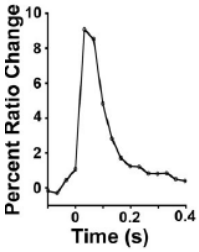
Experiment: Expression of sensor in living cells

Text	Graphics
[Brief explanation of transfection] [Brief explanation of HEK293 cells]	[Cartoon of plasmid into cell, regrown to dendrite]
[Membrane bound response in HEK293 cells to known amount of glutamate (100 μM), using original sensor and optimized sensor.]	 [Change log scale to linear. Change "0N0C" to "original", "8N5C" to "optimized"]

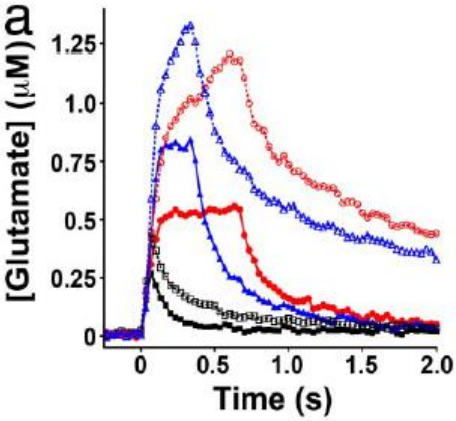
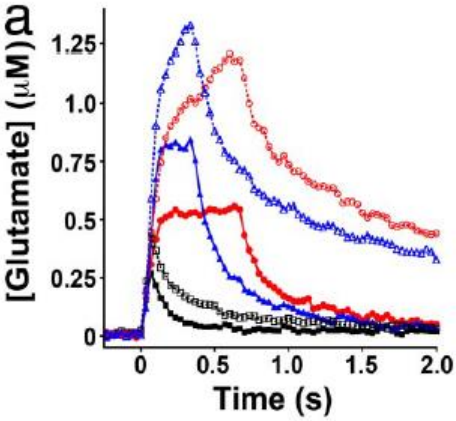
Experiment: Glutamate measurement in dendrites

Text	Graphics
[Transfect membrane-bound sensor gene into neuron cells from rat hippocampus (significance of hippocampus?).	[graphic of brain, hippocampus, cartoon of neural cell culture
[Stimulate cells]	[graphic of external voltage set up]
[CFP/YFP fluorescence from Membrane-bound glutamate sensor in dendrites stimulated for 0.3 seconds (10 action potentials). Record before, during, after. Transient increase in CFP/YFP => transient increase in glutamate. Quantitated and averaged over all dendrite surface.	 ------ ------ ------ 0.3 sec stimulation Erase red line in graph Add color bar
[Concentration of neurotransmitter affected not only by release but also reuptake. Cocaine works largely by inhibiting the reuptake of the neurotransmitter dopamine. [+/- inhibitor of glutamate reuptake: DL-*threo*-β-benzyloxyaspartate (TBOA)]	[cartoon of neurotransmitter reuptake, +/- drug that blocks reuptake]
[Same conditions as before, but with TBOA added to block glutamate reuptake. Glutamate spike persists.]	 ------ ------ ------ 0.3 sec stimulation Leave red line intact

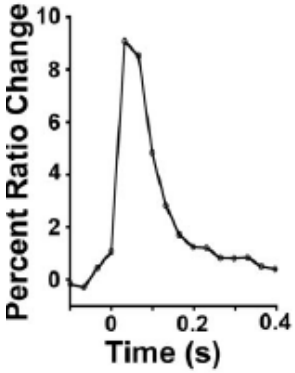
Experiment: Glutamate measurement in dendrites

Text	Graphics
[Same as above, but for just <u>one</u> action potential (averaged over 30 separate repetitions). No TBOA. Show's mostly over after 0.1 seconds. Glutamate transient.	 Add color bar 

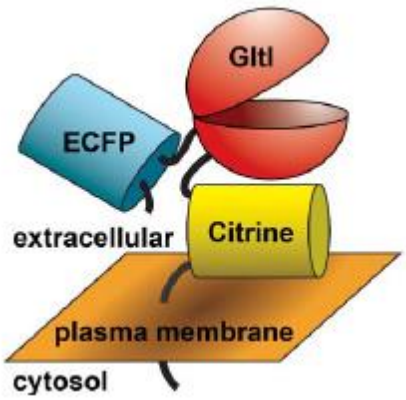
Experiment: Glutamate measurement in response to frequency of action potentials

Text	Graphics
[Nerves may fire at different frequencies. How does that affect the concentration of spillover glutamate? Three conditions: - 1 action potential - 10 action potentials spaced by 67 msec - 10 action potentials spaced by 33 msec Glutamate accumulates to higher level when time of reuptake is smaller.	 [Erase lines with open symbols. Label lines: black = 1 action potential; red = 10 action potentials, 1 per 67 msec; blue = 10 action potentials, 1 per 33 msec.]
[The idea that the difference in glutamate levels is due to a shorter period of reuptake can be tested by blocking reuptake with TBOA. When reuptake blocked, magnitude of glutamate spike for 10 action potentials no longer depends on frequency]	 [Erase lines with closed symbols. Label lines:

Implications: Glutamate function

Text	Graphics
[Glutamate concentration rises rapidly at surface of dendrite and disappears rapidly – 10's of milliseconds--unless reuptake inhibited. Since magnitude of glutamate depends on frequency of stimulation, neighboring cells may be able to respond to the frequency at which a nerve is firing.]	

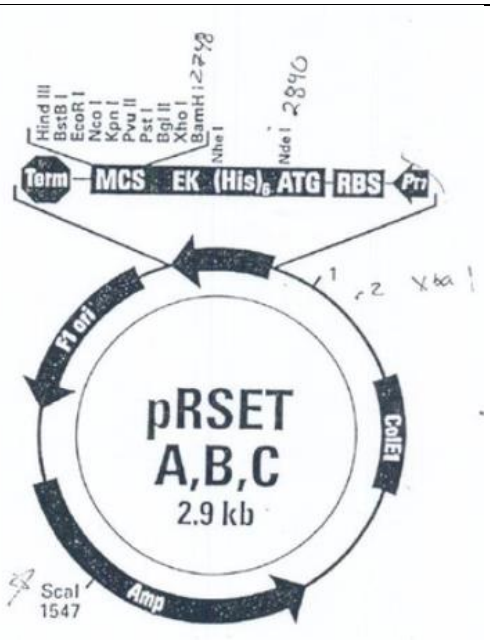
Implications: Sensor design

Text	Graphics
[Two means of optimization: mutagenesis of glutamate binding protein to reduce affinity and truncation of the protein to increase +/- glutamate difference. Optimized sensor far better than original, but difficult to predict best way to truncate the glutamate binding protein to achieve optimization.]	

Protein purification through His tags

Text	**Graphics**
[Explanation of figure]	[Graphic of His-tag-mediated purification]

Overexpression of protein using pRSETB

Text	Graphics
[Explanation of promoter(P_{T7}), ribosome binding site (RBS), His-tag (His_6), EK [???], Multiple cloning sites (MCS), transcriptional terminator (Term), rest of plasmid for replication and selection in E.coli.]	

Directing proteins to a membrane by signal peptides

Text	**Graphics**
[Explanation of figure]	[graphic of signal peptide and passage through membrane]

Transmembrane regions of proteins

Text	**Graphics**
[Explanation of figure]	[Graphic of transmembrane region]

Expression of proteins on the surface of mammalian cells through pDISPLAY

Text	Graphics
[Explanation of promoter (P_{CMV}), signal peptide (IgK Leader), HA [???], Multiple cloning sites, transmembrane region (PDGFR), poly-A tail (BGH pA), rest of plasmid for replication and selection in E.coli.]