

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Global Homeostasis of Excitatory Synapses in Response to Local Optical Stimulation

Permalink

<https://escholarship.org/uc/item/0180h6wz>

Author

Tulyathan, Orapim

Publication Date

2010

Peer reviewed|Thesis/dissertation

Global Homeostasis of Excitatory Synapses in Response to Local Optical Stimulation

by

Orapim Tulyathan

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Biophysics

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Ehud Y. Isacoff, Chair

Professor Mu-ming Poo

Professor Lu Chen

Professor Daniel E. Feldman

Fall 2010

Global Homeostasis of Excitatory Synapses in Response to Local Optical Stimulation

Copyright © 2010

by Orapim Tulyathan

Abstract

Global Homeostasis of Excitatory Synapses in Response to Local Optical Stimulation

by

Orapim Tulyathan

Doctor of Philosophy in Biophysics

University of California, Berkeley

Professor Ehud Y. Isacoff, Chair

Synaptic scaling is a mechanism that neurons use to maintain homeostatic levels of excitability, allowing them to increase their sensitivity when excitatory inputs are weak and to decrease sensitivity at times of elevated excitation. In this way individual neurons and whole neural circuits are thought to maintain a balance between excitation and inhibition, and to operate in a part of their dynamic range that allows for their output to increase and decrease in response to altered inputs. So far, scaling has been studied as cell-wide responses to long-term genetic and pharmacological manipulations of excitability in an entire cell, group of cells, or in whole brain regions. However, under physiological conditions scaling will be elicited not by these kind of global changes in input or excitability, but rather by changes in synaptic drive at a subset of inputs. This thesis sets out to determine whether local excitation elicits scaling responses and, if so, whether the changes would be confined to the site of excitation or would trigger a global response throughout the cell.

Our lab has developed LiGluR, a light-controlled glutamate receptor that rapidly and reliably depolarizes neurons, resulting in calcium influx and inducing action potential firing. In chapter 2 of this thesis I describe the optimization of subcellular LiGluR activation in cultured hippocampal neurons, discussing critical parameters for spatially restricting the illumination. Establishing a targeted illumination system and verifying the spatial resolution of light activation is essential for the experiments later described in chapter 3. The conditions for long-term light activation are also set to take advantage of the noninvasive nature of LiGluR, thus enabling the study of processes that require prolonged, chronic manipulation. The light-activation protocol considers both the light for activation and the light for measuring output, determining maximum power levels for robust photostimulation while maintaining good spatially resolution and cell health. Light activation is carried out on a high resolution scanning confocal microscope so that small features (such as synaptic receptors) can be properly visualized. I also discuss the

expression pattern of LiGluR in neurons and illustrate a co-expression system for driving channel localization to postsynaptic sites.

In chapter 3, I describe LiGluR activation in combination with time-lapse imaging of AMPA receptors (AMPA receptors), a well established expression locus of different modes of synaptic plasticity. LiGluR is an ideal tool to build upon previous studies for its spatial and temporal properties. With targeted illumination I am able to activate LiGluR within a single neuron with sub-cellular resolution. By combining this approach with bath application of TTX, which blocks action potentials without affecting light-induced depolarization, the effect of LiGluR activation is further restricted. Synaptic scaling occurs on the time scale of hours; LiGluR is able to provide the reliable, long-term stimulation necessary to evoke these changes. The non-invasive nature of LiGluR is also ideal for coupling with time-lapse imaging of other fluorescently-tagged proteins that can be expressed within the same cell.

To measure synaptic scaling I monitored the surface expression of AMPA-type glutamate receptors using an ecliptic-tagged GluR2 subunit, a highly pH-sensitive reporter that is fluorescent when the receptor is surface expressed and quenched when it is exposed to the lumen of a vesicle. Expressing this receptor in cultured hippocampal neurons results in punctate surface expression concentrated at spines along the dendritic shaft. The fluorescence intensity increases in hippocampal neurons when culture-wide activity is blocked with TTX, verifying that the system is a faithful reporter of AMPAR accumulation under synaptic scaling conditions. We found that local excitation in a distal sub-region of the dendritic tree induced a global reduction in the surface population of AMPARs at dendritic spines. The loss of AMPARs began within 20-30 minutes and leveled off within 1 hour. This scaling response was blocked by chelating intracellular calcium with BAPTA-AM, blocking voltage dependent calcium channels with cadmium, or inhibiting the activity of calcium/calmodulin-dependent protein kinases with KN-93. Imaging of internal calcium with a fluorescent calcium indicator dye showed that the optical excitation of LiGluR in the distal dendrite does not lead to a calcium rise outside of the illuminated area, indicating that the spread of the homeostatic effect propagates via another means. We consider the possibility that phosphorylated CaMKII, which has been shown to be highly mobile, spreads throughout the dendrite to give a global homeostatic reduction in AMPA receptor surface levels.

Our method employed here for spatially-controlled neuronal stimulation coupled with time-lapse imaging can further be extended to examine other important questions underlying neuronal circuit function.

For Mom and Dad.

Thank you for your constant support.

And for Ali B.

Thank you for your amazing patience.

Table of Contents

Chapter 1: Activity manipulation and synaptic scaling in hippocampal neurons	1
Synaptic scaling introduction	2
AMPA accumulation as an expression locus of synaptic scaling	2
Local versus global activity manipulations	3
Intracellular pathways involved in synaptic scaling	4
Soluble released factors influence synaptic scaling	5
Techniques to manipulate activity level in cultured neurons	7
Use of light-activated receptors to study synaptic scaling	10
References	12
Figures	21
Figure 1. Bi-directional scaling of excitatory synapses	22
 Chapter 2: Subcellular targeting and activation of LiGluR	 24
Summary	25
Introduction	25
Results	26
A targeted illumination system for spatial control of genetically-encoded light activated receptors	26
Functional imaging to measure spatially restricted LiGluR activation	28
Subcellular activation of LiGluR in hippocampal neurons	29
Long-term activation of LiGluR for time-lapse imaging studies with optical readout	30
Co-expression of iGluR6(L439C) with PSD-95 drives LiGluR to postsynaptic sites in dendritic spines	32
Expression of iGluR6(L439C) increases mEPSC frequency but not amplitude	33
Discussion	34
Materials and Methods	36
HEK cell culture and transfection	36
Hippocampal neuron culture and transfection	36
Immunocytochemistry	36

Calcium Imaging	37
Whole-cell patch clamp	37
Image analysis	38
References	39
Figures	44
Figure 1. A targeted illumination system for spatial control of genetically-encoded light activated receptors	45
Figure 2. Functional imaging to measure spatially restricted LiGluR activation	47
Figure 3. Subcellular activation of LiGluR in hippocampal neurons	49
Figure 4. Long-term activation of LiGluR for time-lapse imaging studies with optical readout	51
Figure 5. Co-expression of iGluR6(L439C) with PSD-95 drives LiGluR to postsynaptic sites in dendritic spines	53
Figure 6. Expression of iGluR6(L439C) increases mEPSC frequency but not amplitude	55
 Chapter 3: Global depression of AMPAR surface expression mediated by local LiGluR activation	57
Summary	58
Introduction	58
Results	60
SEP-GluR2 fluorescence represents surface population of receptors, which exhibit canonical synaptic scaling behavior	61
LiGluR activation inhibits TTX-mediated synaptic scaling (indicated by SEP-GluR2 fluorescence)	62
Effect of LiGluR activation on SEP-GluR2 fluorescence requires change in intracellular calcium levels	63
LiGluR-mediated calcium influx requires voltage dependent calcium channel activation to depress SEP-GluR2 fluorescence	64
LiGluR effect on surface AMPAR dependent on Ca ²⁺ /calmodulin-dependent protein kinase activity	66
Discussion	67
Materials and Methods	68
Hippocampal neuron culture and transfection	68

Live cell confocal imaging – ecliptic GFP experiments	69
Live cell confocal imaging – time-lapse experiments	69
Calcium imaging	70
Image analysis	70
References	71
Figures	77
Figure 1. SEP-GluR2 fluorescence represents surface population of receptors, which exhibit canonical synaptic scaling behavior	78
Figure 2. LiGluR activation inhibits TTX-mediated synaptic scaling (indicated by SEP-GluR2 fluorescence)	80
Figure 3. Effect of LiGluR activation on SEP-GluR2 fluorescence requires change in intracellular calcium levels	82
Figure 4. LiGluR-mediated calcium influx requires voltage dependent calcium channel activation to depress SEP-GluR2 fluorescence	84
Figure 5. LiGluR effect on surface AMPAR dependent on Ca ²⁺ /calmodulin-dependent protein kinase activity	86
Appendix I: Synaptic scaling of SEP-GluR1	88
Summary	89
Introduction	89
Results	90
SEP-GluR1 fluorescence intensity scales with activity and NMDAR blockade	90
LiGluR activation does not inhibit TTX-induced synaptic scaling of SEP-GluR1	91
Discussion	92
Materials and Methods	93
Hippocampal neuron culture and transfection	93
Live cell confocal imaging – SEP-GluR1 time lapse experiments with activity and NMDAR blockade	94
Live cell confocal imaging – SEP-GluR1 + LiGluR time lapse experiments	94
Image analysis	94
References	96
Figures	99

Figure 1. SEP-GluR1 fluorescence intensity scales with activity and NMDAR blockade, but not with LiGluR activation	100
---	-----

Acknowledgements

I have many people to thank for the guidance and support that helped me get through graduate school and complete this thesis. First, my adviser, Professor Ehud Isacoff, for taking me on as a student and guiding me through the process of academic research. His excitement about new ideas and scientific expertise made this research possible. Second, my thesis committee, whose important insights and suggestions immensely improved my experiments over the last four years.

Being a part of the Isacoff lab has given me the opportunity to work with very talented, dedicated scientists; I am deeply indebted to my labmates. The team of students and postdocs in the LiGluR group, Stephanie Szobota, Matt Volgraf, Erica Warp, Claire Wyart, Grant Kauwe, Jordan Patti, and Harald Janovjak, have been a tremendous source of knowledge and ideas. Steph has been an essential part of all things light-activated and Erica and Claire provided helpful insights for my experiments. Thank you to Hanson Lee for sharing his neuron expertise and teaching me many experimental techniques. Gautam Agarwal has provided vital assistance in MATLAB programming; like many others in the lab I would be lost in my data analysis without his help. I greatly thank Zhu Fu for her hard work and technical assistance. Holly Aaron, the director of the Molecular Imaging Center, has lent her microscopy expertise on numerous occasions. Thank you to Sandra Wiese, our lab manager, for her help in innumerable areas over the years and for keeping the lab running smoothly. Each of the postdoctoral fellows in the lab: Claire, Francesco, Max, Jordan, Harald, Susy, Einat, Guillaume, and Andreas, have provided advice on everything from data analysis to presentation techniques to career ideas. Many thanks to my fellow graduate students Medha, Robin, Gautam, Steph, Grant, Sarah, Ryan, Erica, and Josh for their support in both scientific and personal matters – I couldn't ask for a better group of colleagues.

Outside of the lab I had a great experience collaborating with scientists from the Chang lab, especially Dylan Domaille. Also, thank you to Shannon DeMaria in the neighboring Ngai lab for countless conversations about calcium imaging and expert proof-reading. Her and Jose have been a vital support through the difficult phases of research and graduate school.

Through the Biophysics Graduate Group I have met great friends and colleagues, and have had invaluable experiences which have shaped me immensely. I especially thank Wendy Hansen and Philip Johnson for their apropos advice and continued friendship. Also, I deeply appreciate the help that Diane Sigman and Kate Chase have given me in navigating the program and the graduate division requirements.

And last but certainly not least, I thank my family and friends for their endless support and motivation over the years. My parents always encourage me and have done everything possible to provide me with the best educational opportunities. I cannot thank them enough for their emotional and financial support throughout all the years. Thank you to my friends who have listened to me when I needed an ear, and for providing occasional, invaluable breaks from science and graduate school. Finally I thank Ali for his infinite patience in the face of my difficult temperament, and for motivating me and helping me in all that I do.

CHAPTER 1:

Activity manipulation and synaptic scaling in hippocampal neurons

Synaptic scaling introduction

Understanding the mechanisms and consequences of activity in neural circuits is critical to the study of neuroscience, as such activity is known to underlie both development of the nervous system and on-going processes such as memory formation. The most widely-studied form of synaptic adaptation is Hebbian plasticity, which includes long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004; Collingridge et al., 2004). Here, synaptic changes operate in a positive feed-back process where the efficacy of individual synapses is rapidly modified in an input-specific manner. For example, synapses that undergo LTP become more excitable and the threshold for further LTP is reduced. Theoretically, neural networks could undergo unconstrained potentiation and lose synapse specificity; therefore a homeostatic compensatory system is necessary to tune the overall sensitivity of the system, constraining runaway activity and preventing saturated synapses from undergoing further potentiation (Pozo and Goda, 2010; Turrigiano, 2008). A complex negative feedback system has been shown in which neurons are able to maintain the network activity level within a set range while also maintaining synapse specificity (Davis, 2006; Turrigiano, 2007).

Several mechanisms of homeostatic compensation have now been identified, revealing the ways in which neurons may sense their own activity level and accordingly adjust their properties to maintain stability (Abbott and Nelson, 2000; Turrigiano and Nelson, 2004). Synaptic scaling is one important mechanism that neurons use to maintain homeostatic levels of excitability, allowing them to increase their sensitivity in times of lowered activity levels and to decrease sensitivity in times of elevated activity (Turrigiano, 2008). These adjustments, which prevent saturation and uncontrollable levels of excitation, have been verified in several systems, including cultured neurons (Thiagarajan et al., 2005; O'Brien et al., 1998), hippocampal slices (McKinney et al., 1999; Kim and Tsien, 2008; Kirov et al., 2004), the *Drosophila* neuromuscular junction (Frank et al., 2006), and in intact animals (Knogler et al., 2010; Desai et al., 2002; Mrsic-Flogel et al., 2007). A canonical example experiment is to suppress all network activity with tetrodotoxin (TTX) which results in a slow compensatory increase in synapse strength. Conversely, enhancing network activity by blocking inhibitory inputs results in a decrease in the strength of excitatory synapses (Turrigiano et al., 1998).

AMPA accumulation as an expression locus of synaptic scaling

AMPA receptors (AMPA) and NMDA receptors (NMDAR) are the two main ionotropic glutamate receptor subtypes present at excitatory synapses in the central nervous system. AMPARs mediate fast synaptic transmission by controlling the postsynaptic depolarization that initiates neuronal firing, while NMDARs are responsible for inducing specific forms of plasticity including LTP and LTD (Malenka

and Bear, 2004). Regulation of AMPAR trafficking and localization, including mechanisms that govern membrane insertion, internalization, recycling, and degradation determines the strength of synaptic transmission (Santos et al., 2009; Barry and Ziff, 2002). AMPA receptors are tetramers composed from a combination of the four subunits GluR1-GluR4, in the hippocampus most AMPARs are hetero-tetramers composed of GluR1/GluR2 or GluR2/GluR3 subunits with a small number of GluR1 homomers (Greger et al., 2007; Wenthold et al., 1996). Receptors lacking the GluR2 subunit are permeable to calcium, receptors containing GluR2 are rendered calcium-impermeable by editing of the Q/R site in >99% of GluR2 mRNAs (Bredt and Nicoll, 2003; Seeburg et al., 1998). In addition to channel properties, the specific subunit composition also determines trafficking to synaptic sites (Shi et al., 2001), recycling (Lee et al., 2004), and the receptor's role in LTP/LTD (Bredt and Nicoll, 2003). The molecular mechanisms underlying these differences are influenced by distinct carboxyl-terminal domains, which interact with different PDZ-binding postsynaptic proteins (Kornau et al., 1995; Dong et al., 1997; Srivastava et al., 1998).

Changes in AMPAR accumulation are also considered a major expression locus of activity-dependent scaling of excitatory synapses (Lissin et al., 1998; Wierenga et al., 2005; Turrigiano, 2008). Previous studies have shown that synapse strength can be bi-directionally scaled by altering the levels of surface AMPA receptors (Figure 1) (Lissin et al., 1998), for example prolonged TTX application resulted in membrane insertion of GluR1 and GluR2 heteromeric AMPARs (Wierenga et al., 2005).

While there is good agreement that AMPAR expression is a reliable measure of synaptic scaling, there are some discrepancies with respect to the subunit composition. Some studies show that activity blockade results in increased GluR1 levels but not GluR2 (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006), while others report synaptic scaling of GluR2-containing AMPARs (O'Brien et al., 1998; Wierenga et al., 2005; Ibata et al., 2008) and recently that the GluR2 subunit itself is required for scaling at all (Gainey et al., 2009).

Local versus global activity manipulations

The majority of studies have examined synaptic scaling by perturbing activity at the network-wide level (O'Brien et al., 1998; Thiagarajan et al., 2005), and a few have made manipulations on a subset of neurons in a culture, for example by expressing an inward-rectifier potassium channel to chronically hyperpolarize only transfected cells (Burrone et al., 2002). Though homeostasis is generally considered to be a global process – a negative feedback system to tune overall sensitivity, some studies have used more spatially restricted manipulations to examine local synaptic changes on a subcellular level. In a recent study a subset of a neuron's presynaptic inputs was blocked by local TTX perfusion, this resulted in no change in GluR2-containing AMPAR accumulation in any part of the neuron (Ibata et al., 2008). This was

consistent with another study that found that inhibiting presynaptic input by chronically expressing the tetanus toxin light chain in hippocampal cultures did not change AMPARs – GluR2 and GluR2/3 accumulation at these synapses did not increase as might be expected, a slight decrease in GluR1 was observed (Harms et al., 2005).

Another study, however, found that inhibiting presynaptic neurons with chronic expression of an inward-rectifier potassium channel resulted in synapses for this neuron having elevated levels of AMPAR GluR1 and GluR2/3 subunits on the postsynaptic side (Hou et al., 2008). Interestingly, addition of local NMDA receptor blockade (by local perfusion) to global activity blockade (bath application of TTX), resulted in increased GluR1-homomers surface expression via a protein-synthesis dependent mechanism (Sutton et al., 2006). It has not been investigated whether synaptic AMPARs are scaled down when activity is increased in a local region of the dendritic tree.

Intracellular pathways involved in synaptic scaling.

A number of molecular mechanisms have been proposed for translating altered neuronal activity levels into scaled AMPAR expression (Turrigiano, 2008). One candidate involves altered intracellular calcium levels since this is an important activity signal for many forms of plasticity (Wayman et al., 2008b; Malenka and Bear, 2004). In hippocampal and neocortical cultures blockade of voltage-dependent calcium channels resulted in scaling up of synaptic strength (Thiagarajan et al., 2005; Ibata et al., 2008).

Elevated levels of intracellular calcium can bind to calmodulin (among other targets) which then activates calcium/calmodulin-dependent protein kinases (CaMKs), initiating a signaling cascade that plays a critical role in neuronal development and plasticity (Wayman et al., 2008a). CaMKII, an important member of this family that is enriched at postsynaptic densities, is maximally activated by calcium influx through NMDA receptors and voltage dependent calcium channels and has been implicated in as a key component of LTP induction and persistence (Lisman et al., 2002). In hippocampal neurons activity blockade increased expression of the β isoform of CaMKII (β -CaMKII), decreasing the ratio of α -to- β CaMKII isoforms in neurons (Thiagarajan et al., 2002). β -CaMKII has also been shown to increase synaptic strength by increasing surface expression of GluR2-lacking AMPA receptors and increasing mEPSC frequency (Groth and Tsien, 2008; Thiagarajan et al., 2007).

A recent study showed that blocking somatic calcium transients with calcium channel blockers was as effective at increasing AMPAR accumulation as blocking postsynaptic firing at the soma with TTX. Both of these scaled up synapses as effectively as blocking firing of the entire culture, suggesting that the somatic calcium level is a key set-point in determining the point where all synapses would be adjusted (Ibata et al., 2008). This study did not examine the effects of locally increasing activity

levels, either at the soma or in the dendrites. The activity blockade decreased levels of CaMKIV (another member of the CaMK family, a transcription regulator most localized at the soma). Considering this in conjunction with upscaled synaptic strength in response to decreased somatic calcium levels suggests a somatic calcium set-point that regulates a transcription-dependent mechanism of synaptic scaling (Ibata et al., 2008).

Another known activity signal, the product of the immediate early gene Arc (also known as Arg3.1) has been shown to modulate a pathway for synaptic scaling. The mRNA product of the activity-regulated gene traffics to dendrites and accumulates at synaptic sites where it undergoes local translation (Bramham et al., 2008). The expression level of Arc protein decreased when activity is blocked, which in turn decreased activation of its selective pathway for AMPA receptors endocytosis and resulted in increased surface GluR1 receptors (Shepherd et al., 2006). Further, overexpression of Arc in cultured neurons decreased surface expression of GluR1 (consistent with previous studies where activity was enhanced by chronic bicuculline treatment) and overexpression of Arc with activity-blockade occluded homeostatic upregulation of GluR1-containing AMPARs (Shepherd et al., 2006).

Soluble released factors influence synaptic scaling

Several molecular pathways have been proposed that enable either a single neuron or an entire network to sense activity level and to tune itself to maintain that set level. While it is likely that multiple pathways operate in conjunction, dissecting different possible mechanisms can give interesting insights into the overall scaling phenomenon and perhaps resolve discrepancies between various studies. Several reports have identified different molecular candidates as mediators of synaptic scaling. A possible candidate molecule could itself be correlated with network activity and be a trigger of a downstream signaling cascade. Another candidate might be a central component of a signaling pathway or a modulator of a pathway necessary for synaptic plasticity, such as a basic receptor trafficking mechanism.

The first soluble released factors shown to influence synaptic scaling is the brain-derived neurotrophic factor (BDNF). BDNF and its receptor, full length TrkB, have been implicated in many important developmental events, including activity-dependent synaptic regulation (Yoshii and Constantine-Paton, 2010). BDNF is a prototypic neurotrophin released in an activity-dependent manner, and when exogenous BDNF is added during activity-blockade scaling up of synaptic strength is inhibited. This effect was not seen with neurotrophin 3 or nerve growth factor (Rutherford et al., 1997). Additionally, incubating cultures with a TrkB-IgG fusion protein (which prevents activation of the BDNF receptor by tightly binding endogenously released BDNF) produced an effect similar to canonical activity blockade (Rutherford et al., 1998).

Another molecule shown to play a role in scaling is the cytokine tumor necrosis factor α (TNF- α). TNF- α is a central mediator in the inflammatory pathway in the CNS, produced in a variety of stress situations from bacterial infection to physical damage (Park and Bowers, 2010) it has already been implicated in a number of other neuronal processes. Exogenous TNF- α added to cultured neurons results in rapidly increased mEPSC amplitude and surface expression of GluR2-lacking AMPAR (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Further, when a soluble form of the TNF- α receptor (TNFR1) was added to scavenge TNF- α during TTX activity blockade neurons were no longer able to scale up mEPSC amplitude. Surprisingly, the source of TNF- α implicated here in scaling neuronal synapses is not produced by the neurons themselves but by glial cells (Stellwagen and Malenka, 2006). When wild-type neurons were plated on glial cultures prepared from TNF- α knockout mice the neurons did not increase mEPSC amplitude in response to chronic activity blockade. Neurons from TNF- α knockout mice were unable to scale synapses when plated on glial cultures also from TNF- α knockout mice, but when the neurons were plated on wild-type glial cultures scaling was rescued (Stellwagen and Malenka, 2006). The glia may be sensing the TTX-mediated activity blockade through the reduction in neuronal glutamate release, and then signaling back to the neurons to increase synaptic strength through release of TNF- α . Interestingly, neither BDNF nor TNF- α are required for scaling down of synapses (Turrigiano, 2008; Pozo and Goda, 2010), suggesting that adjusting synaptic strength in each direction may involve distinct molecular mechanisms.

Recently another signaling pathway has been implicated in the regulation of activity-dependent homeostasis. All-*trans* retinoic acid (RA), which binds to retinoic acid receptor proteins, has previously been shown to play an important role in neuronal development in the embryo (McCaffery et al., 2006) as well as in neurogenesis in the adult hippocampus (Crandall et al., 2004). RA has also been shown to influence dendritic spine formation and morphology in cultured hippocampal neurons (Chen and Napoli, 2008; Liu et al., 2008). The role of RA as a scaling factor was demonstrated in cultured hippocampal neurons and brain slices, where RA treatment rapidly increased mEPSC and surface GluR1 AMPAR subunits (Aoto et al., 2008). When a protein synthesis inhibitor was added RA was no longer able to increase surface GluR1 expression. Further, shRNA knockdown of the RA receptor RAR α blocked synaptic scaling suggesting a pathway in which RAR α senses the RA synthesized in response to lowered activity levels and up scales local translation of GluR1 receptors to increase synaptic strength (Aoto et al., 2008).

Techniques to manipulate activity level in cultured neurons

Typically, homeostatic scaling of excitatory synapses is induced by blocking activity in neuronal cultures with TTX. Increased accumulation of AMPA receptors at synaptic sites can be measured by recording mEPSCs, by imaging fluorescently tagged AMPAR subunits, or by immunocytochemical staining for AMPAR subunits (Turrigiano, 2008). The studies described above have made use these and other molecular biology and electrophysiology tools. Electrophysiological measurements provide a functional readout of a neuron's electrical properties (membrane potential, channel conductance, firing properties, etc.) as well as of the connectivity of the network, for example miniature evoked postsynaptic currents (mEPSC) can be measured by pharmacologically blocking action potential firing, inhibitory inputs, and synaptic transmission. Changes in mEPSC frequency are generally regarded as an indication of changes in presynaptic inputs to the patched cell, while mEPSC amplitude is generally seen as an indicator of postsynaptic glutamate receptor expression.

Another technique to assay molecular changes in neuron properties is to use immunocytochemistry and fluorescence. Antibodies directed against a protein of interest, for example a specific postsynaptic glutamate receptor, are added to sister cultures (one culture that received some sort of activity manipulation and one control culture) then visualized via a fluorescently-coupled secondary antibody. Different protein expression level and localization between the cultures can indicate activity-dependent changes in the network. Both of these techniques rely on population comparisons between different cultures, not longitudinal studies of the same synapses. A third technique, appropriate for studying processes on the time course of hours, is to combine exogenous expression of fluorescently-tagged proteins with live cell time-lapse imaging. Fluorescent intensity can be used to indicate protein levels, though quantitation is difficult, and spatial patterns gives information on protein localization (or perhaps re-localization). This method allows longitudinal study of the same synapses in the same cell before and after activity manipulation. A recent study measured fluorescence intensity changes of eYFP-tagged GluR2 subunits and showed that activity blockade induces AMPA receptors accumulation at synaptic sites much more rapidly than previously thought, on the timescale of hours as opposed to days (Ibata et al., 2008).

The most common induction of synaptic scaling relies on pharmacological agents to manipulate activity levels. As described previously, canonical experiments use bath application of TTX to block culture-wide activity, resulting in scaling up of synaptic strength. Conversely, bicuculline raises the activity level of excitatory neurons by blocking inhibitory inputs and results in scaled down synaptic strength (Turrigiano et al., 1998; O'Brien et al., 1998; Thiagarajan et al., 2005). These techniques have been extended to a local perfusion setup in order to block or raise activity in a subset of cells (Ibata et al., 2008; Sutton et al., 2004). Another method for

targeting a subset of neurons is to express an inward-rectifying potassium channel which leads to chronic hyperpolarization and a dramatic reduction in action potential firing in all transfected cells (Hou et al., 2008). Increasing activity in a local manner was not tested.

The ability to precisely control neuronal activity would be an invaluable tool for the study of how individual neurons and their networks homeostatically adapt to changes in activity. Recent advances in optically controlling neuronal activity have provided tools used to study a wide array of processes in different model systems (Szobota and Isacoff, 2010; Zhang et al., 2007; Kramer et al., 2009) that could be applied here as well. The basis for these techniques is a light-driven ion channel or pump that can depolarize or hyperpolarize a cell. These can be used in conjunction with optical measurement techniques to be a fully remote, noninvasive system.

One approach makes use of naturally occurring proteins from the opsin-based family. For neuronal excitation, the Channelrhodopsin-2 (ChR2) gene was cloned from the unicellular green alga *Chlamydomonas reinhardtii* and expressed in mammalian neurons. ChR2 is a rapidly-gated light sensitive cation channel that reliably depolarizes neurons and induces firing upon illumination with blue light (Boyden et al., 2005). ChR2 has been widely-used to study a variety of neuronal processes, for example to induce long-term potentiation in organotypic slice cultures (Zhang and Oertner, 2007; Zhang et al., 2008), to restore function in retinal degeneration (Lagali et al., 2008; Tomita et al., 2010), to dissect olfactory circuits (Chalasani et al., 2007) or in combination with GCaMP (a genetically-encoded calcium reporter) to map functional circuits in *C. elegans* (Guo et al., 2009), to study the optokinetic response in zebrafish (Douglass et al., 2008), to study primate brain circuits (Han et al., 2009), and to study behavior in freely moving animals (Huber et al., 2008). Recently a channelrhodopsin with a red-shifted action spectrum was identified from *Volvox carteri* (VChR1) and developed for optogenetic stimulation in neurons (Zhang et al., 2008). Also a cation-conducting channel, VChR1 drives spiking when illuminated with 589 nm light; this provides the sufficient spectral separation such that it can also be used in conjunction with ChR2.

To complement the use of ChR2 in optically controlling activity, a halorhodopsin from *Natronomonas pharaonis* (NpHR) was cloned and codon-optimized for neuronal expression. NpHR is a light-driven chloride pump, when expressed in cultured neurons the chloride current hyperpolarizes cells and inhibits firing (Zhang et al., 2007). NpHR has been used to silence activity in brain slices (Tønnesen et al., 2009; Zhao et al., 2008), in retinal cells (Zhang et al., 2009), in *C. elegans* (Liu et al., 2009; Zhang et al., 2007), in zebrafish (Arrenberg et al., 2009; Schoonheim et al., 2010), and in *in vivo* mouse models (Gradinaru et al., 2009; Busskamp et al., 2010). Though there is a small overlap in the excitation spectra for ChR2 and NpHR, the peak wavelength for halorhodopsin activation, 580-nm, is sufficiently red-shifted away from

the ChR2 excitation peak of 460-nm such that the two proteins can be co-expressed for bi-directional optical control of membrane potential (Zhang et al., 2007, 2007).

Another approach makes use of mammalian ion receptors, either native channels in the preparation or genetically-engineered channels that are then exogenously expressed. In the first case, an affinity label attaches a photoswitchable tethered ligand to a targeted native protein, for example a channel blocker can be used to gate a voltage-gated potassium channel. Light-sensitivity is conferred by photo-isomerizing the tethered blocker between two conformations, shortening and elongating the tether to block and unblock the ion channel (Fortin et al., 2008). In the second case an engineered channel is expressed and labeled with a photoswitchable tethered ligand specifically designed to form a covalent bond onto that specific channel. One example, called SPARK, is a photo-isomerizable azobenzene and pore blocker designed to selectively conjugate to an engineered potassium channel. When illuminated with longer-wavelength light (~500-nm) the azobenzene is switched into its longer *trans* conformation, moving the blocker in position to stop channel conduction; illumination with shorter-wavelength light (~380 nm) switches the azobenzene into its shorter *cis* conformation thus retracting the blocker and allowing the channel to conduct (Banghart et al., 2004). In both of these examples, neuronal excitation can be inhibited by optically controlled hyperpolarization.

A different set of tools following this approach couples photoswitchable ligands with mammalian glutamate receptors. The light-activated glutamate receptor LiGluR combines a modified ionotropic glutamate receptor (iGluR6) with a photo-switchable small molecule agonist consisting of a maleimide, azobenzene, and a glutamate analog (MAG) (Volgraf et al., 2006; Gorostiza et al., 2007). MAG is covalently attached to an engineered cysteine on the ligand-binding domain of the receptor. Switching the azobenzene into its *cis* conformation with UV light moves the glutamate into the ligand-binding site, activating the receptor. Visible light switches the azobenzene to its *trans* conformation, deactivating the receptor. The level and duration of stimulation can be varied by the intensity and duration of the illumination, and can be repeated with high fidelity (Volgraf et al., 2006; Gorostiza et al., 2007).

The photostimulation properties of LiGluR can be tuned by adjusting the length of the photoswitch MAG and by changing its anchor point such that the glutamate analog reaches its binding site at different azobenzene lengths. For example, by moving the cysteine-point mutation from residue 439 to residue 486 the channel can now be operated in reverse – 500-nm illumination activates the channel and 380-nm illumination moves the glutamate out of the binding pocket to deactivate the channel (Numano et al., 2009). The conductive properties of the channel can also be engineered, insertion of the GluR0 potassium-selective pore results in the hyperpolarizing light-gated channel called HyLighter (Janovjak et al., 2010). HyLighter expressed in cultured hippocampal neurons or brain slices results in optically-controlled inhibition of neuron firing.

Use of light-activated receptors to study synaptic scaling

Chapter 2 of this thesis describes the optimization of subcellular LiGluR activation in cultured hippocampal neurons, discussing critical parameters for spatially restricting the illumination and examining iGluR6(L439C) protein expression. Establishing a targeted illumination system and verifying the spatial resolution of light activation is critical for the experiments later described in chapter 3. The conditions for long-term light activation are also set to take full advantage of the noninvasive nature of LiGluR, enabling the study of processes that require prolonged, chronic manipulation. The light-activation protocol considers both the 380-nm light for activation and the 488-nm light for measuring output, setting maximum power levels for robust photostimulation while maintaining good spatially resolution. These parameters are set on a high resolution scanning confocal microscope so that small features (such as synaptic receptors) can be properly visualized. I also discuss expression pattern of LiGluR and illustrate a co-expression system for driving channel expression to postsynaptic sites. By co-transfecting LiGluR with PSD-95 light-triggered, depolarization and calcium influx can be targeted to postsynaptic densities.

In chapter 3, LiGluR activation is combined with time-lapse imaging of AMPARs, a well established expression locus of different modes of synaptic plasticity (Bredt and Nicoll, 2003; Malenka and Bear, 2004). A previous study examined the effects of endogenous ionotropic glutamate receptor activation on AMPAR distribution in cultured hippocampal neurons. Upon activation with 100 μ M glutamate bath application, rapid re-localization of GluR1-containing receptors was seen away from synaptic sites to intracellular dendritic shafts and cell bodies (Lissin et al., 1999). Other studies have examined changes in AMPA receptor expression in response to altered activity levels via a culture-wide pharmacological bath application (O'Brien et al., 1998; Ju et al., 2004; Wierenga et al., 2005). LiGluR is an ideal tool to build upon these studies, to examine AMPA receptor expression in response to increased depolarization and calcium influx on a local level. Addition of TTX to the bath solution restricts the LiGluR signal from traveling through the dendritic tree via back-propagating action potentials and also sensitizes neurons to the LiGluR activity manipulation.

To measure synaptic scaling the surface expression of AMPARs was monitored using an ecliptic-tagged GluR2 subunit, a highly-sensitive reporter that is fluorescent when the receptor is surface expressed. Expressing this receptor in cultured hippocampal neurons results in punctate surface expression concentrated at spines along the dendritic shaft. The fluorescence intensity increases in hippocampal neurons when culture-wide activity is blocked with TTX, verifying that the system is a faithful reporter of AMPAR accumulation under synaptic scaling conditions. When combined with LiGluR activation, I found that chronic depolarization and Ca^{2+} influx in a portion of the dendritic tree reduces the surface population of AMPARs at dendritic spines in all parts of the neuron. Loss of AMPARs occurred within the first hour, and puncta

decreased fluorescence intensity at similar rates and to similar levels regardless of whether they were localized to a region that was directly activated by LiGluR or not. The effect of LiGluR activation was blocked by chelating intracellular calcium with BAPTA-AM, blocking voltage dependent calcium channels with cadmium, or inhibiting the activity of calcium/calmodulin-dependent protein kinases with KN-93, indicating an intracellular-calcium dependent mechanism.

References

- Abbott, L. F., and Nelson, S. B. (2000). Synaptic plasticity: taming the beast. *Nat. Neurosci 3 Suppl*, 1178-1183.
- Aoto, J., Nam, C. I., Poon, M. M., Ting, P., and Chen, L. (2008). Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron 60*, 308-320.
- Arrenberg, A. B., Del Bene, F., and Baier, H. (2009). Optical control of zebrafish behavior with halorhodopsin. *Proc. Natl. Acad. Sci. U.S.A 106*, 17968-17973.
- Banghart, M., Borges, K., Isacoff, E., Trauner, D., and Kramer, R. H. (2004). Light-activated ion channels for remote control of neuronal firing. *Nat. Neurosci 7*, 1381-1386.
- Barry, M. F., and Ziff, E. B. (2002). Receptor trafficking and the plasticity of excitatory synapses. *Curr. Opin. Neurobiol 12*, 279-286.
- Beattie, E. C., Stellwagen, D., Morishita, W., Bresnahan, J. C., Ha, B. K., Von Zastrow, M., Beattie, M. S., and Malenka, R. C. (2002). Control of Synaptic Strength by Glial TNFalpha. *Science 295*, 2282-2285.
- Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci 8*, 1263-1268.
- Bramham, C. R., Worley, P. F., Moore, M. J., and Guzowski, J. F. (2008). The immediate early gene *arc/arg3.1*: regulation, mechanisms, and function. *J. Neurosci 28*, 11760-11767.
- Bredt, D. S., and Nicoll, R. A. (2003). AMPA Receptor Trafficking at Excitatory Synapses. *Neuron 40*, 361-379.
- Burrone, J., O'Byrne, M., and Murthy, V. N. (2002). Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. *Nature 420*, 414-418.
- Busskamp, V., Duebel, J., Balya, D., Fradot, M., Viney, T. J., Siebert, S., Groner, A. C.,

- Cabuy, E., Forster, V., Seeliger, M., et al. (2010). Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa. *Science* 329, 413-417.
- Chalasani, S. H., Chronis, N., Tsunozaki, M., Gray, J. M., Ramot, D., Goodman, M. B., and Bargmann, C. I. (2007). Dissecting a circuit for olfactory behaviour in *Caenorhabditis elegans*. *Nature* 450, 63-70.
- Chen, N., and Napoli, J. L. (2008). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *FASEB J* 22, 236-245.
- Collingridge, G. L., Isaac, J. T. R., and Wang, Y. T. (2004). Receptor trafficking and synaptic plasticity. *Nat. Rev. Neurosci* 5, 952-962.
- Crandall, J., Sakai, Y., Zhang, J., Koul, O., Mineur, Y., Crusio, W. E., and McCaffery, P. (2004). 13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice. *Proc. Natl. Acad. Sci. U.S.A* 101, 5111-5116.
- Davis, G. W. (2006). Homeostatic control of neural activity: from phenomenology to molecular design. *Annu. Rev. Neurosci* 29, 307-323.
- Desai, N. S., Cudmore, R. H., Nelson, S. B., and Turrigiano, G. G. (2002). Critical periods for experience-dependent synaptic scaling in visual cortex. *Nat. Neurosci* 5, 783-789.
- Dong, H., O'Brien, R. J., Fung, E. T., Lanahan, A. A., Worley, P. F., and Huganir, R. L. (1997). GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* 386, 279-284.
- Douglass, A. D., Kraves, S., Deisseroth, K., Schier, A. F., and Engert, F. (2008). Escape behavior elicited by single, channelrhodopsin-2-evoked spikes in zebrafish somatosensory neurons. *Curr. Biol* 18, 1133-1137.
- Fortin, D. L., Banghart, M. R., Dunn, T. W., Borges, K., Wagenaar, D. A., Gaudry, Q., Karakossian, M. H., Otis, T. S., Kristan, W. B., Trauner, D., et al. (2008). Photochemical control of endogenous ion channels and cellular excitability. *Nat. Methods* 5, 331-338.

- Frank, C. A., Kennedy, M. J., Goold, C. P., Marek, K. W., and Davis, G. W. (2006). Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. *Neuron* 52, 663-677.
- Gainey, M. A., Hurvitz-Wolff, J. R., Lambo, M. E., and Turrigiano, G. G. (2009). Synaptic scaling requires the GluR2 subunit of the AMPA receptor. *J. Neurosci* 29, 6479-6489.
- Gorostiza, P., Volgraf, M., Numano, R., Szobota, S., Trauner, D., and Isacoff, E. Y. (2007). Mechanisms of photoswitch conjugation and light activation of an ionotropic glutamate receptor. *Proceedings of the National Academy of Sciences* 104, 10865 -10870.
- Gradinaru, V., Mogri, M., Thompson, K. R., Henderson, J. M., and Deisseroth, K. (2009). Optical deconstruction of parkinsonian neural circuitry. *Science* 324, 354-359.
- Greger, I. H., Ziff, E. B., and Penn, A. C. (2007). Molecular determinants of AMPA receptor subunit assembly. *Trends Neurosci* 30, 407-416.
- Groth, R. D., and Tsien, R. W. (2008). A role for retinoic acid in homeostatic plasticity. *Neuron* 60, 192-194.
- Guo, Z. V., Hart, A. C., and Ramanathan, S. (2009). Optical interrogation of neural circuits in *Caenorhabditis elegans*. *Nat. Methods* 6, 891-896.
- Han, X., Qian, X., Bernstein, J. G., Zhou, H., Franzesi, G. T., Stern, P., Bronson, R. T., Graybiel, A. M., Desimone, R., and Boyden, E. S. (2009). Millisecond-timescale optical control of neural dynamics in the nonhuman primate brain. *Neuron* 62, 191-198.
- Harms, K. J., Tovar, K. R., and Craig, A. M. (2005). Synapse-specific regulation of AMPA receptor subunit composition by activity. *J. Neurosci* 25, 6379-6388.
- Hou, Q., Zhang, D., Jarzylo, L., Hugarir, R. L., and Man, H. (2008). Homeostatic regulation of AMPA receptor expression at single hippocampal synapses. *Proc. Natl. Acad. Sci. U.S.A* 105, 775-780.
- Huber, D., Petreanu, L., Ghitani, N., Ranade, S., Hromádka, T., Mainen, Z., and Svoboda, K. (2008). Sparse optical microstimulation in barrel cortex drives

- learned behaviour in freely moving mice. *Nature* 451, 61-64.
- Ibata, K., Sun, Q., and Turrigiano, G. G. (2008). Rapid Synaptic Scaling Induced by Changes in Postsynaptic Firing. *Neuron* 57, 819-826.
- Janovjak, H., Szobota, S., Wyart, C., Trauner, D., and Isacoff, E. Y. (2010). A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing. *Nat Neurosci* 13, 1027-1032.
- Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., Garner, C. C., Tsien, R. Y., Ellisman, M. H., and Malenka, R. C. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci* 7, 244-253.
- Kim, J., and Tsien, R. W. (2008). Synapse-specific adaptations to inactivity in hippocampal circuits achieve homeostatic gain control while dampening network reverberation. *Neuron* 58, 925-937.
- Kirov, S. A., Goddard, C. A., and Harris, K. M. (2004). Age-dependence in the homeostatic upregulation of hippocampal dendritic spine number during blocked synaptic transmission. *Neuropharmacology* 47, 640-648.
- Knogler, L. D., Liao, M., and Drapeau, P. (2010). Synaptic scaling and the development of a motor network. *J. Neurosci* 30, 8871-8881.
- Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.
- Kramer, R. H., Fortin, D. L., and Trauner, D. (2009). New photochemical tools for controlling neuronal activity. *Curr. Opin. Neurobiol* 19, 544-552.
- Lagali, P. S., Balya, D., Awatramani, G. B., Münch, T. A., Kim, D. S., Busskamp, V., Cepko, C. L., and Roska, B. (2008). Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. *Nat. Neurosci* 11, 667-675.
- Lee, S. H., Simonetta, A., and Sheng, M. (2004). Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron* 43, 221-236.

- Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* 3, 175-190.
- Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1998). Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. *Proc. Natl. Acad. Sci. U.S.A* 95, 7097-7102.
- Lissin, D. V., Carroll, R. C., Nicoll, R. A., Malenka, R. C., and Zastrow, M. V. (1999). Rapid, Activation-Induced Redistribution of Ionotropic Glutamate Receptors in Cultured Hippocampal Neurons. *J. Neurosci.* 19, 1263-1272.
- Liu, Q., Hollopeter, G., and Jorgensen, E. M. (2009). Graded synaptic transmission at the *Caenorhabditis elegans* neuromuscular junction. *Proc. Natl. Acad. Sci. U.S.A* 106, 10823-10828.
- Liu, Y., Kagechika, H., Ishikawa, J., Hirano, H., Matsukuma, S., Tanaka, K., and Nakamura, S. (2008). Effects of retinoic acids on the dendritic morphology of cultured hippocampal neurons. *J. Neurochem* 106, 1104-1116.
- Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5-21.
- McCaffery, P., Zhang, J., and Crandall, J. E. (2006). Retinoic acid signaling and function in the adult hippocampus. *J. Neurobiol* 66, 780-791.
- McKinney, R. A., Capogna, M., Durr, R., Gähwiler, B. H., and Thompson, A. S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci* 2, 44-49.
- Mrsic-Flogel, T. D., Hofer, S. B., Ohki, K., Reid, R. C., Bonhoeffer, T., and Hübener, M. (2007). Homeostatic regulation of eye-specific responses in visual cortex during ocular dominance plasticity. *Neuron* 54, 961-972.
- Numano, R., Szobota, S., Lau, A. Y., Gorostiza, P., Volgraf, M., Roux, B., Trauner, D., and Isacoff, E. Y. (2009). Nanosculpting reversed wavelength sensitivity into a photoswitchable iGluR. *Proceedings of the National Academy of Sciences* 106, 6814-6819.
- O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D., and Huganir, R. L. (2004). Activity-dependent trafficking of AMPA receptors to and from dendritic spines. *Neuron* 44, 297-311.

- R. L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21, 1067-1078.
- Park, K. M., and Bowers, W. J. (2010). Tumor necrosis factor- α mediated signaling in neuronal homeostasis and dysfunction. *Cell. Signal* 22, 977-983.
- Pozo, K., and Goda, Y. (2010). Unraveling mechanisms of homeostatic synaptic plasticity. *Neuron* 66, 337-351.
- Rutherford, L. C., Nelson, S. B., and Turrigiano, G. G. (1998). BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron* 21, 521-530.
- Rutherford, L. C., DeWan, A., Lauer, H. M., and Turrigiano, G. G. (1997). Brain-Derived Neurotrophic Factor Mediates the Activity-Dependent Regulation of Inhibition in Neocortical Cultures. *J. Neurosci.* 17, 4527-4535.
- Santos, S. D., Carvalho, A. L., Caldeira, M. V., and Duarte, C. B. (2009). Regulation of AMPA receptors and synaptic plasticity. *Neuroscience* 158, 105-125.
- Schoonheim, P. J., Arrenberg, A. B., Del Bene, F., and Baier, H. (2010). Optogenetic localization and genetic perturbation of saccade-generating neurons in zebrafish. *J. Neurosci* 30, 7111-7120.
- Seeburg, P. H., Higuchi, M., and Sprengel, R. (1998). RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res. Brain Res. Rev* 26, 217-229.
- Shepherd, J. D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R. L., and Worley, P. F. (2006). Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* 52, 475-484.
- Shi, S., Hayashi, Y., Esteban, J. A., and Malinow, R. (2001). Subunit-Specific Rules Governing AMPA Receptor Trafficking to Synapses in Hippocampal Pyramidal Neurons. *Cell* 105, 331-343.
- Srivastava, S., Osten, P., Vilim, F. S., Khatri, L., Inman, G., States, B., Daly, C., DeSouza, S., Abagyan, R., Valtchanoff, J. G., et al. (1998). Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. *Neuron* 21, 581-591.

- Stellwagen, D., Beattie, E. C., Seo, J. Y., and Malenka, R. C. (2005). Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor- α . *J. Neurosci* 25, 3219-3228.
- Stellwagen, D., and Malenka, R. C. (2006). Synaptic scaling mediated by glial TNF- α . *Nature* 440, 1054-1059.
- Sutton, M. A., Ito, H. T., Cressy, P., Kempf, C., Woo, J. C., and Schuman, E. M. (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* 125, 785-799.
- Sutton, M. A., Wall, N. R., Aakalu, G. N., and Schuman, E. M. (2004). Regulation of Dendritic Protein Synthesis by Miniature Synaptic Events. *Science* 304, 1979-1983.
- Szobota, S., and Isacoff, E. Y. (2010). Optical control of neuronal activity. *Annu Rev Biophys* 39, 329-348.
- Thiagarajan, T. C., Lindskog, M., Malgaroli, A., and Tsien, R. W. (2007). LTP and adaptation to inactivity: overlapping mechanisms and implications for metaplasticity. *Neuropharmacology* 52, 156-175.
- Thiagarajan, T. C., Lindskog, M., and Tsien, R. W. (2005). Adaptation to Synaptic Inactivity in Hippocampal Neurons. *Neuron* 47, 725-737.
- Thiagarajan, T. C., Piedras-Renteria, E. S., and Tsien, R. W. (2002). $[\alpha]$ - and $[\beta]$ CaMKII: Inverse Regulation by Neuronal Activity and Opposing Effects on Synaptic Strength. *Neuron* 36, 1103-1114.
- Tomita, H., Sugano, E., Isago, H., Hiroi, T., Wang, Z., Ohta, E., and Tamai, M. (2010). Channelrhodopsin-2 gene transduced into retinal ganglion cells restores functional vision in genetically blind rats. *Exp. Eye Res* 90, 429-436.
- Tønnesen, J., Sørensen, A. T., Deisseroth, K., Lundberg, C., and Kokaia, M. (2009). Optogenetic control of epileptiform activity. *Proc. Natl. Acad. Sci. U.S.A* 106, 12162-12167.
- Turrigiano, G. (2007). Homeostatic signaling: the positive side of negative feedback.

- Curr. Opin. Neurobiol 17, 318-324.
- Turrigiano, G. G. (2008). The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. *Cell* 135, 422-435.
- Turrigiano, G. G., Leslie, K. R., Desai, N. S., Rutherford, L. C., and Nelson, S. B. (1998). Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* 391, 892-896.
- Turrigiano, G. G., and Nelson, S. B. (2004). Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci* 5, 97-107.
- Volgraf, M., Gorostiza, P., Numano, R., Kramer, R. H., Isacoff, E. Y., and Trauner, D. (2006). Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat Chem Biol* 2, 47-52.
- Wayman, G. A., Lee, Y., Tokumitsu, H., Silva, A. J., Silva, A., and Soderling, T. R. (2008a). Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron* 59, 914-931.
- Wayman, G. A., Lee, Y., Tokumitsu, H., Silva, A., and Soderling, T. R. (2008b). Calmodulin-Kinases: Modulators of Neuronal Development and Plasticity. *Neuron* 59, 914-931.
- Wenthold, R. J., Petralia, R. S., Blahos J, I. I., and Niedzielski, A. S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J. Neurosci* 16, 1982-1989.
- Wierenga, C. J., Ibata, K., and Turrigiano, G. G. (2005). Postsynaptic Expression of Homeostatic Plasticity at Neocortical Synapses. *J. Neurosci.* 25, 2895-2905.
- Yoshii, A., and Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Dev Neurobiol* 70, 304-322.
- Zhang, F., Aravanis, A. M., Adamantidis, A., de Lecea, L., and Deisseroth, K. (2007). Circuit-breakers: optical technologies for probing neural signals and systems. *Nat. Rev. Neurosci* 8, 577-581.
- Zhang, F., Prigge, M., Beyrière, F., Tsunoda, S. P., Mattis, J., Yizhar, O., Hegemann,

- P., and Deisseroth, K. (2008). Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri*. *Nat. Neurosci* 11, 631-633.
- Zhang, F., Wang, L., Brauner, M., Liewald, J. F., Kay, K., Watzke, N., Wood, P. G., Bamberg, E., Nagel, G., Gottschalk, A., et al. (2007). Multimodal fast optical interrogation of neural circuitry. *Nature* 446, 633-639.
- Zhang, Y., Holbro, N., and Oertner, T. G. (2008). Optical induction of plasticity at single synapses reveals input-specific accumulation of alphaCaMKII. *Proc. Natl. Acad. Sci. U.S.A* 105, 12039-12044.
- Zhang, Y., and Oertner, T. G. (2007). Optical induction of synaptic plasticity using a light-sensitive channel. *Nat. Methods* 4, 139-141.
- Zhang, Y., Ivanova, E., Bi, A., and Pan, Z. (2009). Ectopic expression of multiple microbial rhodopsins restores ON and OFF light responses in retinas with photoreceptor degeneration. *J. Neurosci* 29, 9186-9196.
- Zhao, S., Cunha, C., Zhang, F., Liu, Q., Gloss, B., Deisseroth, K., Augustine, G. J., and Feng, G. (2008). Improved expression of halorhodopsin for light-induced silencing of neuronal activity. *Brain Cell Biol* 36, 141-154.

Chapter 1 Figures

Figure 1. Bi-directional scaling of excitatory synapses

Figure 1

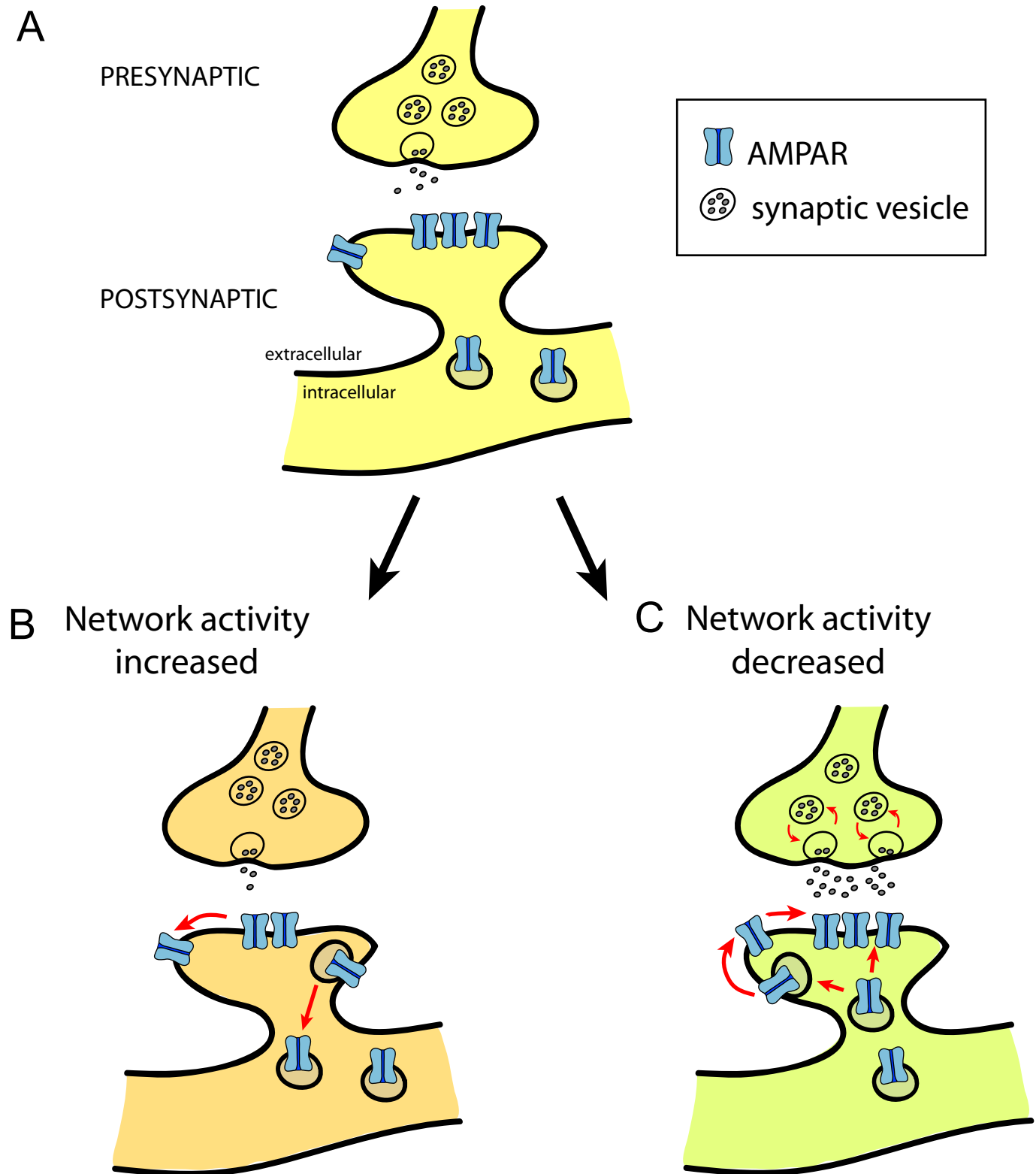


Figure 1. Bi-directional scaling of excitatory synapses

- A). During basal activity level: synaptic transmission occurs between a presynaptic terminal which releases neurotransmitter, and an adjacent postsynaptic terminal where surface receptors are activated.
- B). During elevated levels of activity: the presynaptic terminal decreases release probability of synaptic vesicles while the postsynaptic cell reduces receptor population by endocytosis or lateral diffusion away from synaptic sites.
- C). During lowered levels of activity: the presynaptic terminal enhances vesicle recycling, number of docked vesicles, and release probability while the postsynaptic site increases the number of active surface receptors.

CHAPTER 2:

Subcellular targeting and activation of LiGluR

Summary

LiGluR, with its opto-genetic properties and its noninvasive mode of operation, can be combined with targeted illumination, as a tool for stimulating neurons in a spatially and temporally controlled manner. I describe the optimization of subcellular LiGluR activation in cultured hippocampal neurons, discussing critical parameters for spatially restricting the illumination and examining iGluR6(L439C) protein expression. The light-activation protocol considers both the 380-nm light for activation and the 488-nm light for measuring output, setting maximum power levels for robust photostimulation while maintaining good spatially resolution. Additionally I illustrate a co-expression system for driving channel expression to postsynaptic sites. By co-transfecting LiGluR with PSD-95 light-triggered, depolarization and calcium influx can be targeted to postsynaptic densities.

Introduction

Understanding the mechanisms and consequences of activity in neural circuits is critical to the study of neuroscience, as such activity is known to underlie both development of the nervous system and on-going processes such as memory formation. The ability to precisely control neuronal activity is an invaluable tool for studying how these neuronal networks process and store information. Depolarizing a neuron's membrane potential away from its resting value causes a flux of ions through voltage-gated channels resulting in action potential firing. These action potentials are rapid, all-or-nothing electrical impulses that carry information within a neuron and transmit information to other neurons via chemical neurotransmitters at synaptic sites. We have developed LiGluR, a light-controlled glutamate receptor that rapidly and reliably depolarizes neurons, resulting in calcium influx and inducing action potential firing.

LiGluR combines a modified ionotropic glutamate receptor (iGluR6) with a photo-switchable small molecule agonist consisting of a maleimide, azobenzene, and a glutamate analog (MAG) (Volgraf et al., 2006; Gorostiza et al., 2007). MAG is covalently attached to an engineered cysteine on the ligand-binding domain of the receptor. Switching the azobenzene into its *cis* conformation with UV light moves the glutamate into the ligand-binding site, activating the receptor. Visible light switches the azobenzene to its *trans* conformation, deactivating the receptor. The level and duration of stimulation can be varied by the intensity and duration of the illumination, and can be repeated with high fidelity (Volgraf et al., 2006; Szobota et al., 2007).

Here I discuss combining LiGluR with targeted illumination for subcellular neuronal activation. I show repeated LiGluR activation in a single cell for a time course of hours without inducing toxicity. This non-invasive stimulation is recorded on an

inverted scanning confocal microscope, whose high-resolution imaging capabilities later facilitate studies on a molecular level. Additionally, I discuss expression of iGluR6(L439C) in cultured hippocampal neurons – the localization of the subunit and also physiological effects of overexpressing a glutamate receptor. I provide a method for targeting the receptor to dendritic branches and postsynaptic sites in excitatory neurons. Such targeting allows more physiologically relevant light-controlled depolarization and calcium entry at synaptic sites.

Results

A targeted illumination system for spatial control of genetically-encoded light activated receptors

Developing and optimizing LiGluR has given us an invaluable tool for non-invasive neuronal stimulation. As with all genetic approaches, the gene encoding the iGluR6(L439C) channel must first be introduced into the model system. For cultured hippocampal neurons we use the calcium phosphate-mediated transfection method (Watanabe et al., 1999), which has a typically transfection yield of 3-5%. Since the MAG photoswitch only confers light-sensitivity to kainate receptors with the L439C point mutation (Szobota et al., 2007), only this small subset of cells in the culture are LiGluR positive. We can take advantage of this low expression to selectively activate and study a single neuron, distinguishing it from its hundreds of neighbors (both proximal and synaptic). We can achieve further spatial resolution by targeting the light activation to a portion of the neuron, rather than illuminating the entire cell (Figure 1A).

Single cell and subcellular stimulation with LiGluR has many potential applications. Combining this with an optical readout allows us to exploit the non-invasive nature of the stimulus, enabling study of processes on a longer time scale without loss of cell morphology or health. In the following chapter of this thesis LiGluR is combined with a super-ecliptic tagged GluR2 subunit (SEP-GluR2) to study α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor surface expression (Figure 1B). Previous studies have used fluorescently-tagged proteins (Ibata et al., 2008; Sutton et al., 2004) or immunocytochemistry (Lissin et al., 1999) to examine activity-dependent changes in AMPA receptor expression. To more accurately measure changes in active receptors (that are properly inserted at the membrane) we tag the N-terminus of the GluR2 subunit with a pH-sensitive enhanced ecliptic GFP (Ashby et al., 2004a, 2004b). The enhanced ecliptic (superecliptic) GFP has two point mutations (F64L and S65T) in the original pHluorin sequence that confers increased fluorescence emission while maintaining pH sensitivity (Miesenböck et al., 1998; Sankaranarayanan et al., 2000). Imaging SEP-GluR2 through a time course of local, subcellular LiGluR activation reveals that the global population of surface AMPA

receptors is affected, not only the receptors in immediate proximity of the calcium influx and depolarization (Chapter 3). Live cell imaging of receptor puncta before and after stimulation allows us to normalize the changes in fluorescence intensity of each separate puncta to its initial fluorescence, improving the quality of the analysis.

Imaging synaptic puncta requires a high resolution microscopy system since features are small (typically $\sim 1\text{-}2\ \mu\text{m}$ in diameter) and fluorescent signals can be low. We use a Zeiss LSM 510 NLO Axiovert scanning confocal microscope with a Plan-Apochromat 63x/1.4 oil objective for imaging, with an Argon laser for 488-nm excitation (Berkeley Molecular Imaging Center, UC Berkeley). A Spectra-Physics MaiTai HP laser tuned to 760-nm was frequency doubled to 380-nm illumination to activate LiGluR. An external shutter driver (Uniblitz) triggered by a Digidata acquisition system and pCLAMP software (Axon Laboratory/Molecular Devices) passed illumination through the scan head only during targeted scans. Initial testing of the system was done using kaede-expressing zebrafish larvae (Figure 1C). Kaede protein undergoes a photoconversion when illuminated with UV light, shifting its absorption and emission peaks from green fluorescence to red fluorescence (Ando et al., 2002). Kaede is imaged in two channels for red and green fluorescence, with excitation at 543-nm and 488-nm and emission signals collected using bandpass filters passing 565-615 nm and 510-560 nm light respectively. UV illumination is restricted to the middle segment which, as expected, photoconverts kaede protein in this region by shifting the peak emission from 518 nm to 582 nm (increased intensity in red fluorescent images, Figure 1C first and third panels). The green-to-red fluorescence ratio remains unchanged outside the targeted illumination.

Kaede photoconversion is irreversible (Ando et al., 2002), therefore we could not use it to compare various UV illumination parameters (size of the targeted region, duration, power levels, etc.) on the same sample. The zebrafish-kaede system was also limited to 10x or 20x objectives (due to constrained working distance) while either 40x or 63x oil-immersion objectives are needed for synaptic puncta imaging. We decided to use the photoactivatable dronpa protein for further characterization. Dronpa is switched to its green fluorescent state by UV light and to its neutral non-fluorescent state by $490 \pm 10\text{nm}$ light (Ando et al., 2004; Andresen et al., 2007). This photochromic behavior is particularly useful because fluorescence can be repeatedly switch on and off by light of two different wavelengths. The expression construct used here was a dronpa-actin fusion protein, which provided a bright, immobilized fluorescent signal in cultured hippocampal neurons (Marriott et al., 2008). Spatially restricted UV illumination (380-nm) converted dronpa in that region to its fluorescent state as expected. We determined that for small illumination windows ($\sim 15\mu\text{m}$ or less), the maximum power settings for the UV laser resulted in light diffracting outside the scanned region and activating dronpa throughout the field of view (Figure 1D, left). Using neutral density filters to attenuate the power of the UV light we were able to attain the targeted illumination (Figure 1D, right) needed for future experiments.

Functional imaging to measure spatially restricted LiGluR activation

An important consideration in establishing the targeted illumination system is that different photoactivatable proteins have different light sensitivities. Therefore we next test directly LiGluR activation, with full field-of-view illumination compared to partial field illumination. For this experiment we chose to express LiGluR in HEK293 cells. These cultures are advantageous for a few reasons, 1). their rapid division time yields confluent cultures in 1-3 days, 2). they are compatible with cationic lipid-mediated DNA transfection thus yielding high transfection efficiencies, and 3). they are a proven system for testing light-activated channels and yield reliable, robust calcium responses (Volgraf et al., 2006; Numano et al., 2009; Janovjak et al., 2010).

HEK293 cells were co-transfected with iGluR6(L439C) and dsRed (to visualize transfected cells), loaded with the Fluo-4-AM calcium indicator dye, then labeled with the MAG photoswitch. Fluo-4 loads into all cells in the culture and has a bright green fluorescence (Figure 2A, left). Since neighboring HEK293 cells can become electrically couple via gap junctions (Thomas and Smart, 2005), we choose to measure LiGluR calcium signals from transfected cells isolated from other transfected cells (Figure 2A, right).

In the first stimulus (Figure 2B) the field-of-view is partially illuminated with 380-nm light (covering only cells #1 and 2) while Fluo-4 fluorescence is measured for all cells in the field. As expected, LiGluR activation in cell #1 (entire cell is illuminated) resulted in a large calcium influx. Cell #2 was only partially illuminated therefore the UV stimulus activated some but likely not all LiGluR channels, resulting in a smaller calcium influx (Figure 2B, right). Cells #3-5 located outside this illumination area showed no change in fluorescence. Calcium response traces are shown as change in Fluo-4 fluorescence intensity for each cell, normalized to that cell's initial fluorescence (averaged from five images taken prior to UV pulse). In the second stimulus the entire field of view is illuminated with 380-nm light (Figure 2C, left). LiGluR activation elicited robust calcium responses from all cells (Figure 2C, right). This full field stimulus is given at the end of the experiment, ensuring that all cells are capable of LiGluR-mediated calcium responses and that lack of a response can be correctly attributed to illumination regions.

As discussed previously, we inserted neutral density filters in the path of the Spectra-physics MaiTai laser to attenuate power of the UV light seen on the microscope stage. At lower light intensities we saw better spatial resolution of the UV illuminated region, though we wondered if this power reduction was markedly reducing LiGluR activation. Using this spatial LiGluR HEK293 map we are able to compare these trade-offs and to set an optimal UV power intensity. LiGluR activation for several power settings was measured with a brief pulse of UV illumination over cells #1 and #2 (as in Figure 2B). After each UV pulse the Fluo-4 calcium response was measured for several seconds, followed by a 1 minute rest period for intracellular calcium to clear. The

results are summarized in the bar graph depicting the maximum fluorescence increase measured to each stimulus for each cell (Figure 2D). Cell #1, which is fully illuminated each cycle, had a robust and fairly similar calcium influx in response to all UV stimulations greater than $3 \mu\text{W}/\text{mm}^2$ (Figure 2D, black bars). Cell #2, which is partially illuminated each cycle (covering $\sim 3\%$ of total surface area), had modest calcium influx for UV power settings between $3\text{--}15 \mu\text{W}/\text{mm}^2$ and a somewhat higher calcium influx at the $75 \mu\text{W}/\text{mm}^2$ power setting (Figure 2D, gray bars). This response was closer to the activation level seen when the cell is fully illuminated in the last stimulation cycle. For cells #3-#5 minimal fluorescence change was seen for UV power settings between $3\text{--}15 \mu\text{W}/\text{mm}^2$ though a modest fluorescence increase was seen at the highest power setting ($75 \mu\text{W}/\text{mm}^2$). To measure latent fluctuations in Fluo-4 fluorescence, UV stimulation was omitted altogether for one cycle (bar graph, center point). Fluorescence increases up to $\sim 10\%$ were seen, indicating that there is some noise fluctuations and that low-level intensity changes are not necessarily due to LiGluR activation. To measure the full calcium response of all transfected cells, a full field UV stimulation was given in the last cycle (bar graph, end point) eliciting the maximum calcium response for all cells. This demonstrates that low or absent LiGluR responses in previous cycles are due to intentional targeted illumination off that cell and are not simply due to poor cell health or failed LiGluR activation.

For cells not fully illuminated in the main experiment (#2-#5) a moderate calcium response was still seen at the highest power setting ($75 \mu\text{W}/\text{mm}^2$). Considering that no substantial difference was measured for this power setting for the fully illuminated cell (#1), and taken together with the dropa experiments, this power setting results in UV light diffracting and activating LiGluR channels beyond the user-defined region. For future experiments requiring single cell or subcellular LiGluR activation the UV illumination power is capped at $15 \mu\text{W}/\text{mm}^2$.

Subcellular activation of LiGluR in hippocampal neurons

We next extended our targeted illumination system to LiGluR activation in cultured hippocampal neurons. Neurons were co-transfected with iGluR6(L439C) and either dsRed or eGFP for visualization by calcium phosphate-mediated transfection. In addition to testing with Fluo-4-AM calcium indicator dye we also used the X-Rhod-1-AM calcium indicator dye (Molecular Probes/Invitrogen). In later experiments we use green fluorescent tags to follow proteins of interest, thus requiring the X-Rhod-1 red fluorescent dye to avoid spectral overlap. As with the HEK293 cell experiments, the transfected neuron is identified by fluorescence (Figure 3A) while the calcium dye loads in all cells (Figure 3B). A bright fluorescence indicator is important here because it allows not only identification of the transfected neuron but also visualization of the

extensive dendritic tree. When the full field-of-view is illuminated a robust calcium response is elicited in the LiGluR-positive cell as expected (data not shown).

In optimizing the subcellular illumination protocol the following parameters are considered, 1). size and location of the illuminated region, 2). intensity and duration of the UV light, and 3). duration and frequency of 488-nm imaging light (discussed in the following section). The size of the illuminated region should be large enough to activate a sufficient number of LiGluR channels to elicit a detectable response, but not so large that too many channels are activated and synaptic integration depolarizes the neuron past action potential threshold leading the signal to propagate throughout the cell (Gulledge et al., 2005; Magee et al., 1998). The location of this the region is also important, especially for dendritic stimulation, as illuminating branches too proximal to the soma is more likely to activate voltage-gated ion channels that can raise somatic calcium levels (Magee and Carruth, 1999).

One of the challenges in determining the appropriate illumination window for dendritic stimulation is that dendritic processes are thin. The calcium indicator dye does not fill small volumes well and it therefore is difficult to accurately measure fluorescence changes. Using bright, genetically-encoded fluorescence to visualize the dendrites is useful here for determining a reasonably-sized region where calcium signals can be measured while minimizing overall depolarization. An example of optimal dendritic stimulation is shown in Figure 3C, the left panel maps the illumination area on a small branch of the transfected cell's dendritic tree and the right panel shows the calcium rise in the illuminated branch.

Somatic LiGluR activation is more straightforward as the genetically-encoded fluorescence is easy to identify and the large volume fills well with the calcium indicator dye (and dye signals are easier to measure). LiGluR activation at the soma also seems to be more powerful, typically leading to calcium rises in other parts of the neuron. Positioning the same UV illumination window from before (Figure 3C) over the soma (Figure 3D, left) elicits a larger calcium influx, in parts of the neuron where UV light directly activated LiGluR as well as parts of the neuron outside this region (Figure 3D, right). The false color maps show normalized change in fluorescence intensity immediately following UV stimulation, the same scale is used for both maps (arbitrary units).

Long-term activation of LiGluR for time-lapse imaging studies with optical readout

One advantage of LiGluR is its noninvasive nature. Compared to traditional electrophysiological methods, a neuron can be stimulated for a longer period of time without loss of cell health and membrane integrity. Additionally, the MAG photoswitch can be driven to activate and deactivate the channel for hundreds of cycles without any noticeable loss of current (Janovjak et al., 2010). I examine here the long-term

activation of LiGluR, using functional imaging to monitor channel activation over for 1-2 hours. Cultured hippocampal neurons were transfected with iGluR6(L439C) and dsRed, loaded with the Fluo-4-AM calcium indicator dye and labeled with MAG. The functional imaging technique was needed since whole cell patch clamp recordings are limited to ~40 minutes per single cell. Also, Fluo-4 was chosen for its high fluorescence emission, which allows use at lower intracellular concentrations and thus lower toxicity (Gee et al., 2000). Activating LiGluR every ~1 minute for over 1 hour resulted in robust calcium influxes (Figure 4A) with minimal deleterious effects on cell health. Calcium influx measured at the soma is shown as a change in Fluo-4 fluorescence intensity normalized to the cell's initial fluorescence ($\Delta F/F$). Each full field UV illumination elicits a sharp increase ranging from 50-120%; later peaks have a smaller $\Delta F/F$ due to a slight shift in the baseline over the time course. This drift in the Z-focal plane is manually corrected in later experiments.

Combining long-term neuron stimulation with an optical readout requires balancing the UV light used to activate LiGluR with the illumination to visualize the fluorescently-tagged protein of interest. In experiments described in Chapter 3 of this thesis, a variant of green fluorescent protein is used which requires excitation at 488-nm. Since illumination at longer wavelengths (400 – 600 nm) drives isomerization of MAG to its *trans*-conformation and closes the channel (Volgraf et al., 2006), we examined the effects of 488-nm imaging on LiGluR activation. Functional calcium imaging in cultured hippocampal neurons is setup as before, a LiGluR-positive cell is repeatedly stimulated with a brief pulse of UV illumination over the full field-of-view while Fluo-4 fluorescence is measure. Each cycle differs in the 488-nm imaging protocol used, ranging from constant illumination to illumination at 5-second intervals. Fluorescence traces versus time are superimposed to compare LiGluR-mediated changes in intracellular calcium levels (Figure 4B). In the first cycle Fluo-4 fluorescence was imaged continuously with 488-nm light except during the 500ms pulse of UV (black lines with filled square symbols), resulting in moderate calcium influx (~20% fluorescence increase) after activation which persisted for ~10 seconds. The next cycles then imaged with 488-nm light at 1, 3, and 5 second intervals (black lines with star, open square, and open circle symbols, respectively) resulting in larger calcium influxes (~30% fluorescence increase) that persisted for up to 60 seconds. The last cycle used the same continuous 488-nm imaging as the first cycle, to see that there had been no change in the dye, cell or channel over the experiment and that the different calcium traces were due purely to the different 488-nm illumination protocols. This last cycle (gray line with filled square symbols) had the same result as the first cycle, as expected. This is in agreement with previous experiments showing that LiGluR-induced photocurrents persist in the dark period following UV illumination (Gorostiza et al., 2007), delaying 488-nm illumination thus allows the channel to remain open longer. These results indicate that in order to achieve full, chronic neuronal stimulation, imaging at longer wavelengths following LiGluR activation should be minimized. This

is necessary even when using low-intensity illumination; this experiment for example used minimal 488-nm light intensity (controlled by adjusting the output power of the Argon laser), a setting that was 30 times lower than that of the 380-nm light. Using minimal intensity was possible here because Fluo-4 has high fluorescence emission; this may not be possible in future experiments where small features and/or weakly fluorescing proteins are imaged. If higher excitation power is necessary, the protocol for longer-wavelength imaging should be at longer intervals and delayed from the UV stimulus as much as possible.

Co-expression of iGluR6(L439C) with PSD-95 drives LiGluR to postsynaptic sites in dendritic spines

Previously, we examined the cellular distribution of iGluR6(L439C) by either visualizing a GFP-tagged version of the channel (Szobota et al., 2007) or with immunocytochemical methods using a GluR6/7 antibody. Expression of iGluR6(L439C)-GFP was examined 2-3 days post-transfection by confocal microscopy and was found at high levels at the plasma membrane, distributed fairly evenly throughout the neuron (Szobota et al., 2007). Immunocytochemistry results confirmed that transfected neurons had much higher levels of iGluR6 compared to their untransfected neighbors and that these receptors were expressed at the soma and throughout the dendritic tree (Figure 5A, left). Co-immunostaining with an antibody directed against axonal neurofilaments (anti-SMI 312, Covance, Inc. (Ulfig et al., 1998)) indicates that iGluR6(L439C) is primarily expressed in the dendrites (Figure 5A, right). The GluR6/7 antibody (Chemicon/Millipore Corporation) recognizes native receptors as well iGluR6(L439C), in untransfected neurons the native receptors had a similar cellular localization (levels very low, only faintly visible in image shown, Figure 5A left).

An important feature of LiGluR is the ability to target the channel to certain cell types using specific promoters, or to particular subcellular regions using signaling motifs. For instance, expressing LiGluR in the dendritic tree of excitatory neurons results in its presence along the dendritic branches and concentration at postsynaptic sites. Such targeting allows light-activated depolarization and calcium entry to be locally induced at synaptic sites. A method for driving LiGluR expression to postsynaptic sites is to co-express iGluR6(L439C) with PSD-95. PSD-95, also known as SAP90, is a scaffolding protein enriched at postsynaptic sites; together with its binding partners GKAP, Shank, and Homer, the scaffolding complex constitutes the majority amount of proteins present in the postsynaptic density. PSD-95 contains multiple PDZ binding domains which interact with a number of important synaptic proteins such as adhesion molecules, glutamate receptors, G-protein coupled receptors, and voltage-gated ion channels (Kim and Sheng, 2004; Han and Kim, 2008; Sheng and Hoogenraad, 2007). Since PSD-95 binds the native iGluR6 kainate receptor subunit

(Garcia et al., 1998; Coussen, 2009) we hypothesize that it should also bind the transfected iGluR6(L439C).

Cultured hippocampal neurons were co-transfected with iGluR6(L439C) and PSD-95–GFP, fixed and immunostained to visualize the untagged iGluR6(L439C), then imaged with confocal microscopy. Expression of iGluR6(L439C) was now driven to dendritic branches and concentrated at dendritic spines (Figure 5B, left). The GluR6/7 protein level in transfected cells was again much higher than that of untransfected cells. Expression of PSD-95–GFP was also strong, with the fluorescent signal strongest in the dendritic branches and spines (Figure 5B, right). Previous studies have shown that overexpression of PSD-95 in hippocampal neurons enhances dendritic spine numbers, size, and maturation (El-Husseini et al., 2000; Nikonenko et al., 2008; Sturgill et al., 2009); this enlargement of spines may also help to accommodate increased iGluR6(L439C) at these synaptic sites. The overlay image (Figure 5C) shows good correlation between iGluR6(L439C) and PSD-95–GFP. The magnified view shows that the iGluR6(L439C) is concentrated at spines and overlaps with PSD-95–GFP, though is also present in the dendritic shaft as discussed before. Functional imaging experiments were also performed for this set of neurons (X-Rhod-1-AM was loaded into cells as described above) to verify that PSD-95–GFP co-expression or this localization of iGluR6(L439C) does not interfere with LiGluR function. No difference was seen in LiGluR-mediated calcium signals for these neurons compared to control neurons expressing iGluR6(L439C) with eGFP or dsRed (data not shown). This co-expression system is a robust method for driving LiGluR localization at synaptic sites, while still maintaining high expression levels.

Expression of iGluR6(L439C) increases mEPSC frequency but not amplitude

We overexpress the iGluR6 receptor subunit because MAG conjugation requires the L439C point mutation as an anchor point and therefore cannot confer photosensitivity to endogenous receptors. We wanted to see whether overexpression of iGluR6(L439C) affected latent properties of transfected neurons by measuring spontaneous miniature postsynaptic currents (mEPSC). Two days after transfection we measured spontaneous mEPSCs from neurons expressing either iGluR6(L439C) with YFP or YFP alone as a control. We found no difference in mEPSC amplitude; iGluR6 -YFP co-expressing cells (n=8) had a mean amplitude of 13.13 ± 2.84 pA while YFP expressing cells (n=6) had a mean amplitude of 13.03 ± 5.49 pA (p-value 0.966, unpaired Student's t-test). Surprisingly, mEPSC frequency increased with iGluR6 overexpression though it should be noted that variability here was large; for iGluR6 -YFP co-expressing cells (n=8) the mean frequency was 0.41 ± 0.25 Hz while YFP expressing cells (n=6) had a mean frequency of 0.11 ± 0.09 Hz (p-value 0.011, unpaired Student's t-test). To further test whether the overexpressed iGluR6 subunits led to a modified culture network, we added

6,7-dinitroquinoxaline-2,3-dione (DNQX, an AMPA and kainate receptor antagonist) to the culture media immediately following the transfection to block the kainate receptors. After 2 days of protein expression with DNQX spontaneous mEPSCs were measured as before. Both transfection conditions had similar mEPSCS frequencies and amplitudes as the previous experiment. iGluR6 -YFP co-expressing cells (n=5) had a mean amplitude of 13.58 ± 1.15 pA while YFP expressing cells (n=8) had a mean amplitude of 11.91 ± 5.06 pA (p-value 0.290, unpaired Student's t-test). iGluR6 -YFP co-expressing cells (n=5) had a mean frequency of 0.35 ± 0.17 Hz while YFP expressing cells (n=8) had a mean frequency of 0.07 ± 0.04 Hz (p-value 0.024, unpaired Student's t-test). For each transfection condition neither mEPSC frequencies nor amplitudes were statistically different between DNQX-treated and non-treated cultures. We concluded that overexpressing iGluR6(L439C) minimally affects the network properties of the culture.

Discussion

I have outlined the optimization of subcellular LiGluR activation in cultured hippocampal neurons, discussing critical parameters for the targeted illumination and examining iGluR6(L439C) protein expression. The light-activation protocol considers both the 380-nm light for activation and the 488-nm light for measuring output, setting maximum power levels for robust photostimulation while maintaining good spatially resolution. Additionally I illustrated a co-expression system for driving channel expression to postsynaptic sites. By co-transfecting LiGluR with PSD-95 light-triggered depolarization and calcium influx can be localized to postsynaptic densities.

In the following chapter LiGluR activation is combined with time-lapse imaging of fluorescently-tagged AMPA receptors. AMPA receptors are a well established expression locus of different modes of synaptic plasticity (Bredt and Nicoll, 2003; Malenka and Bear, 2004). A previous study examined the effects of endogenous ionotropic glutamate receptor activation on AMPA receptor distribution in cultured hippocampal neurons. Upon activation with 100 μ M glutamate bath application, rapid re-localization of GluR1-containing receptors was seen away from synaptic sites to intracellular dendritic shafts and cell bodies (Lissin et al., 1999). Other studies have examined changes in AMPA receptor expression in response to various pharmacological agents via a culture-wide bath application (O'Brien et al., 1998; Ju et al., 2004; Wierenga et al., 2005). LiGluR is an ideal tool to build upon these studies, to examining AMPA receptor expression and trafficking in response to single-cell and subcellular stimulation. Establishing a targeted illumination system and verifying the spatial resolution of light activation is critical for this study. The conditions for long-term light activation are also set to maximize the noninvasive nature of LiGluR, enabling the study of processes that require prolonged, chronic manipulation. These

parameters were set on a high resolution scanning confocal microscope so that small features (such as synaptic receptors) can be properly visualized.

The technique of co-expressing LiGluR with PSD-95, a known iGluR6-binding postsynaptic scaffolding protein, effectively drives LiGluR to dendritic spines while maintaining light-driven depolarization and calcium influx functions. However, in the next set experiments examining changes in AMPA receptor surface expression in response to LiGluR, PSD-95 co-expression is not used for two reasons. First, the technical aspect of co-transfecting three large proteins (iGluR6(L439C), PSD-95, and a fluorescently-tagged AMPA receptor subunit) becomes more challenging and transfection efficiencies decrease. Additionally, it decreases the overall expression levels of LiGluR and of the AMPA receptor (since there is a maximum amount of DNA that can be taken up by each neuron regardless of composition). Secondly, PSD-95 directly affects targeting of AMPA receptors to synaptic sites via interaction through stargazin (Chen et al., 2000; Bats et al., 2007) and increasing PSD-95 levels has been shown to increase the number of synaptic AMPA receptors (Schnell et al., 2002). Since overexpression of PSD-95 in hippocampal neurons enhances both clustering and activity of glutamate receptors at postsynaptic sites (El-Husseini et al., 2000), it seems likely that PSD-95 overexpression would interfere with the behavior of the AMPA receptors being studied. Therefore, PSD-95 co-expression is omitted in future experiments. On a technical note, it was important to see here that LiGluR can be successfully co-expressed with other functionally important neuronal proteins, establishing that proteins of interest can be fluorescently tagged and genetically expressed for an optical readout in future experiments. Though a smaller amount of iGluR6(L439C) DNA was used (reduced by 20-50% compared to neurons expressing LiGluR alone), comparable light-activated calcium responses were measured.

The mEPSCs measured in this study had amplitudes similar to other studies, though the frequency of events seemed lower than average. Electrophysiological measurements were made on several different days on several different batches of neuron cultures. Though there was some culture-to-culture variation, overall neurons that received the same treatment were similar enough to be pooled. It's possible that the mEPSC frequency, generally regarded as an indicator of presynaptic inputs to the patched cell, may be somewhat low here due to the young age of the culture (9-10DIV). The mEPSC amplitude, generally an indicator of postsynaptic glutamate receptor expression, is comparable to other reports signifying that the cultures have mature postsynaptic receptor expression. Also, overexpression of the iGluR6 subunit does not affect behavior of this glutamate receptor population since addition of the kainate receptor antagonist DNQX did not change mEPSC amplitudes. This is important since maintaining normal network properties as much as possible before beginning experiments where activity is directly manipulated will ensure more physiologically relevant results.

This chapter describes a spatially-controlled neuronal stimulation setup designed to be coupled with time-lapse imaging; this system is used in the next chapter to study global versus local mechanisms of synaptic scaling, specifically changes in the surface expression of synaptic GluR2-containing AMPA receptors. It is straightforward to then extend this setup to study the behavior of other proteins important in maintaining synaptic function and specificity. This general methodology can also be applied to study other processes mediated by depolarization and changes in intracellular calcium levels, for example local protein synthesis (Bramham, 2008; Steward and Schuman, 2001), Ras activity (Harvey et al., 2008), or CaMKII activation (Rose et al., 2009).

Materials and Methods

HEK cell culture and transfection

HEK293 cells were plated at approximately 6×10^6 cells per coverslip on 25 mm poly-L-lysine coated glass coverslips and maintained in DMEM with 5% fetal bovine serum, 0.2 mg/mL streptomycin, and 200 U/mL penicillin. Cells were co-transfected with 0.9 μ g iGluR6(L439C) and 0.1 μ g dsRed using Lipofectamine 2000 (Invitrogen).

Hippocampal neuron culture and transfection

Dissociated hippocampal neurons prepared from postnatal rats (P0-P5) were plated on poly-L-lysine coated glass bottom dishes (MatTek). Cells were cultured in MEM supplemented with 5% fetal bovine serum, B27 (Invitrogen), GlutaMAX™ (Invitrogen) and serum extender (BD Biosciences). Ara-C (4 μ M) was added after 5 DIV. After 7-9 DIV, cells were transfected with 2 μ g of DNA per 12 mm coverslip by the calcium phosphate method and used 3-7 days later. Cells were co-transfected with either iGluR6(L439C) and dsRed at a 2:1 ratio or iGluR6(L439C) and PSD-95-GFP at a 1:1 ratio.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in DPBS. iGluR6/7 kainate receptor subunits were detected using rabbit monoclonal anti-GluR6/7 [recognizing the C-terminal cytoplasmic domain (Chemicon/Millipore Corporation)]. Axons were identified using the mouse monoclonal antibody SMI 312 [(recognizing highly phosphorylated axonal epitopes of neurofilaments (Covance, Inc.))]. An Alexa647-conjugated anti-rabbit secondary antibody and a Cy3-conjugated anti-mouse secondary antibody were used for

visualization (Invitrogen/Life Technologies). Samples were mounted in Fluoromount-G (Electron Microscopy Sciences) and imaged on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective.

Calcium Imaging

HEK cell experiments were performed 24-48 hours post transfection. Cells were loaded with 5 μ M Fluo-4 AM (Molecular Probes/Invitrogen) for 30 minutes at 37°, washed 3 times to remove any remaining dye from the bath, then de-esterified for 20 minutes at 37°. During the de-esterification process cells were also conjugated with 15 μ M MAG-1 as previously described (Volgraf et al., 2006). Prior to start of experiments cells were treated with 300 mg/l concanavalin A type IV (Sigma) for 10 minutes to block desensitization (Wilding and Huettnner, 1997; Partin et al., 1993). Measurements were performed in an extracellular bath solution containing (in mM): 135 NaCl, 5.4 KCl, 0.9 MgCl₂, 1.8 CaCl₂, 10 HEPES and 10 glucose, pH 7.6.

Hippocampal neuron experiments were performed 3-7 days post transfection. Neuron cultures were loaded with either 5 μ M Fluo-4 AM (Molecular Probes) or 5 μ M X-Rhod-1 AM (Molecular Probes) for 30 minutes at room temperature, washed 3 times to remove any remaining dye from the bath, then de-esterified for 30 minutes. During the de-esterification process neurons were also conjugated with MAG-0 as previously described (Szobota et al., 2007). After MAG conjugation cultures were washed twice with a HEPES-buffered extracellular solution then transferred to new extracellular solution for imaging. The extracellular solution contained (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4.

Time series images were acquired on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective. A Spectra-Physics MaiTai HP laser was used to activate LiGluR with 380-nm light (frequency doubled 760-nm). Five baseline dye images were taken, followed by varying UV illumination protocols depending on the experiment, then fifteen dye images to record changes in intracellular calcium. Images were acquired at 0.07-2 Hz, for Fluo-4 experiments a 488nm Argon laser was used to excite the dye and a 510-560 nm band-pass filter was used to collect fluorescent emission. For X-Rhod-1 experiments a 543-nm HeNe laser was used to excite the dye and a 565-615 nm band-pass filter was used to collect fluorescent emission.

Whole-cell patch clamp

Patch-clamp recordings used an Axopatch 200A amplifier in the whole-cell mode. Recordings were carried out 1–5 days after transfection in hippocampal neurons, cells were voltage-clamped at about –65 mV. Pipettes had resistances of 2–5 M Ω and were

filled with a solution containing (in mM), 135 K-gluconate, 10 NaCl, 10 HEPES, 2 MgCl₂, 2 MgATP, 1 EGTA, pH 7.4. The extracellular recording solution was (in mM) 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. To measure mEPSC action potential firing, inhibitory inputs, and synaptic transmission were blocked with 1μM TTX, 20μM bicuculline, and 50μM D(-)-amino-7-phosphonovaleric acid (AP5). Excitatory neurons were identified morphologically by visualizing cytoplasmic the YFP fill (Rutherford et al., 1997; Watt et al., 2000). Electrophysiological data were recorded with pClamp software (Axon Laboratories) and analyzed using Mini Analysis Program (Synaptosoft Inc.).

Image analysis

Images were exported from Zeiss LSM software for analysis in MATLAB (The MathWorks, Inc). Calcium imaging experiments are quantified by measuring the fluorescence intensity of each cell or region at each time point and then normalizing by the baseline level for that region. Baseline fluorescence is determined by averaging 3-5 initial frames, intentionally taken prior to any manipulation. Regions of interest were identified by a dsRed or eGFP image to visualize transfected cells.

References

- Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002). An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* 99, 12651 -12656.
- Ando, R., Mizuno, H., and Miyawaki, A. (2004). Regulated Fast Nucleocytoplasmic Shuttling Observed by Reversible Protein Highlighting. *Science* 306, 1370-1373.
- Andresen, M., Stiel, A. C., Trowitzsch, S., Weber, G., Eggeling, C., Wahl, M. C., Hell, S. W., and Jakobs, S. (2007). Structural basis for reversible photoswitching in Dronpa. *Proceedings of the National Academy of Sciences* 104, 13005 -13009.
- Ashby, M. C., Ibaraki, K., and Henley, J. M. (2004a). It's green outside: tracking cell surface proteins with pH-sensitive GFP. *Trends Neurosci* 27, 257-261.
- Ashby, M. C., De La Rue, S. A., Ralph, G. S., Uney, J., Collingridge, G. L., and Henley, J. M. (2004b). Removal of AMPA Receptors (AMPA Rs) from Synapses Is Preceded by Transient Endocytosis of Extrasynaptic AMPARs. *J. Neurosci.* 24, 5172-5176.
- Bats, C., Groc, L., and Choquet, D. (2007). The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53, 719-734.
- Bramham, C. R. (2008). Local protein synthesis, actin dynamics, and LTP consolidation. *Curr. Opin. Neurobiol* 18, 524-531.
- Bredt, D. S., and Nicoll, R. A. (2003). AMPA Receptor Trafficking at Excitatory Synapses. *Neuron* 40, 361-379.
- Chen, L., Chetkovich, D. M., Petralia, R. S., Sweeney, N. T., Kawasaki, Y., Wenthold, R. J., Bredt, D. S., and Nicoll, R. A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408, 936-943.
- Coussen, F. (2009). Molecular determinants of kainate receptor trafficking. *Neuroscience* 158, 25-35.

- El-Husseini, A. E., Schnell, E., Chetkovich, D. M., Nicoll, R. A., and Brecht, D. S. (2000). PSD-95 involvement in maturation of excitatory synapses. *Science* 290, 1364-1368.
- Garcia, E. P., Mehta, S., Blair, L. A. C., Wells, D. G., Shang, J., Fukushima, T., Fallon, J. R., Garner, C. C., and Marshall, J. (1998). SAP90 Binds and Clusters Kainate Receptors Causing Incomplete Desensitization. *Neuron* 21, 727-739.
- Gee, K. R., Brown, K. A., Chen, W. N., Bishop-Stewart, J., Gray, D., and Johnson, I. (2000). Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium* 27, 97-106.
- Gorostiza, P., Volgraf, M., Numano, R., Szobota, S., Trauner, D., and Isacoff, E. Y. (2007). Mechanisms of photoswitch conjugation and light activation of an ionotropic glutamate receptor. *Proceedings of the National Academy of Sciences* 104, 10865 -10870.
- Gulledge, A. T., Kampa, B. M., and Stuart, G. J. (2005). Synaptic integration in dendritic trees. *J. Neurobiol* 64, 75-90.
- Han, K., and Kim, E. (2008). Synaptic adhesion molecules and PSD-95. *Prog. Neurobiol* 84, 263-283.
- Harvey, C. D., Yasuda, R., Zhong, H., and Svoboda, K. (2008). The spread of Ras activity triggered by activation of a single dendritic spine. *Science* 321, 136-140.
- Ibata, K., Sun, Q., and Turrigiano, G. G. (2008). Rapid Synaptic Scaling Induced by Changes in Postsynaptic Firing. *Neuron* 57, 819-826.
- Janovjak, H., Szobota, S., Wyart, C., Trauner, D., and Isacoff, E. Y. (2010). A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing. *Nat Neurosci* 13, 1027-1032.
- Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., Garner, C. C., Tsien, R. Y., Ellisman, M. H., and Malenka, R. C. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci* 7, 244-253.
- Kim, E., and Sheng, M. (2004). PDZ domain proteins of synapses. *Nat Rev Neurosci* 5, 771-781.

- Lissin, D. V., Carroll, R. C., Nicoll, R. A., Malenka, R. C., and Zastrow, M. V. (1999). Rapid, Activation-Induced Redistribution of Ionotropic Glutamate Receptors in Cultured Hippocampal Neurons. *J. Neurosci.* *19*, 1263-1272.
- Magee, J., Hoffman, D., Colbert, C., and Johnston, D. (1998). Electrical and calcium signaling in dendrites of hippocampal pyramidal neurons. *Annu. Rev. Physiol* *60*, 327-346.
- Magee, J. C., and Carruth, M. (1999). Dendritic voltage-gated ion channels regulate the action potential firing mode of hippocampal CA1 pyramidal neurons. *J. Neurophysiol* *82*, 1895-1901.
- Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* *44*, 5-21.
- Marriott, G., Mao, S., Sakata, T., Ran, J., Jackson, D. K., Petchprayoon, C., Gomez, T. J., Warp, E., Tulyathan, O., Aaron, H. L., et al. (2008). Optical lock-in detection imaging microscopy for contrast-enhanced imaging in living cells. *Proceedings of the National Academy of Sciences* *105*, 17789 -17794.
- Miesenböck, G., De Angelis, D. A., and Rothman, J. E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* *394*, 192-195.
- Nikonenko, I., Boda, B., Steen, S., Knott, G., Welker, E., and Muller, D. (2008). PSD-95 promotes synaptogenesis and multiinnervated spine formation through nitric oxide signaling. *J. Cell Biol* *183*, 1115-1127.
- Numano, R., Szobota, S., Lau, A. Y., Gorostiza, P., Volgraf, M., Roux, B., Trauner, D., and Isacoff, E. Y. (2009). Nanosculpting reversed wavelength sensitivity into a photoswitchable iGluR. *Proceedings of the National Academy of Sciences* *106*, 6814 -6819.
- O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D., and Huganir, R. L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* *21*, 1067-1078.
- Partin, K. M., Patneau, D. K., Winters, C. A., Mayer, M. L., and Buonanno, A. (1993). Selective modulation of desensitization at AMPA versus kainate receptors by

- cyclothiazide and concanavalin A. *Neuron* 11, 1069-1082.
- Rose, J., Jin, S., and Craig, A. M. (2009). Heterosynaptic Molecular Dynamics: Locally Induced Propagating Synaptic Accumulation of CaM Kinase II. *Neuron* 61, 351-358.
- Rutherford, L. C., DeWan, A., Lauer, H. M., and Turrigiano, G. G. (1997). Brain-Derived Neurotrophic Factor Mediates the Activity-Dependent Regulation of Inhibition in Neocortical Cultures. *J. Neurosci.* 17, 4527-4535.
- Sankaranarayanan, S., De Angelis, D., Rothman, J. E., and Ryan, T. A. (2000). The Use of pHluorins for Optical Measurements of Presynaptic Activity. *Biophysical Journal* 79, 2199-2208.
- Schnell, E., Sizemore, M., Karimzadegan, S., Chen, L., Brecht, D. S., and Nicoll, R. A. (2002). Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proceedings of the National Academy of Sciences of the United States of America* 99, 13902 -13907.
- Sheng, M., and Hoogenraad, C. C. (2007). The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu. Rev. Biochem* 76, 823-847.
- Steward, O., and Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci* 24, 299-325.
- Sturgill, J. F., Steiner, P., Czervionke, B. L., and Sabatini, B. L. (2009). Distinct domains within PSD-95 mediate synaptic incorporation, stabilization, and activity-dependent trafficking. *J. Neurosci* 29, 12845-12854.
- Sutton, M. A., Wall, N. R., Aakalu, G. N., and Schuman, E. M. (2004). Regulation of Dendritic Protein Synthesis by Miniature Synaptic Events. *Science* 304, 1979-1983.
- Szobota, S., Gorostiza, P., Del Bene, F., Wyart, C., Fortin, D. L., Kolstad, K. D., Tulyathan, O., Volgraf, M., Numano, R., Aaron, H. L., et al. (2007). Remote Control of Neuronal Activity with a Light-Gated Glutamate Receptor. *Neuron* 54, 535-545.
- Thomas, P., and Smart, T. G. (2005). HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* 51, 187-200.

- Ulfig, N., Nickel, J., and Bohl, J. (1998). Monoclonal antibodies SMI 311 and SMI 312 as tools to investigate the maturation of nerve cells and axonal patterns in human fetal brain. *Cell Tissue Res* 291, 433-443.
- Volgraf, M., Gorostiza, P., Numano, R., Kramer, R. H., Isacoff, E. Y., and Trauner, D. (2006). Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat Chem Biol* 2, 47-52.
- Watanabe, S. Y., Albsoul-Younes, A. M., Kawano, T., Itoh, H., Kaziro, Y., Nakajima, S., and Nakajima, Y. (1999). Calcium phosphate-mediated transfection of primary cultured brain neurons using GFP expression as a marker: application for single neuron electrophysiology. *Neuroscience Research* 33, 71-78.
- Watt, A. J., van Rossum, M. C., MacLeod, K. M., Nelson, S. B., and Turrigiano, G. G. (2000). Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. *Neuron* 26, 659-670.
- Wierenga, C. J., Ibata, K., and Turrigiano, G. G. (2005). Postsynaptic Expression of Homeostatic Plasticity at Neocortical Synapses. *J. Neurosci.* 25, 2895-2905.
- Wilding, T. J., and Huettner, J. E. (1997). Activation and desensitization of hippocampal kainate receptors. *J. Neurosci* 17, 2713-2721.

Chapter 2 Figures

Figure 1. A targeted illumination system for spatial control of genetically-encoded light activated receptors

Figure 2. Functional imaging to measure spatially restricted LiGluR activation

Figure 3. Subcellular activation of LiGluR in hippocampal neurons

Figure 4. Long-term activation of LiGluR for time-lapse imaging studies with optical readout

Figure 5. Co-expression of iGluR6(L439C) with PSD-95 drives LiGluR to postsynaptic sites in dendritic spines

Figure 6. Expression of iGluR6(L439C) increases mEPSC frequency but not amplitude

Figure 1

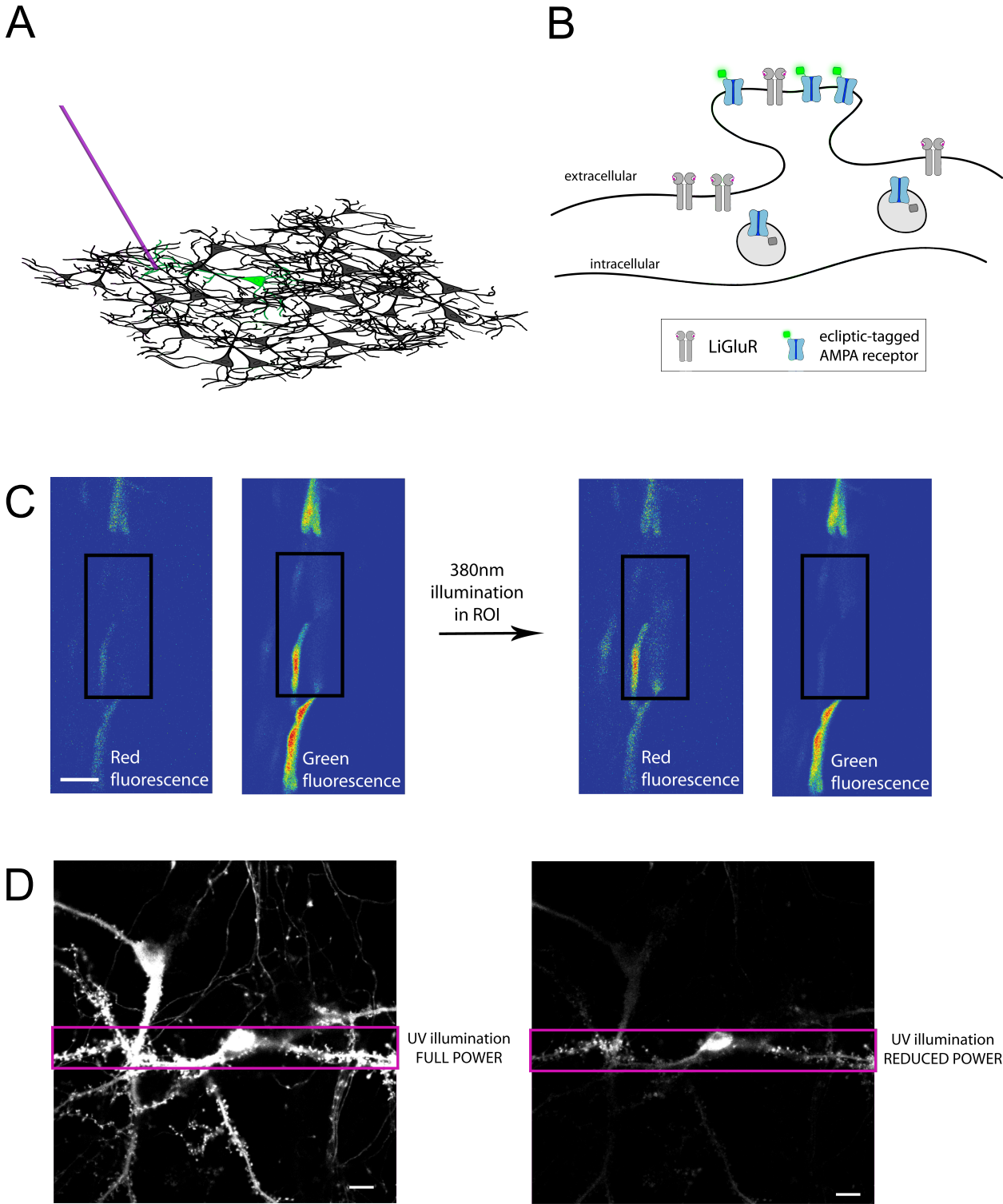


Figure 1. A targeted illumination system for spatial control of genetically-encoded light activated receptors

- A). LiGluR can be expressed in a subset of cultured neurons (shown as green cell), providing a method to probe a single cell within a complex network. Precise, targeted illumination allows further spatial resolution, enabling study of cellular responses to subcellular activation.
- B). LiGluR can be co-expressed with a protein of interest within the same cell. By fluorescently-tagging the protein different properties such as expression levels or localization can be measured as they change in response to LiGluR activation. Co-expressed with an ecliptic-tagged AMPA receptor subunit, LiGluR can be used to assess changes in AMPA receptor surface expression in response to local depolarization and calcium influx.
- C). Spatial characterization of UV laser illumination on high-resolution scanning confocal microscopy using kaede-expressing zebrafish larvae. The segment in the field of view is partially illuminated with 380-nm light, only the kaede protein in the specified region is converted from green fluorescence to red fluorescence. Scale bar is 20 μ m.
- D). Cultured hippocampal neurons expressing dronpa-tagged actin. In both panels the UV illumination to activate the dronpa protein is spatially limited (in the y-axis direction) but the laser power is varied. The entire field of view is scanned with 488-nm light to image the photo-converted protein. At maximum laser power (left) the 380-nm light passing through the confocal scan head diffracts and photo-converts dronpa throughout the entire field. Using a neutral density filter to reduce the power of the transmitted light results in dronpa-actin being converted only in the defined region; the rest of the field remains in the dark state. Scale bar is 10 μ m.

Figure 2

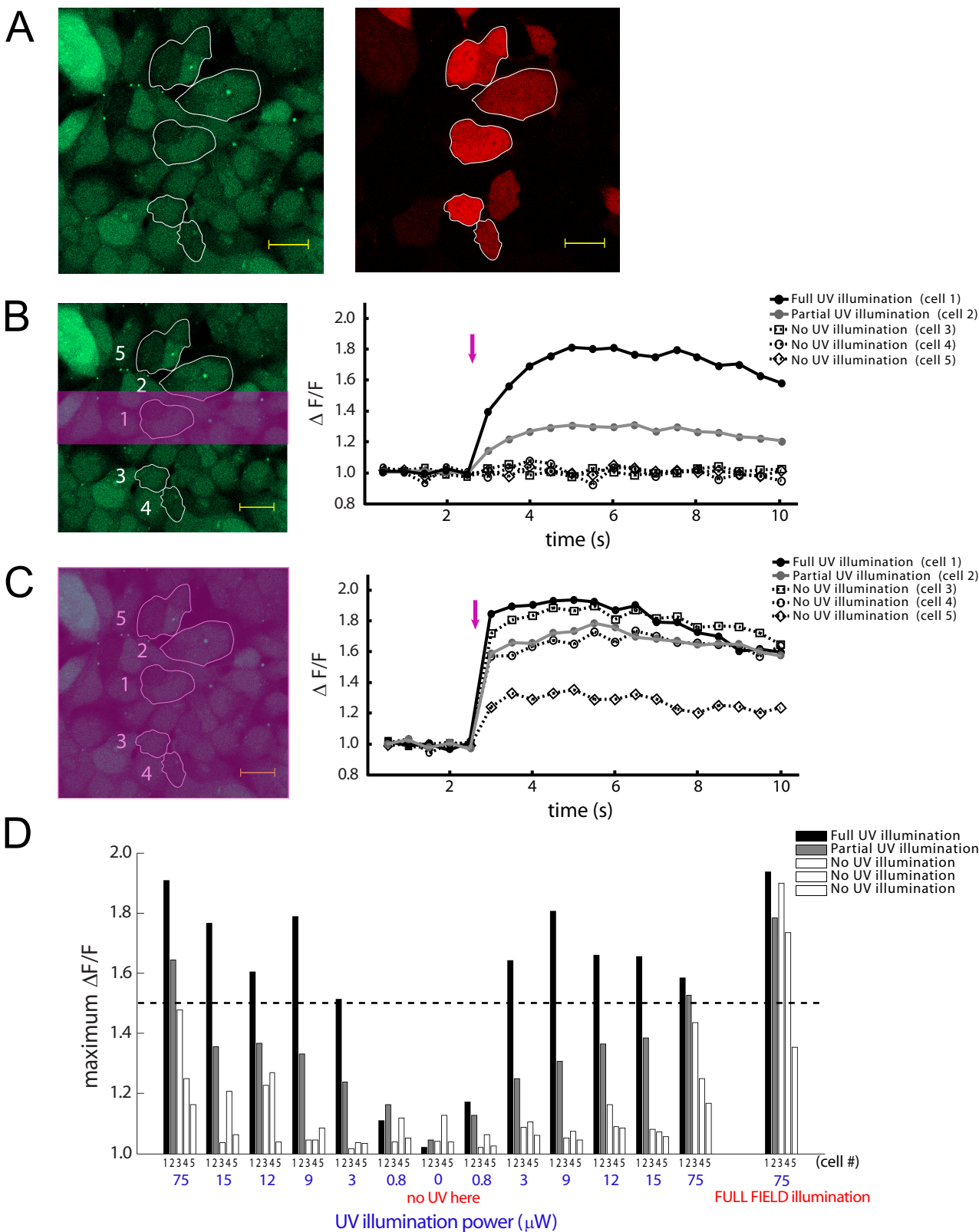


Figure 2. Functional imaging to measure spatially restricted LiGluR activation

- A). HEK293 cells loaded with Fluo-4 calcium indicator dye (green, left). Subset of HEK293 cells co-expressing LiGluR (iGluR6 L439C) and dsRed (red, right). Cells outlined in white as regions for fluorescence measurements. Scale bar is 20 μ m.
- B). Fluo-4 fluorescence image (left), magenta box indicates region of UV illumination, white outlines indicate borders and identities of cells corresponding to intensity trace on right. Fluo-4 fluorescence intensity trace over time (right) showing response to UV illumination (magenta arrow). Cell #1 is completely illuminated with 380-nm light and has large calcium influx, cell #2 is partially illuminated with 380-nm light and has modest calcium influx, cells #3-5 are not illuminated with 380-nm light at all and have no calcium influx. Change in fluorescence normalized to average of five baseline images before UV illumination. Scale bar is 20 μ m.
- C). Fluo-4 fluorescence image (left), magenta box indicates that full field of view is illuminated with UV light, white outlines indicate borders and identities of cells corresponding to intensity trace on right. Fluo-4 fluorescence intensity trace over time (right) showing response to UV illumination (magenta arrow). All cells are completely illuminated with 380-nm light and have large calcium influx. Change in fluorescence normalized to average of five baseline images before UV illumination. Scale bar is 20 μ m.
- D). Bar graph depicting maximum fluorescence change seen per cycle of stimulation. Each group of bars indicates one trial (one UV stimulation), each bar representing the cells as mapped above. All stimulations illuminate only the region shown in (B) except for the last which is the full field as shown in (C). The power of the 380-nm light (indicated in blue text) is varied to see that at very high power settings illumination will diffract and stimulate out-of-field cells. Cycle #7 had no UV illumination at all (indicated in red text) but indicates that there is some minor fluctuations in Fluo-4 fluorescence over time course; these are small compared to direct UV activation for each cell as seen in the last cycle.

Figure 3

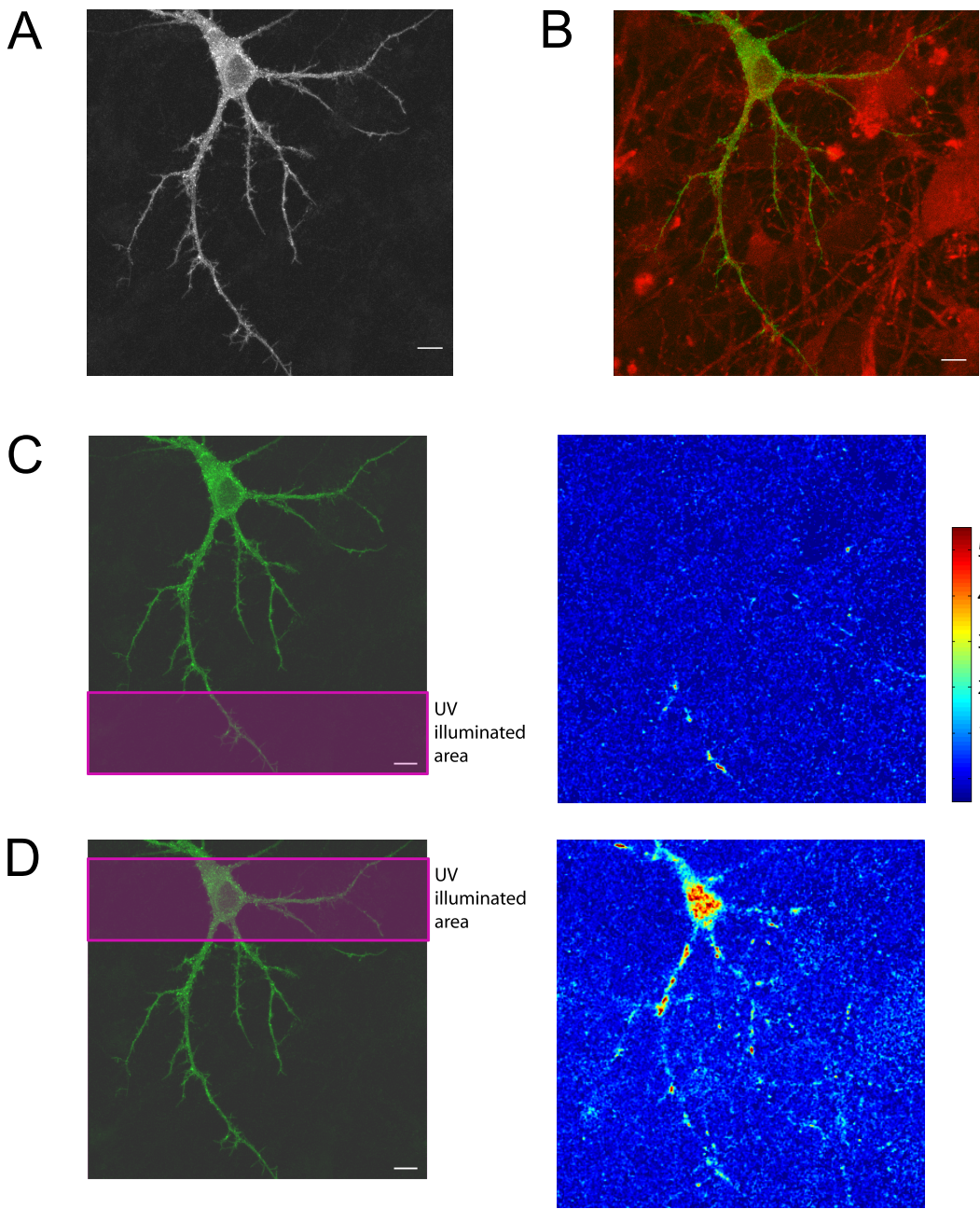


Figure 3. Subcellular activation of LiGluR in hippocampal neurons

- A). LiGluR in cultured hippocampal neuron, green fluorescence (left panel and green in right panel) allows visualization of dendritic processes of the transfected cell while X-Rhod-1-AM dye loaded into all neurons (red in right panel) measures intracellular calcium levels. Scale bar is 10 μ m.
- B). UV illumination (a 142 μ m $\times$ 35 μ m window) over a dendritic region (left) results in calcium influx in dendrites within the area but no detectable calcium increase in the soma or other dendrites (right). Change is fluorescence normalized by initial fluorescence before UV stimulus. Scale bar in arbitrary units.
- C). UV illumination (a 142 μ m $\times$ 35 μ m window) over the soma (left) results in strong calcium influx at the soma with smaller increases through the dendritic tree. Though the same UV illumination window is used here as in (B), a larger portion of the transfected cell is illuminated, leading to a greater rise in intracellular calcium levels. Change is fluorescence normalized by initial fluorescence before UV stimulus. Same scale bar as (B).

Figure 4

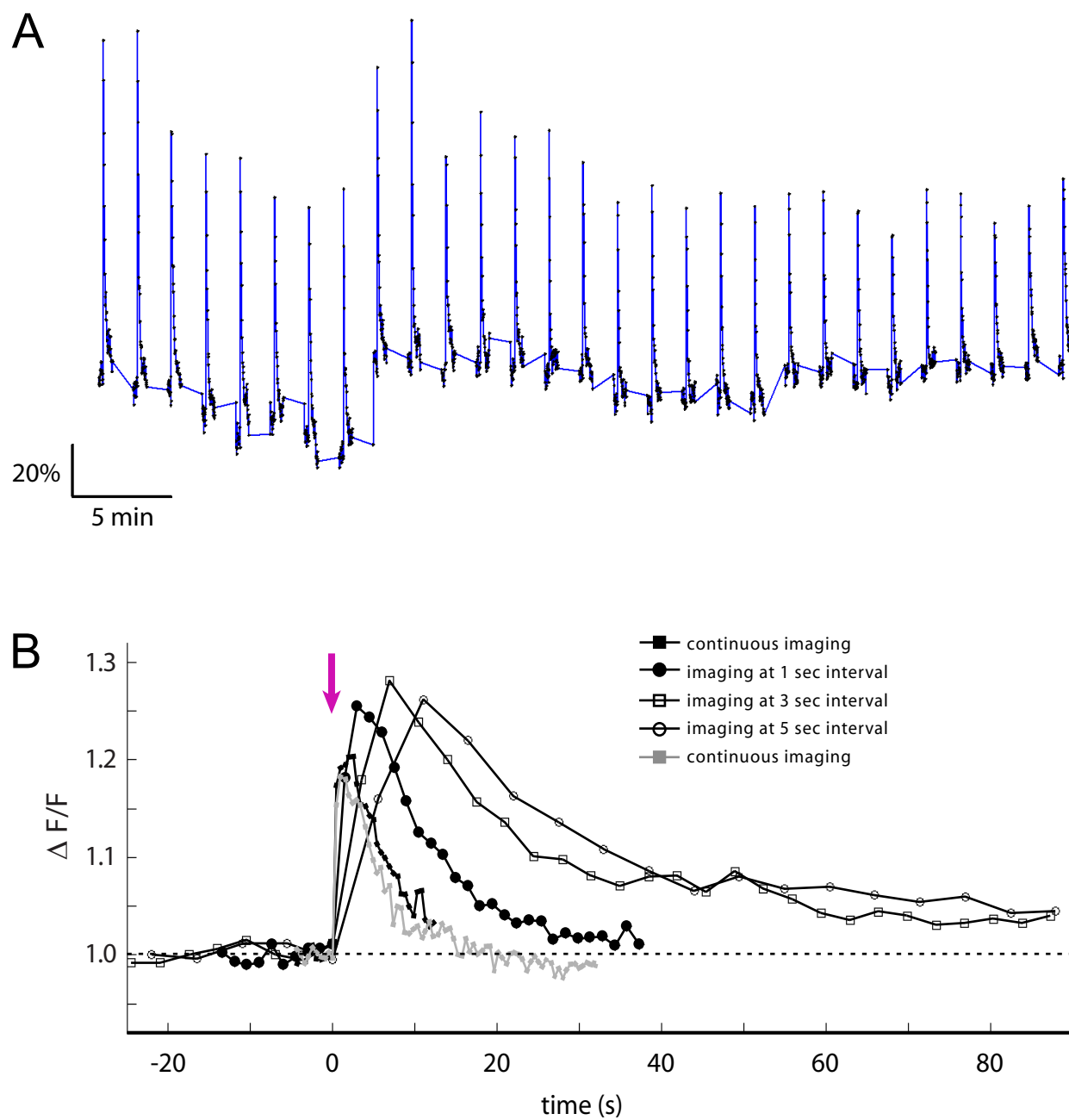
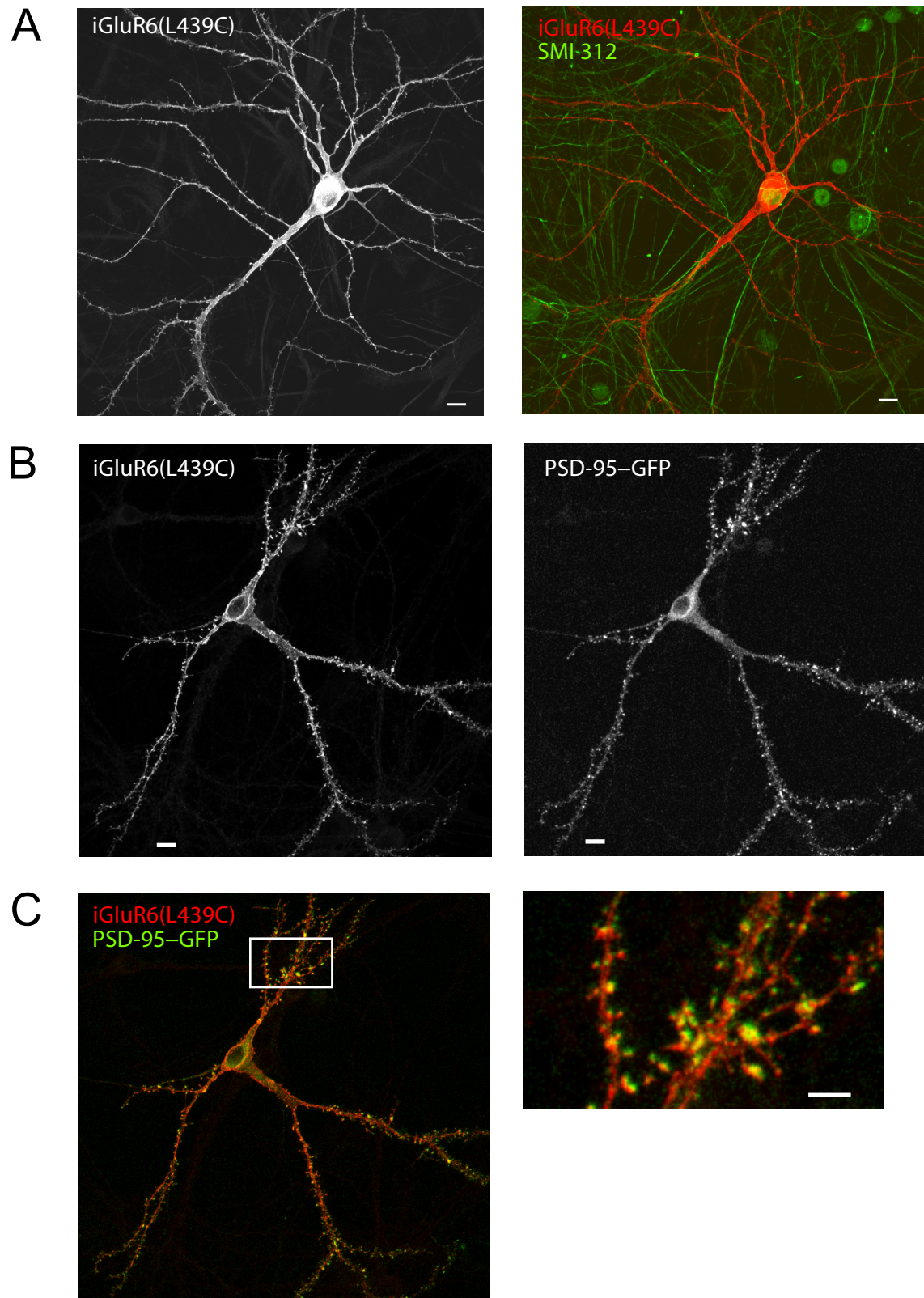


Figure 4. Long-term activation of LiGluR for time-lapse imaging studies with optical readout

- A). Trace of intracellular calcium levels from functional imaging of Fluo-4 calcium indicator dye in hippocampal neuron activated by LiGluR. LiGluR is activated approximately every 2 minutes, with reliable neuron stimulation for over one hour. Change in fluorescence intensity normalized to initial images taken before UV illumination, baseline shift due to slight drift in Z-focus over time.
- B). LiGluR activation for one cell but with Fluo-4 calcium responses measured at different time intervals. Although a low intensity 488-nm light is used to image Fluo-4 (approximately 30× less power than the 380-nm light), this illumination closes the LiGluR channel, therefore less frequent imaging (once every 1, 3, 5 seconds) gives larger and more sustained calcium influx. One cycle of continuous 488-nm imaging is done before and after the interval imaging to check consistency of the system.

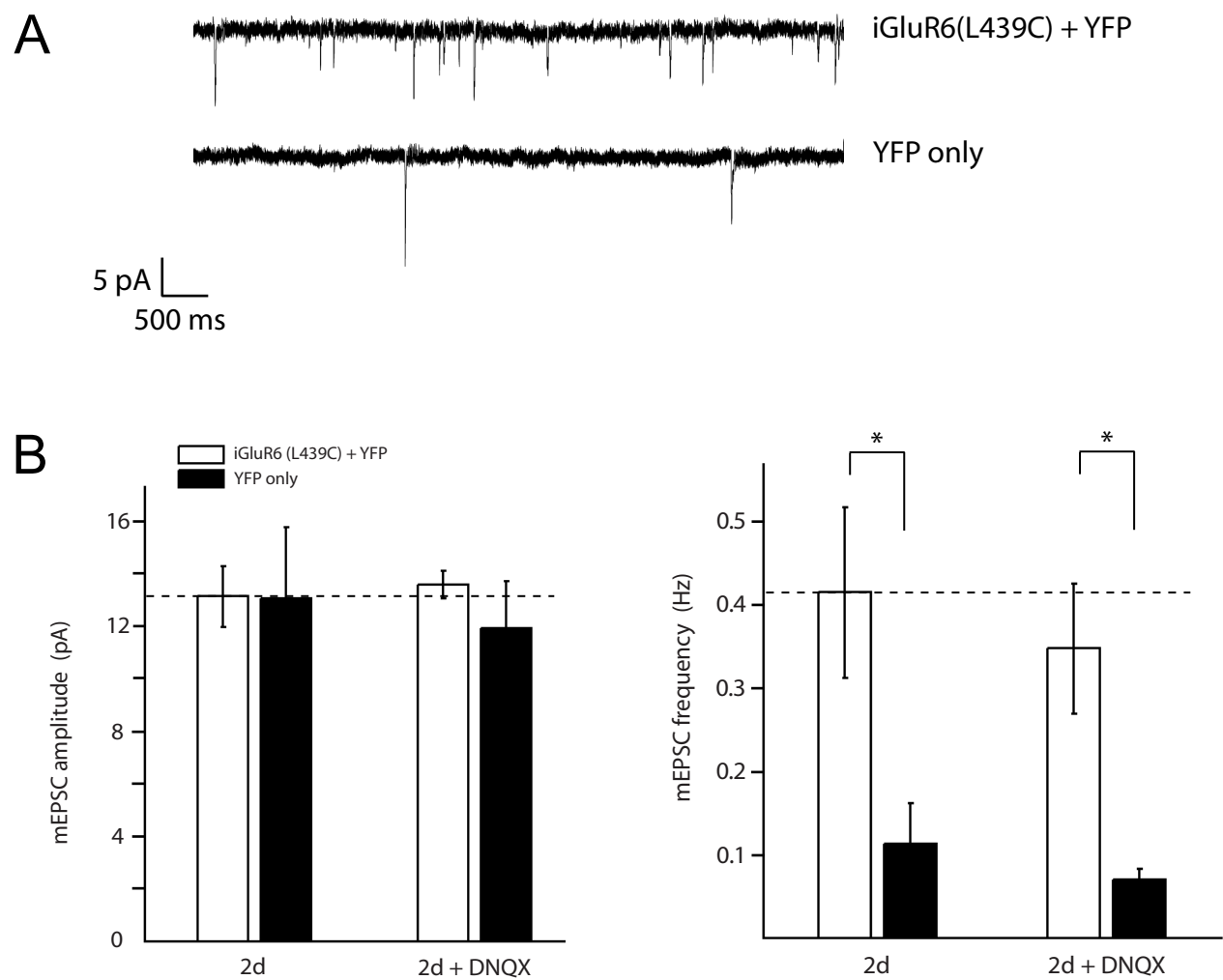
Figure 5



**Figure 5. Co-expression of iGluR6(L439C) with PSD-95 drives
LiGluR to postsynaptic sites in dendritic spines**

- A). Cultured hippocampal neurons were transfected iGluR6(L439C), then fixed and co-immunostained for iGluR6/7 (left and red signal on right) and for axonal neurofilaments using the SMI 312 antibody (green signal on right). iGluR6(L439C) was strongly expressed in the soma and evenly through the dendritic tree. The anti-iGluR6/7 antibody labeled iGluR6(L439C) as well as native receptors, iGluR6(L439C) expressed at such high levels however that only iGluR6(L439C) is visualized at sub-saturating gain settings. Scale bar is 10 μ m.
- B). Cultured hippocampal neurons were co-transfected with PSD-95–GFP (right) and iGluR6(L439C) (left) at 9DIV. Cells were fixed and immunostained for iGluR6/7 at 16DIV. iGluR6(L439C) is present in the dendritic shaft but more strongly concentrated in dendritic spines where it co-localized with PSD-95–GFP. Scale bar is 10 μ m.
- C). Overlay of iGluR6(L439C) and PSD-95–GFP images. Right: detailed view of dendritic spines where PSD-95 and iGluR6 most overlap. Scale bar is 5 μ m.

Figure 6



**Figure 6. Expression of iGluR6(L439C) increases mEPSC frequency
but not amplitude**

- A). Sample traces of mEPSC recordings from cultured hippocamal neuron expressing either iGluR6(L439C) and YFP or YFP alone as a control. Sister cultures from the same preparations were transfected 7DIV and electrophysiological recordings taken 9DIV in solution containing 1 μ M TTX, 20 μ M bicuculline, and 50 μ M AP5.
- B). mEPSCs were recorded from neurons expressing either iGluR6(L439C) + YFP or YFP alone for two days with no difference in mEPSC amplitude (left, first pair of bars) but with increased mEPSC frequency in the iGluR6 expressing cells (right, first pair of bars). Experiment was repeated with DNQX added at the time of transfection (to block effects of kainite receptor overexpression); again no difference was seen in mEPSC amplitude (left, second pair of bars) but iGluR6 overexpression increased mEPSC frequency (right, second pair of bars). For each transfection condition, no difference was seen between non-DNQX and DNQX treatment. * p-value > 0.03, unpaired Student's t-test.

CHAPTER 3:

Global depression of AMPAR surface expression mediated by local LiGluR activation

Summary

Synaptic scaling is a mechanism that neurons use to maintain homeostatic levels of excitability, allowing them to increase their sensitivity when excitatory inputs are weak and to decrease sensitivity at times of elevated excitation. Scaling has been characterized in response to long-term genetic manipulations of excitability in the entire cell or in groups of cells, and also in response to shorter-term pharmacological manipulations of entire cultures or brain regions. The scaling elicited by these global manipulations has been most thoroughly detected as changes in the number of AMPA receptors (AMPA), throughout the entire dendritic tree of the cell. Since natural changes in excitatory drive to a neuron are inhomogeneously distributed in the dendrites, we wondered if local excitation would elicit scaling responses only at the site of excitation or would a global response be triggered. To test this we monitored changes in the surface expression of AMPARs in response to local activation of excitatory iGluR6 kainate receptors. The location and timing of dendritic excitation were precisely defined by using LiGluR: the light-gated version of iGluR6. The experiments were done in the presence of TTX to prevent action potential (AP) firing and to sensitize the neurons to excitation. We found that local excitation in a distal sub-region of the dendritic tree induced a global reduction in the surface population of AMPARs at dendritic spines. The loss of AMPARs began within 20-30 minutes and leveled off within 1 hour. This scaling response was blocked by chelating intracellular calcium with BAPTA-AM, blocking voltage dependent calcium channels with cadmium, or inhibiting the activity of calcium/calmodulin-dependent protein kinases with KN-93. Imaging of internal calcium with a fluorescent calcium indicator dye showed that the optical excitation of LiGluR in the distal dendrite does not lead to a calcium rise outside of the illuminated area, indicating that the spread of the homeostatic effect propagates via another means. We consider the possibility that phosphorylated CaMKII, which has been shown to be highly mobile, spreads throughout the dendrite to give a global homeostatic reduction in AMPA receptor surface levels.

Introduction

Many mechanisms of plasticity have now been identified, revealing the ways in which neurons may sense their own activity level and accordingly adjust their properties to maintain stability (Abbott and Nelson, 2000; Turrigiano and Nelson, 2004). Synaptic scaling is one important mechanism that neurons use to maintain homeostatic levels of excitability, allowing them to increase their sensitivity to low-levels of input and to decrease sensitivity in times of elevated activity (Turrigiano, 2008). These adjustments, which prevent saturation and uncontrollable levels of excitation, have been verified in

several systems, including cultured neurons (Thiagarajan et al., 2005; O'Brien et al., 1998), hippocampal slices (McKinney et al., 1999; Kim and Tsien, 2008; Kirov et al., 2004), the *Drosophila* neuromuscular junction (Frank et al., 2006), and in intact animals (Knogler et al., 2010; Desai et al., 2002; Mrcic-Flogel et al., 2007). A canonical example experiment is to suppress all network activity with tetrodotoxin (TTX), resulting in a slow compensatory increase in synapse strength. Conversely, enhancing network activity by blocking inhibitory inputs results in decreased strength of excitatory synapses (Turrigiano et al., 1998). Previous studies have shown that synapse strength can be scaled by altering the levels of surface AMPA receptors (Lissin et al., 1998), for example GluR1 and GluR2 heteromeric AMPARs insertion was seen in response to prolonged TTX application (Wierenga et al., 2005). Most AMPA receptors are heteromeric, with the specific subunit composition determining channel properties, vesicular trafficking, and synaptic targeting (Greger et al., 2007). GluR2-lacking receptors are permeable to calcium while channels containing the GluR-2 subunit are rendered calcium-impermeable by editing of the Q/R site in >99% of GluR2 mRNAs (Bredt and Nicoll, 2003; Seeburg et al., 1998). The subunit composition also determines trafficking to synaptic sites (Shi et al., 2001), recycling (Lee et al., 2004), and its role in LTP/LTD (Bredt and Nicoll, 2003).

Previous studies have examined synaptic scaling by perturbing activity at the network-wide level (O'Brien et al., 1998; Thiagarajan et al., 2005) or at a single neuron level, for example by expressing an inward-rectifier potassium channel to chronically hyperpolarize only that cell (Burrone et al., 2002). Though homeostasis is generally considered to be a global process – a negative feedback system to tune overall sensitivity, recent studies have used more spatially restricted manipulations to examine local synaptic changes. A subset of a neuron's presynaptic inputs was blocked using local TTX perfusion, resulting in no change in GluR2-containing AMPAR accumulation in any part of the neuron (Ibata et al., 2008). This was consistent with another study that found that inhibiting presynaptic input by chronically expressing the tetanus toxin light chain in hippocampal cultures did not increase AMPARs, GluR2 and GluR2/3 accumulation at these synapses did not change while there was a slight decrease in GluR1 (Harms et al., 2005). Another study, however, found that inhibiting presynaptic neurons with chronic expression of an inward-rectifier potassium channel resulted in synapses for this neuron having elevated levels of AMPAR GluR1 and GluR2/3 subunits on the postsynaptic side (Hou et al., 2008). Both these studies relied on immunocytochemical comparisons between different fixed cultures, not longitudinal studies of the same synapses. Interestingly, addition of local NMDA receptor blockade (by local perfusion) to global activity blockade (bath application of TTX), GluR1-homomers increased surface expression via a protein-synthesis dependent mechanism (Sutton et al., 2006). It has not been investigated whether synaptic AMPARs are scaled down when activity is raised in a local region of the dendritic tree, or whether a significant increase in somatic calcium is required.

Here we examine scaling of excitatory synapses in response to increased depolarization and calcium influx on a local level. We employ the light-activated glutamate receptor, LiGluR, to depolarize cultured hippocampal neurons in a spatially control manner over the course of hours. Addition of TTX to the bath solution restricts the LiGluR signal from traveling through the dendritic tree via back-propagating action potentials and also sensitizes neurons to the LiGluR activity manipulation. By monitoring a fluorescently tagged version of the GluR2 subunit we find that chronic depolarization and Ca^{2+} influx in a portion of the dendritic tree reduces the surface population of AMPARs at dendritic spines in all parts of the neuron. Loss of AMPARs occurred within the first hour, and puncta decreased fluorescence intensity at similar rates and to similar levels regardless of whether they were localized to a region that was directly activated by LiGluR or not. The effect of LiGluR activation was blocked by chelating intracellular calcium with BAPTA-AM, blocking voltage dependent calcium channels with cadmium, or inhibiting the activity of calcium/calmodulin-dependent protein kinases with KN-93.

Results

Experiments were performed on cultured rat hippocampal neurons co-transfected with LiGluR (for subcellular depolarization and calcium influx) and with a super ecliptic pHluorin tagged GluR2 subunit (SEP-GluR2) for assessing changes in surface AMPA receptor populations. The GluR2 subunit has been shown to be required for synaptic scaling (Gainey et al., 2009), and activity-blockade has been shown to enhance GluR2-containing receptors on the rapid timescale studied here (Ibata et al., 2008).

Additionally, activation of ionotropic glutamate receptors has been shown to rapidly alter surface AMPAR expression, redirecting GluR1-containing receptors away from synapses to intracellular localizations (GluR2 not tested) (Lissin et al., 1999).

Expressing LiGluR and SEP-GluR2 in the same neuron allows us to assay changes to the post-synaptic surface AMPARs in response to local depolarization and calcium influx.

Several hours prior to any LiGluR activation or SEP-GluR2 imaging neuron cultures are pre-treated with $1\mu\text{M}$ TTX. This activity blockade increases postsynaptic sensitivity to glutamate, increasing the effectiveness of LiGluR activation (Watt et al., 2000; Desai et al., 1999). Experiments were carried out in an extracellular bath solution containing $1\mu\text{M}$ TTX to continue activity blockade in the culture and also to prevent LiGluR signal propagation via action potential firing. Distal regions of the dendritic branch were illuminated with UV light to produce local LiGluR activation without raising somatic calcium.

SEP-GluR2 fluorescence represents surface population of receptors, which exhibit canonical synaptic scaling behavior

Typically, homeostatic scaling of excitatory synapses is induced by blocking activity in the neuronal cultures with TTX. Increased accumulation of AMPA receptors at synaptic sites is measured by recording mEPSCs, by imaging fluorescently tagged AMPAR subunits, or by immunocytochemical staining for AMPAR subunits (Turrigiano, 2008). A recent study measuring fluorescence intensity changes of eYFP-tagged GluR2 subunits showed that this type of activity blockade induces accumulation of AMPA receptors at synaptic sites much more rapidly than previously thought, on the timescale of hours as opposed to days (Ibata et al., 2008). We performed a similar experiment to confirm that SEP-GluR2 is a faithful reporter of TTX-induced synaptic scaling on this timescale. We began by confirming that SEP-GluR2 fluorescence accurately represents the surface population of GluR2-containing AMPAR. SEP-GluR2 is fluorescent when present on the cell surface and is exposed to the pH ~ 7.4 extracellular environment; the fluorescence dims when present intracellularly due to the acidic environment of the vesicles (Ashby et al., 2004a). We transfected cultured hippocampal neurons with SEP-GluR2 and imaged live cells with confocal microscopy; fluorescence was seen along the membrane with expression primarily in the dendrites and concentrated in bright puncta at dendritic spines (Figure 1A).

To assess whether these GluR2-containing AMPA receptors are a faithful reporter of synaptic scaling, transfected hippocampal cultures were imaged before TTX addition to measure baseline fluorescence, then 1 μ M TTX was added to the extracellular bath solution and the same cells were imaged 60 and 120 minutes later. Control cultures were imaged the same way, with a saline solution added in place of TTX. Fluorescence of individual SEP-GluR2 puncta was measured at each time point and normalized to its initial intensity value ($\Delta F/F$). Cultures where TTX was added increased fluorescence compared to control cultures which showed a slight decrease in fluorescence (Figure 1B). After 60 minutes, $\Delta F/F$ for the TTX condition was 1.12 ± 0.05 ($n=7$ cells) while $\Delta F/F$ for the control was 0.92 ± 0.19 ($n=8$ cells), p -value = 0.0202. At 120 minutes, $\Delta F/F$ for the TTX condition was 1.15 ± 0.11 while $\Delta F/F$ for the control was 0.90 ± 0.18 , p -value = 0.0097. This increase in puncta fluorescence indicates rapid modulation of AMPA receptors in response to activity blockade.

To confirm that the fluorescent puncta imaged at dendritic spines are receptors expressed at the surface, a pH 6.0 solution was briefly washed over neuron cultures. The fluorescence along the edges of the dendrites and at the spines was rapidly and reversibly dimmed in this low pH condition (Figure 1C), confirming that the SEP-GluR2 imaged represents surfaced-expressed receptors (Ashby et al., 2004b). To see what fraction of receptors is expressed at the cell membrane compared to the entire population, neurons were washed with a pH 7.4 solution containing 50mM NH_4Cl solution to collapse the cellular pH gradient. By equilibrating intracellular

compartments to pH 7.4 the entire SEP-GluR2 population can be visualized (Ashby et al., 2004b; Martin et al., 2008). The fluorescence increased most at the soma (not shown) as well as inside the dendritic shafts (Figure 1C). These normally dim regions increased fluorescence intensity by a factor of 2-3 (Figure 1D, blue ROI), compared to dendritic spines, which were minimally affected (Figure 1D, green ROI). Thus experiments that analyze changes in synaptic surface AMPARs only considered SEP-GluR2 fluorescence that is concentrated in puncta at dendritic spines.

LiGluR activation inhibits TTX-mediated synaptic scaling (indicated by SEP-GluR2 fluorescence)

To examine the effect of local depolarization and calcium influx on synaptic scaling we activated LiGluR in a portion of distal dendrites while measuring SEP-GluR2 fluorescence intensity in all regions of the neuron.

To begin the synaptic scaling process and condition the neurons to be more sensitized to the LiGluR manipulation, 1 μ M TTX was added to the neuron cultures approximately 12 hours before beginning LiGluR activation and time-lapse imaging. A small region of the dendrites (generally 10-15% of the dendritic tree) was illuminated with 380-nm light to locally activate LiGluR (Figure 2A). A region 50-100 μ m away from the soma was chosen to ensure that there was no significant rise in the somatic calcium level. In LiGluR activated neurons SEP-GluR2 puncta decreased fluorescence intensity by 17.13% in the UV region and 16.64% in the dark region (n=6 cells, p-values 0.009997 and 0.0189 respectively, for paired Student's t-test with initial values) over the course of 2 hours (Figure 2B), indicating a loss of surface GluR2-containing AMPA receptors. Only SEP-GluR2 fluorescent puncta at dendritic spines were considered in the analysis (Figure 2C), each punctum was tracked separately at each time point and the change in intensity was normalized to the punctum's own initial fluorescence. Interestingly, SEP-GluR2 puncta within the UV-illuminated region and in the non-UV illuminated region (the dark region) behaved similarly, both showing decreased fluorescence intensity in the LiGluR group (Figure 2D) and steady fluorescence intensity in the control group. No significant difference was seen between puncta in the UV region and in the dark region for any of the cells.

Control cultures also co-expressing iGluR6(L439C) and SEP-GluR2 received the same TTX treatment, and were illuminated with the same light protocol however they were not labeled with the photoswitch MAG. This allowed us to assess the effect of repeated UV illumination on the fluorescent protein and overall cell health. No significant difference was detected between SEP-GluR2 puncta in the UV illuminated region and the dark region (Figure 2E). SEP-GluR2 puncta in this control condition slightly increased fluorescence over the course of 2 hours though not significantly compared to their initial level. Fluorescence intensity increased by 10.51% in the UV

region and by 2.81% in the dark region (n=4 cells, p-values 0.1741 and 0.5673 respectively, for paired Student's t-test with initial values). Although time-lapse imaging experiments are performed in the presence of TTX, the fluorescence level is expected to remain mostly stable since the rate of change is greatest in the first few hours of activity blockade (Ibata et al., 2008) and this is 12-15 hours past initial blockade. Compared with this no-MAG control, SEP-GluR2 fluorescence in LiGluR-activated cultures decreased significantly in both the UV region and the dark region (p-value = 0.0053 and 0.0151 respectively, unpaired Student's t-test).

Neuron cultures in a second control condition also co-expressed iGluR6(L439C) and SEP-GluR2, were conjugated with the photoswitch MAG, and received the same TTX treatment as the LiGluR condition. These cultures however, received no UV illumination (and thus no LiGluR activation) in any part of the neuron. This was to ensure that changes in SEP-GluR2 intensity in the LiGluR experiment resulted from either direct (UV region) or indirect (dark region) LiGluR activation and not from simply having MAG conjugated to iGluR6(L439C). As expected, SEP-GluR2 fluorescence intensity did not decrease over the two hour time course (Figure 2F). Neurons in this condition were imaged with the same 488-nm light protocol as the LiGluR condition and the first control condition. After 120 minutes, SEP-GluR2 fluorescence in this second control condition increased by 9.64% and was significantly different from the LiGluR condition (n=11 control cells and 6 LiGluR cells, p-value = 0.0002, unpaired Student's t-test with LiGluR UV region; p-value = 0.0002, unpaired Student's t-test with LiGluR dark region).

For LiGluR samples, after the 2 hour time course, the calcium indicator dye X-Rhod-1 was loaded into the cells to confirm that local UV illumination did not significantly increase somatic calcium (data not shown). These calcium imaging experiments are outlined in Chapter 2 of this thesis.

Effect of LiGluR activation on SEP-GluR2 fluorescence requires change in intracellular calcium levels

Next we investigated whether the effect on surface AMPA receptors by LiGluR activation depends on changes in intracellular calcium levels. As shown previously (Szobota et al., 2007) and Chapter 2 of this thesis, activation of LiGluR depolarizes the neuron and results in a large influx of calcium. To determine whether this rise in cytoplasmic calcium was a key component in causing decreased surface AMPA receptors, we pre-loaded neurons with BAPTA-AM to chelate intracellular calcium (Paschen et al., 2003). Following this, neurons were conjugated with the MAG photoswitch and transferred to the imaging setup for time-lapse experiments with LiGluR activation as before. As the overall health of cultures in this condition was less robust than in the other conditions, data collected past 60 minutes was discarded.

We observed that blocking the rise of cytoplasmic calcium precluded the effect of LiGluR activation on surface AMPA receptors, such that SEP-GluR2 fluorescence no longer decreased over the time course. The fluorescence intensity of puncta slightly increased after 60 minutes (Figure 3A), for the same LiGluR activation and 1 μ M TTX treatment as before. As with previous experiments, there was no distinction between SEP-GluR2 puncta in the UV region with direct LiGluR activation and in the dark region with indirect LiGluR effects (Figure 3B). After 60 minutes puncta in the UV region increased by 4.87% and puncta in the dark region increased by 9.31%, which was not significantly different from each other (n=4 cells, p-value 0.4725, unpaired Student's t-test). Likewise, neither condition significantly changed from its own initial fluorescence (p-values 0.2420 and 0.1578 respectively, paired Student's t-test).

The effect of LiGluR activation on SEP-GluR2 puncta fluorescence occurs within the first hour of activation and is significantly precluded in cells pre-loaded with BAPTA-AM (Figure 3C). For the UV region, LiGluR non-BAPTA-loaded cells (n=6) decreased fluorescence by 9.03% after 30 minutes and by 14.38% after 60 minutes compared to LiGluR BAPTA-loaded cells (n=4) which increased fluorescence by 2.34% and 4.87%, respectively (p-values 0.1444 and 0.0261 at 30 and 60 minutes respectively, unpaired Student's t-test). For the dark region, LiGluR non-BAPTA-loaded cells decreased fluorescence by 10.18% after 30 minutes and by 14.40% after 60 minutes compared to LiGluR BAPTA-loaded cells which increased fluorescence by 5.52% and 9.31%, respectively (p-values 0.0266 and 0.0085 at 30 and 60 minutes respectively, unpaired Student's t-test). This led us to conclude that LiGluR activation affected surface AMPA receptors by increasing cytoplasmic calcium levels.

LiGluR-mediated calcium influx requires voltage dependent calcium channel activation to depress SEP-GluR2 fluorescence

LiGluR activation depolarizes the cell by a current carried primarily by sodium, though the LiGluR channel also conducts a small amount of calcium (Volgraf et al., 2006; Kohler, 1993). Since a large calcium influx is seen upon LiGluR activation, native voltage-gated calcium channels are presumably activated in response to depolarization (Isaeva et al., 1998; Condliffe et al., 2010). Since the LiGluR-mediated decrease in AMPAR surface expression depends on altered cytoplasmic calcium levels, we next tested whether the influx through voltage-dependent calcium channels (VDCC) was a key component. Neuron cultures expressing SEP-GluR2 and LiGluR were conjugated with MAG, imaged with 1 μ M TTX in the bath solution, and partially activated with LiGluR as before. For these experiments cadmium was also added to the bath solution to block VDCC (Chow, 1991; Swandulla and Armstrong, 1989; Taylor, 1988).

Since LiGluR is constructed from the iGluR6 kainate receptor subunit, cadmium should not block current through the LiGluR channel (Brorson et al., 1992, 1994). To

be certain nonetheless, the effect of 100 μ M Cd²⁺ on LiGluR activation was tested prior to time-lapse imaging experiments. Neurons co-transfected with SEP-GluR2 and LiGluR were loaded with the calcium-indicator dye X-Rhod-1, and then labeled with the MAG photoswitch (Figure 4A). LiGluR was activated with full field 380-nm illumination and Ca²⁺ responses for the transfected cell were measured at the soma (Figure 4B). Before addition of cadmium, LiGluR activation produced a ~80% increase in intracellular calcium (Figure 4C). Cadmium (100 μ M) was then added to the recording solution and the same LiGluR activation was applied to the cell. The resulting calcium rise was substantially smaller (~40%, Figure 4D), indicating that a portion of the normal LiGluR calcium response arises from activation of VDCC, while a portion of the calcium influx travels through LiGluR itself. This reduced calcium signal is not a loss of LiGluR function upon repeated stimulation (Szobota et al., 2007; Janovjak et al., 2010) or an artifact of the calcium indicator dye. Previous experiments with this dye and similar dyes showed that LiGluR activation can be measured in similar cycles with little loss of signal amplitude (Chapter 2, Figure 4).

Next, time-lapse imaging experiments were performed – LiGluR was subcellularly activated with 1 μ M TTX and 100 μ M cadmium in the bath solution and SEP-GluR2 fluorescence was monitored. Activating LiGluR with Cd²⁺ blockade prevented the decrease in SEP-GluR2 fluorescence over the course of two hours (Figure 4E). After 60 minutes: UV-region puncta with Cd²⁺ increased fluorescence by 3.60% compared to without Cd²⁺ which decreased by 14.38% (n=6 cells each, p-values 0.0138, unpaired Student's t-test); dark-region puncta with Cd²⁺ increased fluorescence by 0.26% compared to without Cd²⁺ which decreased by 14.40% (n= 6 cells each, p-values 0.0151, unpaired Student's t-test). After 120 minutes: UV-region puncta with Cd²⁺ increased fluorescence by 1.91% compared to without Cd²⁺ which decreased by 17.13% (p-values 0.0246, unpaired Student's t-test); dark-region puncta with Cd²⁺ increased fluorescence by 0.49% compared to without Cd²⁺ which decreased by 16.64% (p-values 0.0243, unpaired Student's t-test).

In the Cd²⁺ condition, as with all preceding cases, no difference was seen between the UV-illuminated regions and the dark regions (p-values = 0.4505 and 0.8447 at 60 and 120 minutes respectively, unpaired Student's t-test). Puncta in neither region were significantly changed from their initial values (UV region: p-values = 0.4549 and 0.6758 at 60 and 120 minutes respectively, dark region: p-values = 0.9459 and 0.8608 at 60 and 120 minutes respectively, paired Student's t-test). These results indicate that the rise of cytoplasmic calcium is important for LiGluR-mediated effects on AMPARs, particularly the large component contributed by VDCC.

LiGluR effect on surface AMPAR dependent on Ca^{2+} /calmodulin-dependent protein kinase activity

Building on the fact that LiGluR activation decreases SEP-GluR2 fluorescence by raising cytoplasmic calcium levels through voltage-dependent calcium channels we next tested whether the family of calcium/calmodulin-dependent protein kinases (CaMKs) is a required component of this effect. Elevated levels of intracellular calcium bind to calmodulin (among other targets) which can then activate CaMKs, initiating a signaling cascade that is critical to neuronal development and plasticity (Wayman et al., 2008). CaMKII, an important member of this family, is enriched at postsynaptic densities and has been implicated in as a key component in LTP induction and persistence (Lisman et al., 2002).

To test the role of CaMKII, neuron cultures co-expressing SEP-GluR2 and LiGluR were set up for time-lapse imaging as before. LiGluR was activated in a portion of the neuron, with $1\mu\text{M}$ TTX in the bath solution along with the CaMKII-specific inhibitor, KN-93. Addition of $5\mu\text{M}$ KN-93 occluded the effect of LiGluR activation on AMPAR surface expression, SEP-GluR2 fluorescence remained stable over the two hour time course (Figure 5A). After 60 minutes: UV region puncta with KN-93 increased fluorescence by 2.22% compared to without KN-93 which decreased by 14.38% ($n=8$ cells and 6 cells respectively, p -values 0.0483, unpaired Student's t -test); dark region puncta with KN-93 increased fluorescence by 3.05% compared to without KN-93 which decreased by 14.40% ($n=8$ cells and 6 cells respectively, p -values 0.0221, unpaired Student's t -test). After 120 minutes: UV region puncta with KN-93 increased fluorescence by 0.66% compared to without KN-93 which decreased by 17.13% (p -values 0.0971, unpaired Student's t -test); dark region puncta with KN-93 increased fluorescence by 1.98% compared to without KN-93 which decreased by 16.64% (p -values 0.0315, unpaired Student's t -test).

Bath application of KN-93 did not produce a difference between behavior of puncta in UV regions versus dark regions (p -values = 0.9127 and 0.8917 at 60 and 120 minutes respectively, unpaired Student's t -test). Puncta fluorescence in neither region were significantly changed from their initial values (UV region: p -values = 0.7644 and 0.9879 at 60 and 120 minutes respectively, dark region: p -values = 0.5068 and 0.6838 at 60 and 120 minutes respectively, paired Student's t -test).

Blocking CaMKII activity precludes the effect of LiGluR activation on SEP-GluR2 fluorescence similarly as blocking calcium directly (Figure 5B,C). Comparing the effects of cadmium and KN-93 bath additions to control cases where LiGluR was not activated at all (either by omitting the MAG photoswitch or by omitting all UV illumination) indicates a fairly complete block of LiGluR-mediated activity. Taken together, that data suggest that LiGluR activation is able to mediate AMPAR surface expression by activating voltage dependent calcium channels, raising cytoplasmic calcium levels, and activating CaMKII.

Discussion

LiGluR is an ideal tool to study local modes of synaptic scaling due to its spatial and temporal properties. By combining targeted illumination and bath application of TTX, LiGluR activation is restricted to a sub-cellular region. As the LiGluR experiments here are performed in the presence of TTX, the effects of depolarization and calcium influx are separated from those of action potentials that would otherwise be generated (Szobota et al., 2007). The non-invasive nature of LiGluR provides reliable, chronic stimulation to study synaptic scaling and is well-suited for coupling with time-lapse imaging of other fluorescently-tagged proteins which can be expressed within the same cell. The UV illumination used to activate the channel did not adversely affect cell health or interfere with photostability of eGFP. The size and location of the UV illumination was chosen to be small and distal such that the somatic calcium level would not be affected, but also large enough such that a reasonable number of puncta could be analyzed per cell. It would be interesting to further reduce the size of the LiGluR activation window to determine whether there is a threshold below which only local changes occur.

A number of molecular mechanisms have been proposed for translating altered neuronal activity levels into scaled AMPAR expression (Turrigiano, 2008), one being changes in intracellular calcium levels since this is an important activity signal in many forms of plasticity (Wayman et al., 2008; Malenka and Bear, 2004). We see that calcium entry is a key component of LiGluR activation-mediated AMPAR reduction. This complements other studies which perform the opposite manipulation, in which synapses are scaled up when voltage dependent calcium channels are blocked (Thiagarajan et al., 2005; Ibata et al., 2008).

A recent study blocked postsynaptic firing at the soma and showed this to be as effective at increasing AMPAR accumulation as blocking firing of the entire culture, suggesting that the somatic calcium level is the key set-point in determining the point where all synapses would be adjusted (Ibata et al., 2008). This study did not examine the effects of locally increasing activity levels, either at the soma or in the dendrites. We therefore focused our study on increasing local calcium influx at portions of the dendritic tree far from the soma, determining that somatic calcium rise is not absolutely necessary to induce AMPAR changes.

A ubiquitous target of calcium at synaptic entry points is CaMKII (Lisman et al., 2002), a kinase which is maximally activated by calcium influx through NMDA receptors and voltage dependent calcium channels. Our results showing that cadmium or KN-93 blockade occluded LiGluR's effect on surface AMPARs suggests an important role for CaMKII. A recent study described the intriguing ability of CaMKII to propagate from a locally activated site (100 μ M glutamate/10 μ M glycine puffed over ~20 synapses in a 20-33 μ m dendritic segment) throughout the entire dendrite and

usually throughout the entire neuron (Rose et al., 2009). This suggests that CaMKII activation could carry some of the LiGluR-mediated effects from areas of direct illumination to other areas of the neuron. Our approach can be extended to follow activated CaMKII instead of AMPAR subunits to test this idea. While KN-93 is primarily used to inhibit CaMKII, off-target effects have been reported on voltage-gated potassium channels (Rezazadeh et al., 2006) and L-type calcium channels (Gao et al., 2006) however, which we can not rule out.

While there is good agreement that AMPAR expression is a reliable measure of synaptic scaling, there are some discrepancies with respect to the subunit composition. Some studies show that activity blockade results in increased GluR1 levels but not GluR2 (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006), while others report synaptic scaling on GluR2-containing AMPARs (O'Brien et al., 1998; Wierenga et al., 2005; Ibata et al., 2008) and recently that the GluR2 subunit itself is required (Gainey et al., 2009). Consistent with these later studies we show a depression of GluR2-containing AMPARs in response to LiGluR activation. We have extended the setup to study ecliptic-tagged GluR1 subunits, monitoring surface expression in response to LiGluR activation. Preliminary results are shown in Appendix 1 of this thesis.

In addition to AMPAR-mediated synaptic scaling, many other forms of homeostatic plasticity have been reported (Sokolova and Mody, 2008; Thiagarajan et al., 2007; Lee et al., 2010). One intriguing idea is whether or not the neuron could sense that LiGluR-activation is the source of increased activity and whether it would be able to down-regulate the LiGluR channel. It has been shown that kainate receptor surface expression is bi-directionally regulated in hippocampal neurons – kainate or NMDA application causes internalization of dendritic iGluR6-containing receptors (Martin et al., 2008). By tagging LiGluR and monitoring its surface expression we could assay the neuron's awareness of our tool, examining if there is decreased local or global surface expression when we chronically activate LiGluR in different regions. Our method employed here for spatially-controlled neuronal stimulation coupled with time-lapse imaging can be extended to study these and other important questions underlying neuronal circuit function.

Materials and Methods

Hippocampal neuron culture and transfection

Dissociated hippocampal neurons prepared from postnatal rats (P0-P5) were plated on poly-L-lysine coated glass bottom dishes (MatTek). Cells were cultured in MEM supplemented with 5% fetal bovine serum, B27 (Invitrogen), GlutaMAX™ (Invitrogen) and serum extender (BD Biosciences). Ara-C (4 μ M) was

added after 5 DIV. After 7-9 DIV, cells were transfected with 2 μ g of DNA per 12 mm coverslip by the calcium phosphate method and used 3-7 days later. Cells were co-transfected with super ecliptic pHluorin tagged GluR2 (SEP-GluR2) and iGluR6(L439C) at a 2:1 ratio.

Live cell confocal imaging – ecliptic GFP experiments

Neuron cultures were imaged in a HEPES-buffered extracellular solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. Cultures were continuously perfused with this solution; with brief washes of either a low pH solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 MES, pH 6.0, or a 50mM NH₄Cl solution to collapse the cellular pH gradient (similar to solution detailed above, replacing equimolar NaCl for NH₄Cl, pH 7.4). Time-lapse images were acquired on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective. Images were acquired at 0.07-2 Hz, a 488nm Argon laser was used to excite the dye and a 510-560 nm band-pass filter was used to collect fluorescent emission.

Live cell confocal imaging – time-lapse experiments

Approximately 12-24 hours before experiments 1 μ M TTX was added to the culture media. For experiments, neurons were transferred a HEPES-buffered extracellular solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. Neurons expressing iGluR6(L439C) were conjugated with MAG-0 as previously described (Szobota et al., 2007). After MAG conjugation cultures were washed three times with extracellular solution and then imaged in new extracellular solution with 1 μ M TTX. For SEP-GluR2 experiments with either LiGluR manipulation or controls without LiGluR manipulation cells from at least two different cultures were used.

Depending on the experiment cadmium (50 μ M) or KN-93 (5 μ M, Tocris bioscience) was also added. Cells from different cultures were used for each of these experiments.

For the BAPTA-AM experiment, neurons were loaded with 10 μ M BAPTA-AM (Tocris bioscience) for 45 minutes, washed 3 times, and then de-esterified for 30 minutes. During the de-esterification process neurons were also conjugated with MAG-0 as previously described (Szobota et al., 2007). Neurons were then transferred to the extracellular solution described above for imaging.

Time-lapse images were acquired on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective. A Spectra-Physics MaiTai HP laser was used to activate LiGluR with 380-nm light (frequency doubled 760-nm)

and a 488-nm Argon laser was used for image acquisition. Three frames were taken as a baseline before any LiGluR activation. Cells were illuminated with UV light for ~1 second every two minutes, over a $40\mu\text{m} \times 140\mu\text{m}$ section of the distal dendrites. A confocal stack imaging the SEP-GluR2 was taken once every 15 minutes.

Calcium imaging

Neuron cultures were loaded with 5 μM X-Rhod1 AM (Molecular Probes) for 20 minutes at room temperature, then de-esterified for 20 minutes. Five baseline images were taken, followed by UV illumination as described above, then fifteen images to record changes in intracellular calcium. Images were acquired through the Zeiss LSM software at 0.5 Hz, a 543-nm HeNe laser was used to excite the dye and a 565-615 nm band-pass filter was used to collect fluorescent emission.

Image analysis

Each LSM confocal stack was imported into MATLAB (The MathWorks, Inc.) as a maximum projected tiff file for compilation and analysis. Motion artifacts in the x-y plane were corrected using DIPimage (www.diplib.org). Punctate SEP-GluR2 features were detected and tracked through the time series using MATLAB scripts from The Matlab Particle Tracking Code Repository (copyright 1997, Daniel Blair and Eric Dufresne). The fluorescence intensity of each punctum at each time point was normalized to its own averaged baseline (3 frames taken prior to any manipulation). Values reported are mean $\pm$ standard error of the mean for the number of neurons indicated. Statistics were performed using the two-tailed, two-sample Student's t test, a p-value of <0.05 was considered statistically significant.

References

- Abbott, L. F., and Nelson, S. B. (2000). Synaptic plasticity: taming the beast. *Nat. Neurosci 3 Suppl*, 1178-1183.
- Ashby, M. C., Ibaraki, K., and Henley, J. M. (2004a). It's green outside: tracking cell surface proteins with pH-sensitive GFP. *Trends Neurosci* 27, 257-261.
- Ashby, M. C., De La Rue, S. A., Ralph, G. S., Uney, J., Collingridge, G. L., and Henley, J. M. (2004b). Removal of AMPA Receptors (AMPA) from Synapses Is Preceded by Transient Endocytosis of Extrasynaptic AMPARs. *J. Neurosci.* 24, 5172-5176.
- Bredt, D. S., and Nicoll, R. A. (2003). AMPA Receptor Trafficking at Excitatory Synapses. *Neuron* 40, 361-379.
- Brorson, J. R., Bleakman, D., Chard, P. S., and Miller, R. J. (1992). Calcium directly permeates kainate/alpha-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptors in cultured cerebellar Purkinje neurons. *Mol. Pharmacol* 41, 603-608.
- Brorson, J., Manzolillo, P., and Miller, R. (1994). Ca²⁺ entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J. Neurosci.* 14, 187-197.
- Burrone, J., O'Byrne, M., and Murthy, V. N. (2002). Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. *Nature* 420, 414-418.
- Chow, R. H. (1991). Cadmium block of squid calcium currents. Macroscopic data and a kinetic model. *J. Gen. Physiol* 98, 751-770.
- Condliffe, S. B., Corradini, I., Pozzi, D., Verderio, C., and Matteoli, M. (2010). Endogenous SNAP-25 Regulates Native Voltage-gated Calcium Channels in Glutamatergic Neurons. *Journal of Biological Chemistry* 285, 24968 -24976.
- Desai, N. S., Rutherford, L. C., and Turrigiano, G. G. (1999). Plasticity in the intrinsic excitability of cortical pyramidal neurons. *Nat. Neurosci* 2, 515-520.
- Desai, N. S., Cudmore, R. H., Nelson, S. B., and Turrigiano, G. G. (2002). Critical

- periods for experience-dependent synaptic scaling in visual cortex. *Nat. Neurosci* 5, 783-789.
- Frank, C. A., Kennedy, M. J., Goold, C. P., Marek, K. W., and Davis, G. W. (2006). Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. *Neuron* 52, 663-677.
- Gainey, M. A., Hurvitz-Wolff, J. R., Lambo, M. E., and Turrigiano, G. G. (2009). Synaptic scaling requires the GluR2 subunit of the AMPA receptor. *J. Neurosci* 29, 6479-6489.
- Gao, L., Blair, L. A. C., and Marshall, J. (2006). CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. *Biochem. Biophys. Res. Commun* 345, 1606-1610.
- Greger, I. H., Ziff, E. B., and Penn, A. C. (2007). Molecular determinants of AMPA receptor subunit assembly. *Trends Neurosci* 30, 407-416.
- Harms, K. J., Tovar, K. R., and Craig, A. M. (2005). Synapse-specific regulation of AMPA receptor subunit composition by activity. *J. Neurosci* 25, 6379-6388.
- Hou, Q., Zhang, D., Jarzylo, L., Huganir, R. L., and Man, H. (2008). Homeostatic regulation of AMPA receptor expression at single hippocampal synapses. *Proc. Natl. Acad. Sci. U.S.A* 105, 775-780.
- Ibata, K., Sun, Q., and Turrigiano, G. G. (2008). Rapid Synaptic Scaling Induced by Changes in Postsynaptic Firing. *Neuron* 57, 819-826.
- Isaeva, E., Fedulova, S., and Veselovskii, N. (1998). Voltage-dependent calcium channels in cultivated neurons of the rat hippocampus. *Neurophysiology* 30, 297-300.
- Janovjak, H., Szobota, S., Wyart, C., Trauner, D., and Isacoff, E. Y. (2010). A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing. *Nat Neurosci* 13, 1027-1032.
- Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., Garner, C. C., Tsien, R. Y., Ellisman, M. H., and Malenka, R. C. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci* 7, 244-253.

- Kim, J., and Tsien, R. W. (2008). Synapse-specific adaptations to inactivity in hippocampal circuits achieve homeostatic gain control while dampening network reverberation. *Neuron* 58, 925-937.
- Kirov, S. A., Goddard, C. A., and Harris, K. M. (2004). Age-dependence in the homeostatic upregulation of hippocampal dendritic spine number during blocked synaptic transmission. *Neuropharmacology* 47, 640-648.
- Knogler, L. D., Liao, M., and Drapeau, P. (2010). Synaptic scaling and the development of a motor network. *J. Neurosci* 30, 8871-8881.
- Kohler, M. (1993). Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: Diversity by RNA editing. *Neuron* 10, 491-500.
- Lee, M., Yasuda, R., and Ehlers, M. D. (2010). Metaplasticity at single glutamatergic synapses. *Neuron* 66, 859-870.
- Lee, S. H., Simonetta, A., and Sheng, M. (2004). Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron* 43, 221-236.
- Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* 3, 175-190.
- Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1998). Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. *Proc. Natl. Acad. Sci. U.S.A* 95, 7097-7102.
- Lissin, D. V., Carroll, R. C., Nicoll, R. A., Malenka, R. C., and Zastrow, M. V. (1999). Rapid, Activation-Induced Redistribution of Ionotropic Glutamate Receptors in Cultured Hippocampal Neurons. *J. Neurosci.* 19, 1263-1272.
- Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5-21.
- Martin, S., Bouschet, T., Jenkins, E. L., Nishimune, A., and Henley, J. M. (2008). Bidirectional Regulation of Kainate Receptor Surface Expression in

- Hippocampal Neurons. *Journal of Biological Chemistry* 283, 36435-36440.
- McKinney, R. A., Capogna, M., Durr, R., Gähwiler, B. H., and Thompson, A. S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci* 2, 44-49.
- Mrsic-Flogel, T. D., Hofer, S. B., Ohki, K., Reid, R. C., Bonhoeffer, T., and Hübener, M. (2007). Homeostatic regulation of eye-specific responses in visual cortex during ocular dominance plasticity. *Neuron* 54, 961-972.
- O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D., and Huganir, R. L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21, 1067-1078.
- Paschen, W., Hotop, S., and AUFENBERG, C. (2003). Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress. *Cell Calcium* 33, 83-89.
- Rezazadeh, S., Claydon, T. W., and Fedida, D. (2006). KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), a calcium/calmodulin-dependent protein kinase II inhibitor, is a direct extracellular blocker of voltage-gated potassium channels. *J. Pharmacol. Exp. Ther* 317, 292-299.
- Rose, J., Jin, S., and Craig, A. M. (2009). Heterosynaptic Molecular Dynamics: Locally Induced Propagating Synaptic Accumulation of CaM Kinase II. *Neuron* 61, 351-358.
- Seeburg, P. H., Higuchi, M., and Sprengel, R. (1998). RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res. Brain Res. Rev* 26, 217-229.
- Shi, S., Hayashi, Y., Esteban, J. A., and Malinow, R. (2001). Subunit-Specific Rules Governing AMPA Receptor Trafficking to Synapses in Hippocampal Pyramidal Neurons. *Cell* 105, 331-343.
- Sokolova, I. V., and Mody, I. (2008). Silencing-Induced Metaplasticity in Hippocampal Cultured Neurons. *J Neurophysiol* 100, 690-697.
- Sutton, M. A., Ito, H. T., Cressy, P., Kempf, C., Woo, J. C., and Schuman, E. M.

- (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* 125, 785-799.
- Swandulla, D., and Armstrong, C. M. (1989). Calcium channel block by cadmium in chicken sensory neurons. *Proc. Natl. Acad. Sci. U.S.A* 86, 1736-1740.
- Szobota, S., Gorostiza, P., Del Bene, F., Wyart, C., Fortin, D. L., Kolstad, K. D., Tulyathan, O., Volgraf, M., Numano, R., Aaron, H. L., et al. (2007). Remote Control of Neuronal Activity with a Light-Gated Glutamate Receptor. *Neuron* 54, 535-545.
- Taylor, W. R. (1988). Permeation of barium and cadmium through slowly inactivating calcium channels in cat sensory neurones. *J. Physiol. (Lond.)* 407, 433-452.
- Thiagarajan, T. C., Lindskog, M., Malgaroli, A., and Tsien, R. W. (2007). LTP and adaptation to inactivity: overlapping mechanisms and implications for metaplasticity. *Neuropharmacology* 52, 156-175.
- Thiagarajan, T. C., Lindskog, M., and Tsien, R. W. (2005). Adaptation to Synaptic Inactivity in Hippocampal Neurons. *Neuron* 47, 725-737.
- Turrigiano, G. G. (2008). The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. *Cell* 135, 422-435.
- Turrigiano, G. G., Leslie, K. R., Desai, N. S., Rutherford, L. C., and Nelson, S. B. (1998). Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* 391, 892-896.
- Turrigiano, G. G., and Nelson, S. B. (2004). Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci* 5, 97-107.
- Volgraf, M., Gorostiza, P., Numano, R., Kramer, R. H., Isacoff, E. Y., and Trauner, D. (2006). Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat Chem Biol* 2, 47-52.
- Watt, A. J., van Rossum, M. C., MacLeod, K. M., Nelson, S. B., and Turrigiano, G. G. (2000). Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. *Neuron* 26, 659-670.

- Wayman, G. A., Lee, Y., Tokumitsu, H., Silva, A., and Soderling, T. R. (2008).
Calmodulin-Kinases: Modulators of Neuronal Development and Plasticity.
Neuron 59, 914-931.
- Wierenga, C. J., Ibata, K., and Turrigiano, G. G. (2005). Postsynaptic Expression of
Homeostatic Plasticity at Neocortical Synapses. J. Neurosci. 25, 2895-2905.

Chapter 3 Figures

Figure 1. SEP-GluR2 fluorescence represents surface population of receptors, which exhibit canonical synaptic scaling behavior

Figure 2. LiGluR activation inhibits TTX-mediated synaptic scaling (indicated by SEP-GluR2 fluorescence)

Figure 3. Effect of LiGluR activation on SEP-GluR2 fluorescence requires change in intracellular calcium levels

Figure 4. LiGluR-mediated calcium influx requires voltage dependent calcium channel activation to depress SEP-GluR2 fluorescence

Figure 5. LiGluR effect on surface AMPAR dependent on Ca^{2+} /calmodulin-dependent protein kinase activity

Figure 1

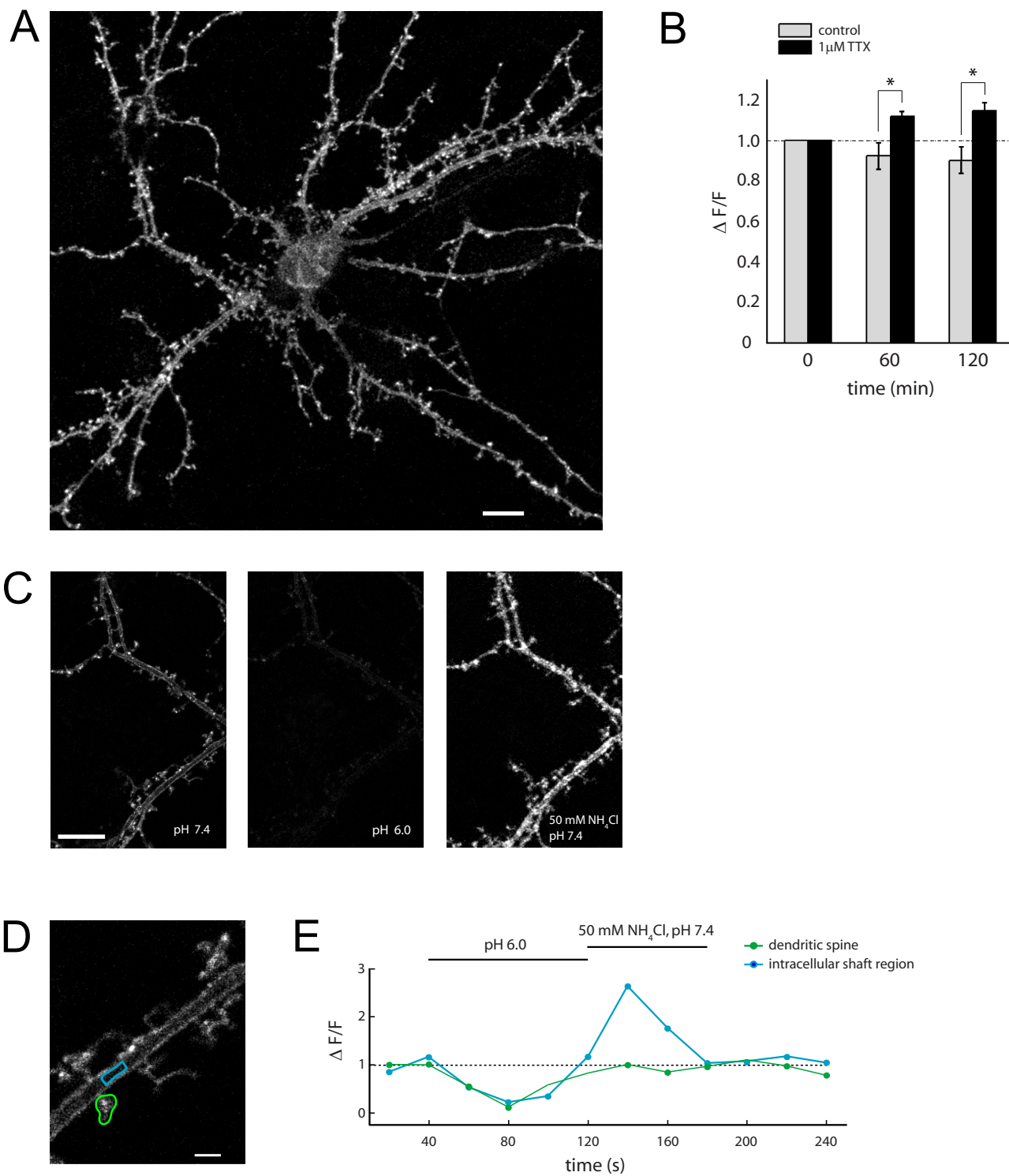
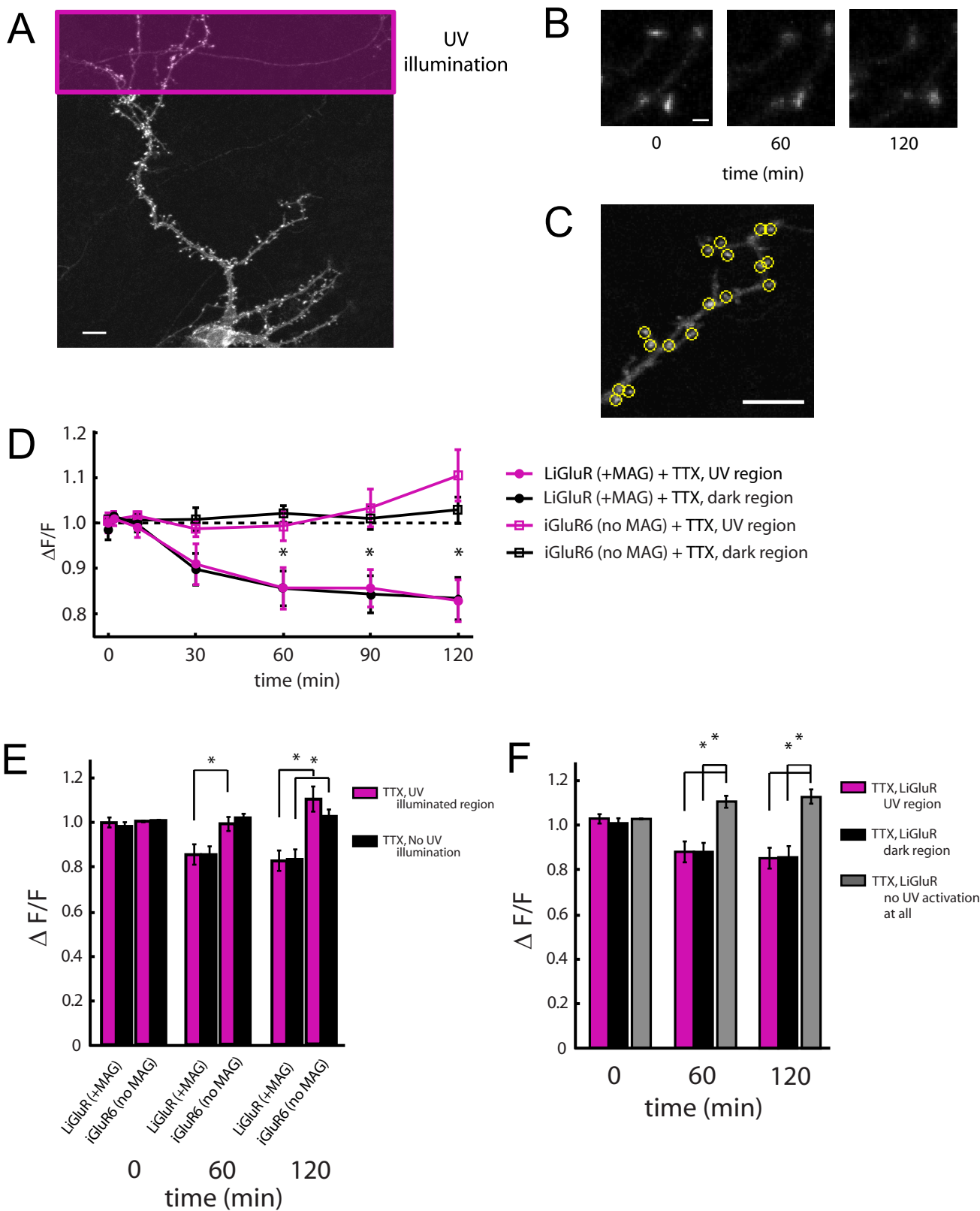


Figure 1. SEP-GluR2 fluorescence represents surface population of receptors, which exhibit canonical synaptic scaling behavior

- A). Example of SEP-GluR2 fluorescence in a cultured hippocampal neuron, SEP-GluR2 is co-expressed with untagged LiGluR (iGluR6 L439C). Scale bar is 10 μ m.
- B). Fluorescence intensity of individual SEP-GluR2 puncta increased in 1 μ M TTX treatment, compared to control cultures. Changes in intensity are normalized to initial fluorescence, n=7 for TTX, n=8 for control. * p-value > 0.05, Student's t-test
- C). SEP-GluR2 fluorescence in pH 7.4 extracellular bath solution are AMPA receptors at cell surface (left panel). Fluorescence is dimmed rapidly and reversibly when an MES-buffered solution at pH 6.0 is washed over the culture (center panel). When the pH gradient is collapsed with a 50mM NH₄Cl solution, the intracellular pH is matched to that of the extracellular and the total population of receptors becomes fluorescent (right panel). Scale bar is 10 μ m.
- D). Regions identified on baseline image (initial pH 7.4) for fluorescence versus time quantification. Scale bar is 2 μ m.
- E). Fluorescence intensity trace for regions identified in (D). Change in fluorescence is normalized to each region's initial value.

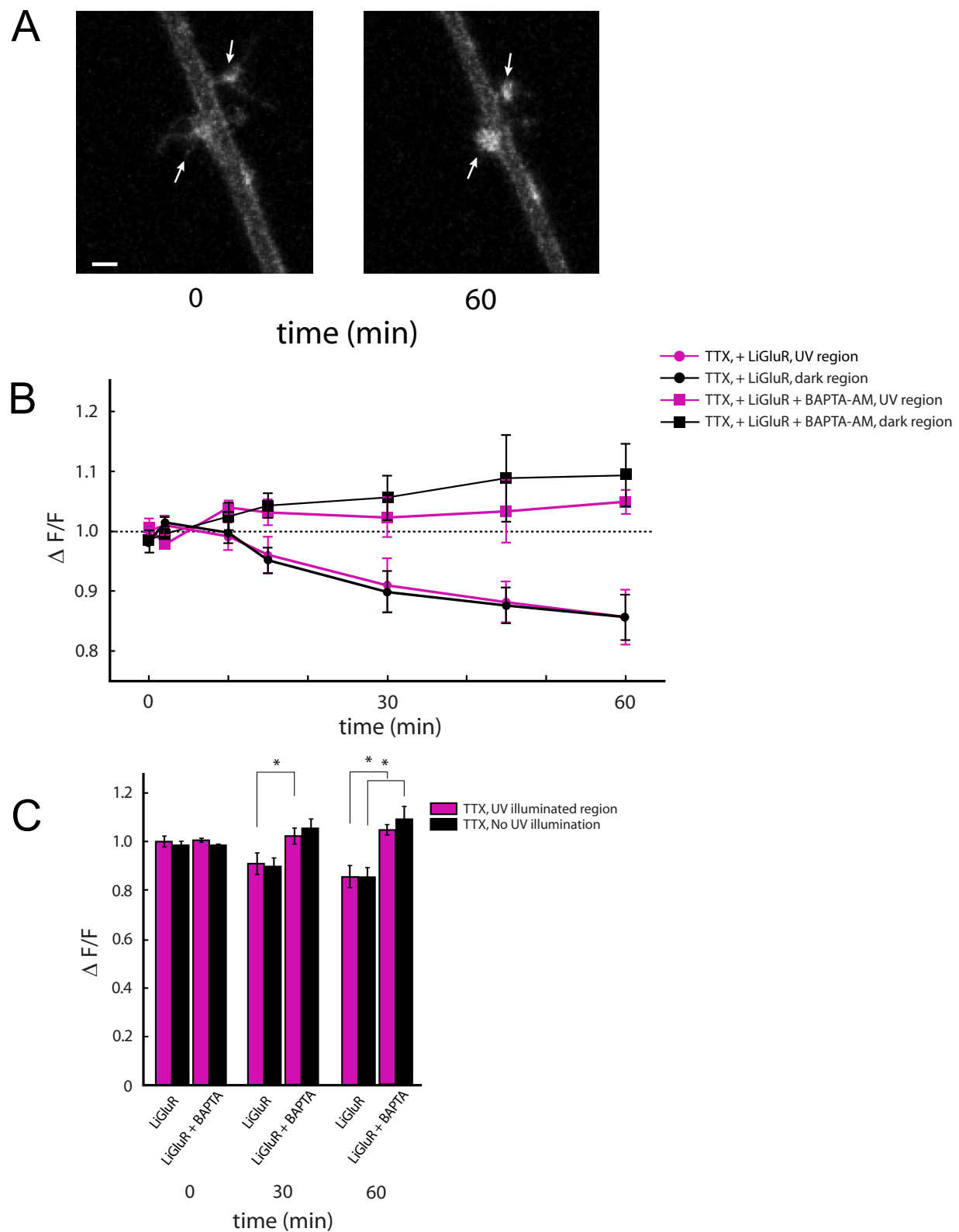
Figure 2



**Figure 2. LiGluR activation inhibits TTX-mediated synaptic scaling
(indicated by SEP-GluR2 fluorescence)**

- A). Time lapse imaging of SEP-GluR2 co-expressed with untagged LiGluR in hippocampal neuron in the presence in 1 μ M TTX. The entire field of view is imaged to assess global changes in SEP-GluR2 but only the indicated top region is illuminated with 380-nm light. Scale bar is 10 μ m.
- B). Example of SEP-GluR2 puncta at dendritic spines whose fluorescence intensity decreases over the course of LiGluR activation. Scale bar is 1 μ m.
- C). SEP-GluR2 puncta are identified (indicated by yellow circles) and tracked over the time course by modified MATLAB scripts. Scale bar is 10 μ m.
- D). SEP-GluR2 fluorescence intensity decreases after LiGluR activation begins at 10 minutes. No significant difference was measured between puncta in the UV region and in the dark region was seen for any of the cells. n=6 cells, * p-values > 0.02, paired Student's t-test with initial values
- E). LiGluR activated cells showed a significant decrease in SEP-GluR2 puncta fluorescence compared to control cells which saw the same chronic UV illumination but was not conjugated with the photoswitch MAG. At the beginning of the experiment 3 images are taken, each punctum's fluorescence is averaged, and this is taken as the baseline. Change in fluorescence at each time point is normalized by the punctum's baseline fluorescence (including at time 0). n=6 for LiGluR cells, n=4 for control cells, * p-values > 0.02, unpaired Student's t-test
- F). LiGluR activated cells showed a significant decrease in SEP-GluR2 puncta fluorescence compared to control cells which were also conjugated with the photoswitch MAG but were not illuminated with 380-nm light anywhere at any point. At the beginning of the experiment 3 images are taken, each punctum's fluorescence is averaged, and this is taken as the baseline. Change in fluorescence at each time point is normalized by the punctum's baseline fluorescence (including at time 0). n=6 for UV/dark treated cells, n=11 for all dark cells, * p-values > 0.001, unpaired Student's t-test

Figure 3



**Figure 3. Effect of LiGluR activation on SEP-GluR2 fluorescence
requires change in intracellular calcium levels**

- A). Example of SEP-GluR2 puncta at dendritic spines for a neuron loaded with BAPTA-AM (10 μ M) and treated with LiGluR activation in 1 μ M TTX, at 0 and 60 minutes. Scale bar is 2 μ m.
- B). SEP-GluR2 puncta of neurons loaded with BATPA-AM slightly increased fluorescence intensity over the course of LiGluR activation (broken lines), compared to neurons not loaded with BATPA-AM (solid lines). Slight increases in puncta fluorescence in both regions were not significant compared to the initial fluorescence. No significant difference was seen between puncta fluorescence in the UV region and in the dark region.
- C). Changes in SEP-GluR2 puncta fluorescence intensity in BAPTA-AM loaded cells are significantly different from non-loaded cells after 60 minutes. At the beginning of the experiment 3 images are taken, each punctum's fluorescence is averaged, and this is taken as the baseline. Change in fluorescence at each time point is normalized by the punctum's baseline fluorescence (including at time 0). n=3 for BAPTA-AM loaded cells, n=6 for control cells, * p-values > 0.03, unpaired Student's t-test

Figure 4

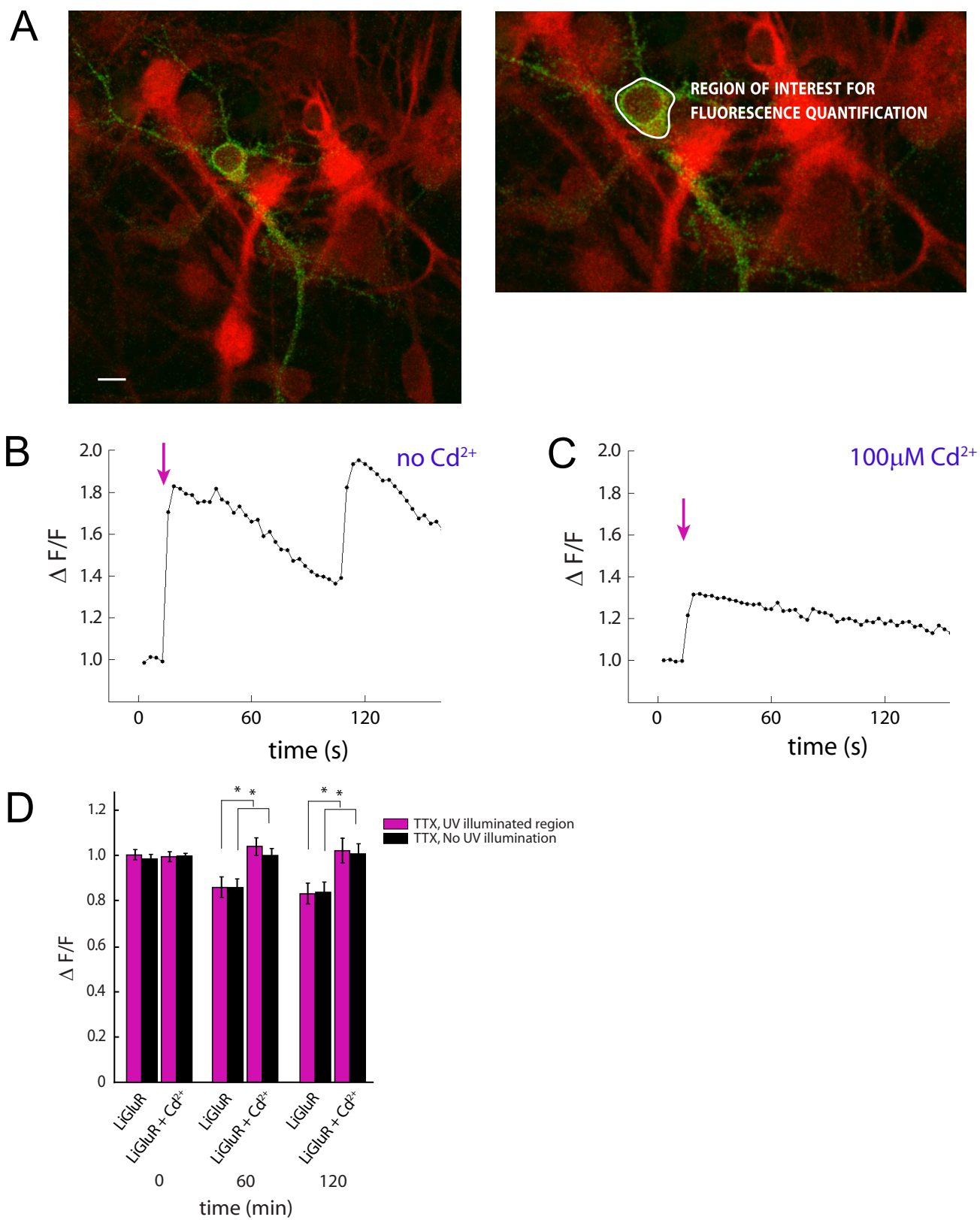
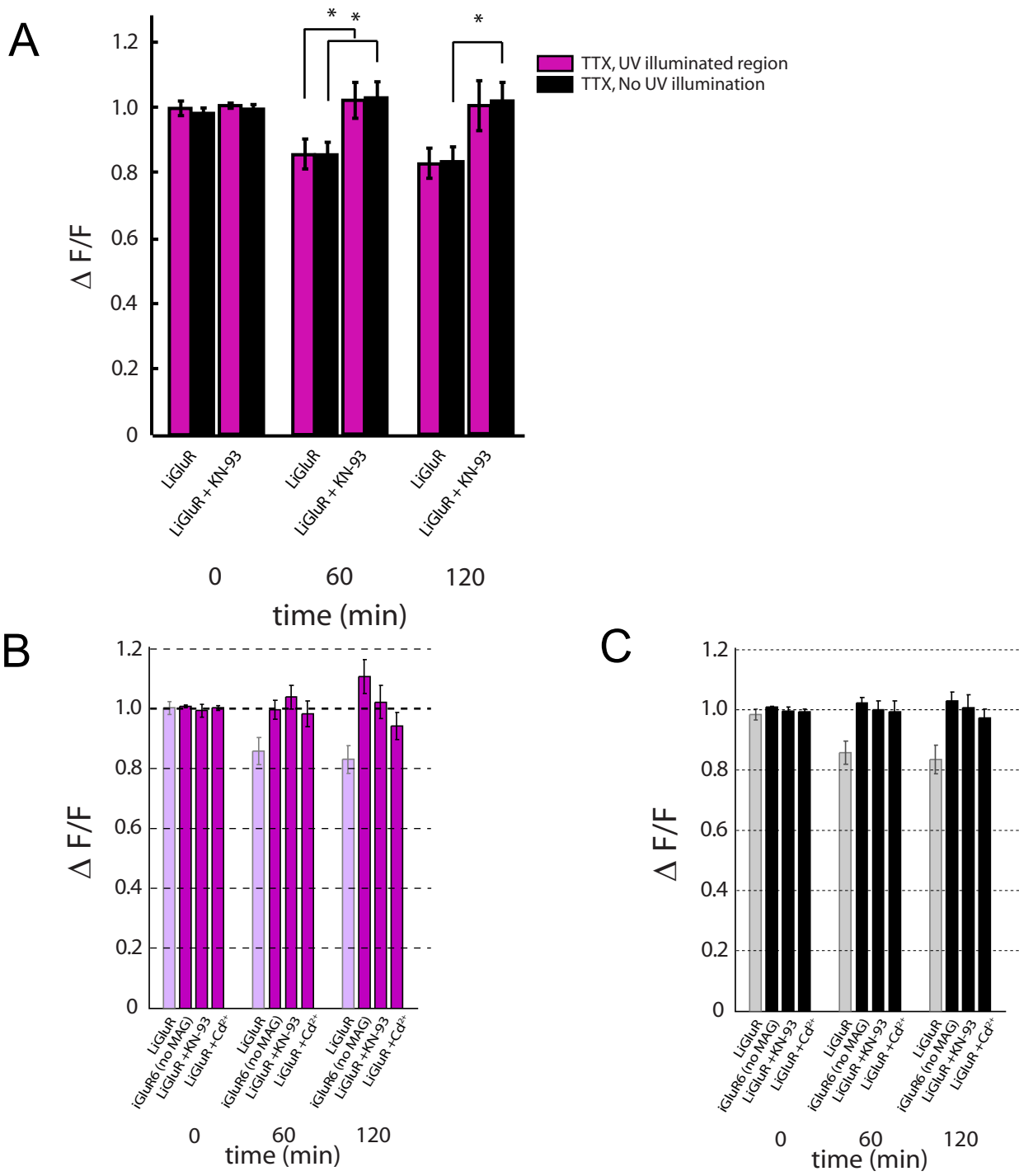


Figure 4. LiGluR-mediated calcium influx requires voltage dependent calcium channel activation to depress SEP-GluR2 fluorescence

- A). Calcium imaging experiment: X-Rhod-1 dye shown in red, SEP-GluR2 fluorescence shown in green (neuron was co-transfected with untagged LiGluR). Right panel identifies region of interest (soma) for fluorescence intensity quantification in (B) and (C). Scale bar is 10 μ m.
- B). Normalized calcium response produced by LiGluR activation, in absence of cadmium. Arrows indicate when UV illumination was applied over entire cell. Changes in fluorescence were normalized to region's baseline intensity, averaged from first 5 images.
- C). Normalized calcium response produced by same LiGluR activation in same cell, with 100 μ M cadmium added to recording solution. Arrows indicate when UV illumination was applied over entire cell. Changes in fluorescence were normalized to region's baseline intensity, averaged from first 5 images.
- D). Cadmium in extracellular bath solution inhibits LiGluR effect on SEP-GluR2 puncta fluorescence. At the beginning of the experiment 3 images are taken, each punctum's fluorescence is averaged, and this is taken as the baseline. Change in fluorescence at each time point is normalized by the punctum's baseline fluorescence (including at time 0). n=6 cells for Cd²⁺ condition, n=6 cells for control, * p-values > 0.03, unpaired Student's t-test

Figure 5



**Figure 5. LiGluR effect on surface AMPAR dependent on
Ca²⁺/calmodulin-dependent protein kinase activity**

- A). KN-93 in extracellular bath solution inhibits LiGluR effect on SEP-GluR2 puncta fluorescence. At the beginning of the experiment 3 images are taken, each punctum's fluorescence is averaged, and this is taken as the baseline. Change in fluorescence at each time point is normalized by the punctum's baseline fluorescence (including at time 0). n=8 cells for KN-93 condition, n=6 cells for control, * p-values > 0.05, unpaired Student's t-test
- B). Blockade with either Cd²⁺ or KN-93 occludes effects of LiGluR activation, leaving SEP-GluR2 fluorescence stable as in the control condition (iGluR6 with no MAG). Normalized change in SEP-GluR2 fluorescence for UV regions across different conditions – no statistical difference between opaque magenta bars (LiGluR condition shown in transparent bars for comparison).
- C). Blockade with either Cd²⁺ or KN-93 occludes effects of LiGluR activation, leaving SEP-GluR2 fluorescence stable as in the control condition (iGluR6 with no MAG). Normalized change in SEP-GluR2 fluorescence for dark regions across different conditions – no statistical difference between opaque black bars (LiGluR condition shown in transparent bars for comparison).

APPENDIX I:

Synaptic scaling of SEP-GluR1

Summary

I compare scaling of different AMPA receptor (AMPA) subunits at excitatory synapses in response to increased depolarization and calcium influx on a local level. Previously we measured changes of synaptic receptors at the plasma membrane by monitoring ecliptic-GFP tagged GluR2 subunits in cultured hippocampal neurons in response to spatially control manner depolarization mediated by LiGluR, a light-activated glutamate receptor. Addition of TTX to the bath solution restricts the LiGluR signal from traveling through the dendritic tree via back-propagating action potentials and also sensitizes neurons to the LiGluR activity manipulation. We found that chronic depolarization and Ca^{2+} influx in a portion of the dendritic tree rapidly reduced the surface population of GluR2-containing AMPARs at dendritic spines in all parts of the neuron. I extend the approach here to monitor ecliptic-tagged GluR1 subunits. Preliminary results show that while these GluR1 subunits scale up in response to activity blockade with NMDAR block, they do not scale down in response to LiGluR activation.

Introduction

AMPA receptors (AMPA) and NMDA receptors (NMDAR) are the two main ionotropic glutamate receptor subtypes present at excitatory synapses in the central nervous system. AMPARs mediate fast synaptic transmission by controlling the postsynaptic depolarization that initiates neuronal firing, while NMDARs are responsible for inducing specific forms of plasticity including LTP and LTD (Malenka and Bear, 2004). Regulation of AMPAR trafficking and localization, including mechanisms that govern membrane insertion, internalization, recycling and degradation, thereby determines the strength of the synapse (Santos et al., 2009; Barry and Ziff, 2002). Most AMPA receptors expressed in the rat hippocampus are heteromeric, composed from a combination of the four subunits GluR1-GluR4. Receptors lacking the GluR2 subunit are permeable to calcium while channels containing GluR2 are rendered calcium-impermeable by editing of the Q/R site in >99% of GluR2 mRNAs (Bredt and Nicoll, 2003; Seeburg et al., 1998). In addition to channel properties the specific subunit composition also determines trafficking to synaptic sites (Shi et al., 2001), recycling (Lee et al., 2004), and its role in LTP/LTD (Bredt and Nicoll, 2003). Changes in AMPAR accumulation are also considered a major expression locus of activity-dependent scaling of excitatory synapses (Lissin et al., 1998; Wierenga et al., 2005; Turrigiano, 2008).

While there is good agreement that AMPAR expression is a reliable measure of synaptic scaling, there are some discrepancies with respect to the subunit composition.

Some studies show that activity blockade results in increased GluR1 levels but not GluR2 (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006), while others report synaptic scaling on GluR2-containing AMPARs (O'Brien et al., 1998; Wierenga et al., 2005; Ibata et al., 2008) and recently that the GluR2 subunit itself is required (Gainey et al., 2009). Consistent with these later studies I previously showed a depression of GluR2-containing AMPARs in response to LiGluR activation. Here I extended the setup to study ecliptic-tagged GluR1 subunits, monitoring surface expression in response to local LiGluR activation. Tetrodotoxin (TTX) is added to the bath solution to restrict the LiGluR signal from traveling through the dendritic tree via back-propagating action potentials and also to sensitize neurons to the LiGluR activity manipulation. Preliminary results show that while these GluR1 subunits scale up in response to activity blockade with NMDAR block, they do not scale down in response to LiGluR activation.

Results

Experiments were performed on cultured rat hippocampal neurons co-transfected with LiGluR for subcellular depolarization and calcium influx and with the super ecliptic pHluorin tagged GluR1 subunit (SEP-GluR1) to assess changes in surface AMPA receptor populations. Previous studies have reported accumulation of GluR1 subunits in response to reduced activity (Sutton et al., 2006; Thiagarajan et al., 2005; Ju et al., 2004), though scaling down was not investigated. Expressing LiGluR and SEP-GluR1 in the same neuron allows us to assay changes to the post-synaptic surface AMPARs in response to local depolarization and calcium influx. Several hours prior to any LiGluR activation or SEP-GluR2 imaging neuron cultures are pre-treated with 1 μ M TTX. This activity blockade increases postsynaptic sensitivity to glutamate, increasing the effectiveness of LiGluR activation (Watt et al., 2000; Desai et al., 1999). Experiments were carried out in an extracellular bath solution containing 1 μ M TTX to continue activity blockade in the culture and also to prevent LiGluR signal propagation via action potential firing. Distal regions of the dendritic branch were illuminated with UV light to produce local LiGluR activation without raising somatic calcium.

SEP-GluR1 fluorescence intensity scales with activity and NMDAR blockade.

As with experiments using the SEP-GluR2 subunit, we began by optimizing expression of SEP-GluR1 with LiGluR. Cultured hippocampal neurons were co-transfected with SEP-GluR1 and iGluR6(L439C) and then imaged with confocal microscopy. Fluorescence was bright and punctate along the dendritic tree and spines, with

background fluorescence in the soma and intracellular dendritic shafts (Figure 1A). To confirm that SEP-GluR1 fluorescence accurately represents surface AMPAR, a low pH extracellular solution was washed over the neurons and puncta fluorescence was eclipsed as expected (data not shown, similar to SEP-GluR2 characterization in Chapter 3). Diffuse fluorescence in soma and other intracellular regions were minimally affected by lowered pH indicating an internal population (Ashby et al., 2004), therefore further experiments only considered punctate SEP-GluR1 fluorescence.

As discussed in Chapter 3, homeostatic scaling of excitatory synapses is typically induced by blocking activity in the neuronal cultures with TTX. Recently, a study reported that NMDAR blockade in addition to TTX accelerated enhanced AMPAR accumulation (Sutton et al., 2006). This increase in mEPSC amplitude was attributed to increased GluR1 surface expression by live labeling of antibodies specific for extracellular epitopes of either GluR1 or GluR2, interestingly no change in GluR2 was observed. We performed a similar experiment to investigate SEP-GluR1 as a faithful reporter of this mode of synaptic scaling on this timescale.

Live cell imaging was performed on cultured hippocampal neurons expressing SEP-GluR1. Baseline fluorescence was measured prior to any manipulation, then 1 μ M TTX and 50 μ M AP5 was added to the extracellular bath solution and the same cells were imaged 60 and 120 minutes later. Control cultures were imaged the same way, with a saline solution added in place of TTX/AP5. Fluorescence of individual SEP-GluR1 puncta was measured at each time point and normalized to its initial intensity value ($\Delta F/F$). Cultures where TTX and AP5 was added increased fluorescence, compared to control cultures which showed a slight decrease in fluorescence (Figure 1B). This increase in puncta fluorescence indicates rapid modulation of AMPA receptors in response to action potential and miniature synaptic transmission blockade.

LiGluR activation does not inhibit TTX-induced synaptic scaling of SEP-GluR1

As shown in Chapters 2 and 3, co-expression of LiGluR with other functional proteins does not adversely affect LiGluR depolarization. This was confirmed for SEP-GluR1 and LiGluR co-expression using the X-Rhod-1 calcium indicator dye (data not shown).

The effect of local depolarization and calcium influx on GluR1 surface expression was studied using a similar setup to the GluR2-monitoring experiments previously described. LiGluR is activated in a portion of distal dendrites in a bath solution containing 1 μ M TTX while measuring SEP-GluR1 fluorescence intensity in all regions of the neuron. A small region of the dendrites (generally 10-15% of the dendritic tree) was illuminated with 380-nm light to locally activate LiGluR. A region 50-100 μ m away from the soma was chosen to ensure that there was no significant rise in the somatic calcium level. To begin the synaptic scaling process and condition the

neurons to be more sensitized to the LiGluR manipulation, 1 μ M TTX was added to the neuron cultures approximately 12 hours before beginning LiGluR activation and time-lapse imaging.

Over the course of two hours of LiGluR activation SEP-GluR1 puncta fluorescence remained primarily stable (Figure 1C). As with previous SEP-GluR2 experiments, there was no distinction between SEP-GluR1 puncta in the UV region with direct LiGluR activation and in the dark region with indirect LiGluR effects. Neither condition significantly changed from its own initial fluorescence. SEP-GluR2 fluorescence changes in response to similar subcellular LiGluR activation are plotted for comparison (Figure 1C, broken lines). These preliminary results suggest that changes to chronic LiGluR activation is mediated, at least in the first two hours, by GluR2-containing receptors.

Discussion

The experiments described here extend the use of LiGluR to study local modes of synaptic scaling, taking advantage of on its spatial and temporal properties. By combining targeted illumination and bath application of TTX, LiGluR activation is restricted to a sub-cellular region. We use here again the ecliptic-GFP tag to monitor GluR1 behavior because it is effectively coupled with LiGluR manipulations for time-lapse imaging and also to give a lateral comparison with the SEP-GluR2 experiments. As expected, the UV illumination used to activate the channel did not adversely affect cell health or interfere with photostability of ecliptic GFP.

These preliminary results suggest that local LiGluR depolarization affects only GluR2-containing AMPARs over a two-hour time course. It is not clear whether SEP-GluR2 fluorescence represents receptors that also contain GluR1 (and vice versa for SEP-GluR1). In the hippocampus most AMPARs are hetero-tetramers composed of GluR1/GluR2 or GluR2/GluR3 subunits with a small number of GluR1 homomers (Greger et al., 2007; Wenthold et al., 1996). The different subunit compositions determine distinct synaptic delivery mechanisms (Shi et al., 2001) as well as recycling pathways (Barry and Ziff, 2002; Santos et al., 2009). The molecular mechanisms underlying these processes were dependent on the different carboxyl-terminal domains, which interact with different PDZ-binding postsynaptic proteins (Kornau et al., 1995; Dong et al., 1997; Srivastava et al., 1998).

A number of experiments remain for this study. An initial control is to perform time-lapse imaging experiments (in the same TTX-containing extracellular bath) but without any LiGluR activation. It is possible that SEP-GluR1 subunits behave differently from SEP-GluR2 subunits. Without LiGluR activation SEP-GluR2 reported constant surface expression over two hours, in some cases fluorescence intensity even increased slightly (though increase is not statistically significant). It's possible that

SEP-GluR1 will have different behavior, perhaps being more or less sensitive to TTX present in solution. The effects of activity and NMDAR blockade shown in Figure 1B are not comparable to SEP-GluR2 experiments or to the LiGluR-activation experiments in Figure 1C because neither of these two cases had AP5 in the extracellular solution.

If SEP-GluR1 fluorescence represents GluR1 homomers this would be reporting the behavior of calcium-permeable AMPARs, a distinct population from those seen in the SEP-GluR2 experiments (which are rendered calcium-impermeable by Q/R editing of GluR2 mRNA (Seeburg et al., 1998; Bredt and Nicoll, 2003)). A straight-forward initial test would be to use antibodies directed against GluR2 or GluR2/3 on SEP-GluR1 expressing cultures. Co-localization between SEP-GluR1 fluorescence and immunostained receptors would indicate GluR1/GluR2 hetero-oligomers. For completeness, antibodies directed against the endogenous GluR1 subunit should be used on SEP-GluR2 expressing cultures. If these assays reveal that SEP-GluR2 fluorescence represents GluR1/GluR2 heteromers (GluR2/GluR3 heteromers is also a possibility) and SEP-GluR1 fluorescence also represents GluR1/GluR2 receptors then time-lapse imaging data should match.

Once further characterization of SEP-GluR1 fluorescence is complete it could be used to study subunit-specific changes of AMPAR expression in response to LiGluR activation. This would complement previous SEP-GluR2 experiments and could elucidate the importance of subunit composition on LiGluR-mediated scaling down of AMPA receptor expression.

Materials and Methods

Hippocampal neuron culture and transfection

Dissociated hippocampal neurons prepared from postnatal rats (P0-P5) were plated on poly-L-lysine coated glass bottom dishes (MatTek). Cells were cultured in MEM supplemented with 5% fetal bovine serum, B27 (Invitrogen), GlutaMAX™ (Invitrogen) and serum extender (BD Biosciences). Ara-C (4 μ M) was added after 5 DIV. After 7-9 DIV, cells were transfected with 2 μ g of DNA per 12 mm coverslip by the calcium phosphate method and used 3-7 days later. Cells were co-transfected with super ecliptic pHluorin tagged GluR1 (SEP-GluR1) and iGluR6(L439C) at a 2:1 ratio.

Live cell confocal imaging – SEP-GluR1 time lapse experiments with activity and NMDAR blockade

For experiments, neurons were transferred a HEPES-buffered extracellular solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. Time-lapse images were acquired on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective. A 488nm Argon laser was used to excite the dye and a 510-560 nm band-pass filter was used to collect fluorescent emission. A baseline fluorescence image was taken for each cell before addition of 1mM TTX and 50mM AP5. A confocal stack imaging the SEP-GluR1 was then taken 60 and 120 minutes later.

Live cell confocal imaging – SEP-GluR1 + LiGluR time lapse experiments

Approximately 12-24 hours before experiments 1 μ M TTX was added to the culture media. For experiments, neurons were transferred a HEPES-buffered extracellular solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. Neurons expressing iGluR6(L439C) were conjugated with MAG-0 as previously described (Szobota et al., 2007). After MAG conjugation cultures were washed three times with extracellular solution then imaged in new extracellular solution with 1 μ M TTX.

Time-lapse images were acquired on a Zeiss LSM 510 NLO Axiovert confocal microscope and a Plan-Apochromat 63x/1.4 oil objective. A Spectra-Physics MaiTai HP laser was used to activate LiGluR with 380-nm light (frequency doubled 760-nm) and a 488-nm Argon laser was used for image acquisition. Three frames were taken as a baseline before any LiGluR activation. Cells were illuminated with UV light for ~1 second every two minutes, over a 40 μ m $\times$ 140 μ m section of the distal dendrites. A confocal stack imaging the SEP-GluR1 was taken once every 15 minutes.

Image analysis

Each LSM confocal stack was imported into MATLAB (The MathWorks, Inc.) as a maximum project tiff file for compilation and analysis. Motion artifacts in the x-y plane were corrected using DIPimage (www.diplib.org). Punctate SEP-GluR2 features were detected and tracked through the time series using MATLAB scripts from The Matlab Particle Tracking Code Repository (copyright 1997, Daniel Blair and Eric Dufresne). The fluorescence intensity of each punctum at each time point was normalized to its own averaged baseline (3 frames taken prior to any manipulation). Values reported are mean $\pm$ standard error of the mean for the number of neurons

indicated. Statistics were performed using the two-tailed, two-sample Student's t test, a p-value of <0.05 was considered statistically significant.

References

- Ashby, M. C., Ibaraki, K., and Henley, J. M. (2004). It's green outside: tracking cell surface proteins with pH-sensitive GFP. *Trends Neurosci* 27, 257-261.
- Barry, M. F., and Ziff, E. B. (2002). Receptor trafficking and the plasticity of excitatory synapses. *Curr. Opin. Neurobiol* 12, 279-286.
- Bredt, D. S., and Nicoll, R. A. (2003). AMPA Receptor Trafficking at Excitatory Synapses. *Neuron* 40, 361-379.
- Desai, N. S., Rutherford, L. C., and Turrigiano, G. G. (1999). Plasticity in the intrinsic excitability of cortical pyramidal neurons. *Nat. Neurosci* 2, 515-520.
- Dong, H., O'Brien, R. J., Fung, E. T., Lanahan, A. A., Worley, P. F., and Huganir, R. L. (1997). GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* 386, 279-284.
- Gainey, M. A., Hurvitz-Wolff, J. R., Lambo, M. E., and Turrigiano, G. G. (2009). Synaptic scaling requires the GluR2 subunit of the AMPA receptor. *J. Neurosci* 29, 6479-6489.
- Greger, I. H., Ziff, E. B., and Penn, A. C. (2007). Molecular determinants of AMPA receptor subunit assembly. *Trends Neurosci* 30, 407-416.
- Ibata, K., Sun, Q., and Turrigiano, G. G. (2008). Rapid Synaptic Scaling Induced by Changes in Postsynaptic Firing. *Neuron* 57, 819-826.
- Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T. J., Adams, S. R., Garner, C. C., Tsien, R. Y., Ellisman, M. H., and Malenka, R. C. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci* 7, 244-253.
- Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.
- Lee, S. H., Simonetta, A., and Sheng, M. (2004). Subunit rules governing the sorting of

- internalized AMPA receptors in hippocampal neurons. *Neuron* 43, 221-236.
- Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1998). Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. *Proc. Natl. Acad. Sci. U.S.A* 95, 7097-7102.
- Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5-21.
- O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D., and Huganir, R. L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21, 1067-1078.
- Santos, S. D., Carvalho, A. L., Caldeira, M. V., and Duarte, C. B. (2009). Regulation of AMPA receptors and synaptic plasticity. *Neuroscience* 158, 105-125.
- Seeburg, P. H., Higuchi, M., and Sprengel, R. (1998). RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res. Brain Res. Rev* 26, 217-229.
- Shi, S., Hayashi, Y., Esteban, J. A., and Malinow, R. (2001). Subunit-Specific Rules Governing AMPA Receptor Trafficking to Synapses in Hippocampal Pyramidal Neurons. *Cell* 105, 331-343.
- Srivastava, S., Osten, P., Vilim, F. S., Khatri, L., Inman, G., States, B., Daly, C., DeSouza, S., Abagyan, R., Valtschanoff, J. G., et al. (1998). Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. *Neuron* 21, 581-591.
- Sutton, M. A., Ito, H. T., Cressy, P., Kempf, C., Woo, J. C., and Schuman, E. M. (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* 125, 785-799.
- Szobota, S., Gorostiza, P., Del Bene, F., Wyart, C., Fortin, D. L., Kolstad, K. D., Tulyathan, O., Volgraf, M., Numano, R., Aaron, H. L., et al. (2007). Remote Control of Neuronal Activity with a Light-Gated Glutamate Receptor. *Neuron* 54, 535-545.
- Thiagarajan, T. C., Lindskog, M., and Tsien, R. W. (2005). Adaptation to Synaptic

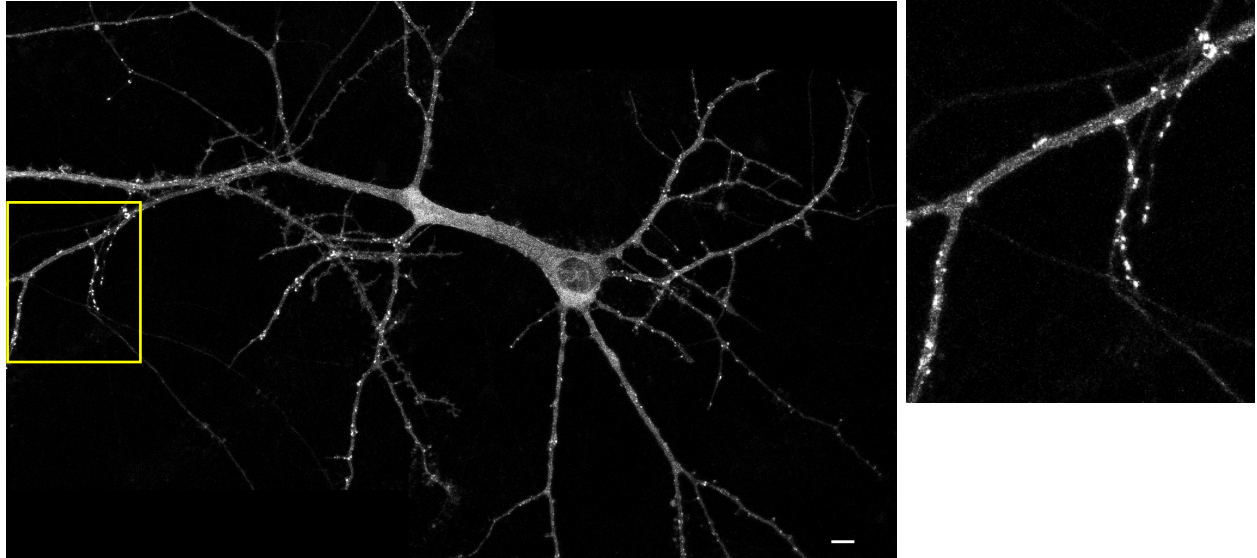
- Inactivity in Hippocampal Neurons. *Neuron* 47, 725-737.
- Turrigiano, G. G. (2008). The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. *Cell* 135, 422-435.
- Watt, A. J., van Rossum, M. C., MacLeod, K. M., Nelson, S. B., and Turrigiano, G. G. (2000). Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. *Neuron* 26, 659-670.
- Wenthold, R. J., Petralia, R. S., Blahos J, I. I., and Niedzielski, A. S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J. Neurosci* 16, 1982-1989.
- Wierenga, C. J., Iyata, K., and Turrigiano, G. G. (2005). Postsynaptic Expression of Homeostatic Plasticity at Neocortical Synapses. *J. Neurosci.* 25, 2895-2905.

Appendix I Figure

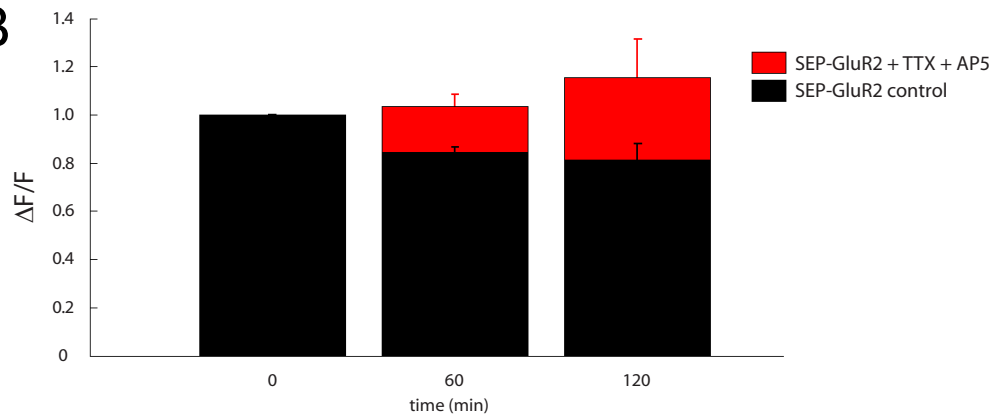
Figure 1. SEP-GluR1 fluorescence intensity scales with activity and NMDAR blockade, but not with LiGluR activation.

Figure 1

A



B



C

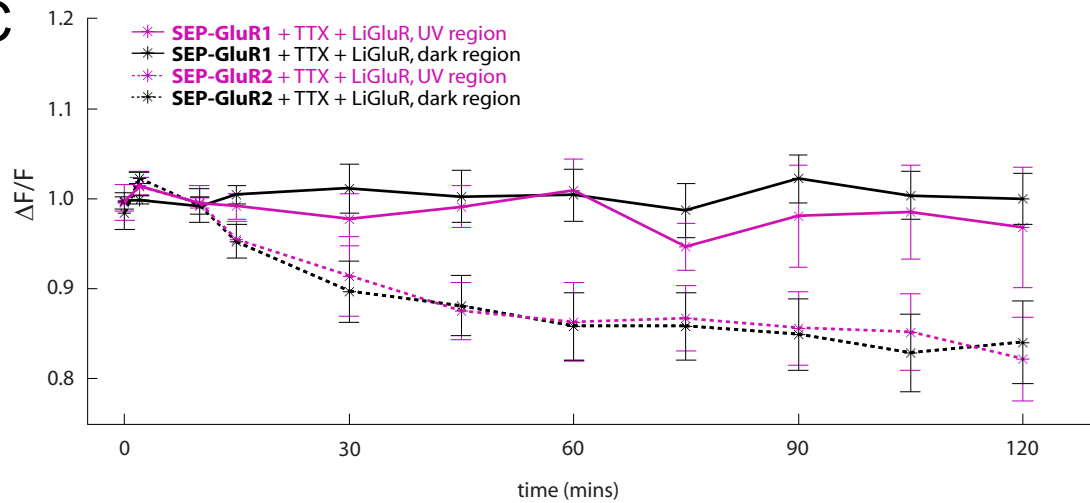


Figure 1. SEP-GluR1 fluorescence intensity scales with activity and NMDAR blockade, but not with LiGluR activation.

- A). Example of SEP-GluR1 fluorescence in a cultured hippocampal neuron, SEP-GluR2 is co-expressed with untagged LiGluR (iGluR6 L439C). Magnified view (right) shows concentrated fluorescence at puncta along dendritic branch. Scale bar is 10 μ m.
- B). Fluorescence intensity of individual SEP-GluR1 puncta increased in 1 μ M TTX + 50 μ M AP5 treatment, compared to control cultures, indicating increased surface GluR1 in response to activity and NMDAR blockade. Changes in intensity are normalized to initial fluorescence.
- C). SEP-GluR2 (broken lines) fluorescence intensity decreases after LiGluR activation begins at 10 minutes while SEP-GluR1 (solid lines) fluorescence intensity remains stable throughout time course. No significant difference was measured between puncta in the UV region and in the dark region was seen for any of the cells.